Interactions between Estrogen and Glucocorticoid Signaling in the Hypothalamus: Effects on Spinogenesis and Male Territorial Aggression

AN ABSTRACT

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BY

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Estrogen and glucocorticoid receptors (ER and GR) are both members of the same subfamily of steroid nuclear receptors, and can both signal classically as ligand-activated transcription factors. However, many responses to estrogen and glucocorticoid exposure occur through the non-classical pathways, which include rapid activation of kinase cascades, activation of membrane-associated receptors, gene regulation through transcription by non-classical transcription factors, and protein regulation by translation and post-translational modification. Male territorial aggression is a hypothalamically-mediated steroid hormone-dependent adaptive behavior in mice. The hypothalamus, which expresses multiple ERs and GRs, is also responsive to estrogen and glucocorticoid treatment at a cellular level. Experiments were conducted to test the effects of estrogen and glucocorticoid interactions on spinogenesis in the ventromedial hypothalamus (VMH) and on male territorial aggression through the resident-intruder paradigm. Studies in male postnatal primary hypothalamic cell cultures demonstrate the expression of classic ERα, the variant ERα-36, and GPR30. PSD-95 protein, a marker for dendritic spines, is increased in response to 12 hours of treatment with the GPR30 agonist G-1 in an ERK/MAPK-dependent manner. Further work in immortalized embryonic hypothalamic cell lines (mHypoE-11 and mHypoE-42) demonstrate non-classical effects of a membrane-limited
glucocorticoid on rapid nuclear translocation of the intracellular GR. Additionally, pharmacological inhibition of the ERK/MAPK pathway results in similar GR translocation in the absence of a ligand. Male postnatal primary hypothalamic cell cultures also respond to glucocorticoid exposure with increased 17β-E synthesis, suggesting crosstalk between GR signaling and estrogen signaling. Spine density in the gonadally intact adult male VMH decreases following suppression of estrogen synthesis with the aromatase inhibitor letrozole, suggesting estrogen is necessary to maintain spine density. In vivo studies in adult male mice demonstrate that estrogen is necessary to maintain basal peripheral CORT synthesis. Behavior testing using the resident-intruder paradigm showed that dexamethasone-suppression of adrenal CORT synthesis increases the amount of time resident mice spent engaged in aggressive bouts, and CORT treatment 20 minutes prior to aggression testing abolished this effect. The findings presented here provide support for the importance of the interactions between classical and non-classical estrogen and glucocorticoid signaling pathways on hypothalamic spinogenesis and male territorial aggression.
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I am incredibly grateful for her help with everything I ever needed. I learned most of the techniques I know from Dr. Clark, and those I knew she improved upon. Dr. Clark also taught me the importance of thinking about the bigger picture; without her I would still be caught up in the minor details.

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<tbody>
<tr>
<td>17β-E</td>
<td>17 beta-estradiol</td>
</tr>
<tr>
<td>Akt</td>
<td>v-akt murine thymoma viral oncogene homolog 1/PKB</td>
</tr>
<tr>
<td>AH</td>
<td>anterior hypothalamus</td>
</tr>
<tr>
<td>ARH</td>
<td>arcuate nucleus of the hypothalamus</td>
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<tr>
<td>BNST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>CamKII</td>
<td>calmodulin kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>3'-5'-cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cav-1</td>
<td>caveolin 1</td>
</tr>
<tr>
<td>cav-2</td>
<td>caveolin 2</td>
</tr>
<tr>
<td>cav-3</td>
<td>caveolin 3</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary (cell line)</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CORT</td>
<td>corticosterone</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP binding protein</td>
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<td>CRH</td>
<td>corticotropin releasing hormone</td>
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<tr>
<td>Dex</td>
<td>dexamethasone</td>
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<tr>
<td>DexBSA</td>
<td>dexamethasone-bovine serum albumin conjugate</td>
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<tr>
<td>E₂BSA</td>
<td>estradiol-bovine serum albumin conjugate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>EB</td>
<td>estradiol benzoate</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ERE</td>
<td>estrogen response element</td>
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<tr>
<td>ERK</td>
<td>extracellular regulated kinase</td>
</tr>
<tr>
<td>ERX</td>
<td>estrogen receptor X</td>
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<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
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<td>ERαKO</td>
<td>estrogen receptor alpha knock out</td>
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<td>ERβ</td>
<td>estrogen receptor beta</td>
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<td>estrogen receptor beta knock out</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GnRH</td>
<td>gonadotropin releasing hormone</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>GPR30</td>
<td>G-protein coupled receptor 30</td>
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<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid response element</td>
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<td>GRα</td>
<td>glucocorticoid receptor alpha</td>
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<td>glucocorticoid receptor beta</td>
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<tr>
<td>HPA</td>
<td>hypothalamus-adrenal-pituatary (axis)</td>
</tr>
<tr>
<td>HSP90</td>
<td>heat shock protein 90</td>
</tr>
<tr>
<td>ICI</td>
<td>ICI 182,780</td>
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<tr>
<td>iGR</td>
<td>intracellular glucocorticoid receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>LH</td>
<td>lateral nucleus of the hypothalamus</td>
</tr>
<tr>
<td>MAGUKS</td>
<td>membrane-activated guanylate cyclases</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>mER</td>
<td>membrane estrogen receptor</td>
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<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
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<tr>
<td>mGR</td>
<td>membrane glucocorticoid receptor</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NR</td>
<td>nuclear receptor</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B/Akt</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PSD</td>
<td>post synaptic density</td>
</tr>
<tr>
<td>PSD-95</td>
<td>post-synaptic density protein 95</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>SON</td>
<td>supra optic nucleus</td>
</tr>
<tr>
<td>SPM</td>
<td>synaptic plasma membrane fractions</td>
</tr>
<tr>
<td>SR</td>
<td>steroid receptor</td>
</tr>
<tr>
<td>vlVMH</td>
<td>ventrolateral division of the ventromedial nucleus of the hypothalamus</td>
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</table>
VMH    ventromedial nucleus of the hypothalamus
VSMC  vascular smooth muscle cells
CHAPTER 1. BACKGROUND AND SIGNIFICANCE

1.1 Introduction

Estrogens and glucocorticoids (GC) are steroid hormones capable of initiating both genomic and non-genomic signaling (Ayroldi et al., 2012; Björnström and Sjöberg, 2005). The estrogen receptors (ER) and glucocorticoid receptors (GR), both members of the same nuclear receptor subfamily, consist of a conserved DNA-binding domain, multiple transactivation domains, and a C-terminal ligand-binding domain (Aranda and Pascual, 2001; Huang et al., 2010). The classic nuclear signaling pathways, through which ERα, ERβ, and GR act as ligand-activated transcription factors, involve receptor dimerization and nuclear localization, followed by receptor binding to cognate enhancer elements such as the estrogen response element (ERE) (Nilsson et al., 2001a), and glucocorticoid response element (GRE) (Almawi and Melemedjian, 2002), located in gene-specific promoter regions. Transcriptional modulation of these genes accounts for many of the long-term effects of estrogens and glucocorticoids, but cannot explain the numerous effects reported to occur within minutes of hormone exposure (Falkenstein et al., 2000). Examination of the specific cellular responses to these hormones at both rapid and chronic time-points has demonstrated a complex system of kinase pathway activation and regulation of genomic effects (Hall et al., 2001). Some of these genomic effects may change cellular responses to the same hormone in the future, through
increased receptor expression (Zárate et al., 2012), or by altering cellular structure (Mukai et al., 2007). Estrogen and glucocorticoids have both been demonstrated to induce dendritic spine changes in neurons through activation of similar kinase pathways (Kramár et al., 2009; Yang et al., 2013b). Additionally, estrogen- and glucocorticoid-initiated pathways can influence each other, and are both necessary for certain behavioral responses, including territorial aggression (Mikics et al., 2004; Ogawa et al., 1997). However, the nature of the interaction between ER and GR signaling is not known, and how the rapid and chronic effects of ER and GR activation may depend on each other has not yet been investigated.

These non-classical effects, which we define to include all estrogen and glucocorticoid effects not directly mediated via receptor binding to their specific hormone response element to modulate transcription, can have long-lasting consequences for gene expression, cell morphology, and behavioral response (McDevitt et al., 2008). The kinase pathways that are required for rapid hormone signaling are traditionally activated by membrane-associated receptors, so the terms rapid signaling and membrane-initiated signaling are often used interchangeably to refer to any effects not due to classic transcription factor activity of the nuclear receptors (Song and Santen, 2006). However, it is important to note that there can be transcriptional consequences of activating these kinase pathways that, while not rapid, are also not products of classical ER (McDevitt et al., 2008) or GR (Komatsuzaki and Murakami, 2005) signaling. Therefore, the net effects of estrogen and glucocorticoids on a cell are due to the convergence of both classical and non-classical signaling (Roepke et al., 2009), and can
be very different depending on sex (Kuo et al., 2010), cell type (Pujols et al., 2002), stage of development (McCarthy, 2008), or part of the cell cycle (Hsu et al., 1992; Matthews et al., 2011). The studies in this dissertation focus on the effects of ER and GR activation on dendritic spines in hypothalamic neurons, and also examine how ER and GR signaling interactions may influence male territorial behavior.

1.2 Aggression

Aggression as agonistic behavior has been defined as “adaptations for situations involving physical conflict or contests between members of the same species”, and is necessary for survival in the wild (Scott, 1966). Understanding pathological aggression first requires identification of the circuitry and molecular mechanisms involved in adaptive, non-pathological aggression, which is currently under investigation in animal models (Anderson, 2012). Since both estrogen (Trainor et al., 2008) and glucocorticoids (Mikics et al., 2004) contribute to aggression in rodents, and because both ERs and GR are expressed in the hypothalamus, this model is ideal for investigating the interactions between these two signaling pathways. The mouse model is ideal because males reliably defend their territory against male intruders, and the availability of genetically modified mice allows for investigation and manipulation of molecular pathways (Natarajan et al., 2009).
1.3 Resident-Intruder Paradigm

The resident-intruder paradigm is an animal model of aggressive behavior based on non-pathological territorial defense by male mice (Koolhaas and Bohus, 1989). This territorial behavior is traditionally observed in males, while females will not display similar aggression unless pregnant or lactating (Albert et al., 1992). The resident-intruder test can be used to measure offensive aggression, violence, defensive behavior, and social stress, depending on the species and strain used, conditions, and timing of the experiment (Koolhaas et al., 2013). To test offensive aggression, the adult resident male mouse must be allowed to establish his cage as his territory by single-housing for a period of at least one week (Bartolomucci et al., 2009; Koolhaas et al., 2013; Teskey and Kavaliers, 1987). The intruder mouse, also male, is group-housed to be less aggressive, of equal or smaller size compared to the resident, but at least 2 months old (Bartolomucci et al., 2009), and has not had a prior interaction with the resident (Koolhaas et al., 2013). Ideally, when the intruder mouse is placed in the resident cage, the resident mouse attacks and wins the social interaction (Koolhaas and Bohus, 1989), which is generally allowed last for between 10 and 15 minutes (Bartolomucci et al., 2009).

Resident-intruder interactions can be scored and evaluated for offensive aggressive behaviors, which include individual acts such as biting, kicking, chasing, dominance grooming, sex, boxing, tail rattling, wrestling, as well as latency to aggressive bout, number of bouts, and total duration of bouts (Koolhaas et al., 2013; Miczek and O’Donnell, 1978). These measures of aggression represent normal, appropriate behavior.
in response to a threat, therefore, the resident intruder paradigm is not a suitable model of pathological aggression. Despite these limitations, the resident intruder paradigm is the most accurate animal model of male aggression available, and it is reasonable to predict human responses to neuroendocrine changes based on the results of these tests (Koolhaas and Bohus, 1989).

1.4 Estrogen Receptor

Both of the classic ERs, ERα and estrogen receptor beta (ERβ) (Li et al., 1997), are found in neurons, and, traditionally, the transcriptional activities of these nuclear receptors have been used to explain the multiple effects of estrogen on neurons (White et al., 1987). ERα and ERβ are coded for by separate genes (Matthews and Gustafsson, 2003). ERα is the 66 kDa protein product of ESR1, located on chromosome 6q25.1a, and consists of 595 amino acids (Ascenzi et al., 2006). ERβ was first detected in peripheral blood lymphocytes (Mosselman et al., 1996), but is highly expressed in the prostate, ovary, uterus, bladder, and lung (Kuiper et al., 1998). ERα and ERβ share some homology in their ligand-binding (55% amino acid homology) and have a high degree of similarity in their DNA-binding (95% amino acid homology) domains, but the N-terminal domain of ERβ is shorter than that of ERα with a sequence homology of only 15% (Kumar et al., 2011). Functionally, these structural differences result in decreased transcriptional activation of the ERE by ERβ, and increased affinity for phytoestrogens as compared to ERα (Le Romancer et al., 2011). Distribution of ERα and ERβ in the brain varies by sex and developmental stage (Canonaco et al., 2008). ERβ expression in the
brain is highest in the hypothalamus and amygdala, regions in which ERα is also expressed (Kuiper et al., 1998). The ventromedial nucleus of the hypothalamus (VMH) predominantly expresses ERα, although a low level of ERβ has been detected by immunohistochemistry in the ventrolateral VMH (vlVMH) (Mitra et al., 2003).

1.5 Glucocorticoid Receptor

The GR belongs to the same nuclear receptor sub-family as ER (Aranda and Pascual, 2001), and shares a similar structural and functional domains (Huang et al., 2010). Unliganded GR exists as a complex with heat-shock protein 90 (HSP90), p23, and a tetratricopeptide protein (Heitzer et al., 2007). The function of this chaperone complex is to prevent DNA binding of the unliganded GR and maintain the proper conformation of the receptor for ligand (Bresnick et al., 1989). GR exists in two isoforms, alpha (GRα) and beta (GRβ). Alternative splicing causes the production of GRβ, but it is generated at much lower frequencies in the brain than GRα (Pujols et al., 2002). GRβ lacks the last 50 amino acids of the GRα carboxy terminus, and instead terminates in a unique 15 amino acid sequence (Pujols et al., 2002). GRβ neither binds glucocorticoids nor has intrinsic transcriptional activity, but has been implicated as a dominant negative inhibitor of GRα activity through formation of a non-functional heterodimer (Oakley et al., 1999). Higher concentrations of GRβ relative to GRα, therefore, result in decreased glucocorticoid sensitivity. However, levels of GRβ protein in the brain are extremely low (Pujols et al., 2002), so the focus of these experiments is exclusively on the activity of GRα.
1.6 Classical Estrogen Signaling

For the purpose of these studies, classical estrogen signaling refers to the genomic effects of ERα or ERβ activation and subsequent transcriptional modulation due to receptor binding to the ERE (McDevitt et al., 2008). 17β-estradiol (17β-E) is the most abundant endogenous estrogen (Dötsch et al., 2001), and is commonly used experimentally to activate all known ERs. Upon ligand binding, both ERα and ERβ dimerize and bind to the ERE, which is located in the promotor region of a diverse pool of genes. ERα has a stronger effect on ERE transcriptional activation than ERβ, although both isoforms of the receptor are capable of initiating transcription in the presence of 17β-E (Hall and McDonnell, 1999). The relative ratios of ERα and ERβ within a cell are also important in determining the cellular response to 17β-E; at low concentrations of 17β-E ERβ functions as a repressor of ERα-mediated ERE transcriptional activation, through the formation of heterodimers that have altered transcription factor function (Hall and McDonnell, 1999).

The specific transcriptional effects of 17β-E-induced protein expression in neurons include up-regulation of syntaxin, spinophilin, and synaptophysin, all of which are important in spine synapse formation (McEwen, 2002). Additional genes for proteins necessary for actin remodeling and spinogenesis, including Cdc42, RhoA, cofilin, and PSD-95 are also upregulated in the brain in response to estrogen (Bethea and Reddy, 2010; Rivera and Bethea, 2012a).
1.7 Classical Glucocorticoid Signaling

Classic GR signaling through transcriptional effects is mediated through ligand binding of the GRE (Stahn et al., 2007). Transactivation of genes for glucose and fat metabolism and transrepression of pro-inflammatory genes result in the metabolic and anti-inflammatory effects of glucocorticoids (Clark, 2007). The synthetic glucocorticoid dexamethasone (Dex) is often used in research because of its increased selectivity for GR over mineralocorticoid receptor, compared to the endogenous GR ligands cortisol and corticosterone (CORT) (Lan et al., 1982). Nuclear localization is the first step required following ligand binding for transcriptional effects of GR; translocation from the cytosol to the nucleus occurs within a few minutes of ligand binding (Savory et al., 1999). However, although unliganded GR is transcriptionally inactive via the GRE due to binding with HSP90 as part of a chaperone complex, an equilibrium between cytosolic and nuclear GR exists in the absence of ligand (Hache et al., 1999). The function of unliganded, and presumably inactive, GR nuclear localization is not entirely understood.

1.8 Nuclear Localization of GR

Nuclear localization of GR traditionally occurs following ligand binding, which exposes a nuclear localization signal (NLS-1) that depends on lysines 513 to 515; deletion of these residues results in cytoplasmic localization of GR when a second NLS, located in the C-terminus (NLS-2) is also deleted (Savory et al., 1999). Nuclear localization is mediated differently by NLS-1 and NLS-2; NLS-1 induced nuclear uptake
occurs quickly, within 10 minutes of Dex treatment. However, NLS-2 mediated GR nuclear localization is much slower, requiring at least 30 minutes of Dex treatment, and up to 2 hours for the maximal effect (Savory et al., 1999). However, even in its unliganded state, GR exists in both the cytoplasm and the nucleus in a state of equilibrium (Hache et al., 1999). Nuclear trafficking is cell cycle-dependent; GR is excluded from the nucleus during mitosis, but accumulates in the nucleus during interphase in transfected HeLa cells (Matthews et al., 2011). Physiological conditions, including elevated pH, salt, heat shock, and laminar shear stress can also induce unliganded nuclear localization (Bresnick et al., 1989; Ji et al., 2003; Sanchez, 1992). The GRα isoform GRα-D associates with GRE-containing promoters even in the absence of ligand, so can be found in the nucleus of glucocorticoid-free cells (Oakley and Cidlowski, 2013), and is expressed in neurons at varying levels at different developmental stages (Sinclair et al., 2011). The cellular effects of unliganded GR in the nucleus are not yet fully understood, but there is evidence from a mouse mammary cell line that unliganded GR is capable of both negatively and positively regulating gene transcription (Ritter and Mueller, 2014).

1.9 Non-Classical Estrogen Signaling

Many effects of 17β-E, including kinase pathway activation, cannot be accounted for by the transcriptional outputs of the classic pathway (Chamniansawat and Chongthammakun, 2010). It is possible that classic ERα and ERβ have functions in addition to being ligand-activated transcription factors (Meitzen and Mermelstein,
2011), or there may be alternate forms of the classic ERs that function non-genomically (Jia et al., 2011). Many of the non-classical effects of estrogen occur too rapidly to involve gene transcription, but instead activate kinase signaling pathways (Abrahám et al., 2004). These estrogen-activated kinase pathways are traditionally initiated at the plasma membrane (Wu et al., 2011), through transmembrane receptors such as receptor tyrosine kinases, or through plasma membrane associated G-proteins.

The first reported rapid effect of $17\beta$-E was an increase in uterine 3′-5′-cyclic adenosine monophosphate (cAMP), which occurred within 15 minutes of $17\beta$-E administration to ovariectomized mice (Szego and Davis, 1967), a time-frame which excludes the participation of gene transcription. More recently, rapid effects of estrogen have been demonstrated in the central nervous system (CNS), and include modulation of kinase pathways, electrophysiological changes, dendrite morphological changes, and behavioral changes. In mature rat cortical neurons, $17\beta$-E rapidly increased ERK, which transiently increased dendritic spine density (Srivastava et al., 2008) within 30 minutes. In rat hippocampal neurons, phospho-cAMP binding protein (pCREB) increased within 1 hour of $17\beta$-E addition, an effect which was blocked by inhibitors to both calmodulin kinase II (CamKII) and ERK pathways (Segal and Murphy, 1998). In the hypothalamus, $17\beta$-E was shown to rapidly depolarize pro-opiomelanocortin (POMC) neurons by activating the protein kinase B (PKB/Akt), extracellular regulated kinase (ERK/MAPK), protein kinase A (PKA) and protein kinase C (PKC) pathways (Kelly and Rønnekleiv, 2008; Malyala et al., 2005). However, the details of how ER activation act on these kinase pathways is unknown. Rapid estrogen effects
on behavior have also been reported, and estrogen is necessary for both male territorial aggression (Clark et al., 2013) and sex behavior (Pfaff et al., 2006). Non-classical effects of estrogen contribute to both of these behaviors, although the molecular mechanisms involved remain unknown.

1.10 Non-Classical Glucocorticoid Signaling

Glucocorticoids are also capable of rapid signaling in the CNS, and there is some degree of overlap between the rapid effects of estrogen signaling and of GC signaling, including spinogenesis and male territorial aggression. Similar to estrogen, glucocorticoids can rapidly modulate a multitude of kinase pathways in neurons, including PKC, PKA, and Akt (Yang et al., 2013b). ERK 1/2 phosphorylation response to GR activation occurs within 1 hour in an anterior pituitary cell line (Revest et al., 2005). In the hippocampus, GR has been detected in the synaptosomal fraction, and inhibition of GR with RU-486 results in the reduction of Akt and ERK phosphorylation within 30 minutes (Chen et al., 2012). Additionally, synaptosomal localization of GR suggests GR plays a role in local signaling at the synapse. GC exposure in the hypothalamus results in suppression of excitatory input to corticotropin releasing homone (CRH) expressing neurons via a post-synaptic release of endocannabinoids, which act as retrograde messengers to glutamatergic pre-synaptic neurons; this same effect is observed with exposure to the membrane-limited dexamethasone-bovine serum albumin conjugate (DexBSA) (Di et al., 2003).
1.11 Membrane Estrogen Receptors

In the CNS, both $^3$H-labeled 17β-E (Towle and Sze, 1983) and a $^{125}$I-labeled membrane-limited conjugate where 17β-E is attached to bovine serum albumin (E$_2$BSA) (Zheng et al., 1996) showed relatively high affinity binding to rat plasma membranes, suggesting the presence of a membrane ER (mER). The identity of the mER is not known, although there are several possible candidates. There may be multiple membrane-associated receptors capable of binding estrogens, expression of which may be cell-type dependent (Gorosito et al., 2008). A mER is defined as any plasma membrane-associated receptor capable of binding estrogens. Traditionally, work has focused on 17β-E binding, since it is the most prevalent endogenous estrogen, although many other estrogens, including 17α-estradiol, have documented cellular effects in the CNS (Pietranera et al., 2014). Candidates for the mER include the G-protein coupled receptor GPR (Filardo et al., 2000), a membrane associated 63 kDa protein ER-X (Toran-Allerand et al., 2002), post-translationally modified ERα and ERβ (Byrne et al., 2012; Li et al., 2003; Meitzen et al., 2013; Wu et al., 2011), or an as yet unidentified receptor.

