DISSERTATION

ESTROGEN RECEPTORS ALPHA AND BETA: OPPOSING ROLES IN HYPOTHALAMIC-PITUITARY-ADRENAL AXIS FUNCTION AND STRESS-RELATED BEHAVIORS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY MICHAEL JAMES WEISER ENTITLED: ESTROGEN RECEPTORS ALPHA AND BETA: OPPOSING ROLES IN HYPOTHALAMIC-PITUITARY-ADRENAL AXIS FUNCTION AND STRESS-RELATED BEHAVIORS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ESTROGEN RECEPTORS ALPHA AND BETA: OPPOSING ROLES IN HYPOTHALAMIC-PITUITARY-ADRENAL AXIS FUNCTION AND STRESS-RELATED BEHAVIORS

Estradiol has reported effects on mood ranging from anxiogenic to anxiolytic and depressant to anti-depressant. These opposing actions of estradiol may be explained by the existence of two distinct estrogen receptor (ER) systems, ER alpha (ERα) and ER beta (ERβ). Furthermore, there exists a sex difference in stress-related psychiatric disorders such as anxiety and depression, for which women are more susceptible than men. Common to the pathology of these disorders is a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis where glucocorticoid negative feedback is impaired leading to chronically high levels of circulating glucocorticoids.

The HPA axis is the main neuroendocrine axis that governs physiological responses to stressors. In rodents, basal and stress-induced activity of the HPA axis is higher in females than in males. This suggests that, if transferable to humans, the sex difference observed in HPA axis function in animal models may help explain the female predisposition for certain psychiatric disorders.

The studies described in this dissertation were aimed at characterizing the distinct roles for ERα and ERβ in HPA axis activity and stress-related behaviors. The studies in Chapter 3 examine the effect of estradiol signaling through ERα or ERβ on glucocorticoid negative feedback of the HPA axis. Results indicate that estradiol impairs glucocorticoid-dependent negative feedback by activating ERα specifically at the level of the paraventricular nucleus (PVN). The studies in Chapter 4 examine the effect of estradiol signaling through ERα or ERβ on anxiety-like and depressive-like behaviors. Results indicate that selective activation of ERα is anxiogenic and depressant, whereas selective activation of ERβ is anxiolytic and antidepressant. Finally, the studies in Chapter 5 examine the effect of estradiol signaling through ERβ on behavior and HPA axis activity induced by glucocorticoid receptor (GR) activation in the central nucleus of the amygdala (CeA). Results indicate that delivery of a GR agonist to the CeA is anxiogenic and augments the HPA axis response to a stressor, and peripheral administration of an ERβ agonist blocks this effect. Collectively, these studies point to an antagonistic relationship between estradiol signaling through ERα and ERβ with respect to HPA axis activity and stress-related behaviors.

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CHAPTER 1

General Introduction

When presented with a real or perceived threat to homeostasis, an animal responds through activation of the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis to maintain homeostasis (for reviews see (McEwen 1998; Swaab, Bao et al. 2005)). Stressor-specific information is relayed from limbic, hypothalamic, and brainstem regions of the central nervous system to the paraventricular nucleus (PVN) of the hypothalamus. This stress-related afferent information is integrated by a small group of neurons in the parvocellular subregion of the PVN. The phenotype of these neurons is characterized by their ability to synthesize and secrete corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). Once stimulated, these neurons release their neuropeptide into the hypophyseal portal system. CRH and AVP then circulate to the anterior pituitary and stimulate corticotrophs to synthesize and secrete adrenocorticotropin hormone (ACTH) into the general circulation. ACTH, in turn, stimulates glucocorticoid synthesis and release from the adrenal cortex. Consequently, the main result of HPA axis activation is the release of glucocorticoids (corticosterone (CORT) in rodents, and cortisol in humans) which then act to mobilize energy stores, induce lipolysis and proteolysis, aid in vasoconstriction, and alter

behavior in an effort to prepare for a potential insult to homeostasis (Munck, Guyre et al. 1984; McEwen and Stellar 1993; Sapolsky, Romero et al. 2000).

Acute activation of the HPA axis is beneficial, including enhancements in learning and memory, immune function, and metabolism. Chronic activation, on the other hand, leads to harmful effects on immune, metabolic, cardiovascular, and brain function (for review see (McEwen 1998)). Therefore, the HPA axis is governed by a tight negative feedback system whereby glucocorticoids act to inhibit further activation through actions at the adrenal gland, anterior pituitary, hypothalamus, and other stress-sensitive brain regions such as the hippocampus (McEwen 1998). In regards to the HPA axis, one of the main effect of elevated glucocorticoids is to decrease the synthesis and release of CRH, AVP, and ACTH (Jingami, Matsukura et al. 1985).

In humans, dysregulation of HPA axis function is a common feature of stress-related psychological disorders such as anxiety and depression. Depressed patients exhibit an impaired ability to respond to a stressor with negative feedback inhibition of the HPA axis resulting in elevated CRH and AVP levels in the PVN, and increased cortisol secretion (Swaab, Bao et al. 2005; Bao, Meynen et al. 2008). This is demonstrated, in depressed individuals, by the inability of the synthetic glucocorticoid dexamethasone (DEX) to suppress the stimulatory actions of CRH administration (Holsboer, Liebl et al. 1982; Arana, Baldessarini et al. 1985; Heuser, Yassouridis et al. 1994). Impaired glucocorticoid-dependent negative feedback leads to higher endogenous glucocorticoid levels and further exacerbates the pathologies associated with depression.

Importantly, epidemiological studies in humans indicate a higher incidence of stress-related psychiatric disorders in women than in men (Weissman, Bland et al. 1993). This discrepancy appears following puberty, suggesting that gonadal steroid hormones might be in part responsible for these sex differences (Angold and Worthman 1993). Interestingly, estradiol is reported to have positive and negative effects on mood. This may be a consequence of two main receptor systems for estradiol, estrogen receptor alpha (ERα) and beta (ERβ). Indeed, animal studies support an anxiogenic and depressant effect of ERα activation, yet an anxiolytic and antidepressant effect of ERβ activation (Walf, Rhodes et al. 2004; Lund, Rovis et al. 2005; Walf and Frye 2005; Hughes, Liu et al. 2008). These results are in agreement with experimental data from transgenic animals in which ERβ knockout animals (βERKOs) display increased anxiety and learned helplessness (Krezel, Dupont et al. 2001; Imwalle, Gustafsson et al. 2005; Rocha, Fleischer et al. 2005).

In rodents, there is also a well-known sex difference in stress reactivity, an effect that is largely due to activational effects of gonadal hormones. Gonad intact female rats have higher basal and stress-induced CORT secretion than gonad intact male rats, a phenomenon that develops after puberty (Viau, Bingham et al. 2005). Additionally, removal of endogenous gonadal hormones via gonadectomy (GDX) results in similar basal and stress-induced CORT secretions in males and females (Viau, Bingham et al. 2005), and treatment of GDX males with estradiol augments stress-induced neuropeptide expression in the PVN as well as increasing plasma CORT levels (Lund, Munson et al. 2004). Estradiol's overall effect on HPA axis activity may be in part due to

impairment in glucocorticoid receptor function thereby impairing glucocorticoid negative feedback (Turner 1990; Burgess and Handa 1992; Turner 1992; Burgess and Handa 1993). If a similar effect is seen in humans, the estradiol-mediated impairment in glucocorticoid-dependent negative feedback may lead to or exacerbate the reported sex differences in stress-related psychological diseases such as depression and anxiety.

Similar to results that are found in behavioral experiments, ERα and ERβ have opposing effects on HPA activity. Peripheral administration of an ERα agonist augments, whereas administration of an ERβ agonist dampens, stress-induced CORT secretion (Lund, Rovis et al. 2005). Furthermore, local delivery of estradiol or an ERα agonist to the PVN increases stress-induced plasma CORT (Lund, Hinds et al. 2006). Conversely, local administration of an ERβ agonist to the PVN decreases stress-induced plasma CORT. These data implicate the PVN region as a main integration hub for estradiol effects on HPA axis activity. Additionally, these data demonstrate contrasting effects of ERα and ERβ activation in the PVN region, where it appears that estradiol's major effects are through the actions of ERα.

Stress-related afferent neuronal input is integrated at the level of the PVN and surrounding region (peri-PVN) (Herman, Tasker et al. 2002). Its proximity to the third ventricle and robust expression of glucocorticoid receptors enable the PVN to weigh stress-related neuronal input with the current endogenous glucocorticoid status. These attributes of the PVN and the aforementioned data from previous studies led to the *hypothesis that estradiol acts via ERα to augment HPA axis activity by releasing the PVN from glucocorticoid-dependent negative feedback constraint*.

In the first research chapter, Chapter 3, I report the effects of estradiol on glucocorticoid negative feedback during the diurnal rise in plasma CORT, and following a psychological stressor (restraint). I utilized the dexamethasone suppression test (DST) in order to determine glucocorticoid negative feedback sensitivity. The DST is a widely used neuroendocrine test that is an effective predictor of depression in humans (Carroll and Curtis 1976), and has been used in rodents as a model for the human test (Lurie, Kuhn et al. 1989). In the DST, the ability of a synthetic glucocorticoid, dexamethasone (DEX), to suppress endogenous glucocorticoid secretion is determined and provides an indication of negative feedback sensitivity of the HPA axis. In the first experiment, young adult ovariectomized (OVX'd) female rats were treated systemically with estradiol benzoate (EB) and subjected to a DST. Indeed, EB impairs DEX suppression of plasma ACTH and CORT at the diurnal peak in secretion and following a restraint stress. Subsequently, to determine if estradiol impairs negative feedback at the level of the PVN, I implanted beeswax pellets containing estradiol, an ERβ agonist, or an ERα agonist to an area just above the PVN via stereotaxic surgery. Results show that local delivery of estradiol or an ERα agonist to the PVN region impairs dexamethasone suppression of plasma ACTH and CORT at the diurnal peak in secretion and following a restraint stress. Conversely, local delivery of an ERβ agonist attenuates diurnal peak and stress-induced plasma levels of ACTH and CORT, and does not impair dexamethasone suppression. These data show that estradiol impairs glucocorticoid dependent negative feedback, and that this occurs through the activation of ERα at the level of the PVN.

The results of Chapter 3 led to the next question: do these receptor subtype specific effects on HPA axis activity translate into behavioral changes? Chronically elevated glucocorticoid levels and a hyperactive HPA axis are associated with psychiatric disorders such as depression and anxiety (Dinan 1994; McEwen 2005; Swaab, Bao et al. 2005). Therefore, in Chapter 4, I report on the effect of various ERα- and ERβ-specific agonists on anxiety-type and despair behaviors in a battery of behavioral platforms. Prior to designing these experiments I surveyed potential ERα and ERβ agonists. The development of ER subtype selective high affinity agonists has provided valuable insight into the biological actions of each receptor (Veeneman 2005; Harris 2007). These ligands include PPT (ERα agonist), DPN and WAY-200070 (ERβ agonists), MPP (ERα antagonist), PHTPP (ERβ antagonist) and plant derived phytoestrogens like genistein, coumesterol, and equol that all exhibit some selectivity for ERβ. DPN is currently the ERβ agonist of choice for *in vitro* and *in vivo* studies. It exists as a racemic mixture of two enantiomers, *S*-DPN and *R*-DPN. Molecular modeling predicts that the *S*-enantiomer is the active form as indicated by a lower theoretical energy state of the *S*-DPN-bound receptor (Sun, Baudry et al. 2003). These data led to the *hypothesis that S-DPN is the biologically active form of the ERβ agonist DPN*.

Results from Chapter 4 establish that recombinant rat ERβ has higher affinity for *S*-DPN than *R*-DPN *in vitro*. Furthermore, agonist treatment of the hypothalamic cell line N-38 transfected with an estrogen response element (ERE)–luciferase promoter reporter construct and an ERβ expression vector reveals that *S*-DPN is a potent activator of ERβ-dependent transcription, whereas *R*-DPN is not. Subsequently, I examined

anxiety-like behaviors using the open field test (OFT) and the elevated plus maze (EPM), or depressive-like behaviors using the forced swim test (FST). Behaviors in the OFT and EPM that are inhibited with typical anti-anxiety drugs like benzodiazepines (BZD) are considered anxiogenic (Time spent next to walls (OFT), time spend in closed arms (EPM), grooming and defecating), and those that are increased with BZD treatment are considered anxiolytic (OFT – time in middle squares, novel item interactions; EPM – open arm entries, time in open arms, rearing, head dips over edge of open arm). Behaviors in the forced swim test that are inhibited with antidepressant drug treatment are considered depressant (time spent immobile in the water), and those that are increased are considered antidepressant (time spent struggling). In these experiments, OVX'd young adult female rats were treated with the ERβ agonists racemic DPN, *S*-DPN, *R*-DPN, and WAY-200070, and the ERα agonist PPT. Rats treated with racemic DPN, *S*-DPN, and WAY-200070 show decreased anxiety-like behaviors in the OFT and the EPM, and decreased depressive-like behaviors in the FST. In concordance with the calculated RBA and transcriptional activity, these results demonstrate that the *S*-enantiomer is the biologically active form of DPN. These studies also indicate, through the use of multiple ERβ-specific ligands, that estrogen's positive effects on mood, including its anxiolytic and anti-depressive actions, are likely due to its actions at ERβ and raise the possibility that selective ERβ agonists can be used in the treatment of mood disorders such as anxiety.

The combined results of Chapters 3 and 4 led to the question: what is the mechanism whereby ERβ activation influences anxiety-type behaviors? Often

associated with stress-related mood disorders is a chronically elevated level of endogenous glucocorticoids. Data from Chapter 3 clearly indicates opposing roles of ERα and ERβ activation on endogenous glucocorticoid levels. The central nucleus of the amygdala (CeA) is a glucocorticoid responsive brain region that is integral to the initiation of fear and anxiety-type behaviors (Davis 1992; Kopchia, Altman et al. 1992; Makino, Gold et al. 1994; Davis 1997). Application of CORT directly to the CeA potentiates anxiety-type behaviors (Shepard, Barron et al. 2000; Shepard, Barron et al. 2003; Myers, Gibson et al. 2005). The cellular actions of CORT are mediated by a two receptor system, high affinity mineralocorticoid receptors (MR), or low(er) affinity glucocorticoid receptors (GR) (Reul and de Kloet 1985). Basal state levels of glucocorticoids occupy primarily MR, whereas elevated glucocorticoid levels result in MR and GR occupation. The CeA has a high level of GR expression and accordingly responds to acutely or chronically elevated CORT (Reul and de Kloet 1985; Swanson and Simmons 1989; Makino, Gold et al. 1994; Watts and Sanchez-Watts 1995; Morimoto, Morita et al. 1996). Furthermore, the CeA expresses ERβ, albeit at a low level (Laflamme, Nappi et al. 1998; Osterlund, Kuiper et al. 1998; Shughrue and Merchenthaler 2001), and has direct projections to the PVN (Marcilhac and Siaud 1997; Palkovits, Young et al. 1998), an area of high ERβ expression. These data led to the *hypothesis that GR activation in the CeA leads to increased anxiety-type behaviors and stress-induced plasma CORT levels, AND that administration of an ERβ agonist can block these effects*.

In the last research chapter, Chapter 5, the effect of ERβ activation on GR mediated behavior was examined. OVX'd young adult female rats were implanted with a beeswax pellet containing the GR agonist RU28362 to an area just above the CeA via stereotaxic surgery and treated peripherally with the ERβ agonist *S*-DPN or vehicle. Results show that GR activation in the CeA increases anxiety-type behaviors on the elevated plus maze and post-EPM plasma CORT levels as compared to controls (blank pellet). Furthermore, peripheral treatment with *S*-DPN blocks this effect and is anxiolytic despite activation of GR in the CeA. This suggests that ERβ may act via modulation of CeA-specific signaling thus altering both behavior and neuroendocrine (HPA axis) outcomes of psychological stressors. Furthermore, ERβ may act to block the enhancing effects of elevated glucocorticoids on fear and anxiety.

The data presented in this dissertation contributes to the understanding of estrogen-mediated signaling on HPA axis function and behavior, particularly though the classic intracellular estrogen receptors, ERα and ERβ. Collectively, these studies point to an antagonistic relationship between estradiol signaling through ERα and ERβ with respect to HPA axis activity and stress-related behaviors. Imbalances in the ERα-to-ERβ signaling ratio may contribute to the neuropathology associated with stress-related psychiatric disorders. Dissecting the individual contributions of ERα and ERβ with respect to HPA axis activity and anxiety- or depressive-type behaviors will provide insight into how fluctuating levels of estrogen, or an abnormal receptor balance, may contribute to stress-related pathologies.

CHAPTER TWO

Literature Review

1. Overview of steroid hormones and actions

Gonadal steroids, the main secretory product of the ovary and testis, possess potent and diverse biological actions. Their chief physiological function is in the development and maintenance of primary (ovaries, uterus, vagina, testicles, prostate, penis, etc) and secondary sex characteristics (voice, body hair, muscle mass, breast, adipose distribution, etc.). Beyond the role of sex hormones in reproductive behavior there is an ever-growing body of evidence indicating their powerful influences on other aspects of brain function. This includes effects on sexual differentiation, cell death, neurogenesis, cell migration, synaptic transmission, neuronal morphology, cognition, behavior (mood, aggression), and psycopathologies (depression, anxiety, post traumatic stress disorder). These complex effects involve genomic and non-genomic mechanisms of altering intracellular (gene transcription, protein expression), intercellular (synaptic transmission), systemic (hormonal), and organismal (behaviors) processes. Early studies examined influences on brain regions and behaviors integral to sexual behavior and sexual competency. This focus has broadened significantly, and it is clear now that gonadal steroids have extensive effects on non-reproductive brain regions and behavior as well (for reviews see (Rubinow and Schmidt 1996; McEwen and Alves 1999)).

Sex steroids are primarily produced by the gonads (testes and ovaries), however synthesis also occurs in the adrenal gland, and locally within certain tissues (adipose, liver, brain). The two main classes of sex steroids are androgens and estrogens. Androgens include testosterone, androstenedione, dihydrotestosterone, and dehydroepiandrosterone. Testosterone is synthesized by the leydig cells of the testis and in small amounts by the thecal cells of the ovary and the adrenal cortex. Testosterone is derived indirectly from cholesterol, as are all steroids, and is readily reduced via 5α-reductase to dihydrotestosterone, the most biologically potent androgen (Figure 2.1). Estrogens include estradiol, estratriol, and estrone. Estradiol is produced by granulosa cells in the ovary (stimulated by follicle stimulating hormone and luteinizing hormone), and in smaller amounts by the sertoli cells of the testis, adrenal gland and breast. As with androgens, local *de novo* synthesis of estradiol may occur within certain tissues (adipose, liver, brain) by steroidogenesis or by aromatization of testosterone to estradiol via aromatase (Figure 2.1). Concentrations of sex hormones at a given tissue are dependent up on synthesis, secretion, percentage bound to sex hormone binding globulin (SHBG), and the degree of local *de novo* synthesis or enzyme activity.

Sex hormone actions can be classified into two broad categories, organizational and activational. Organizational effects are those that permanently influence sexual differentiation of the animal and are dependent upon hormone exposure during development. Activational effects are generally not permanent and require sustained exposure to hormone to maintain. There exist "critical periods" in development, where

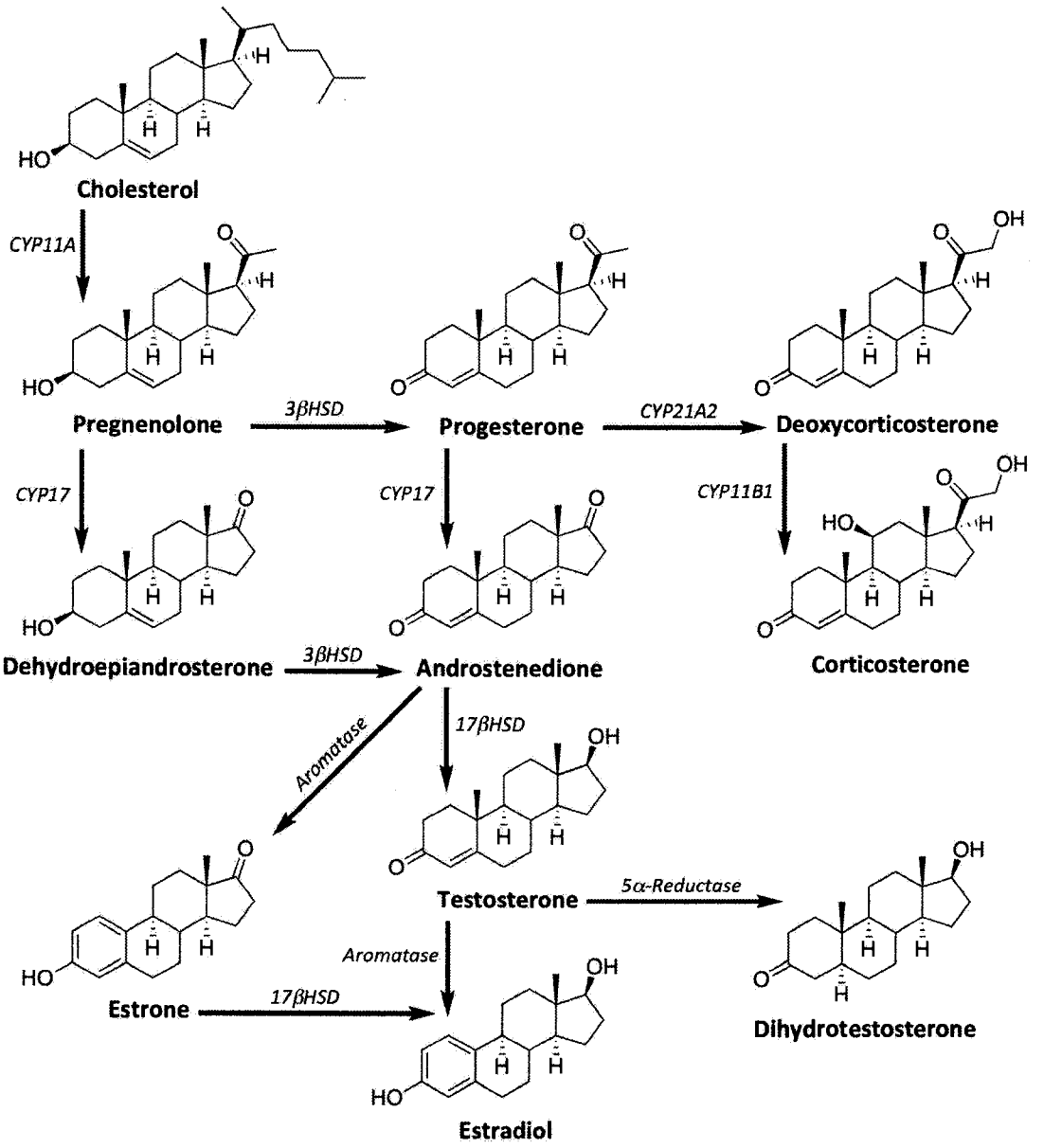


Figure 2.1. Schematic diagram of the major pathways in steroid biosynthesis from the common precursor cholesterol. Steroid hormone common name is provided in bold face under corresponding chemical structure and enzyme responsible for conversion is provided in italic face alongside the arrow depicting direction of reaction. 3α-HSD, 3alpha hydroxysteroid dehydrogenase; 3β-HSD, 3beta hydroxysteroid dehydrogenase; 17β-HSD, 17beta hydroxysteroid dehydrogenase; CYP11A, cholesterol side-chain cleavage enzyme; CYP11B1, 11beta-hydroxylase; CYP17, 17alpha-hydroxylase; CYP21A2, 21alpha-hydroxylase.

exposure to sex hormones lead to long-lasting changes in brain structure and circuitry (Cooke et al 1998, MacLusky and Naftolin 1981). For example, Phoenix and coworkers found that female guinea pigs treated prenatally with testosterone have increased male sexual behavior, and decreased female sexual behavior (Phoenix, Goy et al. 1959). Furthermore, other initial studies indicated that treatment of early postnatal female mice with testosterone could inhibit the activation of ovulation by estradiol later in adulthood (Barraclough and Leathem 1954), indicating that activational and organizational sex steroid effects are interdependent upon each other. The work detailed in this dissertation examines activational sex steroid effects on brain function, however the contribution of organizational effects to sex differences must be considered when exploring activational effects.