1.11.1 Novel Membrane Estrogen Receptors

The novel estrogen receptor X (ERX), a 63 kDa receptor that can be detected with antibodies raised against ERα and ERβ, that is found in the neocortical membrane fraction, and mediates ERK signaling in response to both 17β-E and 17α-estradiol (Toran-Allerand et al., 2002). Although the structure and origin of ER-X are unknown, it
is expressed in ERα knockout mice, and shares homology with the ERα ligand-binding domain (Toran-Allerand et al., 2002).

A second potential source for some or all of the non-classic effects of estrogen is the G-protein coupled receptor 30 (GPR30, also known as GPER1), a former orphan GPCR that was shown to bind 17β-E in a breast cancer cell line, SKBR3, and increase cAMP cells via an increase in adenylyl cyclase activity (Thomas et al., 2005). Cell fractionation and immunocytochemistry revealed endogenous GPR30 to be localized at the plasma membrane in both the SKBR3 (GPER1+, ERα+, ERβ-) breast cancer cell line, and in transfected HEK cells (Filardo et al., 2007). GPR30 is also expressed throughout the brain, including the hippocampus and hypothalamus (Canonaco et al., 2008).

The subcellular localization of GPR30 has been somewhat controversial, and the receptor has been detected in both the plasma membrane (Funakoshi et al., 2006) and the endoplasmic reticulum (Revankar et al., 2005). In the hippocampus, GPR30 was localized to the post-synaptic density of dendritic spines, and associates with PSD-95 through its C-terminal tail (Akama et al., 2013). This association with PSD-95 provides an anchor for GPR30 to the plasma membrane, independent of 17β-E, although in other cells, including transfected HeLa cells, 17β-E can induce membrane localization (Funakoshi et al., 2006). Immuno-electron microscopy analysis of rat hippocampi also revealed exclusive membrane localization (Funakoshi et al., 2006). However, GPR30 can be detected intracellularly in normal mammary gland epithelial cells (Cheng et al., 2011), overexpressed GPR30 in COS7 cells exhibits localization at the endoplasmic reticulum and the Golgi (Revankar et al., 2005), and GPR30 has been shown to localize
to the Golgi apparatus and endoplasmic reticulum in primary hippocampal cultures (Matsuda et al., 2008), suggesting that cell type may determine localization.

Additionally, inclusion of molecular tags may interfere with normal intracellular trafficking of the receptor, which further confounds the localization issue; HeLa cells transfected with Flag-GPR30 show mostly staining at the membrane, while cells transfected with GFP-GPR30 show staining mainly in the endoplasmic reticulum (Funakoshi et al., 2006). In the rat hypothalamus, endogenous GPR30 expression, as tested by immunohistochemistry, is particularly high in the PVN and SON with lower expression in the VMH (Brailoiu et al., 2007), although subcellular localization within these nuclei has not been demonstrated at this time.

GPR30 can signal via both the Gαs and the Gβγ subunits; the Gβγ subunit transactivated the epidermal growth factor receptor (EGFR) leading to downstream activation ERK protein, while simultaneous activation of the Gαs subunit by 17β-E inactivated ERK signaling through activation of adenylyl cyclase and PKA, therefore allowing a short period of cAMP signaling (Filardo, 2002). Mechanisms to limit the timeframe of the effects of cAMP signaling downstream of the GPR30 have been identified as well. GPR30 could also decrease cAMP that was elevated by heterologous ligands, such as Forskolin, through association with membrane-activated guanylate cyclases (MAGUKs) via a C-terminal PDZ domain (Broselid et al., 2014). The MAGUKs act as adaptors for AKAP5 (protein kinase A anchoring protein), which in turn decreased adenylate cyclase activity (Broselid et al., 2014). Furthermore, although GPR30 protein was readily detectable in both the microsomal and plasma membrane subcellular
fractions of breast cancer cell lines, only GPR30 in the plasma membrane fraction bound ligand and activated G-protein signaling, suggesting that only membrane associated GPR30 protein is functional (Filardo et al., 2007). However, GPR30 activation in COS cells initiated intracellular calcium mobilization and nuclear accumulation of PIP3 via EGFR transactivation (Revankar et al., 2005) via a non-membrane initiated signaling mechanism, since calcium flux was not replicated by membrane restricted estradiol derivatives (Revankar et al., 2007).

1.11.2 Membrane ERα

Although there are many candidates for the receptors involved in non-classical ER activation, some of these effects are also dependent on the classic receptors themselves. Many studies have used ICI 182,780 (ICI), a specific ERα and ERβ antagonist (Wakeling and Bowler, 1992), to pharmacologically inhibit kinase activity in response to estrogen exposure; however, ICI also activates GPR30, which may confound some of the earlier results reported with the drug (Filardo et al., 2000). Fortunately, the availability of ERα and ERβ knockout animals, ERαKO and ERβKO, provide a better model to establish the ER dependence of 17β-E-mediated non-genomic signaling, particularly in light of an absence of a specific antibody to the ERβ (Snyder et al., 2010). For example, in female mice a subcutaneous administration of 17β-E caused the ERα- or ERβ-dependent phosphorylation of CREB and ERK within an hour in specific brain regions, including the VMH (Abrahám et al., 2004). A complex brain nuclei-specific and isoform-specific ER dependence was revealed, which may in part be due to the tissue
distribution of the ER isoforms in the brain. In the vlVMH the predominant ER isoform is
the ERα, and CREB phosphorylation requires ERα, but not ERβ (Abrahám et al., 2004). In
the mPOA, either ERα or ERβ can induce pCREB but both act in conjunction to induce
pERK (Abrahám et al., 2004), demonstrating that ER isoforms can regulate rapid
signaling differentially in the same tissue. The regulation in male mice is not currently
known, but the relative ratio of ER isoforms, along with expression of ER variants,
GPR30, and other estrogen responsive receptors all influence the net effect of estrogen
exposure.

The idea that a pool of ERα exists on the plasma membrane has been tested in
multiple studies using 17β-E binding, electron microscopy, immunocytochemistry and
Western blotting have examined the idea that a small percentage (3-5%) of the total
pool of the classical ERα is present on the plasma membrane (Levin, 2009). Consistent
with this idea, immunocytochemistry using minimal fixation in both the breast cancer
MCF-7 cells and the anterior pituitary GH3 cell line, revealed ERα at the membrane in
caveolae in some, but not all cells (Watson et al., 2012). MCF-7 cells with ERα at the
plasma membrane showed increased phospho-ERK (pERK) within 10 minutes of
application of either 17β-E or a membrane limited E2-peroxidase conjugate (Zivadinovic
and Watson, 2005). There is also evidence for mERα-mediated rapid effects of estrogen
in the CNS. ERα was detected in axon terminals, dendritic spines as well as in
astrocytes in the CA1 of proestrus female rats using immunoelectron microscopy
(Milner et al., 2001). In the dorsal CA1 from the female rat, ERα was present in synaptic
vesicles in the axon of some GABAergic basket cells; 17β-E moved these ERα-containing
vesicles towards synapses within 24 hours (Hart et al., 2007). In addition, the majority of this extra-nuclear ERα at synaptosomes and vesicles was phosphorylated though the function of phosphorylation in localization of the ERα remains unknown (Tabatadze et al., 2013). In the guinea pig hypothalamus, ERα was localized to the dendrites and axon terminals (Blaustein et al., 1992). In hypothalamic neurons and astrocytes obtained from both male and female rats, a full-length 66 kDa form of the ERα and a 52 kDa variant has been detected using surface biotinylation and immunocytochemistry (Bondar et al., 2009; Dominguez and Micevych, 2010).

Hydropathicity analysis shows that ERα may have a potential transmembrane domain (Bondar et al., 2009), but the concept that ERα requires a membrane protein to tether it to the plasma membrane is supported by the discovery of caveolin proteins that can anchor the ERα in lipid rafts and caveolae. Caveolin proteins are highly conserved structural proteins that are necessary and sufficient for the existence of caveolae, a subset of lipid rafts (Patel and Insel, 2009), that comprise a restricted compartment for signaling molecules associated with the plasma membrane. Initially, ERα was reported to interact with caveolin 1 (Cav-1) and 2 (Cav-2) in MCF-7 and vascular smooth muscle cells (VSMC) (Razandi et al., 2002), which couple ERα selectively to either other proteins such as the metabotropic glutamate receptors (mGluR) or to processes such as palmitoylation (Meitzen et al., 2013). Caveolins provide a link between ERα and the kinase pathways downstream of mGluRs that may explain how estrogen can influence kinase pathways.
The specific association of ERα with different isoforms of caveolin may determine which mGluR ERα associates with, and, therefore, control the kinase pathways estradiol activates or inactivates. In the hippocampus of the female rat, Cav-1 interacted with mGluR1a so that 17β-E activated downstream Gαq and ERK signaling, which increased CREB activation within 5 minutes of 17β-E application (Boulware et al., 2005). However, Cav-3 tethered ERα in hippocampal neurons to mGluR2/3, which activated Gαi signaling, leading to the inhibition of PKA and the subsequent down-regulation of CREB (Boulware et al., 2007). In hypothalamic astrocytes, the coupling of ERα to mGluR1a resulted in an increase of calcium within 2 minutes of 17β-E application; this was blocked both by the ERα and ERβ antagonist, ICI, and by the mGluR1a antagonist LY 367385 (Kuo et al., 2009), suggesting that ERα or ERβ, along with mGluR1a are necessary for the rapid calcium effect of estrogen. In striatal neurons, Cav-1 facilitated the tethering of ERα to mGluR5 and subsequent Gαq signaling (Meitzen and Mermelstein, 2011). The physiological conditions wherein ERα binds to Cav-1 versus Cav-3 are unknown. The coupling of ERα to mGluRs may also allow downstream signaling to be modulated by sensitivity to two ligands: 17β-E activation of ER, and glutamate activation of mGluR. This is supported by the fact that in hypothalamic astrocytes, glutamate and 17β-E combined elicited a greater calcium increase than either ligand alone (Kuo et al., 2009).

In addition to the role that mGluRs play in linking ERα to Gα proteins, ERα can also directly bind Gα subunits. 17β-E inhibited cAMP production within 5 minutes of addition to GT-1 immortalized GnRH neurons via ERα that is physically tethered to Gαi.
ERα has been demonstrated to tether to Gαi in the caveolae of endothelial cells to increase the activity of nitric oxide synthase (Chambliss and Shaul, 2002), although this mode of regulation of nitric oxide synthase by 17β-E has not been shown in the CNS.

Apart from serving as adaptors, the binding of caveolins to the ER also increases palmitoylation of the ER, a process by which palmitate, a C16 fatty acid is added to an internal cysteine via a thioester bond (Acconcia et al., 2004). Palmitate can associate and insert in the plasma membrane, providing an anchor for ERα. In the human ERα, a canonical palmitoylation site exists at C447; the corresponding site in the mouse ERα is C451 (Adlanmerini et al., 2014) and in rat ERα is C452 (Meitzen et al., 2013). Mutant ERα where the C447 site was mutated to alanine (C447A) did not bind Cav-1, was not localized at the membrane in HeLa cells, and showed decreased pERK activation in response to a 10-minute application of 17β-E (Acconcia et al., 2005), all of which suggest that the palmitoylation site is necessary for an estrogen-mediated rapid effect. In rat hippocampal neuron primary cultures, ERα C452A transfection eliminated CREB activation following 17βE or PPT, a selective ERα agonist, administration (Meitzen et al., 2013), providing evidence that palmitoylation is necessary for mERα effects in neurons as well. When a second palmitoylation site, S522, in the ERα was mutated, localization at the plasma membrane and Cav-1 binding to ERα was also decreased in Chinese Hamster Ovary (CHO) cells (Razandi et al., 2003). A similar effect was observed in rat hippocampal neurons, in which the ERα S522 mutant reduced the ability of 17β-E to activate CREB in (Boulware et al., 2005).
Recently, E₂BSA has been shown to transcriptionally upregulate Cav-1 via a PI3K and ERK pathway within 12 hours in endothelial cells (Tan et al., 2012). This suggests that 17β-E may increase non-classical signaling both by increasing the palmitoylation of ERα within rapid time frames, and via a longer time frame of transcriptional upregulation of Cav-1, both of which increase the amount of mERα available at the plasma membrane to participate in signaling. In hypothalamic astrocytes and neurons, 17β-E increased the amount of both the 52 kDa and 66 kDa mER in the cell membrane within 30 minutes (Bondar et al., 2009; Micevych and Dominguez, 2009). ICI 182,780 or mGluR1a antagonism blocked the increase of mERα, but BSA-conjugated estradiol treatment had no effect on the mERα pool in hypothalamic astrocytes, suggesting cytoplasmic ERα is necessary for rapid trafficking of mERα (Bondar et al., 2009). This increase in mERα may provide a mechanism for potentiation of future membrane-initiated non-classical estrogen effects.

However, in other cell types, estrogen treatment can have the opposite effect, and results in a decrease in mERα. In MCF-7 cells, 17β-E decreased Cav-1 and Cav-2 within 8 hours, thus decreasing the amount of mERα (Razandi et al., 2002). Similarly, 17β-E treatment of hippocampal slices from female rats reduced ERα concentrations in the synaptosomal plasma membrane by depalmitoylation within 20 minutes (Tabatadze et al., 2013). In CHO cells, palmitate incorporation into ERα and association with Cav-1 decreased within 60 minutes in response to 17β-E, suggesting that ERα at the plasma membrane was lowered; consistent with this idea, ERK activation also decreased
(Acconcia et al., 2005). Decreasing ERα at the membrane in these cells may allow for a decrease in non-classical signaling and may mark a shift towards classical signaling.

1.11.3 ERα Variants

One possibility for the source of non-classic estrogen signaling is through variants of the ERα that are able to activate kinase pathways or regulate transcription without ERE involvement. Although the majority of ERα variants have been detected in peripheral tissues, a 52 kDa variant of ERα (ERα-52), detected with a C-terminal antibody that also detects the full-length 66 kDa, was detected in hypothalamic neurons (Domínguez and Micevych, 2010). RT-PCR analysis using a mouse hypothalamic cell line indicated that this receptor is an ERα variant that lacks exon 4 (ERαΔ4) (Domínguez et al., 2013).

Although ERα-52 has also been detected in hypothalamic astrocyte membranes (Bondar et al., 2009), signaling properties and function of the 52 kDa variant remain unknown. A 36 kDa variant lacking the AF-1 and AF-2 domains with a unique C-terminus was detected in human breast cancer cells, coded for by a unique promoter located in intron 1 of the Esr1 gene (Wang et al., 2006) and can be detected by a specific antibody. ERα-36 was localized to the plasma membrane, mediated rapid ERK signaling and is induced by the selective activation of GPR30 with its specific agonist, G-1; however, G-1 has an almost equal affinity for ERα-36, suggesting that some of the responses attributed to the GPR30 might be through ERα-36 (Kang et al., 2010).
A 46 kDa variant observed in endothelial cells is also localized to the plasma membrane (Li et al., 2003) and is composed of a splice variant missing the first 173 amino acids of ERα-66 (Flouriot et al., 2000). ERα-46 cannot be detected with an N-terminus antibody raised to ERα-66, but should be identifiable with a C-terminus ERα antibody. So far, neither ERα-36 nor ERα-46 has been demonstrated in the CNS, though it is possible that some antibodies directed towards the DNA-binding domain or, in the case of the 46 kDa variant, the C-terminal domain of the full-length 66 kDa ERα may detect these forms in studies that use solely immunocytochemistry.

### 1.12 Membrane Glucocorticoid Receptors

Although the identity of the membrane GR (mGR) is controversial, there have been recorded cellular responses to membrane-limited glucocorticoids that suggest, regardless of identity, that a functional mGR does exist (Desquiret et al., 2008; Nogami et al., 2014; Oppong et al., 2014). Similar techniques have demonstrated mGR in CD14+ monocytes has different effects on kinase pathway activation when selectively activated as compared to general GR activation (Strehl et al., 2011). In lung epithelial cells Akt is rapidly activated in response to glucocorticoids, an effect that is dependent on both lipid rafts and cav-1, suggesting mGR involvement (Matthews et al., 2008). In immune cells, p38 MAPK is rapidly activated with DexBSA treatment (Strehl et al., 2011).

These rapid effects of glucocorticoids also occur in the CNS; JNK and p38 MAPK was activated within 10 minutes of glucocorticoid administration in primary hippocampal neurons (Qi et al., 2005). Rapid actions in response to glucocorticoids in
the hypothalamus are also attributed to the mGR, and include endocannabinoid production (Di et al., 2005) and inhibition of glutamate release, an effect that was maintained even with the membrane-limited glucocorticoid DexBSA (Di et al., 2003). Extracts from rat hippocampal synaptoneurosomes show a reduction in Akt and ERK phosphorylation within 30 minutes in response to GR inhibition with RU486 (Chen et al., 2012).

G-protein coupled receptors (GPCRs) are reportedly activated following membrane-limited glucocorticoid treatment, and are responsible for the rapid effects in rat hippocampal primary culture, including Akt, PKA, and PKC activation, and clustering of PSD-95 (Yang et al., 2013b). The release of the CB1 receptor ligand, endocannabinoids by the CRH neuron was dependent on Goα-driven PKA activation in the CRH neuron (Di et al., 2009). However, the release of nitric oxide (NO) that increased GABAergic inhibition (Di et al., 2005) onto the CRH neuron was dependent on Gβγ signaling (Di et al., 2009). GPCR dependent mGR signaling has also been demonstrated in mouse pituitary cells (Maier et al., 2005), and electrophysiology performed on rat hypothalamic slices provides further evidence for GPCR involvement in mGR signaling (Di et al., 2005; Tasker et al., 2005).

1.12.1 Classic GRα as the Membrane GR

Similar to the ERα being considered as a possible mER, the intracellular GR (iGR) that exists as a complex with HSP90, p23, and a tetratricopeptide protein (Heitzer et al., 2007) in the cytoplasm has been proposed as a candidate mGR. The presence of mGR
was detected at very low levels in human lymphocytes and leukocytes using membrane-
impermeable fluorescent liposomes and GR-specific antibodies (Bartholome et al.,
2004). The use of stably transfected siRNA to decrease mGRα protein expression in
CD14⁺ monocytes suggested that both mGR and iGR were derived from the same
transcript (Strehl et al., 2011). However, while plasma membrane association of other
steroid hormone receptors, including ER, is dependent on palmitoylation, mutation of
the homologous sequence in GR did not affect membrane localization (Samarasinghe et
al., 2011), suggesting that other mechanisms must tether mGR to the plasma
membrane. One candidate for anchoring GR to the membrane is through an association
with Cav-1, which has been demonstrated in MCF-7 cells (Vernocci et al., 2013).

Synaptosomal fractions from rat hippocampus contain plasma membrane
associated GR (Komatsuzaki and Murakami, 2005), and GR immunoreactivity has been
observed at the plasma membranes and vesicle membranes of both the hippocampus
and hypothalamus (Liposits and Bohn, 1993). Membrane glucocorticoid receptors were
first observed in the synaptic plasma membrane fractions (SPM) of rat brain via [3H]-
CORT binding assays (Towle and Sze, 1983). Although hypothalamic SPM displayed the
higher binding capacity for glucocorticoids than hippocampal or cortical SPM, iGR
glucocorticoid binding is actually lowest in the hypothalamus, and much higher in
cerebral cortex and hippocampus (McEwen et al., 1976). Synaptosomal fractions from
rat hippocampus contained plasma membrane associated GR (Komatsuzaki and
Murakami, 2005), and GR immunoreactivity was observed at the plasma membranes
and vesicle membranes of the hypothalamus (Liposits and Bohn, 1993). Therefore, in
the PVN, the net rapid negative feedback by glucocorticoids on postsynaptic CRH
neurons is due to a combination of suppression of presynaptic glutamatergic neurons
and excitation of presynaptic GABAergic neurons.

1.13 Hypothalamic Locus of Aggression

Sex-behavior and male territorial aggression are behaviors mediated through
various hypothalamic nuclei, and the hypothalamus is a classically steroid-responsive
area of the brain that is critical for several estrogen-dependent behaviors (Pfaff et al.,
1994). The ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) in mice
has been identified as a specific locus of aggression, and VMHvl activity is required for
mouse aggression (Lin et al., 2011). Stimulation of these neurons can induce aggression,
even against an inanimate object, and optogenetic silencing of these same neurons
inhibits aggression (Lin et al., 2011).

Although circulating levels of 17β-E are very low in male rodents,
neuroestrogens exist in the mammalian male hypothalamus, and are the product of
local testosterone aromatization (Konkle and McCarthy, 2011). The concentration of
these neuroestrogens can be much higher than circulating hormone levels (McCarthy,
2009); because estrogen can be generated locally, the exposure of hypothalamic
neurons to spike in estrogen concentration may occur on a much shorter time-scale
than would otherwise be possible if circulating estrogens were the only endogenous
source of the hormone.
Glucocorticoid signaling in the hypothalamus has been well characterized as part of the Hypothalamus-Pituitary-Adrenal (HPA) axis (Groenink et al., 2002), and glucocorticoids are necessary for hypothalamic behaviors such as aggression (Mikics et al., 2004).

1.14 Role of Estrogens in Aggression

Castration in male rodents removes both testosterone and estrogens and results in loss of aggression in response to territorial intrusion (Nelson and Trainor, 2007). Though most of the studies investigate the role of testosterone in males (Gleason et al., 2009; Soma, 2006), testosterone can be converted to 17β-E by aromatase, which is present in the CNS (Naftolin et al., 1982). Deletion of aromatase in mice abolishes aggression in male mice in a resident-intruder paradigm, though this possibly was due to organizational defects since 17β-E reinstates aggression only when administered before postnatal day 7 (Toda et al., 2001). In California beach mice that were castrated and supplemented with testosterone and an aromatase inhibitor so that they possess testosterone but not estrogen, aggression increased rapidly within 15 minutes of injection with cyclodextrin-conjugated 17β-E (Trainor et al., 2008). This suggests that testosterone is not sufficient to elicit aggressive behavior in a resident-intruder paradigm and that 17β-E is necessary to activate aggression. The short time frame and the inability of cycloheximide to decrease aggression (Laredo et al., 2013) provide evidence that 17β-E acts non-genomically, though most studies in the rat and mouse
have used longer time frames that do not allow distinction between classical and non-classical estrogen signaling.