2. History of sex hormones and neuroendocrinology

Sex hormones

In 1893, the neurophysiologist and physician Charles Edward Brown-Sequard described his discovery that glands "give to the blood, by an internal secretion, principles which are of great importance" (Brown-Sequard 1893). Later he described self-administration of testicular extracts from guinea pigs, which gave him significant increases in muscle strength, mental concentration, and overall vigor (Brown-Sequard 1889). Brown-Sequard also proposed that the ovaries likely contain a similar substance (Olmstead 1946). These "internal secretions", or chemical messengers, became to be known as hormones by the 1910s (Welbourn 1992). Isolations of these hormones from

the adrenal and thyroid gland were followed subsequently by work on secretions by the sex glands, ovaries and testes. Recovery of the animal following testis or ovary removal was the early requirement for a substance to be classified as a sex hormone (Olmstead 1946). Allen and Doisy first described a substance in 1923 isolated from mature follicles that could induce vaginal cornification independent of ovary status (Corner 1964). This hormone was termed oestrin (now oestrogen or estrogen), as it was primarily responsible for the cyclic nature of the female estrous period (from the latin term *oestrus*: frenzy, gadfly). From the discovery of estrogen and isolation in large quantities from cow ovaries and pregnant mare urine (Parkes 1966) developed the field of steroid biochemisty. Characterization of the male sex hormone shortly followed in the 1930s. Early on, the gold standard in male reproductive biology was the castrated rooster. The comb of the rooster provides a convenient external marker for testes state as it falls and atrophies in the absence of gonads (Oudshoorn 1994). Administration of extract from the testes to gonadectomized males resulted in full recovery of the rooster's comb (Parkes 1966). The active component of these extracts was crystallized in 1935 and named testosterone (derived from testis, sterol and ketone) (Freeman, Bloom et al. 2001). Unlike estrogen, testosterone was not available in large quantities from biological sources. In 1929, 20mg of testosterone could be obtained from 40 pounds of bovine testicles (Gallagher and Koch 1929), and thus became available pharmaceutically in 1936. These initial experiments (as described in the references listed) laid the foundation for reproductive endocrinology, and subsequently neuroendocrinology.

Neuroendocrinology "is a branch of the life sciences dealing with neurosecretion and the physiological interaction between the central nervous system and the endocrine system" (Merriam-Webster's Dictionary). In the 1930s, Walter Cannon and Hans Selye developed connections between the psychological and physiological state of the organism in establishing the ideas of "homestasis" and "adaptation" (see later section for discussion). By the end of the 1930s, it was generally accepted that the hypothalamus of the brain exerts control of the secretory properties of the pituitary gland. Hohlweg and Junkmann in 1932 provided the first indication that sex hormones act on a brain "sexual center" and not the pituitary to induce ovulation (Hohlweg and Junkmann 1932). Two decades later, Flerko and Szentagothai determined that this "sexual center" was the preoptic-anterior hypothalamic area (Flerko and Szentagothai 1957).

The beginnings of "neuro" endocrinology

The discovery of blood vessels linking the hypothalamus and the pituitary was described in the early 1930s by Popa and Fielding (Popa and Fielding 1933), and the nature of the flow (brain to pituitary) was first described by Green and Harris in the late 1940s (Green and Harris 1947). Bargmann in 1949 provided the first evidence that nerve fibers extend from the hypothalamus to the pituitary gland (Bargmann 1949), and thus the brain itself could act as an endocrine "gland" (neurosecretion) (Bargmann and Scharrer 1951). These findings led Harris to develop the "chemotransmitter" hypothesis which stated that the neurons of the hypothalamus must produce

substances that are transported to the anterior pituitary (AP) and cause the release of AP hormones into the circulation (Harris 1955). By the 1950s, the labs of Roger Guillemin and Andrew Schally reported that extracts derived from hypothalamus could indeed cause the release of hormones from the anterior pituitary. Guillemin and Schally were responsible for the discoveries of thyrotropin releasing hormone (TRH, thyroid hormone), somatostatin (SS, growth hormone (inhibiting)) gonadotropin releasing hormone (GnRH, luteinizing hormone and follicle stimulating hormone), and the initial work on corticotropin releasing hormone (CRH, adrenocortiocotropic hormone) (Saffran and Schally 1955; Saffran, Schally et al. 1955; Boler, Enzmann et al. 1969; Burgus, Dunn et al. 1969; Guillemin, Burgus et al. 1971; Matsuo, Baba et al. 1971; Burgus, Butcher et al. 1972; Brazeau, Vale et al. 1973; Burgus, Ling et al. 1973). At nearly the same time Rosalyn Yalow developed the technique of radioimmunoassay, which provided a tool to identify blood hormone levels as well as immunological identification of brain cells that express hormones and hormone receptors (Berson and Yallow 1961; Yalow, Glick et al. 1964). It is now well established that steroid and pituitary hormones can act via receptors in the hypothalamus to regulate their own synthesis and secretion. All of these studies provided the groundwork for future studies examining sex hormone effects on brain function.

3. Steroid hormone receptors

Overview

Steroid hormones exert their actions through their respective receptor. Receptors are large proteins that bind to certain molecules (such as a steroid) and through a change in conformation and association with other proteins can alter second messenger pathways or directly modulate gene transcription. However, prior to the 1950s it was presumed that estrogen worked through an enzymatic transhydrogenation pathway. In the late 1950s, Elwood Jensen took an alternative approach and focused on what the tissue does with the hormone rather than what the hormone does to the tissue. He developed a method to radioactively label estradiol, and examined the retention and metabolism of estradiol within tissues. He found that estradiol is retained much longer in uterus and vagina than in liver or blood, and does not undergo transhydrogenation, but rather "some other kind of interaction with a specific receptor in a cellular or nuclear membrane" (Jensen 1962; Jensen, Suzuki et al. 1968). This lead to the discovery by Jensen that estradiol binds it's native receptor, frees it from associated proteins, and converts it to a form that can localize to the nucleus and effect transcription (Jensen, Suzuki et al. 1968). The original "two step" model posited that the receptor resided in the cytoplasm and was transformed and translocated to the nucleus to induce gene expression (Jensen, Suzuki et al. 1968) (Figure 2.2). Subsequently, it was found that the receptor protein for estrogen was localized to the nucleus as determined by studies performed by Greene and Gorski (King and Greene 1984; Welshons, Lieberman et al. 1984; Green, Walter et al. 1986). These original findings by Jensen led

to the later discovery that estrogen receptors belong to a "superfamily" of intracellular receptors that act as ligand-activated transcription factors (Evans 1988).

Characteristics of the steroid receptor superfamily

The steroid/thyroid hormone receptor superfamily consists of three main classes (for reviews see (Evans 1988; Mangelsdorf, Thummel et al. 1995)). Class I includes the thyroid hormone receptor, retinoic acid receptor, vitamin D receptor, and the peroxisome proliferator activated receptor. Class II contains the retinoid X receptor and orphan receptors. Class III comprises of the estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and estrogen related receptor. This superfamily of intercellular receptors has characteristics that are shared across the classes. They exist at a relatively low copy number within the nucleus or cytoplasm of the cell, have high affinity for their respective ligand, exist in multiple forms, and can positively or negatively regulate transcription.

Functional domains of the steroid receptor superfamily.

Members of the nuclear receptor superfamily have a general protein structure that is comprised of functional domains including an immunogenic or trans-activation domain, DNA binding domain (DBD), hinge or linker region, ligand binding domain (LBD), and a protease sensitive region (Figure 2.2). Within each of these functional domains are sub-domains that are important for nuclear localization, dimerization,

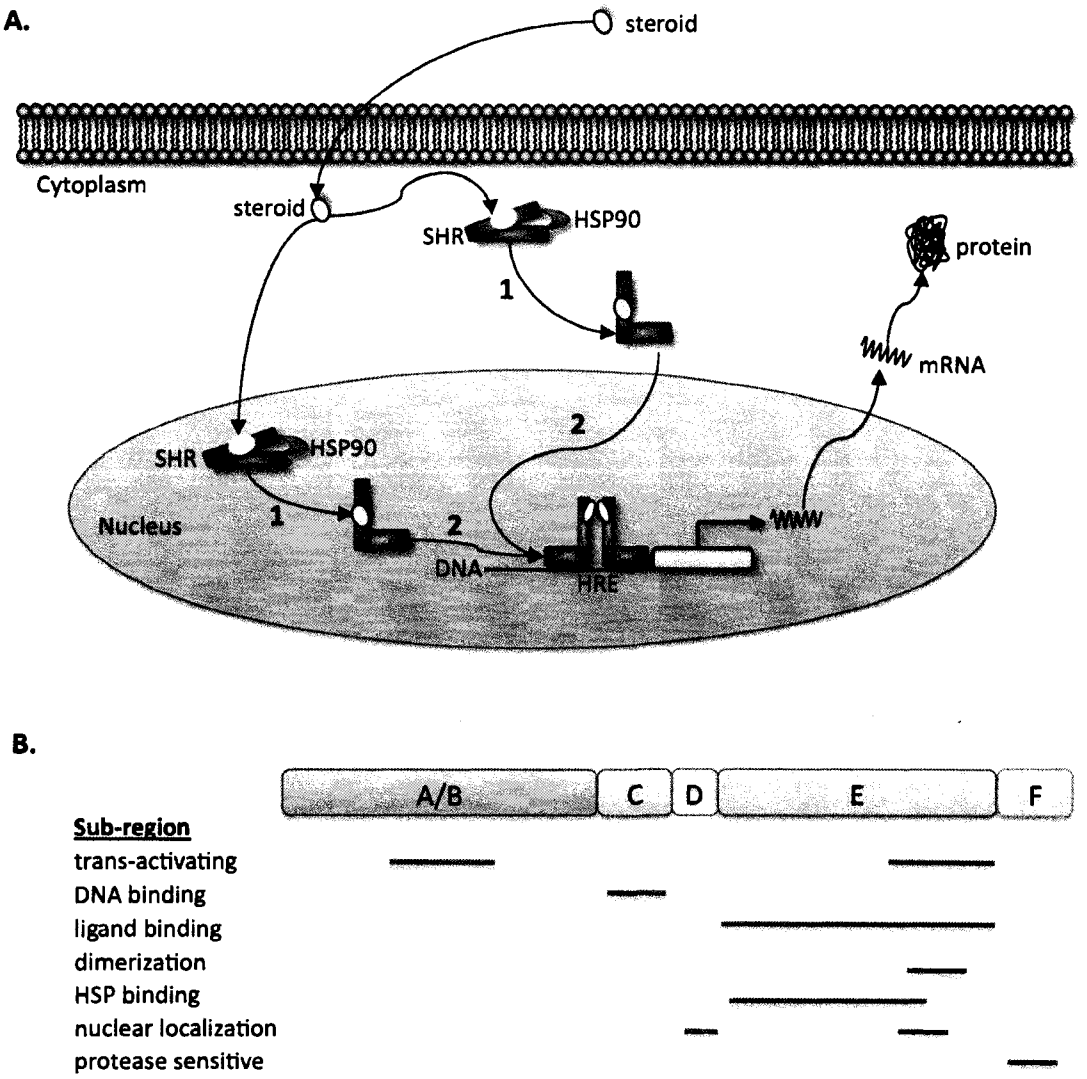


Figure 2.2. Schematic depicition of the general two step mechanism of action and protein structure for the steroid hormone receptor family. *Panel A)* Diagram of steroid hormone activation of gene transcription. Steroid diffuses freely through plasma membrane lipid bilayer into the cyotplasm or the nucleus and binds to a steroid hormone receptor (SHR) associated with heat shock protien (HSP90). This leads to release of HSP90, transformation (1), dimerization, and translocation (2) of the SHR to regulatory regions (e.g., hormone response element, HRE) of DNA to stimulate target gene expression. *Panel B)* Diagram of SHR protein subdomains and general location of subregions within the subdomains.

transcriptional activation, heat shock protein binding (HSP), or silencing. Each of these domains has unique characteristics that effect the ultimate action on gene transcription. The immunogenic domain (A/B), encoded by one exon, is the most variable region and is primarily responsible for the trans-activation ability of the receptor. The DBD (C) contains zinc-fingers (two anti-parallel β strands connected by an α helix bound to a zinc ion) that directly bind to DNA in a specific manner dictated by the sequence of three amino acids within the knuckle of the first zinc finger. In ER these are glutamate, glycine, and alanine, whereas in AR, GR, MR, and PR these are glycine, serine, and valine. The LBD (E) contains a hydrophobic ligand-binding pocket that attracts the ligand, contains a HSP binding domain for association with heat shock protein (HSP), and is responsible for dimerization of receptors. This domain acts as a silencer, inhibiting trans-activational function until ligand binds. The HSP domain binds HSP 90 and HSP 70, which act to mask functional domains such as the DBD, transport the receptor to the nucleus, and promote ligand binding. Once ligand binds to the LBD HSP is released, the receptor undergoes physical transformation and can dimerize. This is made possible by a dimerization motif that includes a regulatory zipper domain containing hydrophobic residues at amino acids 1, 5, and 8. Following dimerization, the receptor complex can bind DNA. Studies utilizing DNA footprinting discovered specific sequences of DNA to which these intracellular receptors bind called hormone response elements (HRE). HREs are a 15-nucleotide consensus sequence comprised of a six nucleotide palindromic sequence separated by three variable nucleotides. For example the glucocorticoid response element (GRE) contains the sequence GGTACAnnnTGTTCT (nnn = three

variable nucleotides). The two DBDs of the receptor dimer each bind to one of the palindromic sequences (HRE halfsite).

Transcriptional activation by steroid hormone receptors

Once the receptor dimer is bound to its respective HRE it can initiate transcription through interactions with coregulatory proteins and the pre-initiation complex (Figure 2.3). Coregulatory proteins are classified as either coactivators or corepressors depending on their overall influence on gene transcription (McKenna, Nawaz et al. 1998; Freedman 1999). These proteins interact with steroid hormone receptors to promote or inhibit associations with transcription factors (general class of proteins that bind to DNA and activate or inhibit polymerase II activity). Coactivators and corepressors can also influence the stability of the pre initiation complex (a complex of general transcription factors that act to promote polymerase II activity at the transcriptional initiation site of a target gene), and alter the structural conformation of DNA through histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity. Transcriptionally inactive DNA is tightly coiled around histone proteins that upon acetylation by HATs disassemble and expose the DNA for transcription. The coactivators that associate with steroid hormone receptors belong to the steroid receptor coactivator (SRC) family. This family includes SRC-1 (p160), SRC-2 (TIF-2, GRIP-1), SRC-3, and creb binding protein (CBP, p300) (Onate, Tsai et al. 1995; Chakravarti, LaMorte et al. 1996; Hong, Kohli et al. 1996; Kamei, Xu et al. 1996; Voegel, Heine et al. 1996; Apostolakis, Ramamurphy et al. 2002). Taken together, ligand-bound steroid receptors

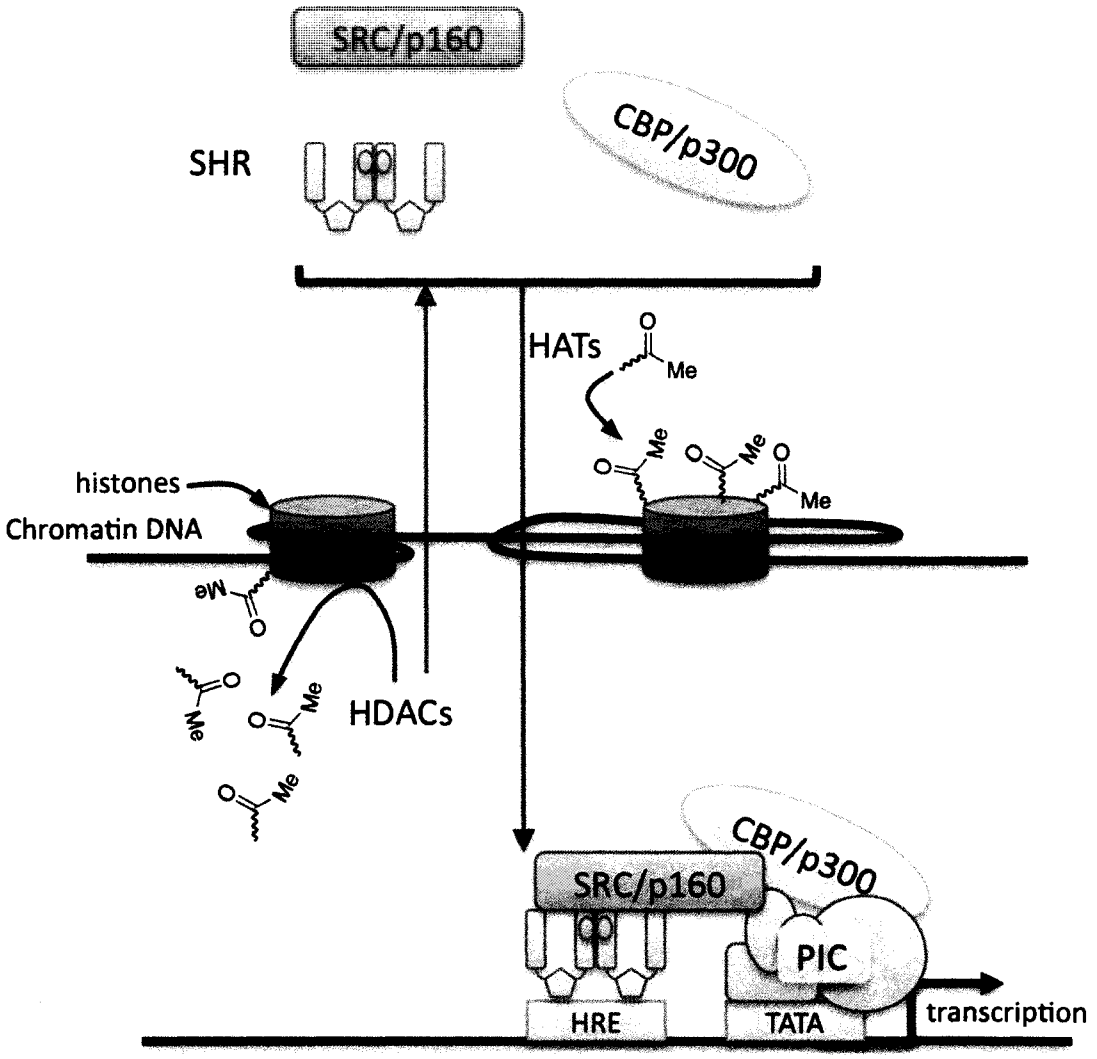


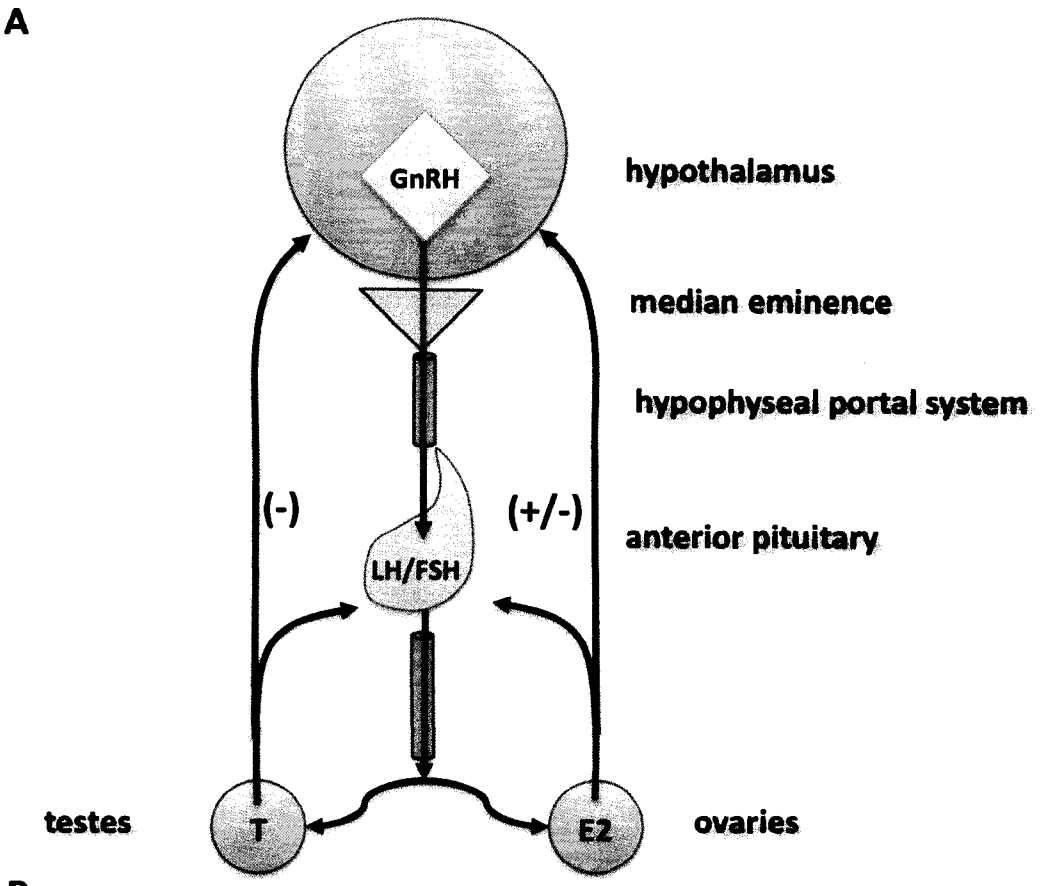
Figure 2.3. Schematic diagram of transcriptional activation by steroid hormone receptors. Steroid-bound receptor recruits coregulatory proteins such as the p160 and p300 family of coactivators. These coactivators have intrinsic histone acetyl transferase (HAT) activity and can acetylate histones associated with inactive chromatin to uncoil DNA and expose regulatory regions and transcription initiation sites of target genes. The steroid-bound hormone receptor can then bind to a regulatory region (e.g., hormone response element, HRE) and associate with coregulatory protein factors (e.g., SRC, CBP) to stimulate transcription through activation of the pre-initiation complex (PIC). Transcription can subsequently be inhibited by the activity of histone deacetylases (HDACs), which act to remove acetyl groups from histones and promote chromatin recoiling.

associate with coactivators/corepressors to promote/inhibit DNA accessibility, binding to a HRE, association with general transcription factors, polymerase II recruitment and target gene transcription.

Transcriptional activation by steroid hormone receptors is not always mediated through binding to an HRE. For example, some members of the steroid hormone superfamily such as ER and GR can influence transcription mediated through an activator protein-1 (AP-1) response element. ER and GR can associate with AP-1 proteins such as Fos and Jun to inhibit or activate AP-1 mediated transcription (Pearce, Matsui et al. 1998; Chinenov and Kerppola 2001; Pak, Chung et al. 2005; Safe and Kim 2008). Adding to this complexity, some nuclear hormones such as ER and PR can activate transcription in the absence of ligand (Tzukerman, Zhang et al. 1990; Power, Mani et al. 1991), or in the presence of other endogenous factors like dopamine (PR) (Mani, Allen et al. 1996; Blaustein 2004).

4. The hypothalamic-pituitary-gonadal (HPG) axis

Reproduction, vital to the survival of species, is controlled by the hypothalamic-pituitary-gonadal (HPG) axis (Figure 2.4a). This axis is comprised of gonadotropin releasing hormone (GnRH)-containing neurons in the hypothalamus (preoptic area and arcuate nucleus) that send their processes to the median eminence to secrete gonadotropin-releasing hormone (GnRH) into the hypophyseal portal system. GnRH release from the median eminence is pulsatile in nature, and results in the pulsatile nature of LH release from the anterior pituitary. This pulsatility is necessary for normal


A
GnRH
hypothalamus
median eminence
hypophyseal portal system
(-)
(+/-)
LH/FSH
anterior pituitary
testes
T
E2
ovaries

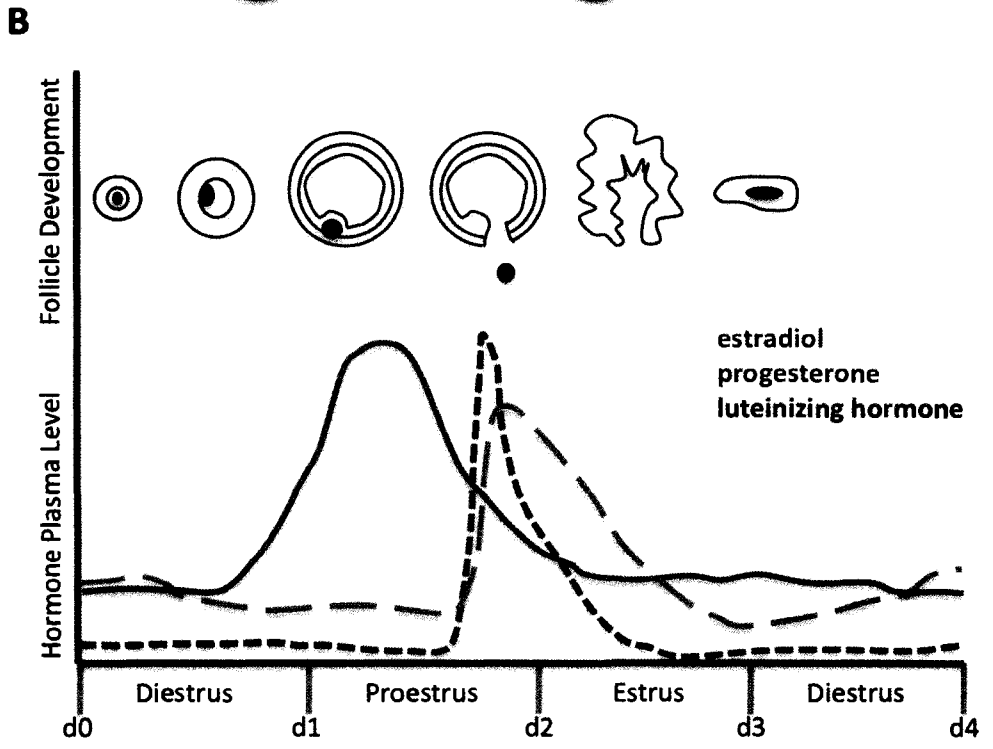

B
Follicle Development
Hormone Plasma Level
estradiol
progesterone
luteinizing hormone
Diestrus
Proestrus
Estrus
Diestrus
d0
d1
d2
d3
d4

Figure 2.4. Schematic diagram of the hypothalamic-pituitary-gonadal (HPG) axis and the rat estrus cycle. *Panel A*) The hypothalamic-pituitary-gonadal axis. Neurons in the hypothalamus secrete gonadotropin-releasing hormone (GnRH) into the hypophyseal portal system where it acts upon gonadotropes within the anterior pituitary to stimulate the synthesis and secretion of luteinizing hormone (LH), and follicle stimulating hormone (FSH) into the general circulation. LH and FSH act to promote follicle maturation, ovulation and estradiol synthesis in the ovary, and spermatogenesis and testosterone synthesis in the testes. Estradiol (E2) and testosterone (T) feed back to inhibit or augment (in the case of estradiol in proestrus) the axis. *Panel B*) The four day rat estrus cycle as depicted by follicle development and plasma hormone levels. Estradiol (solid red line) levels peak during proestrus leading to a surge in LH (short dashed green line) and release of a mature follicle (ovulation). Progesterone (long dashed purple lines) levels peak in late proestrus and early estrus primarily due to the presence of the corpus luteum.

gonadotrope function, since continuous GnRH exposure causes a downregulation of GnRH receptors in gonadotropes and subsequent insensitivity to GnRH secretion. These pulses are typically around 30 minutes apart in the rodent. GnRH subsequently acts downstream on the gonadotropes of the anterior pituitary to stimulate the release of gonadotropins (luteinizing hormone (LH), and follicle stimulating hormone (FSH)) into the general circulation. High frequency and amplitude GnRH pulses preferentially induce the release of LH from the gonadotropins, whereas low frequency and amplitude pulses preferentially induce the release of FSH (Terasawa 1998). Once secreted into the general circulation, LH and FSH can then stimulate the gonads (testis in males, and ovary in female). In the male, LH is responsible for stimulating testosterone synthesis from the Leydig cells, and together with FSH acts to promote spermatogenesis within the seminiferous tubules. In the female, FSH is responsible for the maturation of the follicle, and in combination with LH causes release of the mature follicle and estradiol synthesis by the ovary.

The rat estrus cycle (Figure 2.4b) is a reoccurring chain of physiological events that results in the maturation and release of follicles from the ovary approximately every four days. Plasma estrogen levels remain low through the first day, diestrus, and rise to a peak on the second day, proestrus, when the follicle is nearing maturation. Following the peak in estradiol, LH levels sharply rise (surge) in late proestrus and results in the release of the mature follicle (ovulation). Plasma progesterone peaks in late proestus and early estrus, primarily due to the presence of a corpus lutetium. In the fourth day, diestrus, hormone levels drop and remain low.

5. The hypothalamic-pituitary-adrenal (HPA) axis

History of stress and the HPA axis

The origins of stress physiology and the concept of stress can be traced back to the contributions of Walter Cannon and Hans Selye (for review see (Chrousos, Loriaux et al. 1988)). These pioneers were interested in describing the responses to an acute threat (Cannon), and adaptation in response to chronic challenges (Selye) to an organism. Cannon in 1915 coined the term "fight or flight" in order to describe an organism's response to danger or a threat (Cannon 1915). He developed the concepts of "homeostasis" and "steady state" in 1932 in his book "The Wisdom of the Body":

> The coordinated physiological processes which maintain most of the steady states in the organism are so complex and so peculiar to living beings – involving, as they may, the brain and nerves, the heart, lungs, kidneys and spleen, all working cooperatively – that I have suggested a special designation for these states, homeostasis. The word does not imply something set and immobile, a stagnation. It means a condition – a condition which may vary, but which is relatively constant.
>
> (Cannon 1932)

Walter Cannon described the human body as open system and therefore it requires mechanisms to maintain consistency (homeostasis) in this open state. Further, any tendency towards change in steady state conditions is met with factors that resist change. Homeostasis, as he described, does not occur by chance, but is a result of many

cooperating mechanisms acting in succession or simultaneously to maintain a steady state. Experiments done by Hans Selye in the mid-1930s built upon the groundbreaking work done by Cannon (Selye 1936; Selye 1956). In these experiments, when Selye injected rats with an ovarian extract they developed a triad of symptoms, including enlargement of the adrenal cortex, atrophy of the thymus, and ulcers in the lining of the stomach. Upon injecting extracts of placenta, pituitary, spleen and other organs he observed similar detrimental effects to the animal. To confirm these non-specific effects he injected rats with formalin, a toxic chemical, and again observed enlarged adrenals, atrophied thymus, and ulcers. This led to the hypothesis that the animals were exhibiting signs of a generalized reaction to a damaging stimulus. He initially termed these stimuli "noxious agents" (later referring to them as "stressors") and the general physiological reaction to them as "stress". According to Selye, stress represented "the nonspecific response of the body to any demand", and that over time stress led to illness. As a result of his experiments, Selye developed the General Adaptation Syndrome (GAS) and described it as the "physiological mechanism which raises the resistance to damage". In the first stage, alarm, the body prepares itself for "fight or flight", and involves a heightened state of arousal. In the second stage, resistance, the body builds resistance to the stressor. In the third and final stage, exhaustion, the body becomes less able to resist the stressor, and results in aging and "wear and tear" on the body. In addition, Selye described two components of stress physiology, the set of reactions to a stressor as described by the GAS, and a pathological state derived from continuous, unrelieved stress.

Today, defining stress is somewhat arduous, given the diverse number of physiological and psychological stressors, and vast consequences of these stressors on neural function, physiology, and behavior. As such, Steptoe has proposed that "the effects of stress are manifest in four distinct domains: physiology, behavior, subjective experience, and cognitive function. The physiological effects of stress include alterations in neuroendocrine, autonomic nervous system, and immune function" (Steptoe 2000). Chrousos and Gold define stress as a "state of disharmony, or threatened homeostasis. This adaptive response can be specific or can be generalized and non specific" (Chrousos and Gold 1992). Perhaps a more standard definition of stress is one proposed by Bruce McEwen: "Stress may be defined as a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioral responses" (McEwen 2000).

Following the work of Cannon and Selye, the field of stress physiology moved to find the biological components of the stress response. In 1950, Sayers discovered that the adrenocorticotropin hormone (ACTH) peptide of the anterior pituitary could stimulate glucocorticoid release from the adrenal cortex (Sayers 1950). What regulated the release of ACTH (i.e., a corticotropin releasing hormone) was still unknown. An early model was proposed by Long that systemic blood levels of epinephrine regulated ACTH secretion (the adrenaline model) (Long 1947; Long 1952). However, work by Vogt indicated that glucocorticoid release could still occur in the absence of epinephrine following enucleation of the adrenal medulla (Vogt 1952). Alternatively, Sayers proposed that blood levels of glucocortioids regulated ACTH secretion (Sayers 1950).

There was no doubt that glucocortioids did influence ACTH release, but pituitary transplantations (keeping vasculature intact, but neural control removed) resulted in decreased ACTH and adrenal atrophy. Indeed, neural control of ACTH release became the subsequent focus of the field initiated by Geoffrey Harris and his postulation on "neural control of the pituitary gland" (Harris 1951; Harris 1951; Harris and Jacobsohn 1952). Harris' theory was that blood-born releasing factors (term coined by Schally) originated in the hypothalamus of the brain, passed through the hypophyseal portal vasculature to act upon the anterior pituitary to release hormone (in this case ACTH). Harris showed that pituitary transplants were viable yet nonfunctional (Harris and Jacobsohn 1952). Furthermore, sectioning the pituitary stalk so as to disrupt blood flow from the hypothalamus resulted in severely impaired ACTH secretion (Harris 1950). Harris went a step further and electrically stimulated the hypothalamus in rabbits, and reported a subsequent swift release of ACTH into the general circulation (De Groot and Harris 1950). Several studies ensued detailing hypothalamic lesions and a corresponding lack of ACTH release in response to a stressor (McCann 1953; Porter 1953). These studies established that CRH must be of neural/hypothalamic origin.

The initial attempts on isolating a hypothalamic releasing peptide centered on CRH (Schally and Guillemin), however it turned out to be the more elusive of the hypophysiotropic hormones. This was due in part to work by McCann and Brobeck claiming that AVP was *the* CRH (already shown to induce ACTH release) and there was no need to search any further (McCann and Brobeck 1954). Furthermore, the similarity of ACTH to CRH in size (39 versus 41 amino acids, respectively), likely proved to be

prohibitive in isolating CRH from hypothalamic extracts. It wasn't until 1981, following technological advances in radioimmunoassay, ion exchange, and liquid chromatography, that CRH was isolated and characterized by Wylie Vale (Vale, Spiess et al. 1981). Corticotropin-releasing factor (CRF), as it was initially termed for its ability to cause the release of adrenocorticotropin hormone, has been found throughout the brain and periphery, and has been implicated in a broad array of physiological and psychological processes (Bale and Vale 2004). Thus, it is more appropriately termed CRH to represent its diverse biological actions.

D. deWeid, in collaboration with R.E. Miller, observed an effect of ACTH on behavior (shuttlebox - anxiety) that was independent of its actions on the adrenal gland (glucocorticoid release). This suggested that ACTH had behavioral effects independent of those attributable to glucocorticoids. This led deWied to hypothesize that peptides emanating from the pituitary could act on neural substrates to regulate specific behaviors (aka the Neuropeptide Concept) (for review see (de Weid 1990)). Since then, many studies have provided evidence of direct effects of neuropeptides such as ACTH, arginine vasopressin (AVP), CRH, oxytocin (OT), and prolactin (PRL), on neural substrates and behavior.

Overview of the HPA axis

Presented with a real or perceived threat, an animal will respond to maintain homeostasis through activation of the hypothalamic-pituitary-adrenal (HPA) axis in addition to the sympathoadrenomedullary response (autonomic response). The main

result of HPA axis activation is the release of glucocorticoids (cortisol in humans and most mammals, and corticosterone (CORT) in rodents) from the adrenal cortex into systemic circulation (Figure 2.5). Glucocorticoids act through classic genomic mechanisms (Munck, Guyre et al. 1984; McEwen and Stellar 1993) and non genomic mechanisms (Orchinik, Murray et al. 1991; Moore and Evans 1999) to mobilize energy stores, induce lipolysis and proteolysis, potentiate sympathetic-driven vasoconstriction, suppress reproductive function, and alter behavior in order to maintain homeostasis (Munck, Guyre et al. 1984; McEwen and Stellar 1993; Sapolsky, Romero et al. 2000). Acute activation of this axis is generally beneficial and includes enhanced learning and memory, immune function and metabolism. These generally 'restorative' effects are usually catabolic in nature, and thus detrimental if sustained over long periods of time. Indeed, chronic activation of this axis can result in deleterious effects on immune, cardiovascular, metabolic, and brain function (McEwen 1998).

Stress-related afferent information is relayed from limbic (psychological or emotional stressors) or brainstem (physical or systemic stressors) regions of the CNS to the paraventricular nucleus of the hypothalamus (PVN) (Figure 2.5). The main integrators of stress-related neural information are a discrete subset of neurons in the parvocellular PVN (approximately 4000 in the rat) that synthesize and secrete the ACTH secretagogues CRH and AVP (Swanson 1987). These neurons send their projections to blood vessels in the external lamina of the median eminence, and release CRH and AVP into the hypophyseal portal vasculature. CRH is required for basal and stress-induced ACTH release, whereas AVP mainly acts in synergy with CRH during a stress response to

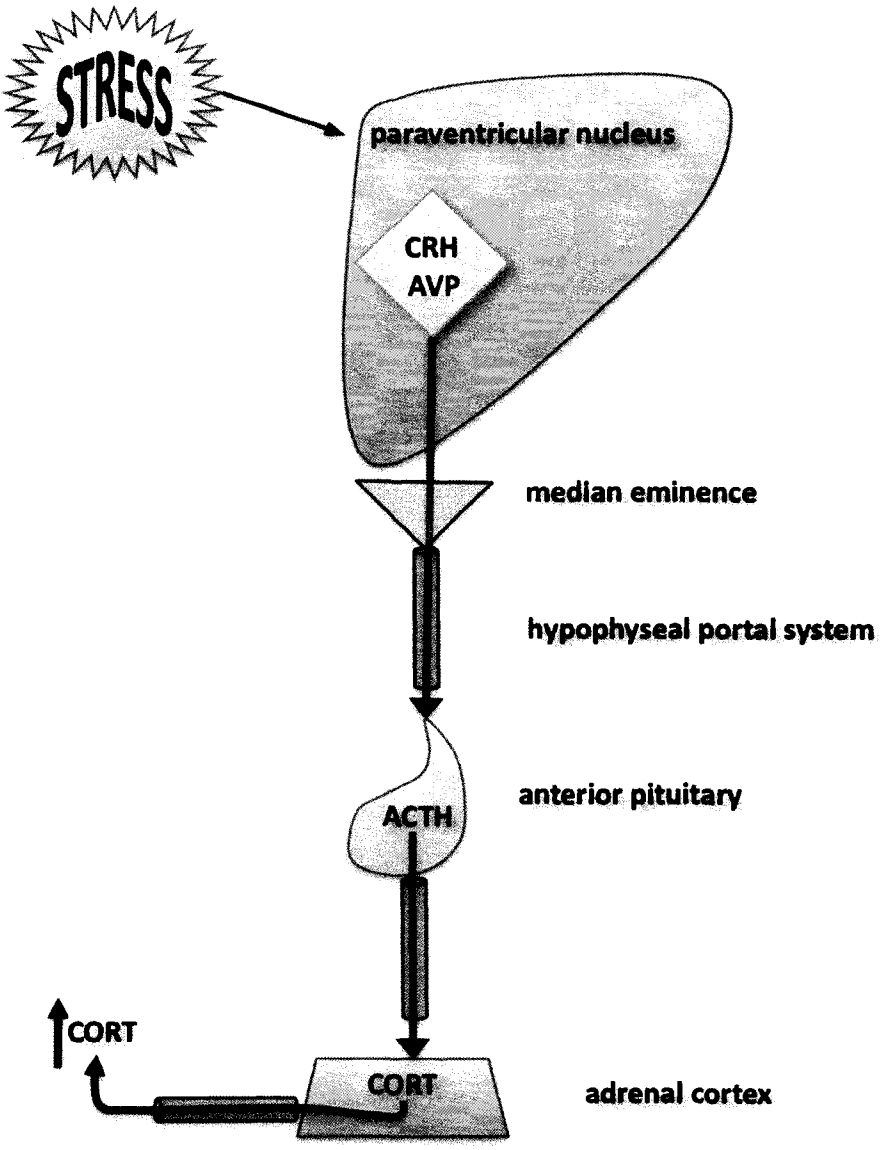


Figure 2.5. Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis. Stress related neuronal information converges on the paraventricular nucleus of the hypothalamus (PVN) to stimulate corticotropin-releasing hormone (CRH) and a small number of vasopressin (AVP) neurons in the medial parvocellular sub-region of the PVN. These neurons send their projections to the median eminence at the base of the hypothalamus where they release CRH and AVP into the hypophyseal portal vasculature. CRH and AVP act synergistically upon corticotrophs of the anterior pituitary to induce the synthesis and secretion of adrenocorticotropin hormone (ACTH) into the general circulation. ACTH, in turn, acts upon the adrenal cortex to increase the synthesis and secretion of corticosterone (CORT) into the general circulation.

increase the gain of the HPA axis (Antoni 1986; Lowry, Estivariz et al. 1986; Whitnall 1993). Once appropriately stimulated by CRH and AVP, the corticotrophs of the anterior pituitary synthesize and release ACTH into the general circulation. ACTH subsequently acts upon the zona fasciculata cells of the adrenal cortex to synthesize and release glucocorticoids into the blood stream. It should be mentioned that, while ACTH is the main stimulator of glucocorticoid release, neural inputs to the adrenalcortex can alter sensitivity to ACTH (Ulrich-Lai and Engeland 2002). The end product of the axis, glucocorticoids, exerts tight negative feedback control by way of its actions at the adrenal gland, pituitary, hypothalamus, and other stress-sensitive brain regions (i.e., hippocampus) (McEwen 1998) (Figure 2.6). This closed-loop system is essential in preventing excessive activation of the HPA axis, and subsequent detrimental effects due to glucocorticoid excess (glucocorticoid negative feedback will be discussed in a subsequent section).

Receptors for corticosterone: mineralocorticoid receptor and glucocorticoid receptor

Glucocortioids exert their molecular and cellular effects through the actions of their receptors. In the late 1960s, Bruce McEwen observed pronounced labeling of cells in the hippocampus, amygdala, dorsal septum and cortical brain regions following injection of adrenalectomized rats with low-levels of tritiated corticosterone (McEwen, Weiss et al. 1968). This provided evidence that there exists CORT binding sites extrinsic to the HPA axis, and indicated that glucocorticoid negative feedback is likely not exclusively intrinsic upon the hypothalamus, pituitary and adrenal cortex. Furthermore,

binding of the low-level ^{3}H-CORT could be abolished by aldosterone, but not by the synthetic glucocorticoid dexamethasone (DEX) (de Kloet 1975). This suggested that there might be more than one type of glucocorticoid receptor. This differentiation was aided by the development of 'pure' glucocorticoids by scientists at Roussel-Uclaf in the early 1980s (Moguilewsky and Philibert 1984). De Kloet and colleagues performed *in vitro* cytosol binding assays with ^{3}H-CORT in the presence of absence of the 'pure' glucocorticoid RU28362, and revealed two types of cytosolic receptors that bind corticosterone but with ten-fold difference in affinity (Veldhuis, Van Koppen et al. 1982; Reul and de Kloet 1985) (de kloet 1985, veldhuis 1982). Type I, or mineralcorticoid receptor (MR) is the high-affinity receptor (K_d~0.5nM, 4°C) and Type II, or glucocorticoid receptor (GR) is the lower-affinity receptor (K_d~5.0nM, 4°C) (Reul and de Kloet 1985). Cloning of both MR (Arriza, Weinberger et al. 1987) and GR (Hollenberg, Weinberger et al. 1985) ensued shortly thereafter.