In rodents, aromatase is expressed in axon terminals throughout the brain (Horvath et al., 1997; Naftolin et al., 1996, 2001), including in the hypothalamus (Wu et al., 2009). In male birds neuroestrogens generated at axon terminals are important in learning in songbirds (Remage-Healey et al., 2009), and in sex behavior in the quail (Balthazart et al., 2004; Cornil et al., 2012). Local 17β-E concentrations in the male rat brain can be much higher than those in serum (Hojo et al., 2008). Together, these data suggest 17β-E generated at the synapse may function as a neurotransmitter in short time-frames (Saldanha et al., 2011). In addition, 17β-E can transcriptionally induce the aromatase gene via ERα-c-jun complex binding to AP-1 elements in the brain-specific promoter of aromatase I.f, expressed in specific hypothalamic neurons (Yilmaz et al., 2009). Therefore, 17β-E can regulate aromatase non-classically via rapid, non-genomic mechanisms, and classically, through transcriptional mechanisms in the male rodent. In CD-1 outbred male mice, ERα concentrations in several areas of the circuit involved in aggression such as the bed nucleus of the stria terminalis (BNST), lateral septum (LS) and anterior hypothalamus (AH) was higher in more aggressive mice (Trainor et al., 2006a). Reduction of ERα in the VMH by adenovirus mediated transfer of shRNA to ERα abolished aggressive behavior in male mice, demonstrating that ERα expression in the VMH is necessary for aggression in adult male mice (Sano et al., 2013).
1.15 Role of Glucocorticoids in Aggression

Though aggression itself is stressful and leads to secretion of glucocorticoids, dominance in an aggressive encounter typically leads to lowering of the CORT level (Bronson and Eleftheriou, 1965). Acute treatment with GR antagonists (Chang et al., 2012; Schjolden et al., 2009), or pharmacological prevention of glucocorticoid synthesis (Mikics et al., 2004), prevented aggression in male mice when presented with territorial challenge. Additionally, acute treatment with CORT before an encounter increased aggressiveness in male rats (Mikics et al., 2004), which suggests CORT is important in regulation the onset of aggressive behavior. Protein synthesis inhibition did not block these acute CORT effects, suggesting that non-genomic signaling by glucocorticoids in the CNS is most likely required to increase aggression (Mikics et al., 2004). Likewise, acute CORT injection also decreased the magnitude of electrical stimulation to the hypothalamus required to elicit attack (Kruk et al., 2004). Importantly, these effects of increased aggression represent normal, appropriate behavioral responses to territorial challenge, and CORT had no effect on aggression within established rat colonies (Mikics et al., 2007).

However, chronic elevated level of glucocorticoids, such as seen in stressed or socially defeated animals, decreased aggression and increased submissiveness in hamsters (Hayden-Hixson and Ferris, 1991a, 1991b), mice (Leshner et al., 1980), and rats (Blanchard et al., 1995, 2001). This is thought to be due a long-term effect due to genomic signaling by glucocorticoids, though the molecular mechanisms by which glucocorticoids facilitate or inhibit aggression are unclear (Haller, 2014).
1.16 Interactions between Estrogen and Glucocorticoid Signaling

Though 17β-E can rapidly increase aggression by modulating neurotransmitter release (Nelson and Trainor, 2007), it may also act through regulation of the HPA axis. In the white-crowned sparrow, neither brain 17β-E concentrations, nor aromatase activity in nuclei involved in aggression, were correlated with aggressive behavior, although CORT did increase rapidly (Charlier et al., 2011). Gonadal status can modify the actions of glucocorticoids on the hypothalamus or can regulate CORT levels itself. 17β-E chronically administered to gonadectomized and adrenalectomized female rats treated with high doses of CORT increased CRH mRNA levels, thus counteracting negative feedback, while DHT treatment to male rats had an opposing effect, and caused a further decrease in CRH levels (Patchev and Almeida, 1996). In female ovariectomized rats, intra-cerebral ventricular, but not systemic, 17β-E injection increased CORT within 30 minutes, an effect mimicked with the ERα selective agonist, PPT, injected into the PVN (Liu et al., 2012). In gonadectomized male rats, CRH mRNA was higher when they were treated with estradiol benzoate (EB) but lower when treated with DHT, implying that estrogens can regulate the HPA axis in males also (Lund et al., 2004). A number of mechanisms may be involved in the rapid increase of aggression by glucocorticoids in response to social challenge, including the regulation of dopamine (Anstrom et al., 2009; Barik et al., 2013), and serotonin (Haller et al., 1998; Summers and Winberg, 2006) neurotransmission. Acute stressors, which increase glucocorticoids, increased neuroestrogen concentrations in the PVN and aromatase
mRNA in the PVN within an hour in female rats, suggesting a possible link between corticosteroid release and neuroestrogen production in the hypothalamus (Liu et al., 2011). Similarly, in male quail, rapid increases in aromatase activity also occurred on restraint stress in the mPOA (Dickens et al., 2013), demonstrating that CORT can modulate neuroestrogen production in both birds and rodents. Furthermore, aromatase protein levels are increased in response to glucocorticoid treatment in a hypothalamic cell line via a transcriptional mechanism (Brooks et al., 2012); protein levels of aromatase peak 48 hours after glucocorticoid treatment, which may have implications for future bouts of aggression by priming the system to produce a larger amount of estrogen in response to future stress. However, despite strong evidence for the necessity of GR signaling for aggression, the molecular mechanisms involved are still not well understood.

1.17 Dendritic Spines

Dendritic spines are protrusion from the plasma membrane of dendrites, and contain the post synaptic density (PSD) in the head region, which is connected to the dendrite via a neck or stalk (Nimchinsky et al., 2002). The PSD is an electron dense region containing receptors, signaling molecules, and other proteins associated with synaptic signaling (Li et al., 2005). PSD-95 is a 95 kDa membrane-associated guanylate kinase (MAGUK) localized to the PSD of glutamatergic synapses that can induce maturation of synapses and increase spine stability (El-Husseini et al., 2000). Spines are plastic, and change in morphology and number throughout development (Cottrell et al.,
Spinophilin is another protein that is highly expressed in dendritic spines, that may be involved in changes in spine density and morphology through its regulatory actions of protein phosphatase 1 (PP1) (Allen et al., 1997). Because most excitatory synapses terminate on spines, spine density is likely correlated with the number of excitatory synapses, represents the potential for a functional increase in activity (Nimchinsky et al., 2002). Although the amount of PSD-95 or spinophilin protein is not an exact measure of spine number, it provides evidence for changes in synaptic terminals that may correlate to spine morphology or density.

### 1.17.1 Estrogen-Mediated Spinogenesis

Since the loss of 17β-E has been associated with a decrease in spine density and memory in female rodents (Li et al., 2004; Luine et al., 1997; Prange-Kiel and Rune, 2006; Woolley, 1998), most of the mechanisms of estrogen-mediated spinogenesis have been investigated in the hippocampus, on either density or morphology over long time frames. Other work has shown Akt-dependent stabilization of PSD-95 within 48 hours in differentiated NG-108 cells, a neural cell line that forms dendritic spines (Akama and McEwen, 2003). 17β-E treatment increases LIM kinase activation, leading to cofilin inactivation in NG-108 cells and hippocampal cell cultures, which permits the actin polymerization necessary for spinogenesis and morphology changes (Yuen et al., 2011). Although the majority of spine studies occur over long time periods, a single study has shown a rapid increase in spines by the ERα agonist, PPT, in CA1 of the hippocampus.
(Phan et al., 2012), which suggests non-classical estrogen signaling may play a role. Details on the receptors involved in spinogenesis or morphology changes in the hypothalamus have not yet been reported.

One study using hypothalamic tissue and primary cultures has demonstrated that 17β-E treatment increases spinophilin expression at 6 hours, and stable spines within 48 hours (Schwarz et al., 2008). This glutamate-dependent increase requires PI3K and ERK signaling, and is blocked by treatment with ICI, which suggests that either ERα or ERβ are necessary for spinogenesis in the hypothalamus (Schwarz et al., 2008). However, ICI also has agonistic activity against GPR30 (Revankar et al., 2005), so it is possible that a more complex interaction between receptors is involved. Blocking spinogenesis in the hypothalamus with cytochalasin D, an actin polymerization inhibitor, reduced lordosis (Christensen et al., 2011), which suggests a functional role for increased spine density in females. The molecular mechanism, as well as the relevance of spinogenesis to aggressive behavior in male rodents is currently unknown.

1.17.2 Glucocorticoid-mediated Spinogenesis

Rapid effects on spinogenesis and spine morphology following GR activation in the hippocampus have also been observed in the hippocampus but not in the hypothalamus. CA1 neurons from male rat hippocampal slices treated with Dex for 1 hour demonstrated a translation-independent increase in spine density, which was lost with co-application of Dex with either the GR antagonist RU-486, or the NMDA receptor blocker MK-801 (Komatsuzaki and Murakami, 2005). The proportion of mushroom-
shaped and thin-type spines was also increased following GR activation (Komatsuzaki and Murakami, 2005). Suppression of PKA, PKC, MAPK, or PI3K signaling completely blocked GR-mediated spinogenesis in CA1 neurons, suggesting that GR signals through convergent kinase pathways to increase actin polymerization, which would allow for spine changes (Komatsuzaki et al., 2012).

1.18 Experimental Rationale

The first set of experiments is designed to determine the effects of specific ER activation on spinogenesis in the hypothalamus. Since ERα is the most abundant ER isoform in the hypothalamus, and because there is no reliable ERβ antibody currently available (Snyder et al., 2010), the experiments examine ERα, as well as GPR30, which is also highly expressed in the hypothalamus, involvement in the net effects of estrogen (Hazell et al., 2009). Specifically, ERα is the most abundant ER isoform in the medial preoptic area (mPOA), BNST, ventromedial hypothalamus (VMH) and arcuate nucleus (ARH) (Laflamme et al., 1998; Mitra et al., 2003; Shughrue et al., 1997). Although the primary hypothalamic cell cultures used in these experiments are not obtained from a particular nucleus, ERs can be pharmaceutically activated or inhibited to define the role each play in contributing to changes in proteins necessary for spinogenesis.

The second set of experiments focuses on the role of an unidentified mGR in nuclear localization of the iGR in a hypothalamic neuronal cell line. Membrane-limited glucocorticoids have not previously been shown to affect iGR localization. mGR interaction with the iGR, either directly or through kinase pathway activation, may
provide a new mechanism for unliganded iGR nuclear localization. The purpose of these experiments is to test the dependence of membrane-initiated iGR nuclear localization, using pharmacologic inhibition and activation, on specific kinase pathways.

The third set of experiments examines the possible interactions between estrogen and glucocorticoid signaling as they pertain to male territorial aggression. Both estrogen signaling and glucocorticoid signaling are required for normal male aggressive behavior, but whether they are part of the same pathway, or represent two parallel pathways is not yet known. The focus of these studies is to determine the dependence of estrogen signaling on glucocorticoid signaling and vice versa (Fig 1.1). Understanding how normal, adaptive male aggression is initiated may help further investigation into pathological aggressive states.

**Figure 1.1:** Hypothetical model of estrogen and glucocorticoid signaling interactions in the hypothalamus. Dotted lines represent unknown interactions that were tested for the purpose of determining how the relationships between classical and non-classical signaling of estrogen and glucocorticoid receptors, may contribute to changes in spinogenesis in the VMH and male territorial aggression in mice.
CHAPTER 2. ESTRADIOL EFFECTS ON SPINOGENESIS IN THE HYPOTHALAMUS

2.1 INTRODUCTION

Male territorial aggression can be induced by stimulation of neurons located in the ventrolateral division of the ventromedial hypothalamus (vlVMH) (Lin et al., 2011), and blocked by ablation of these same neurons (Yang et al., 2013a). ERα is also necessary for male territorial aggression, as demonstrated by the loss of aggression observed with ERα knock out (ERKO) mice (Ogawa et al., 1997). Data from male California beach mice (Peromyscus carlifornicus) suggests that an acute treatment with cyclodextrin-conjugated estradiol (cE2) may increase some aspects of aggression (Trainor et al., 2008). However, the molecular mechanisms of estrogen-dependent, hypothalamically-mediated aggression have not yet been determined.

Hypothalamic neurons express ERα, ERβ, and GPR30 (Canonaco et al., 2008). Classic ERα is a 66 kDa nuclear receptor that dimerizes upon binding estrogen then binds to the estrogen response element (ERE) to regulate gene transcription (Nilsson et al., 2001b). In addition to ERα, the ERβ isoform also binds estrogen and acts as a transcription factor (Kuiper et al., 1996). Within the hypothalamus, ERα is the most abundant ER isoform in the ventromedial hypothalamus (VMH) and arcuate nucleus (ARH) (Laflamme et al., 1998; Mitra et al., 2003; Shughrue et al., 1997), although a low
level of ERβ has been detected in the ventrolateral VMH (vlVMH) (Mitra et al., 2003). However, since ERα is the most abundant ER isoform in most of the hypothalamus, is necessary for aggression in mice (Ogawa et al., 1998) and because there is no reliable ERβ antibody currently available (Snyder et al., 2010), the following experiments focus on the involvement of ERα in the net effects of estrogen on hypothalamic neurons.

Many of the effects of estrogen have been attributed to membrane ERs, by virtue of the rapid cellular response to membrane-limited estradiol conjugates, such as E$_2$BSA (Taguchi et al., 2004). GPR30, a G-protein coupled receptor that is activated by 17β-E, is also expressed in the hypothalamus (Brailoiu et al., 2007; Canonaco et al., 2008; Hazell et al., 2009). Recent identification of ERα of varying molecular weights, including ERα-36 (Chaudhri et al., 2014a), which exists in the plasma membrane in breast cancer (Chaudhri et al., 2014b) and airway epithelial cells (Jia et al., 2011), has raised the possibility that variants of classic ERα may function differently than the full length proteins, and may localize to different subcellular compartments (Dominguez and Micevych, 2010).

Post-synaptic density-95 (PSD-95) is a scaffolding protein localized to the PSD of dendritic spines that increases spine number and maturation in hippocampal neurons (El-Husseini et al., 2000), and can be used as a spine marker (Rivera and Bethea, 2012b). Interestingly, GPR30 has recently been shown to bind PSD-95 in hippocampal spines, presenting further evidence that it may be the ER involved with local regulation of spine density (Akama et al., 2013).
EB, a synthetic estrogen that is biologically more stable than 17β-E (Martínez et al., 2005), increases spinogenesis within 4 hours in the arcuate nucleus of the hypothalamus of female rats (Christensen et al., 2011). Furthermore, inhibition of spinogenesis with cytochalasin D, an actin polymerization inhibitor, blocks lordosis behavior, suggesting that spinogenesis is necessary for a behavioral output (Christensen et al., 2011). The ER responsible for increases in hypothalamic spinogenesis in females is unknown. Within the VMH, 17β-E increases spinogenesis in newborn female mice (Schwarz et al., 2008), but it is currently unknown if spines in the male VMH are similarly affected by ER activation and if spine changes contribute to aggression.

The purpose of these experiments is to identify expression of ERs in primary hypothalamic neurons from postnatal male pups, isolate the specific ER responsible for changes, if any, in dendritic spine-related proteins in males, and test if these changes depend on the ERK/MAPK pathway. Male primary hypothalamic cell cultures grown from postnatal day 1 (PD1) pups were cultured, and experiments were conducted on 7DIV to allow for maturation of neurons for all experiments involving PSD-95 expression, since PSD-95 is a dendritic spine marker. The motivation was to provide a molecular basis from which future studies could explore the causal link between spinogenesis and aggression in males.
2.2 METHODS

2.2.1 Cell Culture

**Primary Hypothalamic Cells from Postnatal Pups:**

Hypothalami were dissected from at least 3 male pups on PD1 (day of birth considered PD0) and placed in ice-cold sterile 1X Hanks’ Buffered Salt Solution (HBSS) (GIBCO®, Pittsburgh, PA). The tissue was digested in 3 ml 1X 0.25% trypsin + 2.21 mM EDTA (CORNING, Manassas, VA) at 37°C for 15 minutes with gentle shaking. Neurobasal A (Invitrogen, Grand Island, NY) + 1% Glutamax (Invitrogen) + 2% B27 (Invitrogen) + 1% Antibiotic Antimycotic Solution (CORNING) (complete Neurobasal medium) plus 5% fetal bovine serum (FBS) (Atlas Biologicals) was added. Cells were triturated with a series of three progressively smaller in diameter fire-polished Pasteur pipets (Fisher Scientific, Pittsburgh, PA) until fully in suspension. Supernatant was plated on poly-D-lysine coated 12 mm glass coverslips, size 0 (Carolina Biological, Burlington, NC), in 24-well plates (CORNING) or in PDL-coated 96-well plates (Falcon, Tewksbury, MA) and kept at 37°C, 5% CO₂ overnight in complete Neurobasal medium, as described above, plus 33% 1X Dulbecco’s Modified Eagle Medium (DMEM) (MediaTech, Manassas, VA) + 5% FBS. Medium was removed and replaced on 2 days *in vitro* (DIV) with fresh complete Neurobasal medium, then cells were maintained at 37°C, 5% CO₂ for 7 DIV. Half of the medium was removed and replaced with fresh complete Neurobasal medium every other day.
mHypoE-11 cell line (Cellutions Biosystems, Burlington, ON):

The murine embryonic hypothalamic cell line mHypoE-11 (N11) was chosen since it has endogenous expression of ERα, ERβ, and GPR30 (Belsham et al., 2004). Cells were plated between passage number 8 (P8) and P30 at a density of 4.5x10⁴ cells/well in 24-well plates containing 12mm glass coverslips, size 0 (Carolina Biological) or in 100mm dishes (Falcon) in 1X DMEM + 5% FBS + 1% Antibiotic Antimycotic Solution (N11 medium). Plates were incubated at 37˚C, 5% CO₂ until approximately 80% confluent. N11 medium was removed, cells were washed once with 1X Dulbecco’s Phosphate Buffered Saline (D-PBS) (GIBCO®), and DMEM 1X + 5% charcoal stripped (CS) FBS (Valley Biomedical, Winchester, VA)+ 1% Antibiotic Antimycotic Solution was added to ensure removal all exogenous hormones for 16 hours at 37˚C, 5% CO₂.

2.2.2 Hormone Treatments

Inhibitors were added either 30 minutes prior to 20 minutes of hormone treatment, or 30 minutes prior to 60 minutes of hormone treatment at 37˚C, 5% CO₂. All hormones or drugs were dissolved in dimethyl sulfoxide (DMSO) except for E₂BSA, which was dissolved in sterile water, then filtered with a 10K Amicon-Ultra 0.5ml filter (Millipore, Billerica, MA), at 14,000 rpm for 10 minutes at room temperature (See Table 2.1 for dilutions). Stock concentrations were made at 1000X and added directly to 5% CS N11 medium.
Table 2.1 Hormones, Specific Agonists and Antagonists for Estrogen Receptors and Kinases

<table>
<thead>
<tr>
<th>Agent</th>
<th>Source</th>
<th>Target</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>Sigma-Aldrich St. Louis, MO</td>
<td>Agonist, all estrogen receptors</td>
<td>10^{-8}M, 10^{-9}M</td>
</tr>
<tr>
<td>17β-Estradiol 6-(O-carboxy-methyl)oxime: BSA (E_2BSA)</td>
<td>Sigma-Aldrich</td>
<td>Agonist, all membrane estrogen receptors</td>
<td>10^{-8}M</td>
</tr>
<tr>
<td>G-1</td>
<td>Tocris Bioscience Minneapolis, MN</td>
<td>Agonist GPR30, agonist ERα-36</td>
<td>10^{-8}M</td>
</tr>
<tr>
<td>G-36</td>
<td>Azano Biotech Albuquerque, NM</td>
<td>Antagonist GPR30</td>
<td>10^{-6}M</td>
</tr>
<tr>
<td>4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT)</td>
<td>Tocris Bioscience</td>
<td>Agonist ERα</td>
<td>10^{-7}M</td>
</tr>
<tr>
<td>1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126)</td>
<td>Fisher Scientific</td>
<td>MEK inhibitor</td>
<td>5x10^{-5}M</td>
</tr>
</tbody>
</table>

2.2.3 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes at room temperature. Fixative was removed, and cells were washed in 1X D-PBS, then incubated in 10% Normal Goat Serum + 0.2% Triton X-100 in 1X PBS (blocking solution) for 1 hour at room temperature. Primary antibodies were diluted (Table 2.2) in block and incubated with cells overnight at 4°C. Cells were then washed in D-PBS, and incubated in secondary antibody diluted (Table 2.2) in blocking solution for 45 minutes at room temperature. After a final series of washes, coverslips were mounted in Vectashield with DAPI (Vector Labs) on glass slides (Fisher Scientific). All fluorescence imaging for
ICC was done on an upright Olympus IX71 microscope, with a Hamamatsu black and white camera and HCImage software (Hamamatsu, Hamamatsu City, Japan). Exposure times were set using cells incubated with secondary antibody only.

PSD-95 immunoreactive puncta were measured manually using ImageJ software (Schneider et al., 2012). Briefly, intensity thresholds were set so discrete puncta could be identified using 600X images of vehicle DMSO treated cells, then 5 pixel diameter regions of intensity were counted on all dendrites for each neuron.

**Table 2.2** Antibody Dilutions for Immunocytochemistry in Primary Hypothalamic Cell Cultures

<table>
<thead>
<tr>
<th>Antibody Name/Target</th>
<th>Source</th>
<th>Host Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα (H-184)</td>
<td>Santa Cruz Biotechnology, Inc. Santa Cruz, CA</td>
<td>Rabbit</td>
<td>1:300</td>
</tr>
<tr>
<td>PSD-95</td>
<td>NeuroMab Davis, CA</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>GPR30 (H-300)</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>Rabbit</td>
<td>1:100</td>
</tr>
<tr>
<td>ERα36</td>
<td>Cell Applications San Diego, CA</td>
<td>Rabbit</td>
<td>1:100</td>
</tr>
<tr>
<td>GFAP Antibody</td>
<td>Cell Signaling Technology® Beverly, MA</td>
<td>Rabbit</td>
<td>1:300</td>
</tr>
<tr>
<td>MAP2</td>
<td>Cell Signaling Technology</td>
<td>Mouse</td>
<td>1:100</td>
</tr>
<tr>
<td>Goat anti-Rabbit IgG (H+L) Secondary Antibody, Dylight 594 conjugate</td>
<td>Thermo Fisher Scientific Pittsburgh, PA</td>
<td>Goat</td>
<td>1:750</td>
</tr>
<tr>
<td>Goat anti-Mouse IgG (H+L) Secondary Antibody, Dylight 488 conjugate</td>
<td>Thermo Fisher Scientific</td>
<td>Goat</td>
<td>1:750</td>
</tr>
</tbody>
</table>
2.2.4 Transfection

Transfection was conducted at the time of plating for cell lines, while cells were in suspension, using the Neon® Transfection System (Life Technologies, Grand Island, NY) according to manufacturer’s instructions. The specific profile for N11 cells was 1600 mV, 10 ms, 2 pulses for a 100 µL transfection tip, at a density of 1x10⁶ cells, which was then distributed evenly between 10 wells in a 24-well plate. Cells were co-transfected with 300ng 3xere-luc, 150ng β-gal-GFP, and 300ng hERα-pSG5, then and allowed to adhere to coverslips overnight. Then cells were changed to 5% CS N11 medium for 16 hours before being treated with hormone for 24 hours. Cells were lysed using BrightGlo Lysis Buffer (Promega, Madison, WI) and assayed for luciferase activity with the BrightGlo Luciferase Assay kit (Promega) using a VICTOR luminometer and accompanying software (PerkinElmer, Waltham, MA). The data were normalized for transfection efficiencies and lysate preparation using the Beta-gal Assay Kit (Promega) according to manufacturer’s instructions.