GR and MR are ligand-activated transcription factors that share similar DNA binding domains, related ligand binding domains, and once activated, bind similar hormone response elements on DNA (Pearce and Yamamoto 1993). The receptors may bind independently or in combination with other transcription factors to DNA motifs in promoters of target genes. These DNA motifs include the consensus glucocorticoid response element (GRE) and negative GREs (nGRE). Some repressive effects of MR and GR are due to transrepression through interaction with factors such as NF-κB and Stat 5 (Gottlicher, Heck et al. 1998; Stoecklin, Wissler et al. 1999). Interestingly, GR can either stimulate or repress AP-1 activity depending on the

presence of c-jun homodimers or c-fos/c-jun heterodimers (Diamond, Miner et al. 1990). However, MR does not repress AP-1 activity (Pearce and Yamamoto 1993), and thus AP-1 function may be a differentiating factor for MR and GR transcriptional effects. Many of MR and GR genomic effects are influenced by interactions with co-regulators such as the SRCs NCoA-1, NCoA-2, TIF2, GRIP-1, and NCoA-3. These co-regulators have highly specific expression patterns in the brain, including the PVN (Meijer, Steenbergen et al. 2000), and likely influence MR and GR transactivation and/or transrepression.

The expression patterns of GR and MR are diverse, and suggest that CORT might have differential effects depending on endogenous levels (Reul and de Kloet 1985). GR is widespread in expression, with highest levels in the lateral septum, dentate gyrus of the hippocampus, central nucleus of the amygdala, and the PVN of the hypothalamus (Reul and de Kloet 1985; Aronsson, Fuxe et al. 1988; Morimoto, Morita et al. 1996). Additionally, lesser amounts of GR expression are seen in the arcuate and ventral medial hypothalamic nuclei (Han, Ozawa et al. 2005). MR expression is less widespread and is highest in the hippocampus, where it is expressed in all pyramidal layers (CA1-CA3) and the dentate gyrus (Herman, Patel et al. 1989). Lesser amounts of MR expression can be found in the hypothalamus (Han, Ozawa et al. 2005). The presence of both receptor types in the hippocampus indicates that this area is important in modulating negative feedback in response to a wide range of circulating CORT levels. Under basal conditions HPA axis activity may be under the greatest influence by the hippocampus, whereas during times of stress, the high circulating levels of CORT may have greatest inhibitory effect directly at the hypothalamus.

Within the PVN, GR have been reported in both CRH and AVP containing neurons although there is much greater evidence for GR presence in AVP (Albeck, Hastings et al. 1994) than in CRH neurons (Uht, McKelvy et al. 1988). In general, MR controls the tone of the HPA axis, and is partially responsible for the initial onset of HPA axis activity. For example, acute blockade of hippocampal MR raises basal HPA axis activity and results in a stronger daily CORT surge (Van Haarst, Oitzl et al. 1997). Furthermore, administration of an MR antagonist facilitates the response to a novel enviroment (Ratka, Sutanto et al. 1989), however this might be due in part to higher basal HPA tone. On the other hand, GR controls the magnitude and duration of the stress response. For instance, acute GR blockade has relatively little influence on basal HPA activity (Ratka, Sutanto et al. 1989; Van Haarst, Oitzl et al. 1996; Van Haarst, Oitzl et al. 1997), yet can increase the length and magnitude of the stress response (Ratka, Sutanto et al. 1989). In summary, MR works to direct the sensitivity and threshold of the HPA axis, while GR facilitates termination and recovery from a stressor.

Stress integration: neurocircuitry of the HPA axis

Hypophysiotropic neurons of the parvocellular PVN, as the main effectors of the stress response, receive stress-related neuronal input from a variety of sources, and integrate that information to produce a relevant response (Figure 2.6). In addition to neural input, it is important to note that these neurons can also respond to the systemic hormonal milieu. Their nerve terminals are able to sample the blood-brain barrier deficient hypophyseal portal vasculature (Antoni 1986; Whitnall 1993), and the PVN is

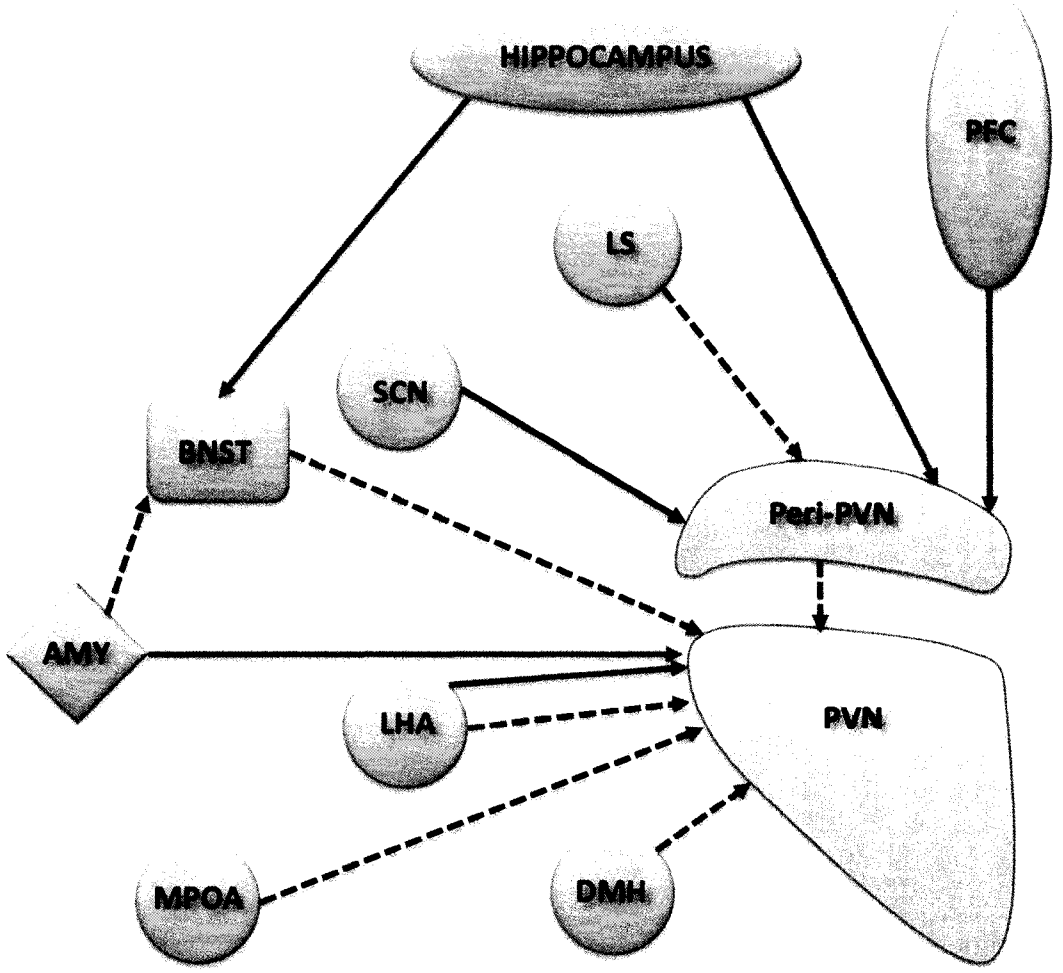


Figure 2.6. Schematic diagram of the neuronal circuitry of the hypothalamic-pituitary-adrenal axis (HPA) axis. Green solid arrows indicate excitatory glutamate connections. Red dashed arrows indicate inhibitory gamma-aminobutyric acid (GABA) connections. Black arrow indicates mixed GABA and arginine vasopressin connections. AMY, amygdala; BNST, bed nucleus of the stria terminalis; DMH, dorsomedial hypothalamus; LHA, lateral hypothalamic area; LS, lateral septum; PFC, prefrontal cortex; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus.

one of the most vascularized regions in the brain (Herman, Mueller et al. 2005). The neurons of the PVN have dendritic arborizations that are mainly confined inside the boundaries of the nucleus (van den Pol 1982; Rho and Swanson 1989), suggesting that direct synaptic modulation occurs within the nucleus. Overall, the PVN receives a limited number of direct inputs, but is surrounded by a shell of nerve fibers and terminals originating from a diverse group of brain and brainstem regions. This outer shell is flush with PVN-projecting GABA (inhibitory neurotransmitter) neurons (Roland and Sawchenko 1993) and suggests that inputs to this region are processed locally prior to contacting the PVN itself.

There are a select group of direct inputs to the parvocellular PVN from the brainstem that are integral in initiating responses to systemic (physiological) stressors. The nucleus of the solitary tract (NTS) and ventrolateral medulla send noradrenergic and adrenergic projections to the PVN. Appropriately, the parvocellular PVN is densely innervated with norepinephrine and epinephrine fibers (Sawchenko and Swanson 1981; Mezey, Kiss et al. 1984; Cunningham and Sawchenko 1988; Cunningham, Bohn et al. 1990). These findings are in line with the observation that the NTS is critical in mediating reflex control of the cardiovascular system, and in relaying information on visceral illness and systemic infection (Ericsson, Kovacs et al. 1994; Lawrence and Jarrott 1996; Seeley, Blake et al. 2000). Perturbations such as these recruit the assistance of glucocorticoids to aid in restoring homeostasis. In addition, the median and dorsal raphe nuclei of the brainstem send serotonergic (5-HT) projections to the parvocellular PVN (Sawchenko and Swanson 1983). Indeed, 5-HT receptors are present in

parvocellular neurons (Lovenberg, Baron et al. 1993; Wright, Seroogy et al. 1995; Zhang, Damjanoska et al. 2002), and serotonin's effects are largely stimulatory on PVN output (Van de Kar and Blair 1999). However, there is a network of 5-HT fibers within and in the surround of the PVN (Sawchenko, Swanson et al. 1983), and therefore the effect of 5-HT is likely different dependent upon where the fiber terminates. Other brainstem regions critical in cardiovascular integration (parabrachial nucleus) and pain (periaquaductal grey) also send direct projections to the PVN (Sawchenko and Swanson 1983; Behbehani 1995; Saper 1995).

Intrahypothalamic inputs comprise a good portion of the direct innervation of the parvocellular PVN (Figure 2.6). Hypothalamic inputs are integral for activation or inhibition of the HPA axis in order to modulate energy balance, reproduction, mineral and water balance, and body temperature. Direct inputs arise from the anteroventral preoptic nucleus, medial preoptic nucleus, lateral hypothalamus, arcuate nucleus, and ventral premammillary nucleus (Sawchenko and Swanson 1983). Stimulation and lesion studies indicate that these inputs are largely inhibitory on PVN output (Okamura, Abitbol et al. 1990; Cullinan, Herman et al. 1993). However, most of these intrahypothalamic regions do express the excitatory neurotransmitter glutamate, and send glutamatergic fibers to the PVN (Ziegler, Cullinan et al. 2002). Furthermore, many of these regions express neuropetides such as dynorphin, enkephalin, proopiomelanocortin atrial natriuretic peptide, which are inhibitory on the HPA axis, and neuropeptide Y and CRH, which are stimulatory (Herman, Prewitt et al. 1996). Therefore, these complex and interconnected hypothalamic inputs can act as a type of

throttle on PVN activity.

In addition to remote hypothalamic input, the parvocellular PVN receives substantial input from neurons in the immediate surround of the nucleus (peri-PVN region) (Roland and Sawchenko 1993) (Figure 2.6). Application of glutamate to these neurons results in GABA dependent inhibition of PVN neurons (Boudaba, Szabo et al. 1996), suggesting that these neurons are generally inhibitory on CRH neurons. Furthermore, microinjection of a wide-spectrum of glutamate receptor antagonist to the peri-PVN results in an elevated CORT response to a restraint stress (Ziegler and Herman 2000). Consistent with their function in modulating PVN activation, these peri-PVN neurons show robust *c-fos* mRNA expression within 30 minutes following a restraint stress (Cullinan, Helmreich et al. 1996; Cole and Sawchenko 2002). Therefore, the peri-PVN is an important gating mechanism for signals originating from forebrain and brainstem locations that project to the surround or shell of the PVN rather than directly to CRH neurons themselves.

The parvocellular PVN also receives a substantial amount of input from limbic areas (Figure 2.6). The BST is the main limbic-related structure with direct innervation of the parvocellular PVN (Sawchenko and Swanson 1983). The BST is a complex group of several subnuclei, of which the fusiform, anterodorsal, and interfasicular subnuclei all have extensive projections to the PVN (Cullinan, Herman et al. 1993; Dong, Petrovich et al. 2001). The majority of theses PVN-projecting neurons are GABAergic (Cullinan, Herman et al. 1993), and lesion studies indicate that these inputs act to inhibit CRH mRNA levels and CORT secretion (Dunn 1987; Herman, Cullinan et al. 1994), suggesting

that the BST is a largely inhibitory influence on the HPA axis. However, selective lesions of the anterior or lateral portions of the BST decrease ACTH release (Dunn 1987; Gray, Piechowski et al. 1993; Herman, Cullinan et al. 1994), suggesting that some areas of the BST may in fact be stimulatory.

The majority of limbic structures (hippocampus, prefrontal cortex, medial amygdala, and lateral septum), and a few hypothalamic structures (SCN and VMH), that modulate HPA axis activity do not directly innervate parvocellular PVN neurons and therefore must act through an intermediary synapse (indirectly) (Figure 2.6). These regions project to areas like the BST and peri-PVN that have direct inhibitory (GABAergic) input to the PVN (Cullinan, Herman et al. 1993; Prewitt and Herman 1998; Dong, Petrovich et al. 2001). The hippocampus and prefrontal cortex have a largely glutamatergic output (Walaas and Fonnum 1980) that is translated into an inhibitory tone on the HPA axis through activation of the BST and peri-PVN regions (Diorio, Viau et al. 1993; Herman, Dolgas et al. 1998; Figueiredo, Bodie et al. 2003). On the other hand, amygdaloid projections to the BST and peri-PVN are GABAergic and thus act to decrease the inhibition of the HPA axis from these regions (Swanson and Petrovich 1998). Interestingly, the SCN influences the PVN through vasopressinergic activation of intermediate GABA neurons in the subparaventricular and peri-PVN regions (Watts, Swanson et al. 1987; Buijs, Hermes et al. 1998). The VMH connects to the PVN by way of the lateral hypothalamus and the peri-PVN (Ter Horst and Luiten 1987) in order to confer anorectic and orexigenic signals to the HPA axis.

In summary, HPA axis circuits allow for weighing of the current internal state

with the environmental situation and learned experiences. Inputs from the limbic system regarding things like spatial and context-related information as well as fear and emotional state can be effectively weighed with the current status of internal homeostasis to result in an appropriate HPA axis response to a real or perceived threat.

Glucocorticoid negative feedback

The HPA axis is governed by a closed-loop negative feedback system mediated by the end product of the axis, glucocorticoids (Figure 2.7). This glucocorticoid-dependent negative feedback control is necessary for the termination of the stress response and reduces the potential for deleterious actions of glucocortioids. Glucocorticoid-mediated HPA axis negative feedback is dependent upon the dose and the duration of exposure (Abe and Critchlow 1980). Normal function of the HPA axis is dependent upon permissive and reactive actions of glucocorticoids (for review see (Sapolsky, Romero et al. 2000)). For example, adrenalectomy (ADX) results in higher PVN neuropeptide synthesis and secretion both at the basal and stressed states. Additionally, feedback can occur either directly at the level of the CRH and AVP neurons of the PVN as well as the corticotrophs of the anterior pituitary, or indirectly though GR and MR containing brain regions that project to the PVN such as the hippocampus and amygdala (Davidson and Feldman 1967; Bohus and Strashimirov 1970; Dallman, Akana et al. 1987; Sawchenko 1987). However, studies have shown that CORT influence on the pituitary is hampered by the existence of transcortin-like molecules present in corticotrophs (de Kloet, Burbach et al. 1977). Furthermore, hippocampal GR exert

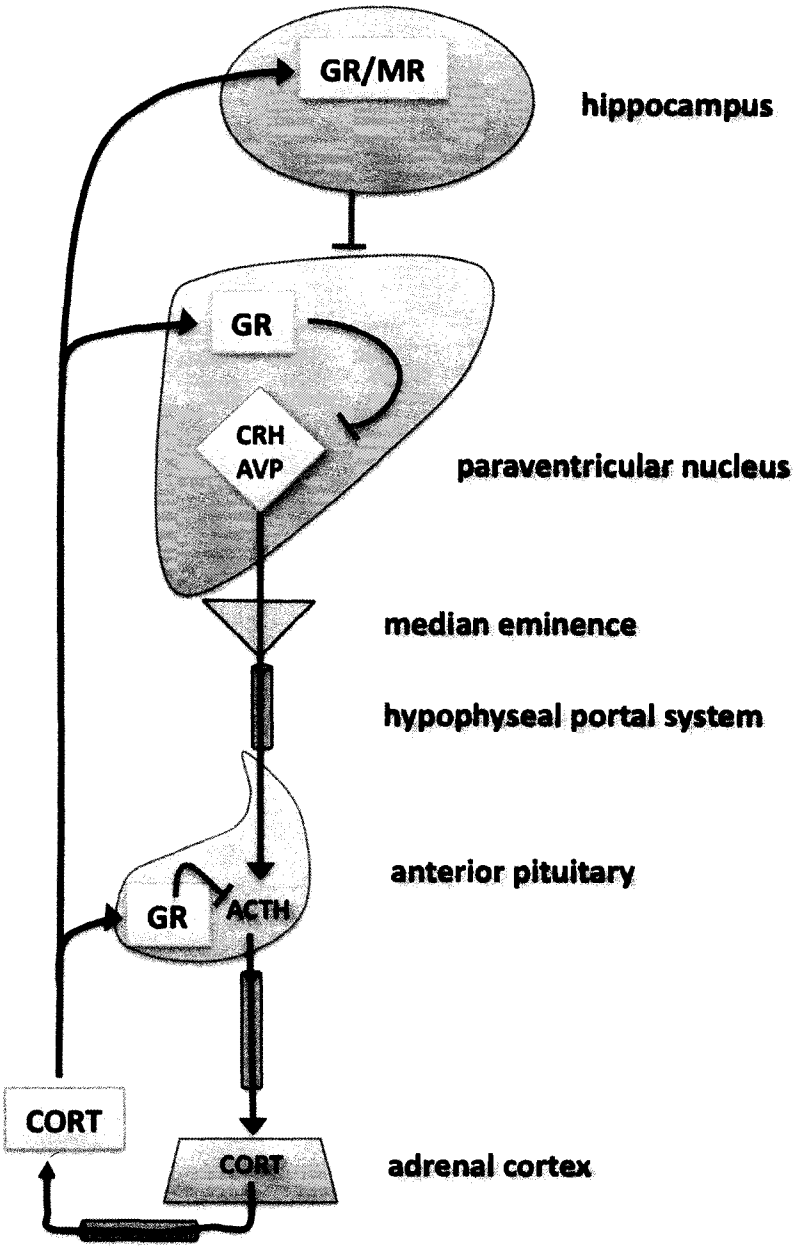


Figure 2.7. Schematic illustration of the negative feedback system of the hypothalamic-pituitary-adrenal (HPA) axis. The end product of the HPA axis, corticosterone (CORT), acts via mineralcorticoid and/or glucocorticoid receptors (MR and GR, respectively) in the hippocampus, paraventricular nucleus, and anterior pituitary to inhibit further synthesis and secretion of corticotropin-releasing hormone (CRH), vasopressin (AVP), adrenocorticotropin hormone (ACTH), and CORT.

negative feedback up on the HPA axis that is secondary to those actions of GR in the PVN (Van Haarst, Oitzl et al. 1997).

Glucocorticoid effects vary according to duration of exposure. Fast feedback occurs within seconds to minutes, whereas delayed feedback occurs within minutes to hours (Dallman, Akana et al. 1987). Fast feedback occurs in a timeframe that is too short for de novo protein synthesis, and must be attributable to non-genomic actions of glucocortioids. These actions have been shown to involve the classical GR and MR as well as endocannabinoids (Widmaier and Dallman 1984; Hinz and Hirschelmann 2000; Di, Malcher-Lopes et al. 2003; Patel, Roelke et al. 2004; Di, Malcher-Lopes et al. 2005). Delayed feedback occurs in a timeframe that likely involves influences on gene transcription and de novo protein synthesis and is attributable to activation of GR and/or MR, and may be direct or indirect in nature.

Influence of glucocortioids on CRH synthesis appears to have several potential mechanisms. For example, glucocorticoids have been shown in inhibit cyclic AMP response element binding protein (CREB) phosphorylation and subsequently interfere with CREB binding to the cyclic AMP response element (CRE) on the promoter of the CRH gene (Legradi, Holzer et al. 1997). The CRH promoter also contains functional negative GRE (nGRE) sights where ligand activated GR can act to inhibit CRH transcription (Malkoski, Handanos et al. 1997; Malkoski and Dorin 1999). Furthermore, glucocorticoids may alter chromatin remodeling and thus transcriptional activity by modulating histone acetylation state and DNA methylation (see previous section on GR) (Ito, Barnes et al. 2000; Banks, Deterding et al. 2001; Sheldon, Becker et al. 2001;

Thomassin, Flavin et al. 2001). GR has also been shown to directly modulate CRH and AVP promoter activity *in vitro* in heterologous cell lines (Guardiola-Diaz, Kolinske et al. 1996; Iwasaki, Oiso et al. 1997; Malkoski and Dorin 1999).

The modulation of ACTH synthesis by glucocorticoids has been studied extensively in the AtT-20 cell line that expresses and secretes ACTH in response to stimulation, and provides an *in vitro* model for the corticotrope (Gumbiner and Kelly 1981). Indeed, glucocorticoids inhibit the synthesis and release of ACTH from the cells. This negative regulation of ACTH synthesis is in part due to the actions of GR at the nGRE and interactions with AP-1 sites contained within the POMC promoter (Drouin, Sun et al. 1993; Boutillier, Monnier et al. 1995; Murphy and Conneely 1997). Additonally, glucocorticoids could inhibit the CRH-stimulated secretion of ACTH through a yet-unidentified mechanism involving second messenger pathways (Tian, Hammond et al. 2001).

Circadian influences on the HPA axis

The HPA axis operates under a circadian rhythm that is both diurnal and ultradian in nature. Basal glucocorticoid secretion peaks daily near the onset of activity (early morning for humans, and early evening for rodents), and can reach levels five to ten times that at the nadir of secretion. Interestingly, ACTH shows a low-amplitude rhythm that varies by up to two fold in plasma concentrations from trough to peak (Dallman, Engeland et al. 1978; Kaneko, Hiroshige et al. 1980; Wilkinson, Shinsako et al. 1981; Akana, Cascio et al. 1986; Kalsbeek, van Heerikhuize et al. 1996). This is mainly

due to a concurrent rhythm in adrenal sensitivity to ACTH (Dallman, Engeland et al. 1978; Kaneko, Hiroshige et al. 1980; Kaneko, Kaneko et al. 1981). Glucocorticoids are also secreted in a pulsatile fashion throughout the day with pulses an hour apart on average (Lightman, Windle et al. 2000). The syncronization between ACTH and CORT pulses is poor, and suggests other adrenal specific mechanisms. Indeed, splachnic nerve transection increases the number of CORT pulses throughout the day (Jasper and Engeland 1997).

There appears to be a delicate interplay between the rhythm of CRH and ACTH secretion with the sensitivity of the adrenal cortex to ACTH and influence of adrenal sympathetic inputs. CRH deficient mice lack a diurnal surge in CORT secretion underlying the importance of CRH in the circadian rhythm (Muglia, Jacobson et al. 1997). AVP, however, is not essential to the diurnal rhythm as evidenced by the fact that deletion of the AVP gene does not eliminate the daily CORT rhythm (Abel and Majzoub 2005). Lesions to the SCN result in an absence of CORT rhythm (Ibuka and Kawamura 1975; Cascio, Shinsako et al. 1987). The SCN sends vasopressinergic projections to the paraventicular and peri-PVN regions (areas with direct inhibitory influence on parvocellular PVN neurons) in addition to the dorsal and lateral parvocellular areas that are rich in pre-autonomic brainstem projecting neurons (Buijs, Hermes et al. 1998). This suggests that the SCN is poised to control CRH and ACTH rhythmicity in addition to tuning autonomic innervation to the adrenal gland.

6. Anatomy of the paraventricular nucleus

Functional subdivisions, neuroendocrine vs non-neuroendocrine, and phenotypes

The paraventricular nucleus (PVN) of the hypothalamus is a well-known diverse collection of neurons well positioned to coordinate the neuroendocrine, autonomic, and behavioral responses to threats of homeostasis (stressors) as well as regulate energy and water balance. The rat paraventricular nucleus is the most comprehensively studied, and consists on the order of 100,000 cell bodies in a volume of approximately $0.5mm^3$ arranged in a wing or triangular shape structure along dorsal portion of the third ventricle in the anterior or supraoptic region of the hypothalamus (Figure 2.8). The PVN was first discovered in the guinea pig as the "subcommissural nucleus" by Ziehen in 1901 (Ziehen 1901). Soon after, in 1904, Cajal made the first illustrations of the guinea pig PVN (Cajal 1904). Then in 1910, Malone coined the name paraventricular nucleus in describing the human PVN (Malone 1910). Fortuyn subsequently described it in the rabbit in 1912 (Fortuyn 1912), and Nissl in the rat in 1913 (Nissl 1913). The initial attempts at a cytoarchitectural organization of the rat PVN was described by Gurdjian in 1927 based upon Nissl staining and comprised of a medial group with small cell bodies, a dense lateral group with medium to large cell bodies, and a dorsal group with unique Nissl staining properties (Gurdjian 1927). The group of densely packed neurons with large cell bodies (later termed magnocellular) was shown to have projections to the posterior pituitary by Bargmann in 1949 (Bargmann 1949). Then, in the early 1980s, Armstrong and Swanson both described in detail two similar versions of the cytoarchitectural (spatial) and chemoarchitechtural (functional)

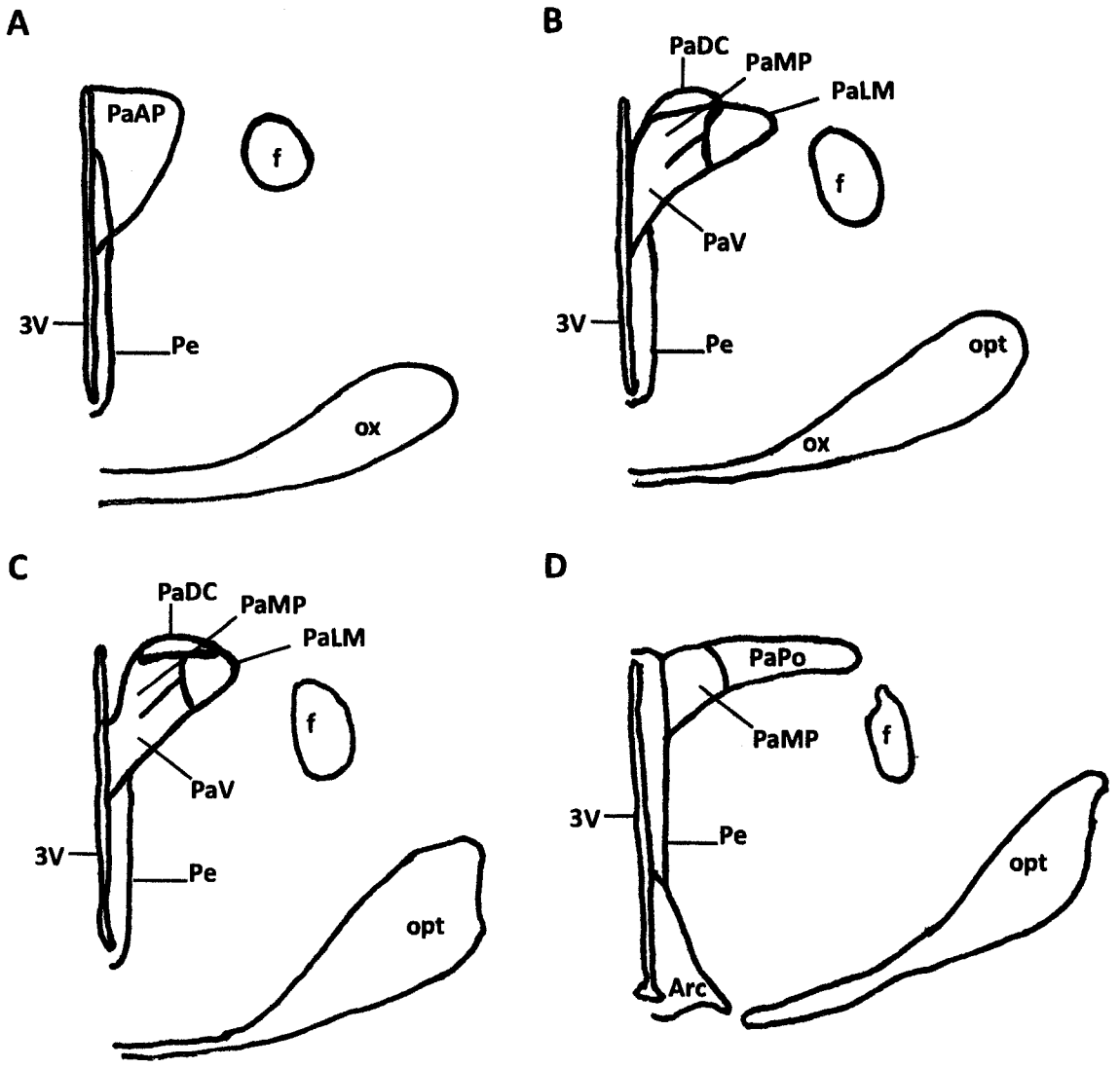


Figure 2.8. Representative drawings of the anatomical organization of the paraventricular nucleus (Pa) of the adult rat. Drawings are adapted from the atlas of G. Paxinos (Paxinos and Watson 1998), and are coordinated to bregma -1.60 (A), -1.80 (B), -1.88 (C), -2.12 (D). 3V, third ventricle; Arc, arcuate nucleus; f, fornix; opt, optic tract; PaAP, anterior parvocellular; PaDC, dorsal cap; PaLM, lateral magnocellular; PaMP, medial parvocellular; PaV, ventral; PaPo, posterior; Pe, periventricular.

organization of the rat PVN based upon tract tracings, golgi impregnations, and immunocytochemistry (Armstrong, Warach et al. 1980; Swanson and Kuypers 1980).

Neurons of the PVN fall into three broad classes. The first, neurosecretory parvocellular neurons, send their axonal projections to the external layer of the median eminence where they secrete hormones (CRH, AVP, TRH, SS) that act as releasing factors for several anterior pituitary hormones (ACTH, TSH, GH) (hypothalamoadenohypophysial system). The second, neurosecretory magnocellular neurons, send their axonal projections to fenestrated capillaries in the posterior pituitary where they secrete hormones into the general circulation (hypothalamoneurohypophysial system). The third, long-projecting descending neurons, send their axonal projections to the brainstem and spinal cord regions involved in autonomic control and somatosensory function. Thus, PVN neurons are largely classified based upon their output, however, they can also be differentiated by their phenotype, afferent input, cell size and density, and dendritic morphology (Armstrong, Warach et al. 1980; van den Pol 1982; Ju, Liu et al. 1986; Swanson, Sawchenko et al. 1986; Rho and Swanson 1989; Kiss, Martos et al. 1991). Armstrong and Swanson characterized the cytoarchitecture of the PVN utilizing different naming paradigms. The nomenclature described in "The Rat Nervous System", 3rd edition, will be used for the purpose of this discussion (Armstrong 2004).

The bulk of PVN magnocellular neurons can be found in two distinct adjoining areas. The medial magnocellular (PaMM) division lies anteromedially within the PVN and contains mainly OT neurons (Figure 2.8). Adjacent yet posterior and dorsal to the

PaMM is the lateral magnocellular (PaLM) division that is comprised of a unique sphere-shaped mass of primarily AVP neurons. This dense sphere of AVP neurons in the PaLM is surrounded by a loop of OT neurons. The majority of these neurons project to the neurohypophysis (neurosecretory). Additionally, there are two groups of accessory magnocellular neurons that are sometimes classified as PVN neurons (Swanson and Kuypers 1980), but are not considered part of the PVN for this discussion. The anterior commissural (AC) magnocellular neurons are 300-400 μm rostral and discontiguous with the main groups of PVN magnocelluar neurons. The periventricular (PeM) magnocellular neurons are closely associated with the AC and start at the same rostral-caudal level, but extend more caudally than the AC. The dendrites of the PeM form a substantial subependymal network (Sofroniew and Glasmann 1981; Ju, Liu et al. 1986). Both the AC and PeM send their axonal projections to the posterior pituitary (Sherlock, Field et al. 1975; Armstrong, Warach et al. 1980; Ju, Liu et al. 1986).

There is a substantial group of small to medium sized neurons in the PVN that send axonal projections to the median eminence. These parvocellular neurons reside in two main areas of the PVN. The anterior parvocellular (PaAP) division extends from the caudal boundary of the AC magnocellular region to the rostral boundary of the PaMM and just lateral to the periventricular area (Figure 2.8). The medial parvocellular (PaMP) division lies lateral to the periventricular area and medial to the PaMM and runs most of the length of the PaMM. Most of the PaAP and PaMP neurons project to the median eminence or to other hypothalamic and extrahypothalamic regions. These other projections include projections to circumventricular organs (subfornical organ and

organum vasculosum of the lamina terminalis), a dorsal projection to the thalamic paraventricular nucleus, a ventrolateral projection to the medial and central nucleus of the amygdala, a rostrovenral projection to the AVPV, and direct intrahypothalamic projections to the anterior hypothalamus, dorso- and ventromedial nuclei, and arcuate nucleus (Luiten, ter Horst et al. 1985; Ter Horst and Luiten 1987; Larsen, Moller et al. 1991). The PaAP and PaMP are very chemically diverse and have been shown to contain AVP, CRH, angiotensin II, atrial naturiuretic peptide, bombesin, cocaine and amphetamine-regulated transcript, cholecystokinin, pituitary adenylate cyclase-activating polypeptide, growth hormone relasing factor, tyrosine hydroxylase, GABA, enkephalins, galanin, neurotensin, somatostatin, thyrotropin-releasing hormone, and vasoactive intestinal peptide (Swanson, Sawchenko et al. 1986; Swanson 1987; Hokfelt, Meister et al. 1990; Hannibal, Mikkelsen et al. 1995; Broberger 1999). The majority of CRH, TRH, and somatostatin in the median eminence originates from the parvocellular PVN (Brownstein, Eskay et al. 1982; Antoni, Palkovits et al. 1983; Kawano and Daikoku 1988; Merchenthaler, Setalo et al. 1989; Kawano, Tsuruo et al. 1991). There is some degree of colocalization between peptides, however some chemoarchitectural trends are seen. Generally, dopamine and somatostatin neurons are found medial to TRH neurons, which are in turn found medial to CRH neurons (Swanson and Kuypers 1980; Kiss, Martos et al. 1991). AVP expression is generally low in the parvocellular PVN, however AVP is colocalized with over half of the CRH neurons (Whitnall and Gainer 1988), and is found in nearly all CRH neurons following adrenalectomy (Kiss, Mezey et al. 1984). The dendritic tress of PaMP and PaAP are largely confined to within the PVN

and typically spread medially toward the third ventricle, or dorsally and ventrally to other subnuclei (van den Pol 1982; Rho and Swanson 1987; Rho and Swanson 1989). There is a group of accessory parvocellular neurons in the periventricular area along the third ventricle and just medial to the PaMP. These periventricular parvocellular (PeP) neurons are sometimes classified as PVN neurons (Swanson and Kuypers 1980), but for the purpose of this discussion they are labeled as an accessory parvocellular area (Armstrong 2004).

The majority of nonsecretory PVN neurons are found in three main regions, the dorsomedial cap (PaDC), the ventral PVN (PaV), and the posterior PVN (PaP) (Figure 2.8). These neurons largely project to autonomic preganglionic and associated nuclei. The PaDC lies dorsal to the PaMP and runs the length of the PaMP. It sends a majority of its projections to the lateral gray horn of the spinal cord (intermediolateral cell column). The PaV is ventral to the PaMP along its entire length and the PaP is largely posterior and lateral to the PaMP and PaMM and directly posterior to the PaLM. The PaV and PaP project to a wide array of brainstem and spinal cord regions including the dorsal vagus motor nucleus, nucleus of the solitary tract, periaqueductal gray, dorsal raphe nuclei, locus coeruleus, parabrachial nucleus, and ventrolateral reticular nucleus (Saper, Swanson et al. 1976; Luiten, ter Horst et al. 1985; Shapiro and Miselis 1985; Hoysoya, Sugiura et al. 1991; Shafton, Ryan et al. 1998; Pyner and Coote 2000). These descending projections may communicate via glutamate, GABA, or various neuropeptides. These peptides include a population of descending AVP and descending OXY neurons (Sofroniew and Schrell 1982), in addition to CRH (Sawchenko 1987; Milner, Reis et al.

1993; Jansen, Wessendorf et al. 1995), angiotensin II (Jansen, Wessendorf et al. 1995), and bombesin (Costello, Brown et al. 1991).

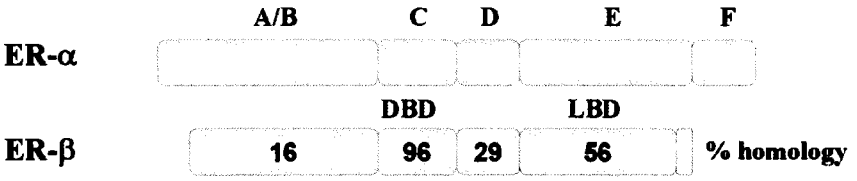
It is currently unknown whether interneurons exist within the PVN. However, there are axon collaterals from PVN neurons that terminate within the PVN. The PaMP has an array of axon collaterals that terminate in the PaMP and the PaLM divisions (van den Pol 1982). Some CRH neurons send recurrent collaterals and could partially explain the existence of CRH-positive synapses on CRH PaMP neurons themselves, in addition to PaMP TRH neurons, magnocellular regions, and the periventricular area (Liposits, Paull et al. 1985; Silverman, Hou-Yu et al. 1989; Hisano, Fukui et al. 1993).

7. Estrogen Receptor Overview

Types of estrogen receptors

The genomic actions of estrogen are mediated by two distinct intracellular receptors that function as ligand-activated transcription factors. These have been termed estrogen receptor alpha (ERα) and beta (ERβ) (Green, Walter et al. 1986; Kuiper, Enmark et al. 1996) (Figure 2.9). For both forms of ER, the binding of estrogen results in receptor dimerization, binding to specific DNA sites in gene promoter regions known as estrogen response elements (ERE), and subsequent modulation of gene transcription (Tsai and O'Malley 1994). ERα and ERβ share similar DNA binding domains (96% homology), similar ligand binding domains (56% homology), and bind to the same hormone response element on DNA (Kuiper, Enmark et al. 1996).

ER Protein Structure:



ER-β Exon Structure:

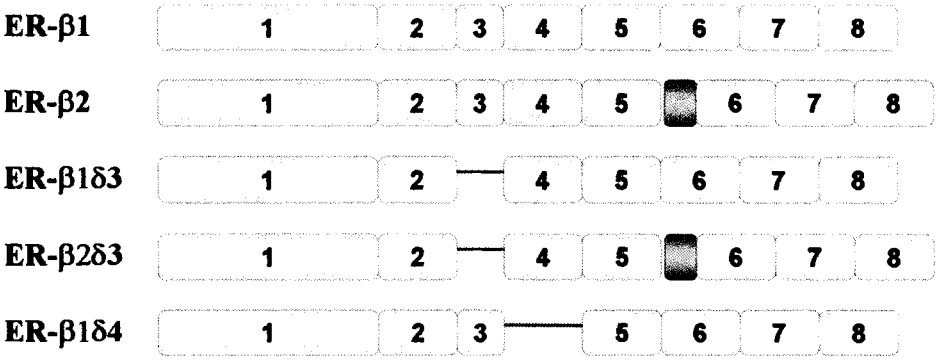


Figure 2.9. Schematic representation of ERα and ERβ protein structure and relative homology, and the exon structure of the five known ERβ splice variants expressed in the rat brain. β2 variants contain a 54 nucleotide (18 amino acid) insertion between exons 5 and 6. Deletions are indicated by a single line, and insertions are indicated by a shaded box. DBD = DNA binding domain, LBD = ligand binding domain. Adapted from Weiser et al. (Weiser, Foradori et al. 2008).

Transcriptional activity of estrogen receptors

Although the classically described mechanism for ER regulation of transcription involves the binding of ERs to an ERE, non-classical mechanisms have also been described. ERs can enhance transcription by modulating the activity of the activator protein complex-1 (AP-1) (Webb, Lopez et al. 1995; Pak, Chung et al. 2005), and it is this non-classical mechanism that diversifies many of the actions of ERs in regulating endogenous promoters. For example, an important difference exists between ERα and ERβ concerning activation through AP-1 sites. ERα is able to activate AP-1 containing promoters in the presence of agonists, such as estradiol or diethylstilbestrol (DES), and the partial agonist/antagonist tamoxifen. In contrast, ERβ is only able to activate transcription from AP-1 sites in the presence of antagonists (Paech, Webb et al. 1997). The AP-1 regulation has been shown to be ER-sensitive in a manner that does not require DNA binding (Webb, Lopez et al. 1995; Webb, Nguyen et al. 1999), but rather through protein:protein interactions with c-Fos, one of the endogenous factors that bind AP-1 elements.

The ability of ERβ to act in a ligand-independent fashion to regulate transcription may provide evidence of its ancient evolutionary roots. Indeed, estrogen receptors have been found in invertebrate species such as *Aplysia californica* and *Octopus vulgaris*. However, although the *Octopus* synthesizes estrogens, such invertebrate ERs do not bind estrogen and are not responsive to estrogens or other steroid hormones. In such cases, ERs are constitutive activators of transcription at an ERE (Thornton, Need et al. 2003; Keay, Bridgham et al. 2006). It appears that like other nuclear receptors, only

later in evolution did the ER attain the ability to bind ligand and exploit the ability to function in a ligand dependent fashion (Escriva, Safi et al. 1997; Thornton 2001). Thus, unlike ERα, ERβ may represent a transitional protein that retains many of the ancient characteristics of the family, such as ligand independence, but has also developed functional versatility by the addition of ligand-dependent activation properties as well.

Differential affinity of estrogen receptors for ligands

Interestingly, although ERα and ERβ share similar ligand binding domains, ERβ possesses a relative binding affinity (RBA) for several steroid hormones that differs from that of ERα (Kuiper, Lemmen et al. 1998). Moreover, subtype-selective high affinity agonists and antagonists allow for the pharmacological examination of receptor-mediated functions in a normal wild-type animal [for review see (Veeneman 2005; Harris 2007)]. For example, propylpyrazoletriol (PPT) has a relative binding affinity (RBA, as compared to estradiol = 100) for ERα of 49 and binding is 410-fold more selective for ERα than ERβ. In contrast, diarylpropionitrile (DPN) has a RBA for ERβ of 18 and binding is 72-fold more selective for ERβ than ERα (Stauffer, Coletta et al. 2000; Meyers, Sun et al. 2001). Transcriptional selectivity of these compounds are greater than that of binding, with DPN having a 170-fold greater relative potency in transcription assays for ERβ (Meyers, Sun et al. 2001). Other ligands, including MPP (ERα antagonist), WAY-200070 (ERβ agonist), PHTPP (ERβ antagonist), and plant-derived phytoestrogens (genistein, coumesterol, and equol) all exhibit some selectivity for ERβ. Interestingly, the dihydrotestosterone metabolite, 5α-androstane, 3β, 17β-diol (3β-Diol), is a

somewhat selective ERβ agonist and can activate ERE-mediated transcription in the presence of ERβ at a much greater selectivity than binding would predict (Handa, Pak et al. 2008) (the role of 3β-Diol in ERβ signaling will be discussed in a subsequent section). These subtype selective agonists provide indispensable tools for use in functional assays.