2.2.5 Western Blot

Cells from 100mm plastes were lysed in 300 µL 1X Radioimmunoprecipitation Assay (RIPA) Buffer (Boston BioProducts, Boston, MA) + 1% Protease Inhibitor Cocktail (Sigma-Aldrich) + 1% Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich) + 1% Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich) for 15 minutes on ice. Lysate was then centrifuged at 14000 xg for 30 minutes at 4°C, and quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA). 20 µg of protein was used per sample, which
were boiled for 7 minutes in 1X Laemelli’s SDS Sample Buffer (Boston BioProducts) immediately before loading.

Samples were run at 125 volts on 4% stacking, 10% separating polyacrylamide gels using the Mini-PROTEAN Tetra Cell system (Bio-Rad), then transferred on ice to non-fluorescent polyvinyl difluoride membranes (LI-COR, Lincoln, NE) at 100 volts for 1 hour. Membranes to be visualized using the were incubated in Odyssey Blocking Buffer (TBS) (LI-COR Biosciences, Lincoln, NE), then incubated in primary antibody diluted (Table 2.3) in Odyssey Blocking Buffer (TBS) overnight at 4°C with gentle shaking. Blots were washed in 1X tris buffered saline (TBS) + 0.2% Tween20 (Sigma-Aldrich) (TBST), then incubated for 1 hour at room temperature with fluorescent secondary antibody diluted (Table 2.3) in Odyssey Blocking Buffer (TBS) + 0.1% SDS (Sigma-Aldrich). Blots were imaged on the Odyssey CLx Imager (LI-COR Biosciences), and analyzed using Image Studio Lite Version 4.0.21 software (LI-COR Biosciences).

Membranes to be visualized with chemiluminescence were incubated in 5% bovine serum albumin (BSA) (Fisher Scientific) in 1X TBS 2% Tween20 (BSA blocking solution) for 1 hour at room temperature. Primary antibody was diluted in BSA blocking solution and incubated at 4°C overnight with gentle shaking. Blots were washed in 1X TBST, then incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. SuperSignal West Femto (Thermo Scientific) was used to visualize immunoreactive bands on a Bio-Rad ChemiDoc™ Imager. Images were analyzed using Quantity One software (Bio-Rad) for densitometric analysis.
Table 2.3 Antibody Dilutions for Western Blotting

<table>
<thead>
<tr>
<th>Antibody Name/Target</th>
<th>Source</th>
<th>Host Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-\textit{Phospho}^{T202/Y204} \textit{ERK}</td>
<td>Phosphosolutions® Aurora, CO</td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>pan \textit{ERK}</td>
<td>BD Biosciences San Jose, CA</td>
<td>Mouse</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-spinophilin</td>
<td>Millipore</td>
<td>Rabbit</td>
<td>1:10,000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Millipore</td>
<td>Mouse</td>
<td>1:100,000</td>
</tr>
<tr>
<td>IRDye 680RD Goat anti-Mouse IgG</td>
<td>LI-COR Biosystems</td>
<td>Goat</td>
<td>1:15,000</td>
</tr>
<tr>
<td>IRDye 800CW Goat anti-Rabbit IgG</td>
<td>LI-COR Biosystems</td>
<td>Goat</td>
<td>1:15,000</td>
</tr>
<tr>
<td>Anti-rabbit IgG, HRP-linked Antibody</td>
<td>Cell Signaling Technology®</td>
<td>Goat</td>
<td>1:20,000</td>
</tr>
<tr>
<td>Anti-rabbit IgG, HRP-linked Antibody</td>
<td>Cell Signaling Technology®</td>
<td>Goat</td>
<td>1:75,000</td>
</tr>
</tbody>
</table>

2.2.6 Statistical Analysis

All statistical analyses were performed using Prism version 5.04 (GraphPad Software, San Diego, CA). A p-value of <0.05 was deemed statistically significant, and 1-way ANOVA with Bonferroni’s post-hoc test was used to compare three or more groups.

2.3 RESULTS

2.3.1 Hypothalamic neurons in primary cell culture express multiple estrogen receptors

Primary hypothalamic cells cultured from male pups on PD1, to ensure mice had experienced the testosterone surge that occurs 12 hours after birth (Flanagan-Cato, 2011), were grown to 7 DIV, at which time both glia and neurons were present, as
shown by positive staining for both glial fibrillary acidic protein (GFAP), and glial marker, and MAP2, a dendrite marker (Fig. 2.1).

Preliminary immunocytochemistry in primary hypothalamic cell culture for ERα, ERα phosphorylated at Serine 118 (pERαS118), ERα-36, and GPR30 demonstrated immunoreactivity for all tested ERs in PSD-95 reactive cells. However, the subcellular localization of each receptor is different; ERα and pERαS118 were expressed predominantly in the nucleus, but also throughout the cytoplasm of the soma and dendrites, while ERα-36 expression was restricted to the neuron soma and nucleus (Fig. 2.2a). Since ERα-36 has not been previously identified in the hypothalamus, we validated the antibody using N11 cell lysate, which revealed a strong immunoreactive band at the expected 36 kDa, a weaker band that may be from ERα-46, but no immunoreactive band at 66 kDa, the expected weight of full length ERα (Fig. 2.2b).

2.3.2 Selective activation of GPR30, but not ERα or ERβ increases spine density in hypothalamic neurons

PSD-95, which is expressed in dendritic spines as part of the post-synaptic density, was selected as a spine marker to determine if selective ER activation may alter spinogenesis. Male pups experience a testosterone surge approximately 12 hours after birth that could potentially alter ER expression in the VMH (Flanagan-Cato, 2011); all male pups were, therefore, used for primary culture at least 24 hours after birth (PD1) to ensure that this natural testosterone surge occurred before
Figure 2.1: Primary hypothalamic cell cultures consist of both glia and neurons. Hypothalami were cultured from P2 male pups to ensure exposure to the testosterone surge that occurs 12 hours after birth. Cultures were grown for 7 DIV, then fixed. Double ICC for GFAP (red), a glial marker, and MAP2 (green), a dendrite marker, was performed to identify the presence of both glia and neurons in these cultures. Image was taken at 200X. Scale bar is equal to 30 microns.
Figure 2.2: Primary hypothalamic cell culture neurons are immunoreactive for ERα, pERS118, ERα-36, and GPR30. Primary hypothalamic cell cultures from male pups were obtained as described in 2.2.1. Immunocytochemistry was performed on 7DIV.
(a) The top row represents ER immunoreactivity from the TXRED channel, the middle row represents PSD-95 immunoreactivity from the FITC channel, and the bottom row represents the pseudo-colored merged images, with ER and GPR30 in red, and PSD-95 in green; the DAPI stains nuclei blue. Images were taken at 600X (ERα, pERα S118, ERα-36) or at 200X (GPR30), scale bar is equal to 30 microns and applies to all images in each sequence.
(b) An immunoblot for ERα-36 on N11 cell lysate revealed immunoreactive bands at 36 kDa and around 46 kDa, but no band at the full length ERα molecular weight of 66 kDa.
sacrifice. Primary hypothalamic cultures were treated on 7 DIV at different time points with vehicle, 17β-estradiol, which activates all ERs, E₂BSA, which selectively activates mERs only, G-1, a specific GPR30 agonist that may also activate ERα-36, or PPT, a selective ERα agonist. PSD-95 immunofluorescence was measured by In Cell Western (ICW); all values were corrected for differences in neuron density using the MAP2 immunofluorescence signal. 17β-estradiol and PPT had no effect on the corrected PSD-95 immunoreactivity, however, E₂BSA and G-1 treatment increased PSD-95 immunoreactivity at 12 hours, though the effect was lost by 24 hours (Fig. 2.3).

In order to confirm that 17β-E remains bound to BSA in the E₂BSA preparation, expression of an ERE-luciferase reporter gene was measured using a luciferase assay in N11 cells, transfected to overexpress human ERα. 17β-E (10⁻⁸M) and PPT (10⁻⁷M) treatment for 24 hours induced a more than 10-fold increase in luciferase activity, but neither E₂BSA nor G-1 were sufficient to increase luciferase activity over vehicle DMSO levels (Figure 2.4).

Although an increase in spinophilin protein, another spine marker (Muly et al., 2004), has been reported in female hypothalamic neurons in response to 6 hours of 17β-E (10⁻⁸M) treatment (Schwarz et al., 2008), we were not able to detect a similar increase in PSD-95 immunoreactivity in male hypothalamic cell culture with the same treatment when measured using an ICW. ICC for PSD-95 immunoreactivity was observed throughout neurons, but not glia, with intense staining in the soma as well as dendrites, although localization to dendritic spines could not be confirmed (Fig. 2.5a). To test the possibility that PSD-95 immunofluorescence in the soma could be masking
Figure 2.3: PSD-95 immunoreactivity is increased in primary hypothalamic cells following 12 hours of either E₂BSA or G-1 treatment. Male primary hypothalamic cells were treated on 7 DIV with 6 hours, 12 hours, or 24 hours of DMSO vehicle, 17β-E (10⁻⁸M), E₂BSA (10⁻⁸M), G-1 (10⁻⁷M), or PPT (10⁻⁷M) (n=3-5/treatment group). ICWs for PSD-95 and MAP2 immunofluorescence were done. The Y-axis represents average PSD-95 immunoreactivity, corrected for total neuron concentration using MAP2 immunoreactivity, and then normalized to the corresponding time-point control (cells treated with DMSO vehicle). Data represent mean ± SEM *p<0.05, t-test compared to corresponding vehicle DMSO value.
Figure 2.4: E₂BSA and G-1 do not activate ERE-induced luciferase expression in the N11 cell line. N11 cells were transfected with an ERE-induced luciferase and β-galactosidase expressing plasmids, prior to treatment with 17β-E (10⁻⁸M), E₂BSA (10⁻⁸M), PPT (10⁻⁷M), and G-1 (10⁻⁸M) for 24 hours. Cell lysate was assayed for luciferase activity, which was then normalized for transfection efficiency and lysate preparation with results from a β–gal assay. Data represent mean ± SEM (n=3) *p<0.05 1-way ANOVA with Bonferroni’s post hoc test compared to DMSO treated cells.
**Figure 2.5**: The number of PSD-95 immunoreactive puncta in male primary hypothalamic cell culture does not change with 6 hours of 17β-E treatment. (a) ICC for PSD-95 (green) protein in male primary hypothalamic cell cultures (7 DIV) revealed immunoreactive puncta in dendrites. Images were taken at 600X, scale bars represent 30 microns. (b) Quantification of PSD-95 immunoreactive puncta localized to primary and secondary dendrites reveals no change in number with hormone treatment. Change this sentence to match others. Data represents mean ± SEM (n=8 DMSO, n=6 17β-E treated neurons). DAPI stain (blue) was used to identify nuclei.
smaller changes in the dendrites, ICC for PSD-95 immunofluorescence localized to
dendrites was done on primary hypothalamic cells treated with 17β-E for 6 hours.
Puncta in dendrites were manually counted, but consistent with our results obtained
using ICW (Fig. 2.3), there was no detectable difference from DMSO vehicle treatment in
PSD-95 immunoreactivity of neurons treated with 17β-E for 6 hours (Fig. 2.5b).

2.3.3 GPR30 activation causes an ERK-dependent increase in PSD-95 Immunoreactivity

To investigate the pathway through which G-1 increases PSD-95
immunoreactivity within 12 hours in male hypothalamic primary cultures, U0126, a
specific MEK inhibitor, at a concentration of 5x10^{-5}M, was applied 30 minutes prior to
application of G-1. We initially tested the efficacy of U0126 at this concentration in the
N11 cell line by measuring phosphorylated ERK, the product of activated MEK. N11 cells
were pre-treated with U0126 for 30 minutes prior to addition of hormones. As can be
seen in the representative blot, basal levels of pERK 1/2 were undetectable following
U0126 treatment, although normalized total ERK 1/2 concentrations remained
unchanged across treatment groups (Fig. 2.6).

Male primary hypothalamic cultures were pre-treated with vehicle DMSO or U0126
(5x10^{-5}M) for one hour, prior to treatment with vehicle (DMSO), E2BSA (10^{-8}M), or G-1
(10^{-8}M) for 12 additional hours. PSD-95 immunofluorescence, normalized to the MAP2
immunofluorescence signal, was measured by ICW. U0126 alone has no effect on PSD-
95 immunofluorescence, but the G-1 induced increase in PSD-95 immunoreactivity is
lost with U0126 pretreatment (Fig. 2.7).
Figure 2.6: U0126 pretreatment inhibits ERK 1/2 phosphorylation within 1 hour of treatment in N11 cells. N11 cells were pretreated with U0126 (10^{-5}M) for 30 minutes prior to 40 minutes of hormone treatment. Immunoreactive bands for pERK 1/2 are undetectable in cells pretreated with U0126, although total ERK 1/2 is unaffected. GAPDH was used as a loading control. The immunoblot was developed using the Odyssey system, with co-incubation of pERK 1/2, ERK 1/2, and GAPDH antibodies on the same membrane. Images were obtained using the Odyssey system; the top image represents the pERK immunoreactivity while the bottom image represents the total ERK immunoreactivity as well as the GAPDH immunoreactivity.
**Figure 2.7:** MEK inhibition blocks the G-1 induced increase in PSD-95 immunofluorescence in primary hypothalamic cell culture. Male primary hypothalamic cells were pretreated with 10^{-5}M U0126, a MEK inhibitor, for 1 hour prior to 12 hours G-1 treatment, followed by double ICC for PSD-95 and MAP2 immunoreactivity. G-1 treatment increases PSD-95 immunofluorescence, but U0126 pre-treatment abolishes this increase. The Y-axis represents the mean PSD-95 fluorescence intensity normalized to MAP2 fluorescence intensity. Data represent mean ± SEM (n=4-12 wells). *p<0.05, t-test compared to DMSO vehicle.
Since G-1 is reported to be an ERα-36 agonist (Kang et al., 2010), as well as a GPR30 agonist, is possible that the G-1 mediated increase in PSD-95 immunoreactivity is partially or completely through ERα-36 activation. To test the dependence of the G-1 effect on GPR30, primary hypothalamic cells were pre-treated with G-36 (10^{-6}M), a specific GPR30 antagonist, at 10^{-6}M for 60 minutes prior to 12 hours of G-1 treatment. The G-1 induced increase in PSD-95 immunofluorescence was lost with G-36 pre-treatment (Fig. 2.8).

2.3.4 PSD-95 protein changes in primary cultures may not represent functional synapses

PSD-95 is a spine marker associated with the post-synaptic density, but functional synapses also require a pre-synaptic neuron to interact with the post-synaptic spine. Synaptophysin is a protein expressed pre-synaptically; co-localization of a pre-synaptic and post-synaptic protein may indicate the presence of a synapse (O’Connor et al., 2009). Dendritic spines require synaptic activity to mature (Frotscher et al., 2000), although it is unknown if our primary hypothalamic cultures can form functional synapses. To test for the existence of synapses, primary hypothalamic cultures were co-incubated with anti-synaptophysin in addition to anti-PSD-95 antibodies to detect colocalization of pre- and post-synaptic structures. However, despite robust PSD-95 staining in hypothalamic neurons, there was much less synaptophysin staining, and very little colocalization (Fig. 2.9a). There was no measurable difference in PSD-95 and
**Figure 2.8**: G-36 pretreatment blocks the G-1 mediated increase in PSD-95 immunoreactivity. Male primary hypothalamic cells were pretreated with $10^{-6}$M G-36, a specific GPR30 inhibitor, for 1 hour prior to 12 hours of G-1 treatment. ICW demonstrates that G-1 treatment alone increases normalized PSD-95 immunofluorescence, but G-36 pre-treatment abolishes this increase. Treatment with G-36 alone does not change PSD-95 immunoreactivity compared to the DMSO control group. The Y axis represents the mean PSD-95 fluorescence intensity normalized to MAP2 fluorescence intensity. Data represents mean with SEM (n=8-12 wells). *p<0.05, t-test compared to DMSO vehicle, #p<0.05, t-test compared to G-1.
Figure 2.9: G-1 treatment of primary hypothalamic cell culture does not increase PSD-95 and synaptophysin colocalization.

(a) ICC for PSD-95 (green) and synaptophysin (red) reveals immunoreactivity in both the soma and dendrites of hypothalamic neurons; yellow indicates regions of colocalization. Images were taken at 600X; merged images are presented. DAPI stains the nuclei blue. Scale bars represent 30 microns.

(b) Analysis of PSD-95 and synaptophysin colocalization reveals no significant difference after 12 hours of vehicle (DMSO) or G-1 treatment. The Y axis represents the average number of PSD-95 and synaptophysin points of colocalization. Data represent mean ± SEM (n=10 DMSO, n=9 G-1 treated neurons).
synaptophysin colocalization following 12 hours of G-1 treatment (Fig. 2.9b) compared to the vehicle treatment.

In addition to PSD-95, spinophilin is another protein used as a spine marker (Muly et al., 2004) that has been used in a single previous study to detect spinogenesis in response to 17β-E in the hypothalamus (Schwarz et al., 2008). ICC for spinophilin in male hypothalamic cell culture revealed spinophilin staining in dendritic spines, but robust staining was also present in glia (Fig. 2.10a), which prevented the use of ICWs to detect changes in spinophilin since the fluorescence signal in spines would likely be masked by glial spinophilin immunoreactivity. N11 cell lysate was tested for expression of spinophilin protein splice variants by Western blot, which revealed immunoreactive bands at both 120 kDa and 89 kDa (Fig. 2.10b). Additionally, 17β-E (10^-8M) treatment had no effect on spinophilin protein expression in N11 cells following 6 hours of treatment (Fig. 2.10c). The presence of spinophilin in both glia and neurons, as well as the presence of multiple immunoreactive bands preclude the use of ICWs with this antibody.

2.4 DISCUSSION

To the best of our knowledge, no other study has attempted to use the technically difficult preparation of mixed neuronal and glial primary hypothalamic cultures from postnatal mice. We selected these culture conditions specifically because we could identify males, and culturing hypothalami from PD1 ensured time for our
Figure 2.10: 17β-E treatment in N11 cells does not change spinophilin protein levels after 6 hours.

(a) ICC for spinophilin in a primary hypothalamic cell culture demonstrated immunoreactivity in both neurons and glia. Image was taken at 200X, scale bar is equal to 30 microns

(b) Immunoreactive bands for spinophilin protein were detected by Western blot at both 89 kDa and 120 kDa in N11 lysate, but only at 120 kDa in olfactory bulb lysate from an adult male mouse (OB), used as a positive control.

(c) N11 cells were treated with DMSO vehicle, 17β-E (10⁻⁸M), E₂BSA (10⁻⁸M), or PPT (10⁻⁷M) for 6 hours. Densitometric analysis of immunoreactive bands showed no difference in spinophilin immunoreactivity with treatment. The Y-axis represents the average density of spinophilin immunoreactivity corrected for loading and lysate preparation with GAPDH, and then normalized to vehicle DMSO. Data represent mean ± SEM (n=6).
cultures that were young enough to be viable, but that had experienced the testosterone surge that occurs 12 hours after birth (Corbier et al., 1992). This early exposure to testosterone, which is converted to estradiol through aromatase activity in the postnatal hypothalamus (Konkle and McCarthy, 2011), is important in masculinization of the brain (Wu et al., 2009). It was also necessary to allow neurons in cultures to form dendrites and mature as much as possible, in order to model functional spinogenesis as closely as possible. Experiments were conducted on 7 DIV to allow sufficient time for neurons to differentiate and mature beyond the commonly used 4 DIV (Konkle and McCarthy, 2011; Schwarz et al., 2008), while still maintaining the health of the fragile cultures. Studies in cortical and hippocampal neurons have demonstrated that neurons are more mature, as measured by appropriate expression and localization of synaptic markers, when maintained for 9 DIV as opposed to 4 DIV (Lattuada et al., 2013). Additionally, this study is unique because we use male-only cultures instead of mixed sex (Dominguez and Micevych, 2010; Schwarz et al., 2008), or female only (Bondar et al., 2009) cultures. In light of recent data that show sexual dimorphism of estrogen actions in the hippocampus (Fester et al., 2012), and in the VMH (Flanagan-Cato, 2011), our use of male-only primary cultures is a more accurate model for in vivo ER activation the adult male mouse.

2.4.1 Estrogen receptor expression in primary hypothalamic cell culture

Spine density in the VMH increases in response to estrogen in peripubertal rats (Segarra and McEwen, 1991), and adult female rats (Calizo and Flanagan-Cato, 2000).
Since the purpose of this study was to identify the specific ER that could regulate spinogenesis in the male hypothalamus, it was important to first identify which ERs are expressed in our male primary hypothalamic cell cultures. Expression of ERα, ERβ, and GPR30 have all been previously identified in the hypothalamus in multiple studies, including one in which all three receptors were detected in hypothalami of PD7 male hamsters through RT-PCR (Canonaco et al., 2008). However, the expression of ERα variants in the hypothalamus is not known. We confirmed expression of ERα using an antibody raised against the N-terminus of the full length ERα (ERα H-184). Subcellular localization, as demonstrated by ICC, shows the presence of ERα immunoreactivity throughout the dendrites, soma, and nucleus. Although nuclear expression of the nuclear receptor ERα is expected, this is the first time ERα expression in hypothalamic dendrites has been confirmed with ICC, although dendritic localization of ERα has also been reported in adult pro-estrous female rats using immunogold labeling in hippocampal sections (Milner et al., 2001).