Distribution of estrogen receptors in brain

ERα and ERβ are expressed throughout the rostral-caudal extent of the brain and spinal cord (Figure 2.10). These receptors have been shown to have overlapping expression patterns with a few exceptions where either ERα or ERβ are not expressed, or one of the receptors is expressed at significantly higher levels compared to the other. Brain regions, including the bed nucleus of the stria terminalis (BNST), medial and cortical amygdaloid nuclei, preoptic area (POA), lateral habenula, periaqueductal gray, parabrachial nucleus, locus ceruleus, nucleus of the solitary tract, spinal trigeminal nucleus and superficial laminae of the spinal cord, express both forms of ER. However there are also striking differences in the expression pattern in certain brain areas. Only ERα is found in the subfornical organ. In contrast, neurons of the olfactory bulb, supraoptic (SON), paraventricular (PVN), suprachiasmatic (SCN), and tuberal hypothalamic nuclei, zona incerta, ventral tegmental area, cerebellum, laminae III–V, VIII, and IX of the spinal cord, and pineal gland contain exclusively ERβ. Although both receptors are expressed by neurons in the arcuate nucleus, ventral medial hypothalamus, and hippocampus, ERα is more abundant in the arcuate nucleus and

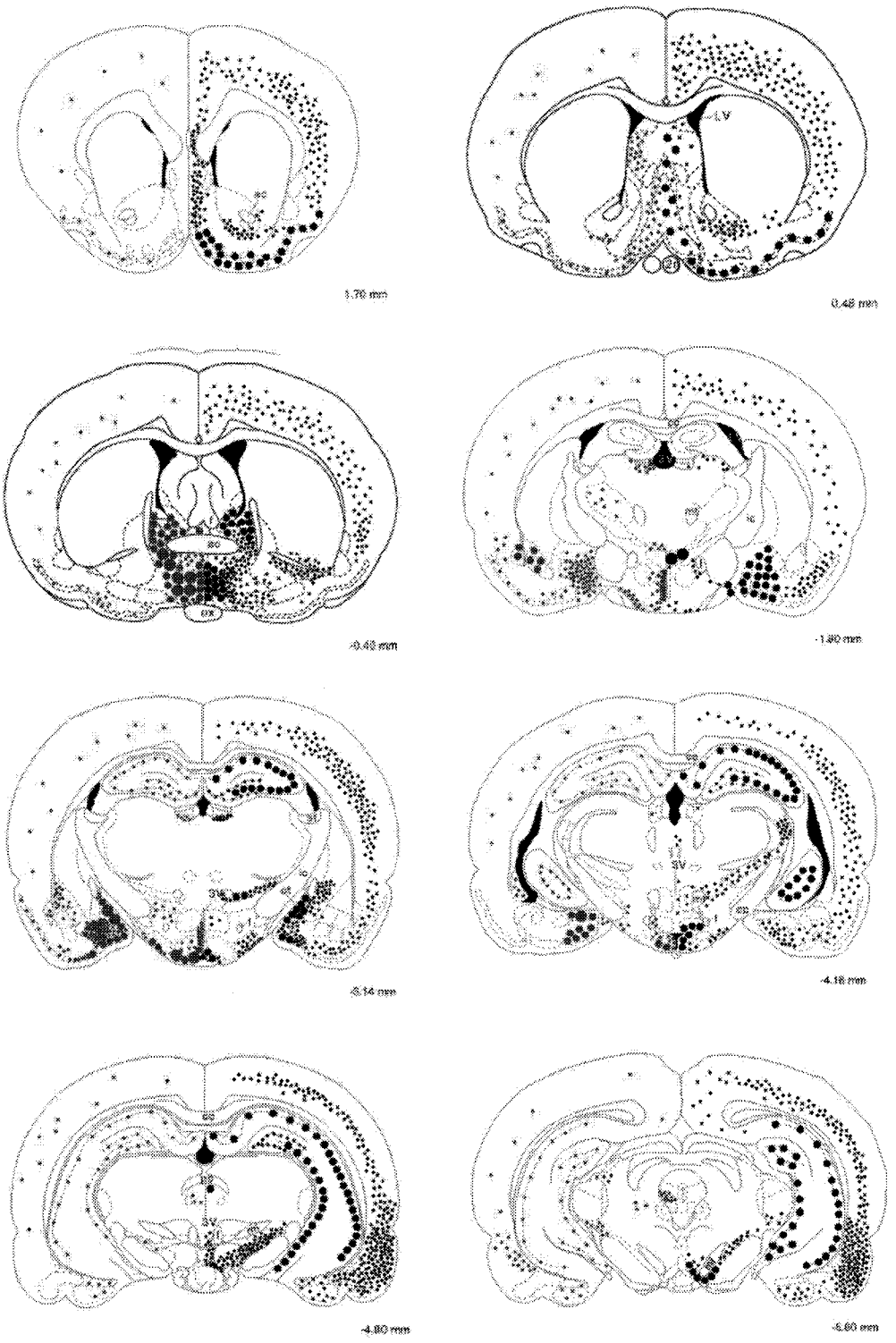


Figure 2.10. Schematic illustration of the relative distribution of ERα and ERβ mRNA expression in the adult rat brain. Density of expression is indicated by density of dots and relative level of expression is indicated by the dot size (red/left, ERα; black/right, ERβ). Adapted from P.J. Shughrue (Shughrue, Lane et al. 1998).

VMH, whereas ERβ is more prevalent in the hippocampus (Shughrue, Komm et al. 1996; Chu and Fuller 1997; Kuiper, Carlsson et al. 1997; Shughrue, Scrimo et al. 1997; Laflamme, Nappi et al. 1998; Hileman, Handa et al. 1999; Mitra, Hoskin et al. 2003). Several studies have also demonstrated that glial cells (both astrocytes and oligodendrocytes) can also express ERα and ERβ (Santagati, Melcangi et al. 1994; Azcoitia, Sierra et al. 1999; Platania, Laureanti et al. 2003; Zhang, Cerghet et al. 2004; Mhyre and Dorsa 2006), although the function of glial ERs are not known.

Hormonal regulation of estrogen receptors

ERβ has been shown to be differentially regulated under a number of physiological conditions. ERβ expression levels in the periventricular preoptic, SON and posterodorsal medial amygdala are strikingly different in pregnant and proestrous females. ERβ mRNA expression in the rat POA and medial basal hypothalamus is highest during the diestrous phase of the estrous cycle (Arteaga-Lopez, Dominguez et al. 2003). In addition, the number of ERβ mRNA expressing cells and ERβ immunoreactive cells are significantly reduced following estrogen treatment of ovariectomized female rats in the external plexiform layer of the olfactory bulb, entorhinal cortex, intermediate part of the lateral septal nucleus, nucleus of the horizontal limb of the diagonal band, lateral, medial and basolateral parts of the amygdala, anteroventral, laterodorsal and lateral posterior parts of the thalamus, medial geniculate nucleus, PVN, BNST, periventricular preoptic, SCN and Purkinje cells in the cerebellum (Osterlund, Kuiper et al. 1998; Patisaul, Whitten et al. 1999; Shima, Yamaguchi et al. 2003). Interestingly, the effect on

ERβ expression is lost in ERαKO animals, implicating estradiol signaling via ERα in the downregulation of central ERβ expression (Nomura, Korach et al. 2003).

The ERα gene is also sensitive to the estrogen state of the animal. Whereas estradiol decreases the brain expression of ERβ globally (Patisaul, Whitten et al. 1999; Shima, Yamaguchi et al. 2003), it appears to have varying effects on ERα expression depending on dose and brain region (Simerly and Young 1991; Osterlund, Kuiper et al. 1998; Prange-Kiel, Wehrenberg et al. 2003; Rose'Meyer, Mellick et al. 2003). For example, during estrous ERα mRNA is expressed in the brain at higher levels than during proestrous (Simerly and Young 1991; Shughrue, Bushnell et al. 1992). Moreover, ovariectomy induces an increase in ERα mRNA in areas such as the VMH, ARC, and MPOA (Simerly and Young 1991; Shughrue, Bushnell et al. 1992). In primary hippocampal cultures, 17β-estradiol treatment has been shown to increase ERα expression, but decrease ERβ (Prange-Kiel, Wehrenberg et al. 2003). On the other hand, a chronic high dose of estradiol increases ERα expression in the arcuate nucleus, VMH and cortical nucleus of the amygdala, but not in other brain regions (Osterlund, Kuiper et al. 1998).

In addition to its cognate ligand, adrenal steroid levels also influence ERβ expression. For example, the synthetic glucocorticoid dexamethasone (DEX) can change the protein and expression levels of ERβ in the PVN and SON of ovariectomized female rats. DEX treatment of ovariectomized female rats increases ERβ expression (Suzuki and Handa 2004). Similarly, Isgor et al. (Isgor, Cecchi et al. 2003) have shown that removal of endogenous glucocorticoids by adrenalectomy reduces ERβ mRNA levels in the PVN

of female rats and that corticosterone replacement reverses this effect. However, in the latter study, up-regulation of ERβ mRNA by adrenal steroids was observed only during proestrous when estrogen levels are high. Downregulation of ERβ has also been seen in the SON following hypernatremia stress (Somponpun and Sladek 2003; Somponpun, Johnson et al. 2004). In summary, the endogenous steroid milieu is a determining factor in the relative abundance of ERα and ERβ in certain brain regions.

8. ERα and ERβ splice variants

ERα splice variants

The ERα gene is highly conserved among mammals. In rat, mouse, and human the ERα gene is preceded by several promoters that can generate several splice variants (Kos, O'Brien et al. 2000). The human ERα gene contains at least seven upstream promoters, the mouse ERα gene contains at least six, and the rat ERα gene is preceded by at least five promoters. Alternative splicing occurs at the first exon within each promoter, resulting in splice variants that differ only in their 5' untranslated region. All splice variants produce the same protein, however the individual mRNAs may differ in their stability and/or ability to be processed. Thus, depending on the promoter utilized, there may more or less protein synthesized depending on the nature of the nacent transcript. This may explain why there are instances where ERα mRNA levels do not correlate well with protein levels (Toran-Allerand, Miranda et al. 1992; Zhou, Shughrue et al. 1995; Pasterkamp, Yuri et al. 1997).

In rat, the ERα promoters include C, 0S, 0N, 0/B, and 0T (Freyschuss and Grandien 1996; Hirata, Koh et al. 1996; Hirata, Koh et al. 1996; Osada, Hirata et al. 2001). 0/B is utilized in the hypothalamus, amygdala, cortex, hippocampus, anterior pituitary, ovary and uterus. Promoter 0N is used in the liver, and both promoters 0/B and 0S operate during development in the neonatal cortex (Freyschuss and Grandien 1996; Hamada, Wada-Kiyama et al. 2005). Promoter 0T is a new member of the family and, while it appears to be utilized in the brain, its function is unknown (Osada, Hirata et al. 2001).

The ERα gene is sensitive to developmental stage is therefore dynamic during development. During the first two weeks of life ERα expression is high, especially within the cortex and hippocampus (Pfaff and Keiner 1973; Sheridan 1979; Shughrue, Stumpf et al. 1990). There is a precipitous decline in overall brain expression of ERα through development into puberty and adulthood (O'Keefe, Li et al. 1995; Prewitt and Wilson 2007). Interestingly, in females, ERα expression is rapidly upregulated in the cortex following an ischemic insult such as middle cerebral artery occlusion (a rodent model for stroke), and has been shown to be responsible for estradiol's neuroprotective effects following focal ischemia (Dubal, Zhu et al. 2001; Westberry, Prewitt et al. 2008). This may represent a shift to a more developmental phenotype, where ERα expression is rescued and aids in preventing neuronal cell death.

Besides estradiol, and ischemia, little has been reported on what regulates ERα expression in the brain. One possible candidate is epigenetic modulation of the ERα gene. Epigenetic modulations include alterations to histone structure (such as

acetylation, methylation and ubiquitylation) and modifications to DNA (such as methylation) that alter the expression of a gene without changing the genetic code or sequence of the DNA itself (Wolffe and Matzke 1999; Klose and Bird 2006). These changes can be passed on from generation to generation, and provide a possible mechanism by which environmental factors (such as maternal care) can influence gene expression over the lifetime of an animal. DNA methylation in particular inhibits transcription by recruiting methyl binding proteins to bind to the methylated DNA that act to inhibit DNA binding proteins as well activate HDACs that deacetylate histones, further condensing chromatin (Rakyan, Preis et al. 2001). Indeed, studies by Melinda Wilson's lab have shown progressive methylation of two promoters (A and C) expressed in the neonatal mouse cortex. They observed a sharp increase in ERα promoter methylation by P10, which corresponds with the decline in ERα expression seen in the developing cortex (Wilson, Westberry et al. 2008). Taken together, ERα expression is a dynamic interplay of specific promoter expression and utilization in addition to epigenetic control of gene responsiveness.

ERβ Splice Variant Characterization and Localization in Brain

Estradiol signaling through ERβ may be more complicated than ERα due to the existence of several splice variants of ERβ. There are five splice variants of ERβ mRNA in the rat described to date, including the originally described wild-type form ERβ (ER-β1), that are thought to arise from alternative splicing of the eight exons which encode ER-β (ER-β1, ER-β2, ER-β1δ3, ER-β2δ3, ER-β1δ4). Transcripts designated ER-β2 possess an in-

frame insertion between exons 5 and 6 that encodes an additional 18 amino acids (AAs) in the ligand binding domain (Chu and Fuller 1997); (Maruyama, Endoh et al. 1998). A deletion of exon 3, which encodes 39 AAs in the carboxyl-terminal half of the DNA binding domain, has been termed ER-β1δ3. ER-β2δ3 is characterized by the addition of 18 AAs inserted between exons 5 and 6 and a deletion of exon 3 (Petersen, Tkalcevic et al. 1998). ER-β1δ4 encodes an ERβ that is missing exon 4 and does not appear to bind estrogen (Price, Butler et al. 2001).

The Handa lab (Price and Handa 2000; Price, Lorenzon et al. 2000) and others (Petersen, Tkalcevic et al. 1998) have shown that splice variants of ERβ mRNA are expressed in multiple tissues and in some cases at levels equivalent to or exceeding those of the full-length mRNA. The high expression level of some of the ERβ mRNA splice variants suggests that if corresponding proteins are expressed, they too would be abundant. Sharma et al. (1999) demonstrated that multiple ERβ variants can be seen with ERβ-specific antisera and Western blot analysis of proteins derived from ovary, a tissue known to express ERβ at high levels (Fitzpatrick, Funkhouser et al. 1999; O'Brien, Park et al. 1999). The distribution of at least one of these splice variants, ER-β2, has been shown in rat brain using anti-peptide antibodies directed against the unique sequence of the insert in the ligand binding domain. These studies have shown a distribution that largely matches that of ER-β1 and indicate high amounts of ER-β2 in SON, cortex and raphe (Chung, Pak et al. 2005). Therefore, the splice variants of ERβ must be considered when assessing receptor function.

ER-β1 is by far the most abundant of the splice variants mRNAs with expression in the lateral septum, SCN, PVN, medial amygdala, hippocampus, cortex and cerebellum with the one exception being the hippocampus. The relative expression levels of ER-β2 and ER-β1δ3 were similar to one another in all ERβ positive brain regions, though both were expressed at a significantly lower level than ER-β1. The isoform that is expressed consistently at the lowest level is ER-β2δ3. In general, the β2 variants are less abundantly expressed than their β1 counterparts (β2 vs. β1 and β2δ3 vs. β1δ3). In the hippocampus, all variants except ER-β1δ4 are expressed at relatively low levels. ER-β1δ4 is also expressed at higher levels in LS and CTX than in the SON, PVN or MA (Petersen, Tkalcevic et al. 1998; Price and Handa 2000; Price, Lorenzon et al. 2000). The respective levels of the others (β1 is the highest; β1δ3; β2δ3 is the lowest) are maintained, similar to the other brain regions.

Binding studies utilizing ^{3}H-estradiol and in vitro transcribed ERβ splice variants have demonstrated that splice variation can provide distinct characteristics to the subsequent forms of ERβ. The relatively high affinity of ER-β1 for estradiol (approx 0.1 nM) is reduced 10 fold by the β2 insertion in the ligand binding domain. In addition, this results in a slower association, and more rapid dissociation of estradiol with ER-β2. Removal of the 3rd exon (δ3 variants) does not seem to have much effect on binding kinetics. Given the changes in estradiol affinity of the β2 splice variant, it is proposed that ER-β2 represents a low affinity form of ERβ that would help extend cellular sensitivity to estradiol with rising levels of estrogen. Such a dual receptor system has been previously proposed for glucocorticoid and mineralocorticoid receptors and their

inhibitory feedback on the HPA axis (Reul and de Kloet 1985; Reul, van den Bosch et al. 1987). Taken together, alternative splicing of nacent ERβ message provides for a diverse group of related receptor proteins that add to the complexity of estradiol signaling.

9. Non-genomic actions of estradiol

It has become increasingly clear that estradiol has rapid cellular effects mediated by actions at the membrane or in the cytosol (non-genomic) in addition to effects on transcriptional regulation of gene expression (classic genomic effects). The mechanisms by which estradiol regulates transcription of target genes has been extensively studied, however the membrane-initiated actions of estradiol are less understood. Estradiol can induce rapid changes in second messanger pathways, such as kinase and phosphatase activation, as well as intracellular ion concentrations, such as calcium. These effects can converge to alter the activation of transcriptional relevant proteins (coactivators, transcription factors, nuclear receptors, etc), and thus may work in tandem with the genomic effects of estradiol to influence gene transcription. Some of the classic estrogen responsive genes such as progesterone receptor (PR), the B-cell lymphoma (Bcl-2) family of pro- and anti-apoptotic genes, oxytocin (OT), OT receptor (OTR), and brain derived neurotropic factor (BDNF) may be regulated by both genomic and non-genomic estradiol-dependent mechanisms. Indeed, it has been shown that estradiol conjugated to BSA (E2-BSA, renders E2 membrane impermeable) can potentiate the transcriptional activity of estradiol (Vasudevan, Kow et al. 2001). Further, E2-BSA has

been shown to induce c-fos expression through MAPK activation in a human neuroblastoma cell line (SK-N-SH), and thus could provide a mechanism by which genes without an ERE-containing promoter could become estrogen-sensitive (Watters, Campbell et al. 1997).

Some of the first evidence of non-genomic actions of estradiol came in 1967 when Szego and Davis observed an increase in cAMP within 15 s of estradiol administration in the uteri of OVX mice (Szego and Davis 1967). Kelly *et al* in 1977 demonstrated a rapid estradiol-dependent increase in neuronal firing in the rat hypothalamus (Kelly, Moss et al. 1977). These were followed by reports on estradiol potentiating excitatory post-synaptic potentials in hippocampal neurons (Foy and Teyler 1983; Smith, Waterhouse et al. 1987), and augmenting glutamate stimulation in the cerebellum (Smith, Waterhouse et al. 1987; Smith 1989). Subsequent studies have confirmed these initial studies indicating rapid changes in neuronal excitability and increases in MAPK, ERK, PKA, PKC, and adenylyl cyclase in response to estradiol or BSA-conjugated estradiol (for reviews see (Schmidt, Gerdes et al. 2000; Vasudevan and Pfaff 2008)). For example, acute estradiol administration potentiates NMDA excitation of hypothalamic neurons (Kow, Easton et al. 2005), whereas in hippocampal cells it augments kainite inward currents in a rapid fashion (Gu and Moss 1996). Acute estradiol can also regulate L-type calcium channels and initiate an Src/ERK signaling cascade in cultured hippocampal neurons (Wu, Wang et al. 2005). These rapid effects can also be observed in estradiol regulation of gonadotrope secretion. Acute estradiol can influence intracellular calcium levels in cultured primate GnRH neurons and

phosphorylated CREB in GT1-7 cells (Abraham, Han et al. 2003; Morales, Diaz et al. 2003; Morales, Diaz et al. 2005; Abe, Keen et al. 2008). In addition, estradiol has been shown to have rapid non-genomic effects on prolactin and LH secretion from the anterior pituitary (Moll and Rosenfield 1984; Christian and Morris 2002). These data indicate that estradiol has many actions too quick to involve *de novo* protein synthesis, and should be considered, especially immediately following acute doses of estradiol or rapid changes in estradiol levels.

The identity of receptors that mediate the rapid effects of estradiol is still somewhat unclear (Watson and Gametchu 1999; Falkenstein, Tillmann et al. 2000; Cato, Nestl et al. 2002; Watson, Campbell et al. 2002). Recent studies have identified an orphan member of the GPCR family, GPR30, that is localized at the plasma membrane, binds estradiol with high affinity, and can couple with $G_{\alpha s}$ to activate adenylyl cyclase (Thomas, Pang et al. 2005; Prossnitz, Arterburn et al. 2008). Some studies claim the existence of GPR30 in the endoplasmic reticulum and suggest that GPR30 may act to increase intracellular calcium in response to estradiol (Revankar, Cimino et al. 2005). Additionally, a novel mER, ER-X, has been found in the neocortex, but has not been definitively characterized as of yet (Toran-Allerand, Guan et al. 2002). On the other hand, the classic ER has been shown to localize to the membrane in some cells (Levin 2005). ICI, an ERα and ERβ antagonist, can block some of the rapid effects of estradiol such as calmodulin kinase activation in hippocampal neurons (Sawai, Bernier et al. 2002) and rapid calcium flux in astrocytes (Chaban, Lakhter et al. 2004). Additionally, estradiol-mediated rapid phosphorylation of ERK in the medial preoptic nucleus is lost in

the double ER knockout mouse (which do not express ERα and ERβ) (Abraham, Todman et al. 2004). Whether these membrane effects of ER are mediated in association with a mER, or alone is unclear. The lack of hydrophobicity and a standard transmembrane structure suggest that the classical ERs need a carrier or tether of some sort to be present at the membrane (Russell, Haynes et al. 2000). ERs may be presented to the membrane by caveolae or lipid rafts, which form specialized plasma membrane signaling platforms (Chambliss, Yuhanna et al. 2000; Chambliss, Yuhanna et al. 2002). ERα has been shown to associate with caveolin-1 in caveolae to support PI3K and adenylyl cyclase signaling (Razandi, Oh et al. 2002). Additionally, in endothelial cells, ERβ in caveolae can activate nitric oxide synthase (NOS) (Chambliss, Yuhanna et al. 2002; Razandi, Oh et al. 2002). In summary, estradiol has rapid effects through classic and non-classic ERs at the membrane or in the cytosol to influence second messenger pathways and ion channel function to regulate neuronal excitability, regulate cell death, and influence transcriptional activity.

10. Androgen Receptors

The Androgen receptor (AR) is similar to ER, as it is a ligand activated transcription factors belonging to the nuclear receptor superfamily. AR is responsible for many of the peripheral and central actions of testosterone (T) and it's 5-α reduced metabolite dihydrotestosterone (DHT). Unlike ER, most unbound AR resides in the cytoplasm (Tyagi, Lavrovsky et al. 2000), and more recent studies have identified AR in axons, dendrites and glial processes (DonCarlos, Garcia-Ovejero et al. 2003; DonCarlos,

Sarkey et al. 2006; Sarkey, Azcoitia et al. 2008). Upon binding to ligand, it typically translocates to the nucleus where it congregates into discrete subnuclear compartments or nuclear foci (similarly to ER) (Htun, Holth et al. 1999; Stenoien, Simeoni et al. 2000; Tyagi, Lavrovsky et al. 2000). It appears that a single AR can undergo several rounds of ligand binding, nuclear translocation, and DNA binding, in contrast to ER, which undergoes proteosomal degradation after engaging in target gene transcription (Nawaz, Lonard et al. 1999; Roy, Tyagi et al. 2001). Thus perhaps ligand inactivation rather than protein degradation is more important in terminating androgen signaling through AR.