Membrane localized ERα-66 and ERα-52 have been detected in biotinylated membrane fractions derived from adult female rat primary hypothalamic astrocyte cultures (Bondar et al., 2009) and mixed male and female embryonic neuron cultures (Domínguez and Micevych, 2010) by immunochemical detection using the H-184 antibody directed towards ERα. mERα has also been detected on the plasma membrane, by Western blotting of membrane fractions, from male embryonic hypothalamic neuron cultures (Gorosito et al., 2008). However, ICC on our male primary hypothalami cultures did not reveal any evidence of membrane localization. It
is possible that although mERα is expressed at the embryonic stage in both mixed male and female (Dominguez and Micevych, 2010), and male hypothalamic neurons in culture (Gorosito et al., 2008), it not present in postnatal hypothalamic cell cultures. Alternatively, standard ICC may not be sensitive enough to detect membrane localized receptors; for example, membrane localization of GR in human peripheral blood mononuclear cells is only detectable when the ICC signal is amplified with fluorescent liposomes (Bartholome et al., 2004). Further testing, perhaps using a biotinylation technique to isolate membrane proteins, is necessary before any conclusions can be made about mERα expression in postnatal male primary hypothalamic cultures.

Our primary male hypothalamic cell cultures were also positive for GPR30 immunoreactivity in the neuron somas. ICC did not reveal plasma membrane localization, consistent with results from a study using the membrane fraction from male embryonic hypothalamic neuron culture, which also failed to detect GPR30 expression in the plasma membrane (Gorosito et al., 2008). As with ERα, a more sensitive technique than ICC, such as surface biotinylation or amplification of the ICC signal, is necessary to test both localization of GPR30 to dendritic spines before any conclusions can be made about GPR30 plasma membrane localization in male postnatal hypothalamic neurons. Co-immunoprecipitation of PSD-95 with GPR30 in the adult female rat hippocampus provides evidence of an interaction between GPR30 and the post-synaptic density (Akama et al., 2013); however, no evidence of GPR30 and PSD-95 immunoreactivity colocalization was visible using ICC in primary male hypothalamic cell cultures.
We also demonstrated immunoreactivity for ERα-36 in both the N11 cell line and in primary hypothalamic neurons. Although ERα-36 has been identified, using subcellular fractionation, in the membrane of airway epithelial cells (Jia et al., 2011) and in breast cancer cell lines (Chaudhri et al., 2014b), membrane localization in neurons has not yet been determined. Subcellular localization of ERα-36 in the hypothalamic neuron appears to be exclusively in the cytoplasm of the soma and in the nucleus, although, once again, plasma membrane expression cannot be ruled out from our results. The expression of ERα-36 is especially interesting as it has been shown to be involved in non-classical estrogen effects on ERK/MAPK signaling (Wang et al., 2006), and is activated by the GPR30 agonist G-1 (Kang et al., 2010).

2.4.2 *Induction of PSD-95 immunoreactivity by GPR30 activation is ERK/MAPK dependent*

Non-classical effects of estrogen contribute to increases in spinogenesis and changes in dendritic morphology observed in neurons (Micevych and Christensen, 2012; Sanchez et al., 2012). Although the specific ERs required for estrogen-mediated spinogenesis in the hypothalamus have not been previously identified, our results suggest that GPR30 activation, and subsequent ERK/MAPK activity is necessary for an increase in PSD-95 protein in male hypothalamic cell culture. One proposed mechanism for estrogen-mediated spinogenesis in the VMH is through an ERK-dependent pathway that was investigated in mixed-sex embryonic hypothalamic rat cultures and slices from postnatal female rats (Schwarz et al., 2008). 17β-E activated Akt pre-synaptically,
followed by glutamate release and subsequent N-methyl D-aspartate (NMDA) receptor activation post-synaptically, which then caused increased ERK phosphorylation in the post-synaptic neuron. An increase in spinophilin protein in the post synaptic neuron was dependent on ERK/MAPK signaling (Schwarz et al., 2008). These data suggest that the increase in spines, which was observed in Golgi-stained sections from postnatal female rats, was dependent on ERK/MAPK signaling (Schwarz et al., 2008). The G-1 mediated increase in PSD-95 immunoreactivity in male postnatal primary hypothalamic cultures in our study is also dependent on MAPK, which provides further support for the dependence of spinogenesis on ERK/MAPK signaling in the rodent hypothalamus, regardless of sex. In hippocampal neurons, both MAPK and Akt activation have been documented in response to GPR30 stimulation (Tang et al., 2014). Interestingly, Akt activation in neuroblastoma cells can stimulate PSD-95 protein synthesis in a transcriptionally-independent manner (Akama and McEwen, 2003). In our primary male hypothalamic cultures treatment with E₂BSA for 12 hours transiently increased PSD-95 immunoreactivity, suggesting the non-classical effect is through selective activation of membrane ERs. Despite our inability to confirm membrane localization of an ER with ICC, the increase in PSD-95 immunoreactivity in response to E₂BSA suggests a mER is present and functional in male primary hypothalamic cell cultures. Results from our ERE-luciferase assay confirm that our preparations of E₂BSA are plasma membrane-limited, in agreement with our previous studies in neuroblastoma cells (Clark et al., 2014).
G-1 treatment was sufficient to induce an increase in PSD-95 immunoreactivity that is similar to that obtained with E₂BSA; it is probable that G-1 is activating the same membrane ERs, either GPR30 or ERα-36, as E₂BSA. Our use of the specific GPR30 antagonist, G-36, suggests that the G-1 mediated increase in PSD-95 immunoreactivity is through GPR30 and not ERα-36. This result could be further confirmed by the use of GPR30 knockout mice for hypothalamic culture, to verify that GPR30 is necessary for the G-1-mediated effect on PSD-95. Prior to this study, GPR30 activation has not been shown to increase spinogenesis in any brain region. 17β-E increases spine density in the VMH of adult female rats in a population of non-ERα immunoreactive neurons (Calizo and Flanagan-Cato, 2000); therefore, our study, albeit in males, implies that GPR30 is a plausible candidate for the ER through which 17β-E induces spinogenesis in the female VMH. PPT, a selective ERα agonist, had no effect on PSD-95 immunoreactivity, which suggests that simultaneous activation of both classic ERα and mERα has no net effect on PSD-95 protein expression. Similarly, activation of all ERs with 17β-E has no effect on PSD-95 immunoreactivity, supporting the idea that selective membrane ER activation, in the absence of intracellular ER activation, is necessary to increase PSD-95 protein expression. Since 17β-E also has no effect on spinophilin expression in N11 cells, it is possible that activation of multiple ERs may have opposing effects on the same signaling pathways. For example, GPR30 activation inhibits the activation of ERK/MAPK induced by ERα in the uterus (Gao et al., 2011), and 17β-E both activates and inhibits CREB signaling in rat hippocampal neurons (Boulware et al., 2005). Therefore, the relative expression of different ERs is likely important in the regulation of estrogen signaling.
The expression of ERs in postnatal primary hypothalamic culture may be different than in adult male mice since ER expression changes throughout development (Charitidi and Canlon, 2010; Wilson et al., 2011). Interestingly, development is not the only factor controlling ER expression; the localization and expression of ERs in the hypothalamus changes with hormone exposure (Domínguez and Micevych, 2010).

2.4.3 PSD-95 as a synaptic marker

Although spinophilin has also been used previously as a marker for spine density (Schwarz et al., 2008), we were not able to show an increase in the N11 cell line with treatment at 6 hours. N11 cells do not form dendrites, so it is not unexpected that spine-associated changes may not occur as they would in primary neurons. However, it is impractical to use our mixed neuronal and glial primary cultures to detect changes in spinophilin because the antibody for spinophilin also detects a protein present in glia; any changes in spinophilin protein detected with Western blotting could not be definitively attributed to neurons. PSD-95 protein expression was restricted to neurons, which made it a more reliable marker for dendritic spines. The loss of the G1-mediated effect at 24 hours may be a due to the culture conditions, in which stabilization of new spines does not occur like it does in vivo by activity-dependent reinforcement (De Roo et al., 2008). The presence of PSD-95 in puncta located on dendrites provided initial evidence that PSD-95 may be located in spines. Double ICC on treated primary cultures for both PSD-95 and the pre-synaptic protein synaptophysin provided more evidence that functional synapses may be forming in our
primary hypothalamic cell cultures. However, there was very little colocalization
detected, even with G-1 treatment, which supports our idea that any new spines that
may have been forming are not being stabilized. This may explain the transient increase
in PSD-95; further experiments in vivo are necessary to test the effects of G-1 on the
hypothalamus. For example, Golgi-Cox impregnation of adult male brains would
provide a conclusive answer to the question of whether G-1 treatment induces
spinogenesis, and would allow for differentiation between hypothalamic nuclei, each of
which may respond differently to GPR30 activation.

2.4.4 Conclusion

In conclusion, we have demonstrated that in addition to ERα and GPR30, the
variant ERα-36 is expressed in the somas of hypothalamic neurons cultured from
postnatal male pups. For the first time, we show that selective activation of mERs or the
GPR30 increases PSD-95 immunoreactivity, in an ERK/MAPK dependent manner, in the
male hypothalamus.
CHAPTER 3. MEMBRANE GLUCOCORTICOID EFFECTS ON GR NUCLEAR TRANSLOCATION

3.1 INTRODUCTION

CORT (CORT), the primary endogenous glucocorticoid in rodents, is released from the adrenal cortex following stressful experiences, including social challenge by an intruder of the same species (Summers and Winberg, 2006). The normal, adaptive behavioral response to this territorial intrusion is aggression (Koolhaas and Bohus, 1989), however, inhibition of CORT synthesis with metyrapone reduces aggressive behavior (Mikics et al., 2004). Since male territorial aggression is mediated through neurons located in the VMHvl (Lin et al., 2011; Yang et al., 2013a), and GR is highly expressed in the hypothalamus (Brooks et al., 2012; Liposits and Bohn, 1993), it is possible that the increased aggression as a result of glucocorticoid signaling occurs via GR activation in hypothalamic neurons.

GR is a member of the same nuclear receptor subfamily as the ER (Aranda and Pascual, 2001), and shares similar structural and functional domains (Huang et al., 2010). As a nuclear receptor, liganded GR can modulate transcription as a co-activator or repressor, and can directly bind DNA at the GRE (Oakley and Cidlowski, 2013). However, even in its unliganded state, GR exists in both the cytoplasm and the nucleus in a state of equilibrium (Hache et al., 1999). Nuclear trafficking is cell cycle-dependent (Matthews et al., 2011), and physiological conditions, including elevated pH, salt, heat
shock, and laminar shear stress can also induce unliganded nuclear localization (Bresnick et al., 1989; Ji et al., 2003; Sanchez, 1992). Hormone treatment rapidly induces nuclear localization of the receptor-ligand complex (Hache et al., 1999), an effect that also occurs in response to the synthetic glucocorticoid Dex (Htun et al., 1996). The mechanism for ligand-initiated nuclear translocation is not completely understood, but is dependent on HSP90 (Czar et al., 1997), receptor dimerization (Robertson et al., 2013), nuclear importins (Freedman and Yamamoto, 2004), and exposure of a nuclear localization signal (Matthews et al., 2011), and results in classical transcription regulation from the GRE. However, the function of unliganded GR in the nucleus is not understood.

Non-classical effects of glucocorticoids include ERK/MAPK (Kumamaru et al., 2008; Oppong et al., 2014), p38 MAPK (Strehl et al., 2011) and PI3K (Limbourg et al., 2002) modulation in a variety of cell types, and occur within minutes of treatment. Other rapid responses to glucocorticoids, including endocannabinoid production (Di et al., 2005) and inhibition of glutamate release, an effect that was maintained with the membrane-limited glucocorticoid DexBSA (Di et al., 2003) have been recorded in the hypothalamus, and have been attributed to a membrane glucocorticoid receptor (mGR) (Tasker et al., 2005). Electrophysiology performed on rat hypothalamic slices provide evidence for GPCR involvement in mGR signaling (Di et al., 2005; Tasker et al., 2005). These short time-frames and cellular responses to membrane-limited glucocorticoids suggest a functional mGR exists, although its identity is currently unknown (Desquiret et al., 2008; Nogami et al., 2014; Oppong et al., 2014).
The presence of membrane localized classic GRα was detected at very low levels in human lymphocytes and leukocytes using GR-specific antibodies (Bartholome et al., 2004). Neurons in the rat hippocampus contain plasma membrane associated GR in synaptosomal fractions (Komatsuzaki and Murakami, 2005), and GR immunoreactivity in the hippocampus and hypothalamus has been observed at the plasma membranes and vesicle membranes (Liposits and Bohn, 1993).

GR exists as two isoforms, glucocorticoid receptor alpha (GRα) and glucocorticoid receptor GR beta (GRβ), although GRβ is not transcriptionally active, and is generated at much lower frequencies than GRα in the brain (Pujols et al., 2002), so the focus of these experiments is exclusively on the activity of GRα (GR). The purpose of this study is to elucidate the mechanism by which membrane-initiated iGR nuclear translocation occurs. Therefore, these experiments are designed to first demonstrate the membrane-limited glucocorticoid effect on intracellular GR nuclear localization in the mHypo-E11 (N11) and mHypoE-42 (N42) hypothalamic neuron cell lines, both of which endogenously express GR and secondarily to test the involvement of kinase pathways on rapid iGR nuclear translocation.
3.2 METHODS

3.2.1 Cell Culture

Primary Hypothalamic Cells from Postnatal Pups:

Hypothalami were dissected from at least 3 female pups on PD1 (day of birth considered PD0) and placed in ice-cold sterile 1X Hanks’ Buffered Salt Solution (HBSS) (GIBCO®, Pittsburgh, PA). The tissue was digested in 3 ml 1X 0.25% trypsin + 2.21 mM EDTA (CORNING, Manassas, VA) at 37°C for 15 minutes with gentle shaking. Neurobasal A (Invitrogen, Grand Island, NY) + 1% Glutamax (Invitrogen) + 2% B27 (Invitrogen) + 1% Antibiotic Antimycotic Solution (CORNING) (complete Neurobasal medium) plus 5% fetal bovine serum (FBS) (Atlas Biologicals) was added. Cells were triturated with a series of three progressively smaller in diameter fire-polished Pasteur pipets (Fisher Scientific, Pittsburgh, PA) until fully in suspension. Supernatant was plated on poly-D-lysine coated 12 mm glass coverslips, size 0 (Carolina Biological, Burlington, NC), in 24-well plates (CORNING) or in PDL-coated 96-well plates (Falcon, Tewksbury, MA) and kept at 37°C, 5% CO₂ overnight in complete Neurobasal medium, as described above, plus 33% 1X Dulbecco’s Modified Eagle Medium (DMEM) (MediaTech, Manassas, VA) + 5% FBS. Medium was removed and replaced on 2 days in vitro (DIV) with fresh complete Neurobasal medium, then cells were maintained at 37°C, 5% CO₂ for 7 DIV. Half of the medium was removed and replaced with fresh complete Neurobasal medium every other day.
mHypoE-11 cell line (Cellutions Biosystems, Burlington, ON):

Cells were plated between passage number 8 (P8) and P30 at a density of 4.5x10⁴ cells/well in 24-well plates containing 12mm glass coverslips, size 0 (Carolina Biological) in 1X DMEM + 5% FBS + 1% Antibiotic Antimycotic Solution (N11 medium). Plates were incubated at 37°C, 5% CO₂ until approximately 80% confluent. N11 medium was removed, cells were washed once with 1X Dulbecco’s Phosphate Buffered Saline (D-PBS) (GIBCO®), and DMEM 1X + 5% charcoal stripped (CS) FBS (Valley Biomedicals, Winchester, VA)+ 1% Antibiotic Antimycotic Solution was added to ensure removal all exogenous hormones for 16 hours at 37°C, 5% CO₂.

mHypoE-42 cell line (Cellutions Biosystems):

Cells were plated between P2 and P20 at a density of 4.5x10⁴ cells/well in 24-well plates containing 12mm glass coverslips, size 0, or in 96-well plates (Falcon) in 1X DMEM + 5% FBS + 1% Antibiotic Antimycotic Solution + 0.2% Plasmocin Prophylactic® (Invivogen, San Diego, CA) (N42 medium). Plates were incubated at 37°C, 5% CO₂ until approximately 80% confluent. N42 medium was removed, cells were washed once with 1X D-PBS, and DMEM 1X + 5% CS FBS + 1% Antibiotic Antimycotic Solution was added to ensure removal all exogenous hormones for 16 hours at 37°C, 5% CO₂.

3.2.2 Hormone Treatments

Inhibitors or activators were added either 15 minutes prior to 20 minutes of hormone treatment (See Table 3.1 for concentrations), or 30 minutes prior to 60
minutes of hormone treatment at 37°C, 5% CO₂. All hormones or drugs were dissolved in dimethyl sulfoxide (DMSO) except for DexBSA, which was dissolved in sterile water, then filtered with a 10K Amicon-Ultra 0.5ml filter (Millipore, Billerica, MA), at 14,000 rpm for 10 minutes at room temperature.

Table 3.1 Hormones, Kinase Activators and Inhibitors

<table>
<thead>
<tr>
<th>Agent</th>
<th>Source</th>
<th>Target</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>Sigma-Aldrich</td>
<td>Agonist, all GRs</td>
<td>10⁻⁵M, 10⁻⁶M, 10⁻⁷M, 10⁻⁸M</td>
</tr>
<tr>
<td>Cyclodextrin-conjugated CORT</td>
<td>Sigma-Aldrich</td>
<td>Agonist all GRs</td>
<td>10⁻⁶M</td>
</tr>
<tr>
<td>1,4-pregnandien-9α-fluoro-16α-methyl-11β,β,21-triol-3,20-dione 21-hemisuccinate :BSA 37:1 (DexBSA)</td>
<td>Steraloids, Inc. Newport, RI</td>
<td>Agonist, all mGRs</td>
<td>10⁻⁶M, 10⁻⁷M, 10⁻⁸M, 10⁻⁹M</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Sigma-Aldrich</td>
<td>Translation inhibitor</td>
<td>10⁻⁶M</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>Tocris Bioscience</td>
<td>Transcription inhibitor</td>
<td>10⁻⁶M</td>
</tr>
<tr>
<td>Geldanamycin</td>
<td>Tocris Bioscience</td>
<td>HSP90 inhibitor</td>
<td>1x10⁻⁴M</td>
</tr>
<tr>
<td>SC-79</td>
<td>Tocris Bioscience</td>
<td>Akt activator</td>
<td>1.1x10⁻⁵M</td>
</tr>
<tr>
<td>10 DEBC*HCl</td>
<td>Tocris Bioscience</td>
<td>Akt inhibitor</td>
<td>5x10⁻⁶M</td>
</tr>
<tr>
<td>PP2</td>
<td>Tocris Bioscience</td>
<td>Src inhibitor</td>
<td>5x10⁻⁵M</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>Tocris Bioscience</td>
<td>Membrane permeable calcium chelator</td>
<td>5x10⁻⁶M</td>
</tr>
<tr>
<td>SB 202190</td>
<td>Tocris Bioscience</td>
<td>p38 MAPK inhibitor</td>
<td>1x10⁻⁵M</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>Tocris Bioscience</td>
<td>p38 MAPK activator</td>
<td>1x10⁻⁵M</td>
</tr>
<tr>
<td>Letrozole</td>
<td></td>
<td>Aromatase inhibitor</td>
<td>10⁻⁹M</td>
</tr>
<tr>
<td>1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126)</td>
<td>Fisher Scientific</td>
<td>MEK inhibitor</td>
<td>5x10⁻⁵M</td>
</tr>
</tbody>
</table>
3.2.3 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes at room temperature. Fixative was removed, and cells were washed in 1X D-PBS, then incubated in 10% Normal Goat Serum + 0.2% Triton X-100 in 1X PBS (blocking solution) for 1 hour at room temperature. Primary antibodies were diluted (Table 3.2) in block and incubated with cells overnight at 4°C. Cells were then washed in D-PBS, and incubated in secondary antibody diluted (Table 3.2) in blocking solution for 45 minutes at room temperature. After a final series of washes, coverslips were mounted in Vectashield with DAPI (Vector Labs) on glass slides (Fisher Scientific). All fluorescence imaging for immunocytochemistry was done on an upright Olympus IX71 microscope with a Hamamatsu black and white camera and HCImage software (Hamamatsu City, Japan), and confocal images were taken with a Nikon A1 microscope. Exposure times were set using cells incubated with secondary antibody only and kept consistent within the experiment.

Table 3.2 Antibody Dilutions for Immunocytochemistry in N11, N42, and Primary Hypothalamic Cell Culture

<table>
<thead>
<tr>
<th>Antibody Name/Target</th>
<th>Source</th>
<th>Host Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR (M-20)</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>Rabbit</td>
<td>1:300</td>
</tr>
<tr>
<td>PSD-95</td>
<td>NeuroMab</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-Rabbit IgG (H+L) Secondary Antibody, Dylight 594 conjugate</td>
<td>Thermo Fisher Scientific</td>
<td>Goat</td>
<td>1:750</td>
</tr>
<tr>
<td>Goat anti-Mouse IgG (H+L) Secondary Antibody, Dylight 488 conjugate</td>
<td>Thermo Fisher Scientific</td>
<td>Goat</td>
<td>1:750</td>
</tr>
</tbody>
</table>
3.2.4 Cell Profiler Intensity Analysis

Cell Profiler Cell Image Analysis Software (www.cellprofiler.org) was used to measure the mean intensity of the fluorescence signal per nucleus, cytoplasmic region, and the whole cell (Carpenter et al., 2006). The DAPI image was used to identify the nuclear compartment, from which the cytoplasm is designated as a pre-set distance of 10 pixels from the border of the nucleus. In primary cell culture experiments the filter displaying PSD-95 fluorescence is used to isolate neurons. The neuron nuclei outlines were overlaid on the TXRED image to measure the fluorescence intensity of the protein of interest.

3.2.5 Transfection

Transfection was conducted at the time of plating for cell lines, while cells were in suspension, using the Neon® Transfection System (Life Technologies, Grand Island, NY) according to manufacturer’s instructions. The specific profile for N42 cells was 1600 mV, 10 ms, 2 pulses for a 100 µL transfection tip, at a density of 1x10⁶ cells, which was then distributed evenly between 10 wells in a 24-well plate. Cells were co-transfected with 300ng GRE-TK-Luc, 150ng β-gal-GFP, then and allowed to adhere to coverslips overnight. Then cells were changed to 5% CS N42 medium for 16 hours before being treated with hormone for 24 hours. Cells were lysed using BrightGlo Lysis Buffer (Promega, Madison, WI) and assayed for luciferase activity with the BrightGlo Luciferase Assay kit (Promega) using a VICTOR luminometer and accompanying software (PerkinElmer, Waltham, MA). The data were normalized for transfection efficiencies.
and lysate preparation using the Beta-gal Assay Kit (Promega) according to manufacturer’s instructions.

3.2.6 In Cell Western:

Cells in were fixed in 4% paraformaldehyde for 15 minutes at room temperature in 96-well plates and then permeabilized with 1X D-PBS+0.1% Triton X-100. Cells were incubated with Odyssey Blocking Buffer (PBS) (LI-COR Biosystems) for 1 hour at room temperature. Cells were incubated with primary antibodies diluted (Table 3.3) in Odyssey Blocking Buffer (PBS) overnight at 4°C with gently shaking. Cells were washed in 1X D-PBS + 0.1% Tween20, then incubated with secondary antibody diluted (Table 3.3) in Odyssey Blocking Buffer (PBS) + 0.2% Tween20 for 1 hour at room temperature with gently shaking. Plates were then imaged on an Odyssey CLx Imager (LI-COR Biosciences), and analyzed using Image Studio Lite Version 4.0.21 software (LI-COR Biosciences).

3.2.7 Statistical Analysis

All statistical analyses were performed using Prism version 5.04 (GraphPad Software, San Diego, CA). A p-value of <0.05 was deemed statistically significant, and 1-way ANOVA with Bonferroni’s post-hoc test was used to compare three or more groups.
Table 3.3 Antibody Dilutions for Western Blotting

<table>
<thead>
<tr>
<th>Antibody Name/Target</th>
<th>Source</th>
<th>Host Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-PhosphoT202/Y204 ERK</td>
<td>Phosphosolutions®</td>
<td>Rabbit</td>
<td>1:100</td>
</tr>
<tr>
<td>pan ERK</td>
<td>BD Biosciences San Jose, CA</td>
<td>Mouse</td>
<td>1:300</td>
</tr>
<tr>
<td>Phospho-Akt (Ser473) (D9W9U)</td>
<td>Cell Signaling Technology®</td>
<td>Mouse</td>
<td>1:100</td>
</tr>
<tr>
<td>Akt Antibody</td>
<td>Cell Signaling Technology®</td>
<td>Rabbit</td>
<td>1:200</td>
</tr>
<tr>
<td>Phospho-p38 MAPK (The180/Tyr182) (D3F9) XP®</td>
<td>Cell Signaling Technology®</td>
<td>Rabbit</td>
<td>1:800</td>
</tr>
<tr>
<td>P38α/β (A-12)</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>Mouse</td>
<td>1:100</td>
</tr>
<tr>
<td>IRDye 680RD Goat anti-Mouse IgG</td>
<td>LI-COR Biosystems</td>
<td>Goat</td>
<td>1:800</td>
</tr>
<tr>
<td>IRDye 800CW Goat anti-Rabbit IgG</td>
<td>LI-COR Biosystems</td>
<td>Goat</td>
<td>1:800</td>
</tr>
</tbody>
</table>

3.3 RESULTS

3.3.1 Membrane-limited glucocorticoid initiates iGR nuclear translocation

Nuclear localization of the GR was tested in mHypoE-11 (N11) cells in response to Dex, which crosses the plasma membrane, and in response to DexBSA, which is membrane-limited (Strehl et al., 2011). Immunocytochemistry for GR was performed on fixed cells that had been treated for 20 minutes with a range of concentrations of either vehicle DMSO, Dex, or DexBSA (Figure 3.1a). For each treatment group, nuclear localization was measured as a ratio between the nuclear and cytoplasmic fluorescence intensity of GR immunoreactivity which was then normalized to the ratio from vehicle treated cells (Fig. 3.1b). As expected, Dex treatment increased the nuclear localization...
at all tested concentrations. DexBSA also nuclear localization at concentrations greater than $10^{-8}$M, to the same degree as Dex treatment despite being membrane-limited.

To confirm that free Dex is not present in our preparations of DexBSA, we tested transcriptional activation in the presence of this membrane-limited conjugate. After transfection of N11 cells with a GRE luciferase reporter, cells were treated for 1 hour with vehicle DMSO, Dex ($10^{-6}$M), or DexBSA ($10^{-7}$M) and luciferase activity assayed 23 hours later. If free Dex was present in the DexBSA solution, then it would be expected that luciferase activity increases due to GRE activation by Dex-bound GR. As expected, Dex treatment results in increased luciferase activity, but DexBSA treatment does not change luciferase activity compared to vehicle (Fig. 3.2).

To test the dependence of the observed increase in nuclear GR compared to cytoplasmic GR on transcriptional or translational mechanisms, N11 cells were also pretreated with the protein synthesis inhibitor cycloheximide and the transcriptional inhibitor actinomycin D prior to treatment with Dex and DexBSA. There was no decrease in Dex- or DexBSA-mediated iGR nuclear localization following inhibition of transcription or translation (Fig. 3.3).

### 3.3.2 HSP90 inhibition, and Akt activation inhibit GR nuclear translocation

DexBSA, despite being membrane-limited, induces GR nuclear translocation within the same timeframe as an equivalent amount of Dex, but may do so through regulation of different pathways than liganded GR. To test the dependence of DexBSA-
Figure 3.1: Dex and DexBSA treatment induce nuclear localization of GR within 20 minutes in the N11 cell line.
(a) ICC for GR localization (red), and DAPI staining to identify nuclei (blue) following 20 minutes of hormone treatment. Images were taken at 200X. Scale bar is equal to 100 microns and applies to all images.
(b) Dose response for Dex and DexBSA for 20m in N11 cells at the indicated concentrations. The nuclear to cytoplasmic ratio of GR fluorescence intensity was quantified using Cell Profiler as described in 3.2.4. Data represent mean ± SEM. *p<0.05, 1-way ANOVA with Bonferroni's post hoc test, compared to DMSO vehicle.
Figure 3.2: DexBSA does not activate GRE-induced luciferase expression in the N11 cell line. N11 cells were transfected with a GRE-induced luciferase and β-galactosidase containing plasmids, then treated with Dex (10^{-6}M), DexBSA (10^{-7}M) for 1 hour. Medium was changed and 23 hours later cells were lysed. Cell lysate was then assayed for luciferase activity, which was normalized for transfection efficiency and lysate preparation with results from a β-gal assay. Data represent mean ± SEM (n=3). *p<0.05, 1-way ANOVA with Bonferonni’s *post hoc* test, compared to DMSO vehicle.
**Figure 3.3:** Transcription and translation inhibition have no effect on Dex- or Dex BSA-mediated GR nuclear translocation. N11 cells were pretreated with 5x10^{-5}M cycloheximide (CHX), a translation inhibitor or, 5x10^{-5}M actinomycin D (ActD), a transcription for 30 minutes prior to 20 minutes of hormone treatment. The nuclear to cytoplasmic ratio of GR fluorescence intensity was quantified using Cell Profiler. Data represent mean ± SEM, normalized to the positive control (Dex-treated cells).
mediated GR nuclear translocation on the function of the chaperone HSP90, mHypoE-42 (N42) cells were treated with geldanamycin for 30 minutes prior to 20 minutes of hormone treatment. ICC for GR immunofluorescence revealed that N42 cells respond to Dex and DexBSA treatment with an increase in GR nuclear translocation that is similar to that seen using the N11 cell line, and GR nuclear translocation in response to CORT (CORT) is the same as the response to Dex (Fig. 3.4). There was also a decrease in DexBSA-mediated GR nuclear translocation in cells pre-treated with geldanamycin compared to DexBSA treatment alone and this reduction was similar to the reduction in nuclear translocation observed with concomitant geldanamycin and Dex treatment (Figure 3.4).

To test the dependence and sufficiency of kinase pathways for GR nuclear translocation cells were treated with kinase inhibitors and activators, prior to the addition of Dex and Dex-BSA. Akt, a kinase found in the PI3 kinase pathway was selected because glucocorticoids can increase PI3K activity (Limbourg et al., 2002). N42 cells were pretreated with the specific Akt agonist SC79 and the Akt inhibitor 10 DEBC*HCl for 30 minutes prior to Dex and DexBSA treatment. Inhibition of Akt had no effect on either Dex or DexBSA mediated iGR nuclear translocation, but Akt activation decreased DexBSA-mediated iGR nuclear translocation (Fig. 3.5). A similar trend was observed in Dex treated cells, although reduction in GR translocation was not significantly different between SC79 pretreated cells and Dex treated cells alone (p=0.08).
Figure 3.4: HSP90 inhibition decreases Dex and DexBSA mediated GR nuclear translocation. N42 cells were pretreated with geldanamycin an HSP90 inhibitor, 30 minutes prior to 20 minutes of hormone treatment. The nuclear to cytoplasmic ratio of GR fluorescence intensity was quantified using Cell Profiler. Data represent mean ± SEM (n=3), normalized to the positive control (Dex-treated cells). *p<0.05, t-test between indicated groups.
Figure 3.5: Akt activation decreases DexBSA mediated GR nuclear translocation. N42 cells were pretreated with SC79, an Akt activator, or 10 DEBC*HCl (DEBC) an Akit inhibitor, 30 minutes prior to 20 minutes of hormone treatment. ICC for GR was analyzed using Cell Profiler. Data represent mean ± SEM (n=3), normalized to the positive control (Dex-treated cells). *p<0.05, t-test between indicated groups.
3.3.3 MEK inhibition induces GR nuclear translocation

The ERK/MAPK pathway has also been demonstrated to be activated in response to glucocorticoid treatment (Kumamaru et al., 2008; Oppong et al., 2014). Although a pharmacological activator for MEK is not available, we used U0126, a specific MEK inhibitor, to test the dependence of GR nuclear translocation on the MAPK pathway. Pretreatment with U0126 did not affect Dex or DexBSA mediated GR nuclear translocation, but U0126 alone was sufficient to induce GR nuclear translocation to the same degree as Dex treatment (Figure 3.6).

3.3.4 Src, p38 MAPK and calcium signaling have no effect on GR translocation

The signal transduction molecules Src, p38 MAPK, and calcium were also tested for involvement in GR translocation. Pharmacological inhibition of the p38 MAPK pathway using anisomycin D treatment had no effect on iGR nuclear translocation, and neither did treatment with the specific p38 MAPK activator SB 202190 (Fig. 3.7a). Src inhibition with PP2, likewise, had no effect on translocation, and calcium chelation with the membrane permeable agent BAPTA-AM also failed to affect Dex or DexBSA-mediated iGR nuclear translocation (Fig. 3.7b).

3.3.5 ERK, Akt and p38 MAPK are not activated in response to Dex or DexBSA

Since inhibition of MEK induced iGR nuclear localization, it is possible that Dex and/or DexBSA down-regulate MEK as an initial step preceding nuclear localization of
Figure 3.6: U0126 treatment induces GR nuclear translocation. N42 cells were pretreated with U0126, a MEK inhibitor, 30 minutes prior to 20 minutes of hormone treatment.
(a) ICC for GR demonstrates nuclear localization in cells treated with U0126. Images were taken at 200X. Scale bar is equal to 30 microns and applies to all images.
(b) ICC for GR was analyzed using Cell Profiler. Data represent mean ± SEM (n=3), normalized to the positive control (Dex-treated cells). *p<0.05, 1-way ANOVA with Bonferonni’s post hoc test between indicated groups.
Figure 3.7: p38 MAPK activation and inhibition, and Src and calcium signaling have no effect on GR nuclear translocation.
(a) N42 cells were pretreated with anisomycin, a p38 MAPK activator, or SB 202190, a p38 MAPK inhibitor for 30 minutes prior to 20 minutes of hormone treatment.
(b) N42 cells were pretreated with PP2, a src inhibitor, or BAPTA-AM, (BAPTA) a cell permeable calcium chelator, for 30 minutes prior to 20 minutes of hormone treatment. ICC for GR was analyzed using Cell Profiler. Data represent mean ± SEM (n=3), normalized to the positive control (Dex-treated cells).
the iGR. To test this idea, in cell westerns (ICWs) for phosphorylated ERK 1/2 were performed on N42 cells treated with Dex or DexBSA for 10, 20, 30, 45, or 60 minutes. However, there was no evidence that either hormone treatment decreased ERK phosphorylation; instead, there was a trend towards an increase in ERK 1/2 activation with Dex and DexBSA at 10, 20, and 30 minutes of treatment, although this increase is not significant compared with vehicle (Fig. 3.8). ICWs for Akt phosphorylation (S473) and p38 MAPK phosphorylation were conducted in the same manner, and neither demonstrated any significant change in protein phosphorylation following treatment with Dex or DexBSA (Fig. 3.9).

3.3.6 Classical GR is not visible in the plasma membrane of N11 cells

To test the possibility that the mGR is classic GRα, N11 cells were transfected with a membrane-localized green fluorescent protein (mGFP). Transfected cells were then treated with either DMSO vehicle or 1 µM Dex, followed by fixation ICC for GR without permeabilization, to preserve membrane proteins, 60 minutes post hormone treatment. Confocal microscopy images show no evidence of colocalization between mGFP and GR immunoreactivity, in either vehicle or Dex treated cells (Fig. 3.10).

3.3.7 GR nuclear translocation occurs in response to Dex in primary hypothalamic cells

Primary hypothalamic cell cultures from PD1 female mice were cultured for 7 DIV, prior to treatment with DMSO, Dex (10^{-6}M), or DexBSA (10^{-7}M) for 20 minutes. In order to identify neurons in this mixed culture, Double ICC for PSD-95, a neuronal marker and for
Figure 3.8: ERK phosphorylation does not change with Dex or DexBSA treatment. N42 cells were treated with DMSO for 60 minutes or Dex or DexBSA for 10, 20, 30, 45, or 60 minutes.
(a) ICWs for phosphorylated ERK 1/2 (pERK) were done using the Odyssey system. Data represent mean ± SEM DMSO (n=3).
(b) Image from the Odyssey system showing merged pERK (green) and total ERK (red) on the left, and the pERK fluorescence signal alone on the right.
Figure 3.9: Akt and p38 MAPK phosphorylation do not change with Dex or DexBSA treatment. N42 cells were treated with DMSO for 60 minutes, or Dex or DexBSA for 10, 20, 30, 45, or 60 minutes. ICWs were done using the Odyssey system.
(a) ICW for phosphorylated Akt (Ser473) (pAkt) corrected with total Akt signal.
(b) ICW for phosphorylated p38 MAPK (pp38 MAPK) corrected with total p38 MAPK signal. Data represent mean ± SEM, normalized to DMSO (n=3).
Figure 3.10: GR on the plasma membrane cannot be detected with ICC for GR M-20. N11 cells were transfected with membrane localized GFP (mGFP) for 24 hours prior to 1 hour treatment with either DMSO or 1µM Dex. ICC was done on non-permeabilized cells for GR. The top row represents GR immunoreactivity from the TXRED channel, the middle row represents mGFP fluorescence from the FITC channel, and bottom are pseudocolored and merged. Images were taken with a confocal microscope, at 600X.
GR was performed. The majority of GR immunofluorescence was localized to the nucleus in even DMSO-treated cells, however, Dex treatment for 20 minutes increased nuclear localization of GR significantly. DexBSA treatment for 20 minutes was insufficient to induce a change in the nuclear to cytoplasmic ratio of GR immunofluorescence in PSD-95 immunoreactive female hypothalamic neurons (Fig. 3.11).

3.4 DISCUSSION

Ligand binding of the GR initiates nuclear translocation, which is the first step of the classical signaling pathway, followed by transcriptional activity of the dimerized GR through the GRE (Aranda and Pascual, 2001). However, some conditions can induce nuclear localization of the unliganded GR, including heat shock (Sanchez, 1992), and shear stress (Ji et al., 2003). Under shear stress conditions such as those experienced by aortic endothelial cells, unliganded GR can function similarly to the liganded receptor by increasing transcription of genes downstream of the GRE (Ji et al., 2003). A non-classical rapid signaling pathway, the p38 MAPK pathway, is thought to increase GR transcriptional activation from the GRE, possibly via the phosphorylation of the GR at serine 211 (pGR S211) in leukemia cell lines (Miller et al., 2005), and in osteosarcoma and lung cancer cell lines (Chen et al., 2008). However, pGR S232 in rat cortical neuron primary cultures (orthologous to human pGR S211) is less transcriptionally active (Kino et al., 2007), suggesting that the classical effects of GR phosphorylation are cell-type dependent (Galliher-Beckley et al., 2011). These studies imply that integrated signaling,
Figure 3.11: Primary hypothalamic neurons respond to Dex treatment with GR translocation. Primary hypothalamic cell cultures from female pups were obtained as described in 3.2.1. The experiment began on 7DIV. Cells were treated with DMSO, Dex ($10^{-6}$M) or DexBSA ($10^{-7}$M) for 20 minutes prior to fixation, followed by ICC for PSD-95 (to identify neurons, green) and GR (red). DAPI staining was used to identify nuclei (blue).
(a) Representative images of neurons in each treatment group.
(b) Nuclear to cytoplasmic GR fluorescence intensity. Data represent mean ± SEM (n=19 DMSO, n=20 Dex, n=19 DexBSA). *p<0.05, t-test between Dex and DMSO treated cells.
through post-translational modification of GR by phosphorylation, couples non-classical signal transduction cascades to transcription, which may be as important for GR physiology as it is for estrogen physiology (Clark et al., 2014). However, in mammary cells, nuclear localization of the unliganded GR results in involution through activation of apoptotic pathways, while liganded GR has the opposite effect of inducing growth and differentiation, resulting in lactation (Ritter and Mueller, 2014). This presents the possibility that the transcriptional activity of liganded and unliganded GR in the nucleus may determine the final cellular response to glucocorticoids, depending on the concentration of glucocorticoids available to bind GR.

3.4.1 Membrane initiated GR nuclear translocation

Our results demonstrate that, within the time-frame of hormone addition (up to 1 hour) Dex appears to remain bound to DexBSA in our DexBSA treated wells, since there is no significant activation of the GRE with DexBSA treatment. Therefore, effects of DexBSA treatment that are observed within this time-frame can be attributed to a membrane GR. Dex induces nuclear translocation at all tested concentrations; $10^{-6}$M was chosen to correlate with results from electrophysiology experiments on hypothalamic slices (Di et al., 2005). The unique result of our study is that DexBSA at concentrations greater than $10^{-8}$M was sufficient to induce nuclear localization, and this increased nuclear localization in the N42 cell line suggests that membrane-initiated actions are sufficient to induce translocation of the iGR, even in its unliganded form. Since genomic effects of GR require nuclear localization, rapid translocation may be
significant in terms of future transcriptional events. CORT, the predominant glucocorticoid in mice, has the same effect in N42 cells on nuclear translocation as Dex and DexBSA, which suggests these synthetic hormones are acting in a similar matter to the endogenous ligand *in vivo*.

Although DexBSA treatment induced nuclear localization of GR within 20 minutes in the hypothalamically-derived N11 and N42 cell lines, similar treatment of primary hypothalamic cell cultures was not sufficient for GR nuclear localization. There are a few possible explanations for this discrepancy, including the likely possibility that only certain neurons in the hypothalamus respond to DexBSA treatment, because different cells may express different amounts of mGR. The N11 and N42 cell lines were derived from embryonic hypothalamic neurons (Dalvi et al., 2011), but the hypothalamic nuclei from which they originated is unknown. Our primary hypothalamic cell cultures were generated from the entire hypothalamus of PD1 female mice, and represent all hypothalamic nuclei. A marker for a particular type of neuron may be helpful in determining which nuclei may respond to DexBSA treatment. Alternatively, longer treatment with DexBSA may be necessary to induce nuclear localization in primary cell culture as compared to immortalized cell culture. The majority of GR in our primary hypothalamic cell cultures is nuclear, even in the vehicle treated groups, which may be another reason that detecting a further increase in nuclear localization with hormone treatment is difficult.
3.4.2 MAPK inhibition induces GR nuclear localization in the absence of ligand

Our results indicate that MAPK inhibition by U0126 has similar results to Dex or DexBSA treatment, resulting in translocation of GR to the nucleus. Experiments using lung cancer cell lines have demonstrated that Dex treatment can decrease baseline ERK 1/2 phosphorylation within 24 hours (Greenberg et al., 2002); however, to be involved in Dex- or DexBSA-mediated GR nuclear translocation, ERK activation would have to occur in hypothalamic cells within 20 minutes. Our results did not show any change in phosphorylation of ERK 1/2 with treatment at any tested time point between 10 minutes and 60 minutes. In epidermal keratinocytes, a potentially similar response to MEK inhibition has been reported; U0126 pretreatment re-sensitized previously glucocorticoid insensitive cells, resulting in the increased transcription of multiple GRE regulated genes following glucocorticoid treatment (Onda et al., 2006), suggesting MEK inhibition does not interfere with GR-GRE interactions. Our data uniquely suggest that MEK inhibition, and subsequent ERK-MAPK inhibition, results in nuclear translocation of the GR in the absence of glucocorticoids. ERK/MAPK phosphorylates human GR at S203 (pGR S203) (Galliher-Beckley and Cidlowski, 2009), and pGR S203 was found to be exclusively localized to the cytoplasm, even following Dex treatment, in both osteosarcoma and lung cancer cell lines (Wang et al., 2002). Additionally, transfection of human airway smooth muscle cells with the phosphorylation mutant, GR S203A, in which the serine residue was replaced with alanine, resulted in increased GR nuclear localization and GRE-mediated transcription (Bouazza et al., 2014). Therefore, inhibition of basal ERK activation with U0126 treatment may reduce mouse pGR S212
(orthologous to human pGR S203), allowing GR nuclear localization, which may explain the rapid accumulation of GR in the nucleus following U0126 treatment.

Likewise, MAPK signaling has been implicated in GR localization in human epithelial cells, in which siRNA-mediated reduction in p38 MAPK, or treatment with a p38 MAPK inhibitor, increased GR nuclear translocation, even in the absence of a GR ligand (Bouazza et al., 2014), which suggests a decrease in classical GR signaling upon p38MAPK activation. Multiple studies have confirmed that p38 MAPK phosphorylates GR at S211 (Galliher-Beckley and Cidlowski, 2009), but p38 MAPK activation also increases GR phosphorylation at S203 in human airway smooth muscle (Bouazza et al., 2014), which may represent a common mechanism between ERK/MAPK- and p38 MAPK-mediated regulation of GR localization.

DexBSA treatment reportedly increased p38 MAPK activation in CD14+ monocytes (Strehl et al., 2011), which would appear to suggest that DexBSA treatment may inhibit nuclear localization of GR. Our data do not show evidence of p38 MAPK signaling affecting GR nuclear localization in hypothalamic neurons, suggesting that although p38 MAPK or ERK/MAPK signaling may not be a universal pathways for nuclear localization of the GR, basal activity of MAPK signaling pathways in cells might be important in determining the localization of the GR without ligand.