Following the isolation of AR in 1970 by Mainwaring, studies performed by Stumpf and Sar utilized radiolabeled T and DHT to examine androgen sensitive brain regions (Sar and Stumpf 1973; Sar and Stumpf 1977). These experiments indicated that androgens have a high affinity for regions of the brain like the hypothalamus that control hormonal (gonadotropin release) and behavioral (copulatory) components of reproduction. Later studies examining the mRNA and protein expression profile for AR in the brain found that AR is expressed within brain areas involved in reproduction such as the MPOA, VMH, ARC, and BST (Handa, Reid et al. 1986; Simerly, Chang et al. 1990; McLachlan, Tempel et al. 1991; Roselli 1991). However, the expression of AR in extrahypothalamic brain regions such as lateral septum, amygdala, hippocampus, and cortex suggests a role for AR signaling outside of reproduction. Indeed, a role for androgens has been implicated in cognition, learning and memory, stress, and mood.

11. Effects of gonadal hormones on anxiety- and depression-like behaviors

Evidence that ERβ plays a role in anxiety- and depressive-type behaviors

Studies performed on βERKO mice provided an initial clue for a role of ERβ in anxiety- or depressive-like behaviors (Krezel et al., 2001). In the forced swim test (FST), a model for depression (Porsolt, Le Pichon et al. 1977), estradiol-treated wild type mice showed less depressive-like behaviors (more time struggling and less time immobile) than controls, however, this effect of estradiol was lost on βERKO mice suggesting that estradiol's antidepressant actions are mediated through ERβ (Rocha, Fleischer et al. 2005). In the elevated plus maze (EPM), a test to model anxiety-like behaviors (Handley and McBlane 1993), two separate studies indicate increased anxiety-like behaviors in βERKO mice relative to their wild-type counterparts (Krezel, Dupont et al. 2001; Imwalle, Gustafsson et al. 2005).

Anatomical evidence supporting a role for ERβ in depressive-like behaviors comes from studies showing that greater than 90% of ERβ-IR neurons in the dorsal raphe and periaqueductal gray also express tryptophan hydroxylase (TPH), the rate limiting enzyme in serotonin synthesis (Nomura, Akama et al. 2005). This finding correlates with the behavioral effects seen following treatment of rodents with ERβ selective ligands (see below), while the existence of ERβ in neuropeptidergic neurons (discussed below) provides a potential link between the neuroendocrine actions of these ligands and their behavioral effects.

Pharmacological studies using ER selective agonists.

Studies utilizing subtype-specific ER agonists have concurred with the experiments using knockout animals (Lund, Rovis et al. 2005). In the open field test, ovariectomized females treated with the ERβ agonist diarylpropionitrile (DPN) spent more time in the middle of the arena, had more novel item interactions and a greater number of rears as compared to controls. The total number of square-crossings remained consistent suggesting an activity-independent decrease in anxiety-type behavior in DPN treated animals. In the light/dark box, DPN treated animals had a significantly longer latency to enter the dark portion of the box. Furthermore, in the elevated plus maze, DPN treated animals had more entries and time spent on the open arms of the maze, a greater number of rears and head dips which are signs of anxiolysis whereas they also exhibited fewer anxiogenic behaviors such as numbers of fecal boli, and time spent grooming as compared to controls. Treatment of gonadectomized males with DPN produced similar effects on anxiety-type behaviors in the EPM. These behavioral effects have since been replicated with the use of other ERβ agonists as shown by the data presented in this dissertation. The effects of DPN are prevented by concomitant treatment with the ER antagonist tamoxifen, indicating an ER-mediated mechanism. Interestingly, treatment with the ERα agonist propylpyrazoletriol (PPT) was anxiogenic on the EPM and OF, and depressant in the FST. Such data could help explain why estrogen has been reported to have both anxiogenic and anxiolytic effects (Palermo-Neto and Dorce 1990; Leret, Molina-Holgado et al. 1994).

Other studies have further confirmed the anxiolytic actions of ERβ agonists. DPN treated ovariectomized females exhibit less depressive-like behavior in the FST and

horizontal crossing task (Walf, Rhodes et al. 2004), as well as decreased anxiety in the EPM (Walf and Frye 2005). Administration of ERβ-selective ligands directly to the hippocampus decrease depressive and anxiety-type behaviors, suggesting a possible role for ERβ in the hippocampus (Walf and Frye 2006). Furthermore, the Flinders Sensitive Line (FSL) of rat, a strain selectively bred for depression, exhibit decreased immobility in the FST and increased social interaction following DPN treatment, both signs of anxiolysis (Overstreet, Osterlund et al. 2006). Recent studies have shown the ERβ agonist WAY-200070 to be anxiolytic in the four-plate test, and anti-depressant in the tail suspension test when peripherally administered to male mice (Hughes, Liu et al. 2008). Therefore, the similarities seen across the three behavioral paradigms (open field, elevated plus maze, and forced swim test), and amongst the different ERβ agonists, all implicate a positive role for ERβ in mood. On the other hand, ERα activation appears to have an opposite effect, and suggests that the behavioral response to estrogen is dependent upon an integration of its effects mediated through ERα and ERβ.

Androgens and mood

The effects of estradiol on mood have been well studied, however the role of androgens in mood is less understood. In male rodents, removal of the endogenous source of androgen by gonadectomy (GDX), causes increased anxiety- and depressive-type behaviors which are reversed by systemic testosterone treatment (Slob, Bogers et al. 1981; Adler, Vescovo et al. 1999; Frye and Seliga 2001). Administration of DHT

also leads to reductions in anxiety- and depressive-like behaviors (Frye and Wawrzycki 2003; Edinger and Frye 2005). Testicular feminized mutation (tfm) male mice, which have an mutation in the AR gene rendering the resulting protein nonfunctional, displace increased anxiety in the elevated plus maze and open field arena that can not be reversed by androgen treatment (Rizk, Robertson et al. 2005; Zuloaga, Morris et al. 2008). In men, chemical castration as a result of prostate cancer treatment is associated with increases in anxiety (Almeida, Waterreus et al. 2004). Similarly, aging in men is associated with a concomitant decline in androgen levels that may lead to a host of behavioral symptoms that overlap greatly with those of major depression (Amore 2005). Taken together, these data indicate that androgens are predominantly anxiolytic and antidepressant in nature.

Potential mechanism for androgen's effects on mood

In studies not described in detail within this dissertation, I examined a potential molecular mechanism to explain androgen's positive effects on stress and mood in collaboration with Tracy Bale's lab at the University of Pennsylvania (Weiser, Goel et al. 2008). Dysregulation of CRH signaling, in particular, plays a key role in the development of depression and anxiety (Heuser, Bissette et al. 1998; Arborelius, Owens et al. 1999; Reul and Holsboer 2002). The receptor for CRH has been termed CRHR and it exists in two different types -R1 and -R2. Both receptors belong to the G-protein coupled receptor family of membrane receptors. Since the original cloning of CRHR2 (Lovenberg, Liaw et al. 1995; Perrin, Donaldson et al. 1995) several splice

variants have been discovered. These include CRHR2α, found predominantly in brain, CRHR2β, found primarily in peripheral tissues, and CRHR2γ, which, to date, has only been found in humans (Lovenberg, Chalmers et al. 1995; Kostich, Chen et al. 1998). Highest areas of CRHR2 expression in the brain include the olfactory bulb, lateral septum, BNST, VMH, medial and posterior cortical nuclei of the amygdala, and midbrain raphe nuclei (Van Pett, Viau et al. 2000). CRHR1 expression is much more widely spread throughout the brain including most hypothalamic areas such as the MPOA, MPN, PVN, SCN, ARC, DMH, and limbic regions such as the hippocampus, medial septum, amygdala (all nuclei), and BNST (Van Pett, Viau et al. 2000).

Several additional endogenous ligands have been discovered for the CRF/urocortin receptor network of CRHR1 and CRHR2 since the discovery of the CRH peptide in 1981 (Vale, Spiess et al. 1981). These ligands include urocortin (Ucn), urocortinII (UcnII, stresscopin-related peptide), and urocortinIII (UcnII, stresscopin) (Donaldson, Sutton et al. 1996; Lewis, Li et al. 2001; Reyes, Lewis et al. 2001). CRH has a 10-fold higher affinity for CRHR1 than CRHR2, whereas Ucn has equal affinity for both receptors, and UcnII and UcnIII are selective for CRHR2 (Hsu and Hsueh 2001). While CRH may only activate CRHR2 during highly stressful events, UcnII and UcnIII may be regulating CRHR2 basal activation. Interestingly, unlike the other urocortins, UcnIII terminal fields overlap well with areas of CRHR2 expression and include the dorsomedial VMH, lateral septum, BNST, and MeA (Li, Vaughan et al. 2002). Thus, UncIII may be the endogenous ligand of choice for CRHR2.

Both receptors for CRH, CRHR1 and CRHR2, have integral roles in regulating stress sensitivity and alterations in receptor expression can be linked to behavioral disorders [for review see (Bale and Vale 2004)]. CRHR2 has been implicated in regulating anxiety-type behaviors and is expressed in stress-responsive brain regions, some of which also contain AR (Van Pett, Viau et al. 2000). Interestingly, the CRHR2 promoter contains EREs and AREs, which suggests a potential role for sex hormones in the modulation of CRHR2 expression (Catalano, Kyriakou et al. 2003). Therefore, we hypothesized that androgen may exert its effects through actions on CRHR2 and consequently examined the regulation of CRHR2 mRNA and receptor binding in the male rat forebrain following androgen administration (Weiser, Goel et al. 2008). Those studies indicate that androgen increases CRHR2 mRNA in specific stress-responsive brain regions. These changes in CRHR2 mRNA also correspond with changes in CRHR2 binding within the lateral septum. Furthermore, androgen increases CRHR2 expression in primary hippocampal cell cultures, an effect that is blocked with concomitant treatment with the AR antagonist flutamide. This suggests that androgen regulation of CRHR2 expression is mediated specifically through androgen receptor activation, at least in the hippocampus. Thus, the CRHR2 gene may be a target for AR-mediated regulation and these data suggest a potential mechanism for androgen modulation of stress and stress-related disorders.

12. Effects of gonadal hormones on hypothalamic-pituitary-adrenal axis activity

Sex differences in HPA axis activity

Gonadal steroid hormones play a vital role in modulating hypothalamic-pituitary-adrenal (HPA) axis function. It has now been established that basal and stress-induced adrenal steroid secretion is greater in females than in males (Critchlow, Liebelt et al. 1963; Kitay 1963; Handa, Burgess et al. 1994), and that the activational effects of gonadal steroids play an integral role in this sex difference (Sencar-Cupovic and Milkovic 1976). In females, ovariectomy reduces stress-induced CORT and ACTH, and this is reversed by estrogen treatment (Burgess and Handa 1992; Handa, Burgess et al. 1994; Suzuki, Lund et al. 2001). However, this is not always the case as several groups have reported that estrogen can inhibit responses to stress (Young, Altemus et al. 2001; Figueiredo, Dolgas et al. 2002; Ochedalski, Subburaju et al. 2007). This discrepancy may be due in part to opposing actions of estradiol signaling through ERα and ERβ (as described in the subsequent section).

In contrast, in males, gonadectomy increases stress-induced CORT and ACTH, an effect is reversible with testosterone or dihydrotestosterone (DHT) treatment (Bingaman, Magnuson et al. 1994; Handa, Burgess et al. 1994; Handa, Nunley et al. 1994; Viau and Meaney 1996; Suzuki, Lund et al. 2001; Viau, Lee et al. 2003; Viau and Meaney 2004). Furthermore, treatment of gonadectomized male rats with estrogen increases stress-induced *c-fos* mRNA, CRH hnRNA, AVP hnRNA, and CORT, whereas DHT treatment inhibits the response when compared to control animals (Lund, Munson et al. 2004). Thus, available evidence suggests that estrogen increases the gain of the HPA axis, while testosterone and its non-aromatizable metabolite DHT decrease the gain of the HPA axis.

Localization of estrogen receptors in stress-sensitive brain regions

Clues to determining how estradiol modulates HPA axis activity can be gleaned by identifying the locations and phenotypes of ER expressing neurons. Information from our laboratory and others has indicated that ERβ is expressed within several different phenotypes of neurons particularly relevant to the HPA axis. ERβ immunoreactivity (IR) has been found in populations of corticotropin-releasing hormone (CRH), vasopressin (AVP), oxytocin (OXY) and prolactin (PRL) containing neurons in the hypothalamus. ERβ-IR is co-localized with OXY-IR within the medial parvocelluar PVN (84% of OXY neurons) (Hrabovszky, Kallo et al. 2004; Suzuki and Handa 2004). Additionally, ERβ-IR is co-localized with CRH-IR within the medial parvocellular PVN (13% of CRH neurons), and ERβ mRNA is found in CRH-IR neurons of the caudolateral PVN (60-80% of ERβ neurons) (Laflamme, Nappi et al. 1998; Suzuki and Handa 2004). ERβ-IR has also been observed within AVP-IR in the parvocellular magnocellular PVN (66% of AVP neurons) (Hrabovszky, Kallo et al. 2004; Suzuki and Handa 2004). PRL-IR neurons of the parvocellular magnocellular PVN also contain ERβ-IR (85% of PRL neurons) (Suzuki and Handa 2004). ERα, on the other hand, is not expressed in the PVN, but is found in brain regions that send direct and indirect projections to the PVN such as the peri-PVN region, BNST, MPOA, amygdala, and hippocampus (Shughrue, Lane et al. 1998; Suzuki and Handa 2005). ERβ, is also expressed in these stress-sensitive brain regions (except for the peri-PVN) indicating that both receptors are well positioned to exert direct (ERβ) and indirect (both) influences on the HPA axis.

Pharmacological studies using selective agonists

The role of ERβ in HPA axis regulation has been explored with the use of ER subtype-selective compounds. For example, studies from our laboratory show that treatment of ovariectomized females with the ERβ selective agonist DPN causes a significant decrease, while treatment with the ERα selective agonist PPT results in a significant increase in stress-induced ACTH and CORT (Lund, Rovis et al. 2005). This is consistent with the behavioral effects seen following treatment with these compounds.

It is likely that sex hormones have influence on all the components of the HPA axis, including the hypothalamus (Handa, Nunley et al. 1994; Viau and Meaney 1996; Viau, Lee et al. 2003), pituitary (Coyne and Kitay 1969; Coyne and Kitay 1971; Viau and Meaney 2004), and adrenal gland. However, the results of studies described in this dissertation suggest that the role of estradiol in HPA axis regulation may be indirect through alteration of glucocorticoid dependent HPA axis negative feedback, in addition to a direct action upon CRH and AVP neurosecretory neurons of the PVN. In studies performed by Lund *et al*, wax pellets containing the ERβ agonist DPN, ERα agonist PPT, or estradiol were implanted near the PVN of gonadectomized male rats. Stress-induced *c-fos* mRNA, and plasma CORT and ACTH levels were measured. Similar to what was observed following peripheral administration, DPN decreased while PPT and estradiol increased stress-induced *c-fos* mRNA and serum CORT levels, and these effects could be blocked with concomitant treatment with tamoxifen (Lund, Hinds et al. 2006). These results suggest that attenuation of HPA reactivity via ERβ or augmentation via ERα is

mediated via neuronal populations in and/or around the PVN. While it has been well established that ERβ is the dominant ER expressed by neurons within the PVN, recent studies have indicated that ERα transcript and immunoreactivity are present near the PVN (peri-PVN) and sparsely within the PVN (Laflamme, Nappi et al. 1998; Suzuki and Handa 2005). Thus, the augmentation of the HPA axis seen with systemic and local treatment with estradiol and PPT may be via ERα expression in neurons surrounding the PVN.

Potential link between HPA axis effects and behavioral effects

Behavioral actions of ERβ activation may be explained by direct effects on regulation of neuropeptides that are involved in the stress response. ERβ has been shown to drive CRH and AVP promoter activity in vitro (Shapiro, Xu et al. 2000; Miller, Suzuki et al. 2004; Pak, Chung et al. 2007), and subtype selective ligands alter CRH, adrenocorticotropin hormone (ACTH), and corticosterone (CORT) responses to a stressor (see below). Both ERα and ERβ have been shown to coordinate with CBP at a CRE to induce CRH transcription in an amygdala-derived neuronal cell line (Lalmansingh and Uht 2008). Other possible mechanisms by which estradiol regulates mood may include ERβ regulation of oxytocinergic neurotransmission. Oxytocin has powerful anxiolytic properties (Uvnas-Moberg, Ahlenius et al. 1994; McCarthy, McDonald et al. 1996; Windle, Shanks et al. 1997; Mantella, Vollmer et al. 2003; Amico, Mantella et al. 2004) that can be augmented by estradiol (McCarthy, McDonald et al. 1996; Ochedalski,

Subburaju et al. 2007), and ERβ has been found in most oxytocinergic neurons (Suzuki and Handa 2005).

The influence of estrogen upon the HPA axis appears to be a delicate interplay between estrogen's actions at ERβ and ERα. These findings implicate ERs particularly around and within the PVN as a site of action for estrogen on HPA axis output. Further studies investigating the mechanisms of ERα and ERβ action on neurosecretory neurons of the PVN is certainly warranted.

Androgens and stress

Evidence from gonadectomized and hormone-replaced male animals suggests that androgens have an inhibitory effect on HPA activity (Handa, Nunley et al. 1994; Viau and Meaney 2004). Treatment with DHT decreases stress-responsive corticosterone and ACTH release, decreases stress-induced cellular activation in the hypothalamus, and decreases CRH levels in the hypothalamus (Bingaman, Magnuson et al. 1994; Lund, Munson et al. 2004; Seale, Wood et al. 2004). Moreover, GDX males have higher stress-induced CORT and ACTH, which is also reversible via testosterone or dihydrotestosterone (DHT) treatment (Handa, Nunley et al. 1994; Viau and Meaney 2004). Gonadectomy is not accompanied by changes in pituitary sensitivity to CRH (Handa, Nunley et al. 1994) or changes in levels of circulating corticosteroid binding globulin (CBG; (Lund, Munson et al. 2004)) suggesting that the actions of androgens on HPA axis reactivity to stress are mediated centrally. Basal CRH expression is lower in males than in females, and gonadectomy in males causes an increase in basal CRH

expression that can be restored by dihydrotestosterone propionate (DHTP) treatment (Haas and George 1988; Bingaman, Magnuson et al. 1994).

The existence of AR in the PVN indicates a potential direct role for androgens in HPA axis regulation. However, the majority of AR-expressing neurons in the PVN are spinal cord projecting and not neurosecretory (Bingham, Williamson et al. 2006). This suggests that AR activation in the PVN might alter the autonomic response to a stressor, but does not directly influence neuropeptide expression. However, AR is expressed in stress sensitive brain regions such as the hippocampus, BNST, and MPOA that project to the PVN (Williamson and Viau 2007). Indeed, studies performed by Viau *et al* have indicated that testosterone implants near the MPOA of GDX'd male rats reduces the ACTH and CORT response to a restraint stress (Viau and Meaney 1996). However, this study is limited in interpretation due to the possibility of local aromatization of testosterone to estradiol and subsequent activation of ER in the region. In summary, androgens are largely inhibitive of HPA axis reactivity.

Are DHT actions mediated by metabolism to 3β-Diol and linked to ERβ?

Adding to the complexity of androgen signaling is the recent discovery that the DHT metabolite 5α-androstane-3β, 17β-diol (3β-Diol) binds ERs, with a higher affinity for ERβ than ERα, and exhibits only weak affinity for AR (Kuiper, Lemmen et al. 1998). Traditionally, studies examining AR biology utilized the non-aromatizable androgen dihydrotestosterone. However it has become increasingly clear that DHT can be irreversibly metabolized to 3β-Diol, among other metabolites, by a number of p450

enzymes including 3α hydroxysteroid dehydrogenase (3α-HSD), 3β hydroxysteroid dehydrogenase (3β-HSD) and 17β hydroxysteroid dehydrogenase (17β-HSD) (Jin and Penning 2001; Weihua, Lathe et al. 2002; Gangloff, Shi et al. 2003; Torn, Nokelainen et al. 2003). Therefore, biological effects once classified as androgen-dependent may be in part or wholly through an ERβ-dependent mechanism. The enzymes necessary to convert DHT to 3β-Diol are present in the brain suggesting that some of androgen's central actions such as regulation of the HPA axis, HPG axis, and behavior may be attributed to activation of ERβ, and not AR (Guennoun, Fiddes et al. 1995) (Figure 2.11).

Similar to that of DHT, 3β-Diol has potent effects on the HPA axis. Peripheral treatment of GDX male mice with 3β-Diol results in reduced plasma CORT and ACTH following restraint (Lund, Munson et al. 2004). This effect is mimicked by DHT, however it is not blocked by concomitant treatment with the AR antagonist flutamide. Furthermore, the ERβ agonist DPN mimicks the effect of 3β-Diol, and interestingly, the ER antagonist tamoxifen abolishes the effect of 3β-diol. These data suggest that DHT's influence on HPA axis reactivity may be a result of 3β-diol's ability to bind ERβ. Interestingly, mRNAs for 3α-HSD and 17β-HSD are present in the PVN of the hypothalamus, suggesting that local metabolism of DHT may integrate the cellular response to DHT. To address this possibility, Lund *et al* administered 3β-diol bilaterally to the PVN of castrated males by stereotaxic implantation of hormone-containing beeswax pellets (Lund, Hinds et al. 2006). In these studies, administration of 3β-Diol to the PVN mimicked the effects seen with peripheral injection. Furthermore, co-treatment with tamoxifen inhibited this effect, while flutamide had little effect.