3.4.3 Kinase pathway involvement in GR nuclear localization

HSP90 inhibition with geldanamycin reduced the amount of Dex and DexBSA-mediated GR nuclear translocation. Studies in COS and HeLa cells have shown that
geldanamycin blocks GR-mediated transcriptional activity (Bamberger et al., 1997), and results from experiments with mouse fibroblasts have demonstrated that geldanamycin blocks GR nuclear translocation (Czar et al., 1997). Although the inhibition of Dex-mediated nuclear translocation was expected based on these published studies, the effect of HSP90 inhibition on DexBSA-mediated GR nuclear localization was not yet known. Our studies suggest that HSP90 activity is necessary for both ligand- and membrane-initiated GR nuclear translocation.

Akt activation with SC79 treatment inhibited the effects of both Dex and Dex-mediated GR nuclear translocation, although Akt inhibition had no effect. Again, these results suggest that Dex- and DexBSA-mediated GR nuclear translocation may occur through similar or overlapping pathways. Akt is part of the PI3K pathway, which can be activated at the plasma membrane by receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs) that respond to growth factors and cytokines (Liu et al., 2009). In postnatal rat primary hippocampal culture, membrane-initiated glucocorticoid signaling occurs through GPCR activation (Yang et al., 2013b). Furthermore, rapid Akt phosphorylation in response to glucocorticoids has been reported in human epithelial cells, even in the presence of a transcription inhibitor (Limbourg et al., 2002), but we were not able to detect any change in Akt phosphorylation in response to Dex or DexBSA treatment in the N42 cell line. However, glucocorticoid-mediated Akt phosphorylation occurs downstream of GPCR activation, the lack of response in our hypothalamic cell line may be due to differences in GPCR expression or association with GR between the hippocampus, in which $G_{\alpha i/o}$ signaling activates PI3K (Yang et al., 2013b).
and the hypothalamus, in which predominantly G\(_{\alpha}\)s signaling in response to glucocorticoids has been detected (Di and Tasker, 2008).

### 3.4.4 Identity of the mGR

The identity of the mGR is still unknown, although membrane localized GR immunoreactivity has been detected in lymphoma (Gametchu and Watson, 2002), breast cancer (MCF-7) cell lines (Vernocci et al., 2013), and transfected HEK 293T cells (Strehl et al., 2011). We were not able to confirm plasma membrane localization of the classic GR using the GR M20 antibody in the N11 cell line. However, studies have suggested that standard ICC is not sensitive enough to detect the small amount of GR on the plasma membrane (Vernocci et al., 2013); alternatively, GR M20 may not be an appropriate antibody to detect mGR if the epitope detected by this antibody is not available in the mGR.

### 3.4.5 Conclusion

Membrane-limited DexBSA increases nuclear localization of iGR in the N11 and N42 cell lines to the same degree as Dex or CORT treatment in 20 minutes. Although a complete molecular mechanism for this membrane-initiated, unliganded nuclear translocation is still unknown, it is transcription- and translation-independent, but dependent on HSP90. Nuclear translocation of the iGR, in response to Dex or DexBSA does not depend on ERK/MAPK, Akt, p38 MAPK, Src, calcium signaling, transcription, or translation. Nuclear localization is part of the classic GR signaling pathway, and is
necessary for GRE regulation of transcription; however, we have demonstrated that non-classical signaling, through both an unidentified mGR, and through inhibition of the ERK/MAPK pathway, can induce unliganded iGR nuclear translocation. The function of unliganded GR remains to be determined, but may represent a mechanism for finely controlling transcriptional output in response to different cellular conditions.
CHAPTER 4. INTERACTIONS BETWEEN ER AND GR ON HYPOTHALAMIC AGGRESSION AND SPINOGENESIS IN THE VMH

4.1 INTRODUCTION

Male territorial aggression is a robust response in mice between a resident mouse and a conspecific intruder male mouse that can be used as a model for normal, adaptive aggression (Koolhaas et al., 2013), and can be used as a bioassay for the actions of estrogen (Ogawa et al., 1997) and glucocorticoids (Mikics et al., 2004) in the CNS. The ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) in mice has been identified as a specific locus of aggression (Lin et al., 2011). Stimulation of glutamatergic neurons in the VMHvl can induce aggression, even against an inanimate object, and optogenetic silencing of these same neurons inhibits aggression (Lin et al., 2011).

17β-E is necessary for aggressive behavior in males, which has been demonstrated in tests using transgenic mice that lacked the gene for aromatase (Cyp19), which abolished aggression by male mice in a resident-intruder paradigm (Toda et al., 2001). Results from our lab support the necessity of estradiol signaling in aggression; the aromatase inhibitor letrozole, administered in adulthood, reduced aggression in male mice (Clark et al., 2013). Furthermore, injection of 17β-E 20 minutes prior to an aggressive bout was sufficient to restore aggression (Clark et al., 2013). This
short timeframe suggests that non-classical effects of estradiol are sufficient to restore aggression.

Glucocorticoids are also necessary for aggressive behavior in males, which has been shown by studies on rainbow trout (Schjolden et al., 2009) and in lizards (Summers et al., 2005) treated with the GR antagonist, RU486, for 30 minutes results in a reduction in territorial aggression. In male rats, CORT (CORT), the endogenous glucocorticoid in rodents, synthesis inhibition with metyrapone 20 minutes prior to the resident-intruder test reduced offensive behavior (Mikics et al., 2004). Serum CORT increases when the hypothalamic attack area, which includes the VMH, is electrically stimulated, and CORT injection in adrenalectomized rats was sufficient to restore hypothalamic aggression within 10 minutes (Kruk et al., 2004). Additionally, injection of CORT 2 minutes prior to the aggressive encounter increased aggression, even following treatment with the protein synthesis inhibitor cycloheximide (Mikics et al., 2004). These data support the necessity and sufficiency of non-classical glucocorticoid signaling for aggression, and since CORT administration has no effect on aggressive behavior within established rat colonies (Mikics et al., 2007), CORT-mediated aggression appears to be part of a normal and adaptive response.

Although the molecular mechanisms that drive aggression are not completely understood, studies with metyrapone (Mikics et al., 2004) revealed dependence on glucocorticoid signaling, and transgenic mice (Ogawa et al., 1997) and studies with aromatase inhibitors (Clark et al., 2013; Trainor et al., 2006a) have demonstrated a dependence on ligand-dependent ERα signaling, and 17β-E for normal male aggression.
However, it is currently unknown if these two steroid signaling pathways occur in parallel, or if they are dependent on each other.

Despite low serum levels of estrogens in males, 17β-E can be synthesized locally in the hypothalamus (Konkle and McCarthy, 2011) from testosterone through aromatase cytochrome P450 (Simpson et al., 2002) to yield concentrations of 17β-E in the male brain that are higher than circulating levels in females (McCarthy, 2009). Cyp19, the gene coding for aromatase, is located downstream of a GRE (Simpson et al., 2002), and in the murine hypothalamic cell line mHypo-E42 (N42), treatment with Dex for 2 hours was sufficient to increase aromatase mRNA expression (Brooks et al., 2012). 

*In vivo* studies in the male Japanese quail (*Coturnix japonica*) correlated increased circulating serum CORT to a transient increase in aromatase activity in the VMH (Dickens et al., 2011). These results suggest that CORT, through the GR, can increase aromatase activity, and therefore regulate 17β-E in the brain, although this has yet to be confirmed in rodents. Conversely, estrogen may also regulate CORT synthesis. EB treatment for 4 days in the paraventricular nucleus of the hypothalamus (PVN), increased CORT synthesis via ERα in the adult female rat adrenal cortex in response to restraint stress compared to vehicle treated rats (Weiser and Handa, 2009).

Although it is unknown if spinogenesis in the hypothalamus is associated with aggression, lordosis in female rats, which is another hypothalamically driven and estrogen-dependent social behavior, requires spinogenesis (Christensen et al., 2011). Spine density and dendrite morphology in the VMH can be influenced by 17β-E (Christensen et al., 2011; Griffin and Flanagan-Cato, 2008) in females, but the effect of
17β-E in the male VMH is unknown. The effect of glucocorticoids on spine density in the hypothalamus has not been evaluated, but glucocorticoids can induce spinogenesis in both the male rat hippocampus (Komatsuzaki et al., 2012) and the mouse cortex (Liston and Gan, 2011). We speculate that both glucocorticoids and estrogens may drive aggression in male mice through regulation of hypothalamic spine density. The focus of these studies is to determine if estrogen and glucocorticoid signaling may interact to affect dendritic spine density in the hypothalamus and male aggressive behavior toward an intruder.

We designed experiments to first test the ability of glucocorticoids to stimulate estradiol synthesis in hypothalamic cells, and on the ability of estradiol to stimulate peripheral glucocorticoid synthesis in vivo, to test for a possible positive feedback loop between the two steroid hormones. The second experiment is intended to measure aggression, using the resident-intruder paradigm, to investigate the dependence of aggression on peripheral CORT synthesis. Finally, we measured spine density in the male VMH to test for dependence on estradiol synthesis and regulation by systemic CORT administration.

4.2 METHODS

4.2.1 Primary Hypothalamic Cells from Postnatal Pups

Hypothalami were dissected from at least 3 female pups on PD1 (day of birth considered PD0) and placed in ice-cold sterile 1X Hanks’ Buffered Salt Solution (HBSS) (GIBCO®, Pittsburgh, PA). The tissue was digested in 3 ml 1X 0.25% trypsin + 2.21 mM
EDTA (CORNING, Manassas, VA) at 37°C for 15 minutes with gentle shaking. Neurobasal A (Invitrogen, Grand Island, NY) + 1% Glutamax (Invitrogen) + 2% B27 (Invitrogen) + 1% Antibiotic Antimycotic Solution (CORNING) (complete Neurobasal medium) plus 5% fetal bovine serum (FBS) (Atlas Biologicals) was added. Cells were triturred with a series of three progressively smaller in diameter fire-polished Pasteur pipets (Fisher Scientific, Pittsburgh, PA) until fully in suspension. Supernatant was plated on poly-D-lysine coated 12 mm glass coverslips, size 0 (Carolina Biological, Burlington, NC), in 24-well plates (CORNING) or in PDL-coated 96-well plates (Falcon, Tewksbury, MA) and kept at 37°C, 5% CO₂ overnight in complete Neurobasal medium, as described above, plus 33% 1X Dulbecco’s Modified Eagle Medium (DMEM) (MediaTech, Manassas, VA) + 5% FBS. Medium was removed and replaced on 2 days in vitro (DIV) with fresh complete Neurobasal medium, and cells were maintained at 37°C, 5% CO₂ for 7 DIV. Half of the medium was removed and replaced with fresh complete Neurobasal medium every other day.

4.2.2 Serum CORT and Media Estradiol Levels

Trunk blood from mice was collected immediately after rapid decapitation. Serum was isolated from coagulated whole blood that was centrifuged for 15 minutes at 2000 xg. Medium from treated primary hypothalamic neurons was collected prior to cell fixation. Both sera and media samples were assayed at the University of Virginia (UVA) Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA) for 17β-E and CORT using ELISA.
4.2.3 Drug and Hormone Treatment

Letrozole (U.S. Pharmacopeial Convention, Rockville, MD) was heated to 55°C for 48 hours until completely dissolved in distilled, deionized water that contained 2% propylene glycol (Fisher Scientific, Pittsburgh, PA) to a final concentration of 70 µM. The vehicle control was water was distilled, deionized water that contained 2% propylene glycol. Cyclodextrin-conjugated estradiol (Sigma, St. Louis, MO) was dissolved in sterile saline (VetOne, Cambridge, ON) to a final concentration of 3 mg/ml. 2 mg/ml CORT (Sigma) was dissolved in sesame oil (Sigma) that contained 10% ethanol. Water-soluble Dex (Sigma) was dissolved in sterile saline to a concentration of 0.2 mg/ml. All solutions were stored at room temperature prior to use.

4.2.4 Experimental Timelines

All experiments outlined in Figure 4.1 were conducted on adult male C57BL/6J mice (8-12 weeks; Charles River Inc), housed singly on a 12L: 12D (lights off at 11:00 AM) and allowed access to water and food ad libitum.

The choice of time-frames and drug treatments

Classical estrogen and glucocorticoid signaling through gene transcription and translation into proteins, requires a minimum of 30 to 60 minutes (Cato et al., 2002), but may continue to produce effects on protein expression for days. Therefore, any effects of estrogen or glucocorticoids that occur in less than 30 minutes must be non-classical effects. We elected to test for non-classical effects of CORT and estrogen using
a timeframe of 20 minutes to ensure that any results are from activation of the non-
classical pathways. Additionally, aggression has been shown to be increased in response
to both 17β-E in beach mice (Trainor et al., 2008) and CORT in rats (Mikics et al., 2004)
within 20 minutes, so we were confident that this timeframe would be sufficient to
measure aggression due to non-classical effects.

Letrozole was selected instead of fadrazole, which has been used in a number of
studies of aggression in rodents (Laredo et al., 2013; Trainor et al., 2006a, 2008),
because it is a more potent aromatase inhibitor (Demers, 1994). Additionally, letrozole
has been demonstrated to cross the blood brain barrier (Miyajima et al., 2010).

**Experiment A** (Fig. 4.1a) was designed to test if peripheral basal CORT levels required
the presence of 17β-E. Therefore, adult male mice were separated randomly into two
groups: a group that received vehicle control water and a second group that received
water to which had been added letrozole, as per the formulation stated above. After 2
weeks of this treatment, mice were injected with 30 µl of either vehicle saline or cE2 (3
mg/kg) subcutaneously and sacrificed rapidly using Decapicones (Fisher Scientific); the
time of sacrifice was chosen to be 2 to 4 hours after the beginning of their light cycle
(1:00 AM to 3:00 AM), at time points when baseline CORT levels are at their lowest
(Romero and Reed, 2005). Trunk blood was collected and sera obtained from this was
used to measure CORT using ELISA (See Section 4.2.2).

**Experiment B** (Fig. 4.1b) was designed to test if CORT administration could affect
neuromorphology i.e. spine density in the VMH of male mice. Adult male mice were
allowed to drink either vehicle control water or water to which letrozole had been added (See Section 4.2.3) for 2 weeks. At the end of this regimen, half the mice were subcutaneously injected with 30 µl of CORT (2 mg/kg) dissolved in sesame oil that contained 10% ethanol, as per the time-points shown in Fig.4.1b. Vehicle treated animals received 30 µl of sesame oil that contained 10% ethanol. 20 minutes after the third injection, mice were rapidly sacrificed by decapitation and brains collected for Golgi-Cox staining (See Section 4.2.5).

**Experiment C** (Fig. 4.1c) was designed to test if peripheral CORT levels was sufficient to induce aggression in male mice. Dex, a synthetic glucocorticoid specific for the GR, does not cross the blood-brain-barrier (Meijer et al., 2014), but can act on the anterior pituitary to suppress adrenocorticotropic hormone (ACTH) secretion, effectively blocking peripheral corticosteroid production (Cole et al., 2000). Dex-suppression can be used as an experimental tool to test behavior in the absence of peripheral CORT synthesis. In male mice, a dose of 200 µg/kg body weight is sufficient to completely suppress CORT production, even following stress (Bartolomucci et al., 2004). Water-soluble Dex (Sigma) was dissolved in sterile saline to a concentration of 0.2 mg/ml. 30 µl (200 µg/kg) Dex or sterile saline was injected subcutaneously 6 hours prior to behavior testing. 2 mg/ml CORT (Sigma) was dissolved in sesame oil (Sigma) plus 10% ethanol. 30 µl CORT (2 mg/kg) or sesame oil plus 10% ethanol was injected subcutaneously 20 minutes prior to behavior testing, or prior to
Figure 4.1: Timelines for hormone and drug treatments in mice.

(a) Experiment A: Mice were allowed to drink water containing letrozole, an aromatase inhibitor, or water with vehicle for 2 weeks, followed by a subcutaneous injection of vehicle saline or cE2, 20 minutes prior to sacrifice. Serum was then obtained from trunk blood and was used to assay for CORT.

(b) Experiment B: Mice were allowed to drink water with letrozole or water with vehicle for 2 weeks, followed by a series of injections of vehicle (sesame oil with 10% ethanol) or CORT at 72 hours, 24 hours, and 20 minutes prior to sacrifice. Brains were subjected to Golgi impregnation.

(c) Experiment C: All mice were subjected to the resident-intruder paradigm so that they could experience aggressive encounters. 2 weeks after this encounter, mice were injected subcutaneously with vehicle or Dex. 6 hours after Dex suppression, mice were again injected with either vehicle or CORT, 20 minutes prior to testing for aggressive behavior in a resident-intruder paradigm.
sacrifice, depending on the experiment. All mice were initially exposed to intruders in a single 15 minute resident-intruder test (See Section 4.2.7) so that there would be similar levels of aggression, regardless of treatment. Mice were then allowed to rest for 2 weeks to avoid inter-test confounds. At the end of 2 weeks, mice were injected subcutaneously with 30 µl of either Dex (200 µg/kg) or vehicle (saline) to suppress peripheral CORT levels. After 6 hours, Dex-treated mice were either injected subcutaneously with 30 µl of CORT (2 mg/kg) or vehicle, 20 minutes prior to testing for aggression. After 6 hours, vehicle-treated mice were injected subcutaneously with 30 µl of vehicle, 20 minutes prior to testing for aggression.

4.2.5 Golgi-Cox Staining

Following treatment regimens, animals were anesthetized with isoflurane and decapitated. Brains were dissected, and one hemisphere of each animal was placed in Golgi-Cox solution (FD NeuroTechnologies, Ellicott City, MD). Following a 10-14 day impregnation in Golgi-Cox solution, 100µm coronal sections were taken using a cryostat (Leica Microsystems, Chicago, IL) and mounted onto gelatin-coated slides. The section thickness allowed for the preservation of the majority of the dendritic arbor, while remaining thin enough to delineate fine processes from individual neurons. Slides were allowed to dry for 4 days in the dark, and then were developed according to the Rapid Golgi Kit’s instructions, dehydrated through a series of ethanols, cleared with xylene, and coverslipped using Permount mounting medium (Fisher Scientific).
4.2.3 Imaging for Golgi Impregnated Brains

For Golgi-Cox stained tissue, images were collected on an Olympus X71 microscope, with a Hamamatsu black and white camera, using HCImage software (Hamamatsu, Hamamatsu City, Japan). Golgi-Cox stained neurons will be imaged using brightfield microscopy at 1000x magnification, and z-stacks were obtained by manually adjusting the fine focus. Individual images were merged into stacks using ImageJ software before manually counting dendritic spines and measuring dendrite lengths (ImageJ, National Institutes of Health, Bethesda, MD) by an observer blind to experimental status.

4.2.7 Testing for Aggression Using the Resident-Intruder Paradigm

The resident-intruder paradigm is an animal model of aggressive behavior based on non-pathological territorial defense (Koolhaas and Bohus, 1989), and can be used to measure offensive aggression (Koolhaas et al., 2013). Male C57/BL 6 mice (Charles River, Inc., 10-15 weeks of age) were housed on a 12 hour light-dark schedule (lights on at 11:00 AM). Resident males were single housed and given vehicle or letrozole dissolved in water, and intruder mice were group housed an allowed to drink regular water *ad libitum*. Mice were acclimated to the testing room for 90 minutes prior to the first injection. Intruder mice were placed in the home cages of resident mice, and the interaction was recorded for 15 minutes using Elgato Video Capture software. Intruders were counterbalanced across experimental subjects so that residents never fought intruders from the same cage again.
4.2.8 Analysis of Aggressive Behavior

Interactions were scored from recordings, beginning with placement of the intruder into the resident cage, and ending 15 minutes later. The following actions were scored: attacks, bites, tail rattles, boxing, lunges, chases, sex, dominance grooming, kicks, and wrestling. An offensive bout began with any of the above behaviors and ended when the resident mouse stopped all aggressive activity for a period of at least 3 seconds (Koolhaas et al., 1980). The analyst was blind to the experimental treatment regimen.

4.2.9 Statistical Analysis

All statistical analyses were performed using Prism version 5.04 (GraphPad Software, San Diego, CA). A p-value of <0.05 was deemed statistically significant, and 1-way ANOVA with Bonferroni’s post hoc test was used to compare three or more groups. Outliers were designated as values more than 2 standard deviations from the mean, and were excluded from analyses in Experiment C.

4.3 RESULTS

4.3.1 Letrozole in drinking water is sufficient to induce weight gain in male mice

Experiment A was designed to test the requirement of 17β-E in the maintenance of baseline serum CORT levels in male mice. Although letrozole is an established aromatase inhibitor (Bhatnagar et al., 1990), most studies administer this drug by injection to rodents (Li et al., 2014), which produces a stress response that is a potential
experimental confound when investigating the role of CORT in aggressive behavior or spinogenesis. Therefore, we administered letrozole dissolved in drinking water. To test the efficacy of this novel route of administration, we measured both the amount of water consumed, and the body weight of the mice at the beginning and end of the treatment period, since letrozole treatment has been shown to increase body weight in male mice (Eshet et al., 2004). Mice on both vehicle and water containing letrozole drank the same average amount of water per day (Fig. 4.2a), but letrozole-treated mice gained more weight than vehicle-treated mice (Fig. 4.2b). This confirmed that these male mice were receiving an adequate dose of letrozole through drinking water, since similar weight gain has been reported in male mice injected with letrozole (Eshet et al., 2004).

4.3.2 Estrogen synthesis is necessary to maintain baseline CORT levels

The first link between estrogen and glucocorticoid signaling we tested was the dependence of CORT synthesis on the presence of 17β-E in male mice. In order to determine if 17β-E is required to maintain baseline levels of peripheral CORT in adult male mice, mice were treated with letrozole for 2 weeks, to completely stop endogenous 17β-E synthesis by inhibiting aromatase activity. In order to test if an injection of 17β-E could acutely regulate CORT synthesis, letrozole treated mice were injected subcutaneously with either vehicle saline or cyclodextrin-conjugated 17β-E (cE₂) 20 minutes prior to sacrifice. We chose to administer cE₂ 20 minutes prior to serum
Figure 4.2: Mice treated with letrozole drink similar amounts as vehicle, but gain more weight in a 3 week period. Male C57/BL6 mice were given either letrozole-treated or vehicle water (n=15 Vehicle, n=23 Letrozole) for 3 weeks.
(a) There was no difference in water consumption by vehicle-treated or letrozole-treated mice.
(b) Letrozole-treated mice gained more weight over a 3 week period of treatment than vehicle treated mice. Body weight was measured before and after the water treatment Data represent mean ± SEM. *p<0.05, t-test compared to vehicle treated group.
Figure 4.3: Baseline serum CORT is reduced with letrozole treatment, and partially restored with rapid cE2. Mice were allowed to drink water with either vehicle or letrozole for 2 weeks. Subsequent to this, mice were given an injection of vehicle saline (Veh+Veh groups and Let+Veh groups) or cE2 (Let+cE2) 20 minutes prior to sacrifice. Serum obtained from animals (n=5/treatment group) was assayed for CORT, as described in 4.2.4. Data represent mean ± SEM. *p<0.05, 1 way ANOVA with Bonferonni’s post hoc test compared to the Veh+Veh group.
collection to limit signaling by cE₂ to the rapid, non-classical pathways that we could separate from longer term genomic actions of the classic pathway. Mice treated with letrozole had significantly less serum CORT than control mice, and a rapid dose of cE₂ partially restored serum CORT levels (Fig. 4.3).

4.3.3 Dexamethasone increases estradiol secretion by hypothalamic cells

After establishing that 17β-E could influence baseline CORT, we next tested the effects of GR activation on 17β-E synthesis. Since Dex treatment for 48 hours has been increases aromatase protein in an immortalized hypothalamic neuronal cell line (N42) (Brooks et al., 2012), and increases aromatase activity in the male quail hypothalamus (Dickens et al., 2011), we tested if this could lead to increased 17β-E in primary hypothalamic cell cultures. Medium collected from primary hypothalamic cultures that were treated with 1µM Dex contained three times as much 17β-E as medium collected from cells treated with DMSO (Fig. 4.4).

4.3.4 Estradiol synthesis is necessary to maintain baseline dendritic spine density in the VMH

Since Dex treatment increased the concentration of 17β-E from hypothalamic cultures over 48 hours (Fig. 4.4), and 17β-E increases spinogenesis in the VMH of female postnatal mice (Schwarz et al., 2008), we speculated that CORT, the endogenous glucocorticoid in mice, may also increase spine density in the VMH of male mice in vivo. To test the ability of CORT to increase spinogenesis in an estrogen-dependent manner in
Figure 4.4: Medium from primary hypothalamic cultures treated with Dex has increased $17\beta$-E. Female primary hypothalamic cell cultures were grown for 7DIV prior to treatment with vehicle DMSO or 1 µM Dex for 48 hours. Medium was then collected and assayed for $17\beta$-E. Cultures treated with Dex had a 3-fold increase in medium estradiol compared to vehicle treated cells. Data represent mean ± SEM. *$p<0.05$ compared to DMSO vehicle treated cultures.
the VMH, adult male mice were administered letrozole in their drinking water for 2 weeks before receiving a series of injections of CORT or saline. The injection schedule (Fig. 4.1b) was designed to allow sufficient time for CORT to signal classically by transcriptionally upregulating aromatase in a GRE-dependent manner. Therefore, CORT was administered at 72 and 24 hours prior to tissue collection. However, since non-classical CORT signaling may also be critical in activation of aromatase (Dickens et al., 2011), a third injection of CORT was administered 20 minutes prior to tissue collection. Long-term treatment with letrozole significantly reduced the spine density of neurons in the VMH. However, CORT treatment alone did not significantly increase spine density, and it was not sufficient to rescue the decrease in spine density seen in the VMH of letrozole treated mice (Fig. 4.5).

### 4.3.5 Dexamethasone suppresses peripheral CORT production within 6 hours

To test the effects that blocking peripheral CORT synthesis may have on aggression, we confirmed that the timing of Dex administration and dose of Dex was sufficient to reduce serum CORT to undetectable levels. Dex suppression of peripheral CORT production was tested by subcutaneous injection of Dex (200 µg/kg) to adult male mice. At 6 hours post-injection, control mice that received an injection of saline had basal CORT levels consistent with unstressed mice (Cole et al., 2000), and mice treated with Dex had serum CORT levels below the detectable range (Fig. 4.6).
Figure 4.5: Letrozole treatment decreases spine density in the VMH of adult male mice. Mice were administered vehicle or letrozole in their drinking water for 2 weeks prior to receiving 3 injections of vehicle or CORT at 72h, 48h, and 20m prior to tissue collection. The Vehicle/Vehicle group represents mice that received vehicle in the drinking water and vehicle control injections at subsequent time points. The Vehicle/CORT group represents mice that received vehicle in the drinking water but CORT injections at subsequent time points. The letrozole/CORT group represents mice that received letrozole in the drinking water but CORT injections at subsequent time points (n=6 mice/treatment group).

(a) Brain tissue was processed using the Golgi-Cox method as described in 4.2.5, then imaged and analyzed for spine density in the VMH. Images were taken at 1000X, scale bar represents 5 microns and applies to all images.

(b) Primary dendrites emanating from VMH neurons have fewer spines per 10 microns in mice in the Letrozole/CORT group compared to those of vehicle treated mice (Vehicle/Vehicle group).

(c) Spine density is also decreased in the Letrozole/CORT group on secondary dendrites in the VMH compared to both Vehicle/Vehicle and the Vehicle/CORT groups. Data represent mean ± SEM. *p<0.05 1-way ANOVA with Bonferroni’s post hoc test compared to Vehicle/Vehicle, and #p<0.05 compared to Vehicle/CORT, treated animals.
Figure 4.6: Dexamethasone (Dex) reduces peripheral basal levels of CORT in male mice to below detectable levels. Mice were administered vehicle or letrozole in their drinking water for 2 weeks prior to receiving a dexamethasone injection, 6 hours prior to sacrifice. The Veh+Veh group represents animals that received vehicle in their drinking water for 2 weeks, prior to a vehicle injection, 6 hours before sacrifice. The Veh+Dex group represents animals that received vehicle in their drinking water for 2 weeks, prior to a Dex injection, 6 hours before sacrifice. The Let+Dex group represents animals that received letrozole in their drinking water for 2 weeks, prior to a Dex injection, 6 hours before sacrifice. Data represent mean ± SEM.
4.3.6 Dexamethasone may alter the quality of aggressive behavior

In order to establish the experience of aggressive behavior in all mice, regardless of subsequent treatments, mice were first exposed to intruders in their home cages for 15 minutes in the absence of drug or hormone treatments. To test the effects of peripheral glucocorticoids on aggression, mice were injected with either Dex (200 µg/kg) to block adrenal CORT synthesis, or vehicle saline, 6 hours prior to administration of an acute injection of vehicle oil or CORT (2 mg/kg), 20 minutes before testing for aggression in a resident-intruder paradigm that lasted for a period of 15 minutes. In Dex-treated mice, there was a trend toward an increase in total length of aggressive bouts (p=0.0512) that was not observed in Dex-treated mice that were also given CORT 20 minutes prior to testing (Fig. 4.7a). The total length of bouts that included at least one attack was increased in mice given Dex, but this increase was completely abolished when Dex treatment was followed by CORT given 20 minutes prior to testing (Fig. 4.7b). However, despite the increased amount of time that Dex-treated mice spent engaged in a bout or attack, the latency to first bout (Fig. 4.8a) and latency to first attack (Fig. 4.8b) were not different between any treatment groups.

Analysis of a number of different parameters of aggressive behavior revealed that dominance grooming, in which the resident mouse forcibly groomed the intruder mouse, showed a trend toward an increase in the Dex treated group that was abrogated by CORT administered 20 minutes prior to the behavior (Fig. 4.9a). Other agonistic behaviors, including chases, tail rattles, and the number of bites were not significantly different between treatment groups (Fig. 4.9b,c,d).
Figure 4.7: Treatment with Dexamethasone to suppress peripheral CORT levels increases aggression in male mice. Mice were given a series of 2 injections before aggression testing, one at 6 hours prior (vehicle saline or Dex) to suppress peripheral CORT, and one 20 minutes prior (vehicle oil or CORT). (n=5 mice/treatment group). (a) Mice in the Dex plus vehicle treated group trended (p=0.0516) toward an increase in total length of aggressive bouts, but this trend was lost when CORT was administered 20 minutes before testing. (b) The total length of bouts that included at least one attack is increased with Dex injection, but the effect is lost when CORT is given, 20 minutes prior to testing. Data represent mean ± SEM. *p<0.05, t-test compared to control (Veh+Veh).
Figure 4.8: Dex treatment does not affect latencies to bout or attack. Mice were given a series of 2 injections before aggression testing, one at 6 hours prior (vehicle saline or Dex) and one 20 minutes prior (vehicle oil or CORT). (n=5 mice/treatment group). Dex treatment, followed by vehicle or CORT treatment, given acutely prior to behavioral testing had no effect on (a) latency to bout or (b) latency to attack. Data represent mean ± SEM.
Figure 4.9: Dexamethasone treatment results in a trend towards an increase in dominance grooming. Mice were given a series of 2 injections before aggression testing, one at 6 hours prior (vehicle saline or Dex) and one 20 minutes prior (vehicle oil or CORT). (n=5 animals/treatment group).

(a) Dex treatment alone trended toward an increase in dominance grooming ($p=0.12$). Dex treatment, followed by vehicle or CORT, given acutely prior to behavioral testing, had no effect on (b) number of bites, (c) number of chases, or (d) number of tail rattles. Data represent mean ± SEM.
4.4 DISCUSSION

4.4.1 Positive feedback loop between estrogen and glucocorticoid signaling

Both estradiol and glucocorticoid signaling are necessary for normal male territorial aggression, and our results suggest that non-classical estrogen signaling increases peripheral basal CORT synthesis within the rapid timeframe of 20 minutes. EB administered to the PVN of adult female rats over a longer timeframe of 4 days also results in an increase in serum CORT, although this increase is following restraint stress (Weiser and Handa, 2009) and the mechanism for this increase remains unknown.

Glucocorticoids can modulate estradiol synthesis through classical regulation of aromatase protein levels in 48 hours (Brooks et al., 2012) and non-classical effects on aromatase activity in 15 minutes (Dickens et al., 2011) in the quail hypothalamus. Our results demonstrate that 48 hours of Dex treatment can increase medium estradiol from primary hypothalamic cell cultures, suggesting that glucocorticoids can increase long-term neuroestrogens in the rodent hypothalamus. Testosterone, the substrate for aromatase-mediated estradiol synthesis (Wu et al., 2009) must be synthesized by the hypothalamic cell cultures, since gonadal testosterone is not available in culture.

Hippocampal neuron cultures from adult rats synthesize estrogen de novo under serum- and steroid-free conditions (Rune and Frotscher, 2005). Our results provide evidence that murine hypothalamic cells are also able to synthesize estrogen de novo.

Together, our results suggest the existence of a positive feedback loop between estrogen and glucocorticoid signaling in the mouse. Estradiol may increase peripheral CORT synthesis, which would result in increased serum CORT concentrations that could
presumably feed back on the hypothalamus to increase estradiol synthesis and potentially loop back to signal for increased peripheral CORT synthesis in the adrenal cortex. However, confirmation of this feedback loop would require testing the ability of estradiol to increase serum CORT concentration above normal baseline levels, and that estradiol synthesis localized to the hypothalamus is sufficient to increase adrenal CORT synthesis in mice. Conclusive evidence the role of non-classical signaling in this model would require measurements of hypothalamic neuroestrogen within 30 minutes, or following cycloheximide exposure, in response to CORT treatment.

4.4.2 Systemic CORT is insufficient to increase spinogenesis in the VMH

Postnatal female mice have less hypothalamic neuroestrogen than males (Konkle and McCarthy, 2011), and have fewer dendritic spines on VMH neurons than males at birth; however, the number of spines can be increased to equal male numbers within 6 hours of estradiol treatment (Schwarz et al., 2008). Our results show a clear decrease in spine density in the VMH of adult male mice treated with the aromatase inhibitor letrozole for 2 weeks. This is consistent with the reported decrease in hippocampal spine density in both ovariectomized and gonadally intact adult female mice treated with letrozole (Zhou et al., 2010). Our data provide evidence, for the first time, that 17β-E plays a role in maintaining spine density in the VMH of male mice. Additionally, letrozole treatment, while blocking endogenous estrogen synthesis, also results in increased serum testosterone and growth hormone (Eshet et al., 2004). While the increased testosterone in the absence of estrogen does not affect male territorial
aggression (Trainor et al., 2006b), its effects on spinogenesis in the VMH have not been tested. However, testosterone has been reported to increase spinogenesis in the hippocampus of adult male rats (Hatanaka et al., 2014), so it is unlikely that testosterone is responsible for the letrozole-induced decrease in spine density in the VMH. Growth hormone deficiency results in decreased male aggression (Sagazio et al., 2011), but the effects of increased growth hormone on aggression and spinogenesis are unknown.

Despite the fact that our primary hypothalamic cell cultures responded to Dex treatment with an increase in aromatase activity, as measured by an increase in estradiol production, and the fact that estradiol treatment has been shown to increase spinogenesis in the VMH (Schwarz et al., 2008), our in vivo experiment failed to demonstrate an increase in spine density in the VMH following systemic CORT treatment. There are several possible reasons for this discrepancy. First, spines in the male VMH may be at maximal density in gonadally intact mice, and, therefore, CORT is unable to further increase spinogenesis. In support of this argument, much of the work on spinogenesis has been conducted in female rodents in which neuroestrogen concentrations are lower than in males (Konkle and McCarthy, 2011), and spine density in untreated animals is also lower than in males (Rune and Frotscher, 2005; Schwarz et al., 2008). Treatment with estradiol increases spinogenesis in these female animals, but only to increase total spine number equal to that in untreated males (Schwarz et al., 2008). Letrozole treatment, as expected, reduced spine density in the VMH of male mice in our study, and since CORT treatment alone did not restore spine density to
vehicle levels, it is possible that either the dose or time-course of CORT treatment was not sufficient to stimulate the concentration or duration of neuroestrogen in the VMH necessary for spinogenesis. Alternatively, it is also possible that the in vitro effects of GR activation on aromatase are not present in vivo, or that adult male mice respond differently than the postnatal female mice used for primary hypothalamic culture.

4.4.3 Dex-mediated changes in aggression

Dex, while completely blocking peripheral CORT production through inhibition of ACTH release from the anterior pituitary (Babic et al., 2014), has independent effects on aggression (Brain et al., 1971) that are not typical of those reported with metyrapone, a CORT synthesis inhibitor, which results in decreased aggression (Kruk et al., 2004). One possible explanation for the difference between the metyrapone-mediated effect and the Dex-mediated effect on aggression is that metyrapone, which is able to cross the blood brain barrier, unlike Dex (Meijer et al., 2014), would inhibit any de novo local CORT synthesis in the hypothalamus. Since intracerebroventricular injection of CORT increases aggression in adult male rats (Mikics et al., 2004), a Dex-mediated increase in central CORT could be responsible for the increased aggression in Dex-treated male mice. However, the decrease in peripheral CORT resulting from Dex suppression at the pituitary of ACTH removes negative feedback of CORT on hypothalamic neurons (Cole et al., 2000), which could result in an increase in local hypothalamic CORT synthesis following Dex treatment.
Dex treatment has also been correlated with increased aggression in mature male mice (Brain et al., 1971), possibly from increased central corticotropin releasing hormone (CRH) due to loss of negative feedback by circulating CORT (Bartolomucci et al., 2004) that results from Dex suppression of adrenal CORT synthesis. In male Syrian hamsters, treatment with a CRF1 receptor antagonist, SSR125543A, reduces aggression (Farrokhi et al., 2004), CRH infused directly into the amygdala of male rats resulted in increased aggression (Elkabir et al., 1990), which supports the possibility that the increase in aggression seen with Dex treatment in mice is mediated by an increase in central CRH. However, knockout of the CRH receptor CRFR-1 in mice, results in no deficits in intermale aggression (Gammie and Stevenson, 2006), which suggests that CRH may be sufficient, but is not necessary for normal male aggression.

Interestingly, the Dex-mediated effects on aggression in our mice were abolished by subsequent CORT treatment. Dex-treatment increased the total time mice spent engaged in aggressive bouts that included at least one attack, and increased the number of bouts that included dominance grooming. However, subsequent CORT treatment 20 minutes prior to behavior testing completely abolished both aggressive behaviors in Dex treated mice, and returned aggression back to the same levels as vehicle treated mice. It is possible that if the increase in Dex-mediated aggression is via increased hypothalamic CRH, then CORT treatment could restore the negative feedback on hypothalamic neurons, and return CRH to normal concentrations, which may explain the loss of increased aggression we see in our CORT treated mice.
4.4.4 Conclusion

Crosstalk between estrogen and glucocorticoid signaling pathways has the potential to influence both aggression and spinogenesis in the VMH through regulation of neuroestrogen and peripheral CORT synthesis. A loss of estradiol signaling reduces basal peripheral CORT, but acute treatment with 17β-E can rapidly increase peripheral CORT. Glucocorticoid signaling also increases estrogen synthesis in hypothalamic cells, possibly through a classical transcriptional increase in aromatase protein, and a non-classical, rapid, increase in aromatase activity. Together, these data suggest a positive feedback loop between non-classical estrogen signaling and both classical and non-classical glucocorticoid signaling. Our results also demonstrate, for the first time, that basal estradiol synthesis, through aromatase activity, is necessary to maintain normal spine density in the adult male VMH. CORT treatment alone was not sufficient to increase spine density in the VMH of adult male mice, or to restore the spine loss induced by letrozole treatment.

Although peripheral CORT synthesis is completely inhibited by Dex suppression, Dex increased the total time spent engaged in an aggressive bout in the resident-intruder paradigm. However, CORT administered peripherally 20 minutes prior to testing was sufficient completely inhibit the Dex effect, suggesting that Dex has independent actions on aggression that are not due to inhibition of peripheral CORT synthesis.
CHAPTER 5. SUMMARY OF FINDINGS

Non-classical signaling can be initiated by the classical receptors ERα, ERβ, and GR, or by membrane receptors, such as mGR, mERα, and GPR30, or variant nuclear receptors, including ERα-36 and ERα-52. These receptors can rapidly activate kinase signaling cascades, such as ERK/MAPK, p38 MAPK, and PI3K, resulting in and regulation of transcription, translation, and post-translational modifications of proteins. Non-classical signaling can be coupled to classical signaling through direct phosphorylation of classical nuclear receptors, which modulates the transcriptional effects of the classical pathway. Hence, nuclear receptor non-classical signaling is diverse, and plays a crucial role in determining the net cellular response to hormone exposure. The results of these studies demonstrate the importance of non-classical signaling, and provide evidence of interactions between two steroid hormone signaling pathways.

Non-classical signaling through GPR30 increases expression of the spine marker PSD-95 in primary hypothalamic neurons from postnatal male mice. We have demonstrated that in addition to ERα and GPR30, the variant ERα-36 is expressed in the somas of hypothalamic neurons cultured from postnatal male pups. For the first time, we show that selective activation of mERs or the GPR30 increases PSD-95 immunoreactivity, in an ERK/MAPK dependent manner, in the male hypothalamus.
The non-classical and classical signaling pathways converge to induce GR nuclear translocation. Membrane-limited DexBSA increases nuclear localization of iGR in the N11 and N42 cells lines to the same degree as Dex treatment in 20 minutes. This membrane initiated signal occurs within 20 minutes, and is transcription- and translation-independent, but dependent on chaperone protein HSP90 function. Nuclear translocation of the iGR, in response to Dex or DexBSA does not depend on ERK/MAPK, Akt, p38 MAPK, Src, calcium signaling, transcription, or translation. Nuclear localization is part of the classic GR signaling pathway, and is necessary for GRE regulation of transcription; however, we have demonstrated that non-classical signaling, through both an unidentified mGR, and through inhibition of the ERK/MAPK pathway, can induce unliganded iGR nuclear translocation. The function of unliganded GR remains to be determined, but may represent a mechanism for finely controlling transcriptional output in response to different cellular conditions.

In addition to the interactions between classical and non-classical pathways, steroid hormone signaling can affect other hormone responses. Crosstalk between estrogen and glucocorticoid signaling pathways has the potential to influence both aggression and spinogenesis in the VMH through regulation of neuroestrogen and peripheral CORT synthesis. A loss of estradiol signaling reduces basal peripheral CORT, but acute treatment with 17β-E can rapidly increase peripheral CORT. Glucocorticoid signaling also increases estrogen synthesis in hypothalamic cells, possibly through a classical transcriptional increase in aromatase protein, and a non-classical, rapid, increase in aromatase activity. Together, these data suggest a positive feedback loop
between non-classical estrogen signaling and both classical and non-classical glucocorticoid signaling. Our results also demonstrate, for the first time, that estradiol synthesis, through aromatase activity, is necessary to maintain normal spine density in the adult male VMH. CORT treatment alone was not sufficient to increase spine density in the VMH of adult male mice, or to restore the spine loss induced by letrozole treatment.

Dex signaling, which completely suppressed peripheral CORT synthesis, altered the aggressive response of male mice by increasing the total time spent engaged in an aggressive bout in the resident-intruder paradigm. CORT, administered peripherally 20 minutes prior to testing, was sufficient to reduce time spent in an aggressive bout back to the same time as vehicle treated mice, suggesting that rapid, non-classical GR signaling can inhibit the effects of Dex-mediated GR activation. It is likely that Dex-mediated GR activation is limited to the periphery, including the anterior pituitary, and CORT-mediated non-classical GR signaling occurs through both peripheral and centrally located GR.

In conclusion, we propose, based on the results of these studies, that classical and non-classical estrogen and glucocorticoid signaling pathways interact to influence spinogenesis in the VMH and to regulate hypothalamically-driven aggression in male mice (Fig. 5.1). Further research into the detailed molecular mechanisms of these signaling pathways will provide important insight into the role of non-classical hormone action on synaptic organization and, ultimately, on the regulation of social behaviors.
Figure 5.1: Summary model of interactions between estrogen and glucocorticoid signaling in the hypothalamus. Classical and non-classical signaling through both estrogen and glucocorticoid receptors interact to contribute to spinogenesis in the VMH and male territorial aggression in mice.
BIOGRAPHY

Jennifer Renée Rainville was born July 22, 1982 in Rome, New York to Martha Trim Rainville and Norman George Rainville. She has two younger brothers, Nicholas Gene and Alex Bradley. She lived in Minnesota until the age of 6, then she spent the remainder of her childhood in St. Albans, VT. She graduated from Bellow’s Free Academy in 2000, then attended St. Olaf College in Northfield, MN, where she received a Bachelor of Arts, with Major in Biology and a Concentration in Biomedical Studies, in 2004. After attending the University of Vermont College of Medicine for one year, she withdrew to pursue a career in teaching. She taught middle school science at Arden Cahill Academy in Gretna, LA, before returning to school at Tulane University, where she earned a Master of Science in Cell and Molecular Biology in 2010. She entered the Ph.D. program in Cell and Molecular Biology in the fall of 2010, where she joined the lab of Dr. Nandini Vasudevan.