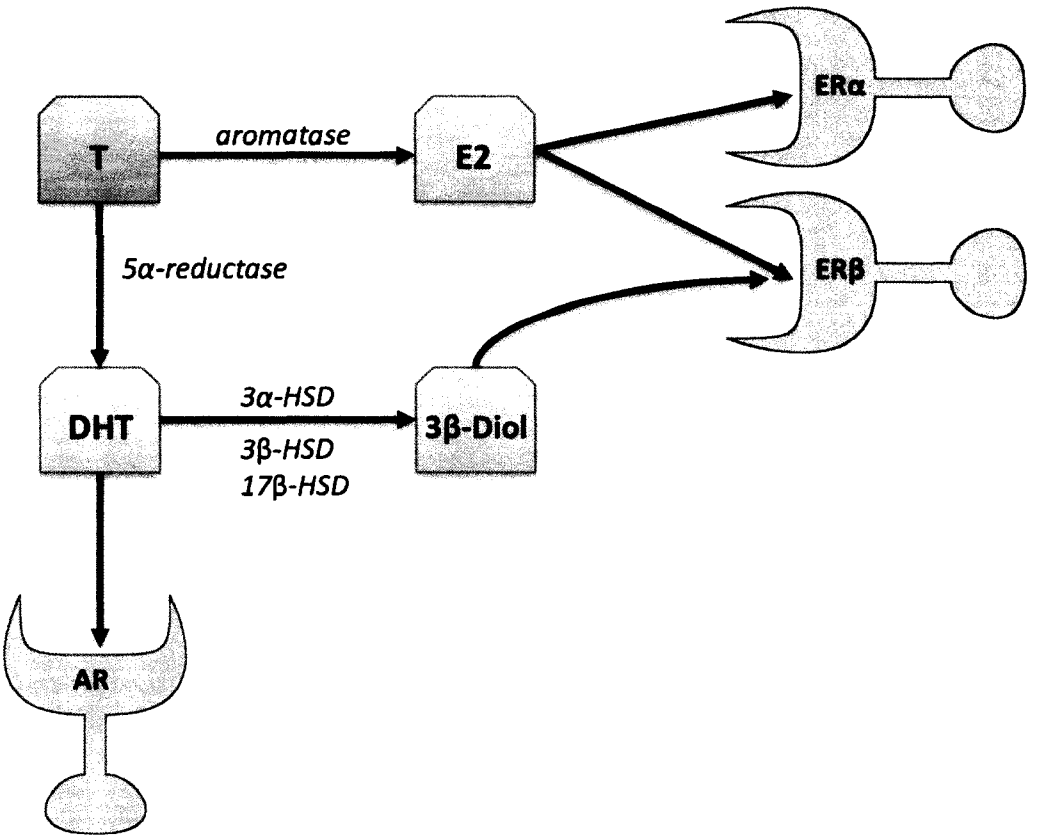


Figure 2.11. Schematic illustration of testosterone metabolite interactions with estrogen receptors. Testosterone (T) is converted to the estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) ligand estradiol (E2) by aromatase. T is also converted by 5α-reductase to the androgen receptor (AR) ligand dihydrotestosterone (DHT). DHT is converted to the ERβ ligand 5α-androstan-3β, 17β-diol (3β-Diol) by one of several enzymes. 3α-HSD, 3alpha hydroxysteroid dehydrogenase; 3β-HSD, 3beta hydroxysteroid dehydrogenase; 17β-HSD, 17beta hydroxysteroid dehydrogenase.

Interestingly, flutamide and tamoxifen only partially blocked the effects of locally administered DHT, suggesting that DHT and/or its metabolites can modulate HPA axis reactivity through binding to either AR or ERβ. Thus, DHT signaling may be an integration of systemic DHT levels, local *de novo* DHT synthesis and metabolism, and relative local AR and ERβ expression levels.

3-β-Diol's systemic and local actions on HPA axis function raise the question of whether or not it can modulate neuronal function via classical genomic actions of ERβ. To this end, Pak et al demonstrated that 3β-Diol can activate transcription through ERβ at a classical ERE in the hippocampal-derived neuronal cell line HT-22 (Pak, Chung et al. 2005). 3β-Diol enhances ERβ-dependent transcription to a greater extent than does estradiol, which is intriguing given that 3β-Diol has a lower affinity than estradiol for ERβ (K_i = 1.7nM and 0.1nM respectively) (Pak, Chung et al. 2005; Pak, Chung et al. 2007). Beyond activation of a simple ERE-driven reporter gene, 3β-Diol can activate known estrogen sensitive target genes. Provided that ERβ is found in AVP neurons of the PVN and SON (Laflamme, Nappi et al. 1998; Suzuki and Handa 2005), AVP can potentiate the actions of CRH on corticotrophs (Rivier and Vale 1983), and estradiol augments the stress response (Handa, Burgess et al. 1994), one such target gene may perhaps be AVP. Indeed, 3β-Diol can increase AVP promoter activity in the presence of $ER\beta_1$ and $ER\beta_2$ (Pak, Chung et al. 2007). Interestingly, 3β-Diol had no effect in the presence of ERβ1δ3, an ERβ splice variant that does not bind DNA, suggesting that 3β-Diol exerts its effect through receptor:ligand DNA binding. Furthermore, 3β-Diol activation of the AVP promoter can be abolished by inhibition of the coactivator GRIP1, suggesting that 3β-

Diol-bound ERβ can recruit similar coactivators as estradiol-bound ERβ (Pak, Chung et al. 2007). However, ERβ can selectively recruit coactivators depending on the ligand bound (Kraichely, Sun et al. 2000), indicating that 3β-Diol may have vastly different genomic effects from estradiol depending on the splice-variant expressed and the coactivators and corepressors recruited to the promoter.

The remaining question becomes whether the molecular and systems level actions of 3β-Diol are translated into changes at the organism level. Previous studies, including those described in this dissertation have consistently shown anxiolytic effects of ERβ agonist treatments. Accordingly, 3β-Diol decreases behavioral measures of anxiety in the elevated plus maze similarly to DPN when administered to OVX adult female rats. Whether this is a direct effect of the reduced hormonal response to a stressor seen in these animals, or an effect of ERβ activation on brain circuitry essential for modulating anxiety state is unclear.

13. Mood disorders

Overview

Anxiety and depression have an overwhelming and immeasurable impact on society both economically and socially. They are multifaceted and debilitating psychiatric illnesses that often share similar symptoms and have a high degree of comorbidity (Freeman, Freeman et al. 2002; Nutt, Ballenger et al. 2002; Nutt and Stein 2006). Depression is often reported in anxiety patients, and vice versa. Additionally, the symptoms of anxiety and depression may respond to the same treatments

suggesting similar underlying neurobiological pathologies (Kendler, Heath et al. 1987; Roy, Neale et al. 1995). However, relatively little is known about the etiology and underlying pathology of anxiety and depression.

Anxiety

Approximately 25% of adults will suffer from one or more forms of anxiety disorder in their lifetime, and the yearly economic cost in the U.S. is estimated at $40 billion per year (Gordon and Hen 2004). Fear is a normal emotion and an adaptive mechanism of alarm that acts to prepare an animal to respond to threatening stimuli. Chronic and excessive levels of fear lead to pathological anxiety, which is associated with suffering and distress. Symptoms of anxiety include fatigue, tension, worrying, lack of concentration and sleep, and ability to experience pleasure. There are at least five forms of anxiety: 1) generalized anxiety, where the stimulus is unknown and the symptoms are continuous; 2) panic, involving random episodic panic due to unknown cause; 3) phobia, involving irrational fear-based avoidance of anything (people, situations, things, places, etc); 4) post-traumatic stress disorder (PTSD), involving debilitating anxiety-causing memories of past trauma; and 5) obsessive-compulsive disorder (OCD), where incessantly obsessive thoughts cause ritualistic behaviors to alleviate these anxiety-causing thoughts. Current treatment options for anxiety are limited, and include benzodiazepines, which modulate GABA synaptic transmission, and selective serotonin reuptake inhibitors (SSRIs), which modulate the serotonergic system.

The heterogeneity of anxiety disorders provides a challenge in designing

appropriate animals models with predicative ability in one or more areas of anxiety. Most animal models can be classified according to the nature of the adverse stimulus used, and the response elicited. The stimulus may be physically adverse (shock, heat, thirst) or psychologically adverse (exposed environment, light, predator), and the response may be learned (conditioned) or innate (unconditioned). Typically the best models for anxiety are those that do not explicitly involve pain or discomfort (i.e., exposure to a brightly lit arena, or a predator) and illicit spontaneous or natural reactions such as flight, freezing, and avoidance. These 'ethological' models attempt to mimic natural situations where anxiety-type behaviors might be elicited. Ethological models also reduce the occurrence of confounds due to differences in perception and motivation as a result of variability in learning and memory, nociception, thirst, and hunger. These models examine the natural conflict between the animals innate desire to explore with the innate desire to avoid exposed or adverse environmental factors. The most robust and extensively studied ethological anxiety models include open field, elevated plus maze, and light/dark box.

The open field model, originally developed by Hall in the 1930s, consists of a brightly lit open arena divided into squares and measures the tendency of the animal to spend time along the walls away from the center and monitors levels of urination and defecation (Hall 1934). Variations on this model include a circular or square arena and introduction of novel objects in the center squares (for review see (Prut and Belzung 2003)). Anxiogenic behaviors include time spent along the walls, grooming, defication, and urination. Anxiolytic behaviors include time spent in middle, latency to exit middle,

interactions with novel objects, and rearing. Treatment with benzodiazepines or SSRIs reduces the occurance of angiogenic behaviors and increases anxiolytic behaviors, yet they do not affect overall locomotion in the model as measured by total square crossings.

The light/dark paradigm, originally developed by Crawley in 1981, is another model for anxiety that is based on the innate aversion of rodents to brightly lit areas (Crawley 1981; Hascoet and Bourin 1998). This model examines the behavior of an animal that is allowed to freely explore a box consisting of two chambers where one is open, lit, and clear/white, and the other is closed, dark, and black. Animals placed in the light chamber typically move quickly into the closed chamber and spend little time in the exposed light chamber. Anxiolytic behaviors measured include time spent in open chamber, number of transtions between chambers, and rearing. Anxiogenic behaviors include latency to enter dark chamber, grooming, and defecation. However, treatment with anxiolytic drugs reduces the anxiety associated with the brightly lit and exposed area and increases time spent exploring the light chamber. The light/dark box has been used widely in screening anxiolytic drugs in mice due to its high throughput ability (Costall, Jones et al. 1989).

The elevated plus maze (EPM) is one of the most popular, extensively characterized, and pharmacologically validated models for anxiety. The EPM was developed by Handley and Mittani based on the pioneering work of Montgomery on animal fear and exploration (Montgomery and Monkman 1955; Handley and Mithani 1984). There have been several variations on the EPM developed, including the

elevated T-maze, zero maze, and the unstable elevated exposed plus maze (Jones and King 2001). The EPM is in the shape of a plus and consists of four arms of equal width and length elevated from the ground. Two opposing arms are open (do not have walls) and are connected to two closed (walled) arms by an intermediary middle square. The open arms combine elements of exposure, light, novelty, and elevation. As with the open field and light/dark box, this model examines the natural conflict between the desire to explore and the natural aversion to open places. Animals taken from their home cage will generally display behavior characterized with open-arm avoidance and preference for the closed arm. Anxiolytic behaviors include open arm time, open arm entries, head dip (where animal dips its head over the edge of the arm), and rears. Anxiogenic behaviors include closed arm time, grooming, and defecation. Some investigators characterize the locomoter component of the model to be measurable by total entries (Lister 1987) or closed entries (Rodgers and Johnson 1995). By measuring open-to-total ratios where the percent open arm time or percent open arm entries with respect to total time or total entries, one may address potential confounds presented by specific or non-specific alterations in locomotion and individual variability. The EPM has reported variability between labs suggesting that testing paradigms must remain consistent within and across studies. Factors that influence EPM outcomes include the animals themselves (strain, sex, age), housing conditions, diet, handling, time of day, illumination, routes of drug administration, maze construction, and prior exposure to maze apparatus (Rodgers and Cole 1993; Griebel, Belzung et al. 2000; File 2001; Wahlsten, Metten et al. 2003).

Depression

Approximately 12-17% of the U.S. population is affected by depression at some point in their life (Kessler, McGonagle et al. 1994; Kessler, Chiu et al. 2005). The global burden of disease study by the World Bank estimates that depression is the single most costly disease of middle-life (Ustun, Ayuso-Mateos et al. 2004). Furthermore, it is predicted that depression will be the second leading cause of disability by the year 2020, behind only ischemic heart disease (Murray and Lopez 1997). However, depression is associated with an increased risk for heart disease, further exacerbating its burden (Glassman and Shapiro 1998). The economic impact of depression is far-reaching, including cost of treatment, worker productivity, and premature morbidity and mortality (Panzarino 1998; Thompson and Richardson 1999; Pincus and Pettit 2001; Greenberg, Kessler et al. 2003).

Antidepressants are currently the treatment of choice even though their exact mechanism of action is not well understood. Furthermore, resistance to antidepressants is observed in 30% of depressed patients, and treatment can take a substantial length of time to be effective. The development of mood disorders is likely a combination of many factors that include genetics and environmental influences during both development and in adulthood. Much of the preclinical work on depression has focused on the relationship between stress and depression (Bao, Meynen et al. 2008), as stress is a main trigger for depressive episodes. An underlying physiological and psychological loss of control over stress sensitivity and responsiveness appear to be

important factors in the development of depression. In addition to the severe episodic and sustained mood disturbances, there exist cognitive, autonomic, and endocrine abnormalities in depression. Most hypotheses regarding depression center around dysregulation of the HPA axis and implicate CRH and glucocorticoid actions on, or stemming from brain regions such as the hippocampus, amygdala, nucleus acumbens and discrete regions of the hypothalamus (Dinan 1994). These brain regions are essential in regulating biological processes that are abnormal in depressed patients such as circadian rhythm, energy, metabolism, eating, sleeping, motivation, and behavioral responses to adverse or rewarding stimuli. The working hypotheses of depression implicate processes that are dependent upon genetically or environmentally influenced abnormalities in monoamines, neurotropins, stress or neurogenesis.

The monoamine hypothesis predicts that development of depression is due to insufficient neurotransmission by serotonin, norepinephrine, and dopamine (Brown and Gershon 1993; Charney 1998). This impairment is likely a result of decreased synthesis or increased uptake/degradation of the neurotransmitters, altered function or expression of the neurotransmitter receptors, or abnormal post-synaptic cell response to the neurotransmitter. Most antidepressants operate under this hypothesis and act to inhibit neurotransmitter uptake (tricyclics, TCAs; serotonin reuptake inhibitors, SSRIs) or degradation (monoamine oxidase inhibitors, MAOIs). These treatments present a key biological confound whereby treatment increases synaptic neurotransmitter levels, but elevations in mood can take weeks following treatment. Therefore, chronic changes in monoamine neurotransmission may induce slow-developing alterations in neuronal

plasticity.

The neurotropins hypothesis links the development of depression with a decreased function or expression of brain derived neurotrophic factor (BDNF) (Duman, Heninger et al. 1997; Altar 1999). Numerous studies have linked hippocampal atrophy seen in depressed patients to decreased expression and function of BDNF and its receptor TrkB (Duman, Heninger et al. 1997; Altar 1999; Vaidya and Duman 2001; Wong and Licinio 2001; Nestler, Barrot et al. 2002). Chronic treatment with antidepressants can reverse this and induce increased levels of BDNF in the hippocampus (Russo-Neustadt, Ha et al. 2001). Since BDNF and/or TrkB knockout mice do not survive past early postnatal periods several studies have utilized heterozygous knockout mice where BDNF and TrkB signaling are impaired (Saarelainen, Hendolin et al. 2003; Monteggia, Barrot et al. 2004; Chen, Jing et al. 2006). These studies do not show an increase in anxiety-type or depressive-type behaviors in these animals, but do show impairment in efficacy of antidepressant treatment. This implicates BDNF in the mechanism of antidepressant action, but not in the etiology of depression per se.

The discovery of progenitor cell proliferation and differentiation (neurogenesis) in certain brain regions like the hippocampus and olfactory bulb led to the neurogenesis hypothesis of depression (Duman, Malberg et al. 2000; Jacobs, Praag et al. 2000; Henn and Vollmayr 2004), whereby changes in monoamine and neurotrophin signaling are funneled into an end result of reduced neurogenesis. Many factors have a positive influence on neurogenesis such as antidepressant treatment, voluntary exercise, exposure to an enriched environment, and estrogen (Kempermann, Kuhn et al. 1997;

Charney 1998; Tanapat, Hastings et al. 1999; van Praag, Christie et al. 1999). However, animals that exhibit depressive-type behaviors in the forced swim test (an animal behavioral test for depression) do not differ in hippocampal neurogenesis in comparison to normal animals (Vollmayr, Simonis et al. 2003). Therefore, neurogenesis may play an important role in antidepressant treatment, but perhaps is secondary to other overriding factors in the physiopathology of depression.

Dysfunctional glucocorticoid signaling and glucocortcoid receptor function has long been implicated in the pathogenesis of stress-related psychiatric disorders like depression and anxiety (Arborelius, Owens et al. 1999; Biondi and Picardi 1999; Brown, Rush et al. 1999; Holsboer 2000). The observation of impaired HPA axis function in these individuals (Holsboer and Barden 1996; Zobel, Yassouridis et al. 1999) has led to the stress or glucocorticoid hypothesis of depression. Whether these impairments are directly to blame for the etiology of depression or are merely as a result of a depressive state remains to be determined. This hypothesis proposes that elevated glucocorticoids as a result of deficient feedback control of HPA activity are responsible for the cascade of molecular and cellular events that leads to this disease state. An alternative version of this hypothesis, the CRH hypothesis, claims that the HPA axis dysregulation seen in depression is due to a primary upregulation of hypothalamic CRH (Arborelius, Owens et al. 1999; Holsboer 2000). Yet another alternative version of this hypothesis, the AVP hypothesis, states that HPA axis hyperactivity in depression is AVP-driven rather than CRH-driven (Purba, Raadsheer et al. 1995; van Londen, Goekoop et al. 2001; Keck, Welt et al. 2003). The role of the HPA axis in depression and the specific effects of

glucocorticoids and GR function in depression will be discussed in a subsequent section.

Examining the molecular and cellular correlates of depression in humans is rather difficult given the *in vivo* limitations and limited supplies of tissue. Therefore several animal models for depression have been developed such as chronic stress, learned helplessness, and despair in rats as well as social separation in primates (Willner 1984; Kalueff, Wheaton et al. 2007). Human symptoms of depression as classified in the "Diagnostic Statistical Manual IV" (DSM IV) have been correlated to specific symptoms and behaviors in rodents (for review see (Gass, Reichardt et al. 2001)). For example, the "core symptoms" of sadness, anhedonia (decreased pleasure-seeking behavior), and fatigue can be associated with despair (forced swim test) and decreased consumption of sugar solution (anhedonia) in rodents. "Associated symptoms" like hopelessness, lack of concentration, and extreme sensitivity can be linked to anxiety (elevated plus maze) and neophobia (abnormal fear of novelty; open field, light-dark box) in rodents. "Vegetative symptoms" such as insomnia, altered appetite and loss of sex drive can be correlated to sleeping, eating, and sexual behavior as well as HPA axis output in the rodent.

Chronic mild stress models predict that persisting stress states that cannot be controlled may lead to a depressive state (Willner 1997). These models utilize moderate stressors over a significant period of time such as social isolation, intruder, wet bedding or chow, food deprivation, altered light levels and noise (Katz, Roth et al. 1981; Katz and Sibel 1982; D'Aquila, Newton et al. 1997). The main readouts of these experiments include increased anhedonia, or lack of preference between a sugar solution (pleasure) and normal water, decreased locomotion, body weight, food consumption, and

increased despair. Abnormalities in these categories are comparable to those seen in human depression (Willner, Benton et al. 1998). The effects of chronic stress can be reversed with antidepressant treatment further indicating its usefulness in mimicking the human condition.

The concept of learned helplessness is attributed to Martin Seligman who, in 1967, proposed that exposure to unpredictable and uncontrollable stress leads to a depressive state with the hallmark symptom of decreased avoidance to adverse stimuli (helplessness) (Seligman and Maier 1967). In the original model, rats were exposed to uncontrollable footshocks and tested the subsequent day on their ability to terminate the shocks by pressing a lever. Normal animals will quickly learn to press the lever to cease the shocks, however an abnormal (helpless) animals display less motivation to stop the adverse stimulus (Edwards, Johnson et al. 1986). These abnormal animals display defects in cognition, appetite, sleep patterns, sex behaviors and locomotion (Adrien, Dugovic et al. 1991). Antidepressant treatment will increase active coping (lever pressing in this case) and decrease the passive coping (helplessness) in this paradigm (Sherman, Sacquitne et al. 1982). Glucocorticoids play a major role in this model, as evidenced by impaired suppression of CORT following dexamethasone treatment in helpless animals (Greenberg, Edwards et al. 1989). Normal function of the HPA axis is important for incorporation of a learned response following a stress (Edwards, Harkins et al. 1990; Henn and Vollmayr 2004).

The despair model is attributed to Porsolt, who in 1977 described "a new behavioral method for inducing a depressed state in mice" (Porsolt, Le Pichon et al.

1977). He was examining mouse behavior in a water maze when noticed that some of the mice stopped swimming after a period of time and remained passively floating (Porsolt, Bertin et al. 1979). From this observation he devised what is known as the Porsolt forced swim test (FST), where animals are placed into an inescapable cylinder of room temperature water and observed for struggling, swimming, and immobile behaviors. Porsolt described the state of immobility as "despair" and "reflecting a state of lowered mood" (Porsolt, Bertin et al. 1978). This despair may be characterized as an adaptive response to an inescapable situation, and is a passive coping mechanism derived from an unwillingness to maintain effort in the inescapable situation. Porsolt observed that a large range of antidepressants reduced the amount of time the animals spent immobile in the water. Currently, the FST is regarded as the most reliable animal model for depression (Holmes 2003), and is consistently reliable across laboratories (Borsini and Meli 1988).

HPA axis and mood disorders

Manfred Bleuler was the first scientist to correlate changes in hormones, mood and behavior with psychiatric disorders (Bleuler 1919). Bleuler observed a correlation between irregular thyroid function, excessive cortisol levels and the incidence of psychiatric disorders, especially depression. Abnormalities in the HPA axis are now well documented in many mental disorders including anxiety and depression (Ehlert, Gaab et al. 2001; Varghese and Brown 2001; Barden 2004). It is well known that stressors are key instigators for the onset of depressive episodes (Kendler, Karkowski

et al. 1999; Paykel 2001), and the HPA axis is regarded as the 'final common pathway' for the numerous symptoms of major depressive disorder (Bao, Meynen et al. 2008). Patients with depression often exhibit elevated plasma cortisol, impaired dexamethasone suppression, decreased GR expression and function, increased hypothalamic CRH and AVP, augmented adrenal sensitivity to ACTH, and/or enlargement of the pituitary and adrenal glands (Krishnan, Doraiswamy et al. 1991; Dinan 1994; Raadsheer, van Heerikhuize et al. 1995; Rubin, Phillips et al. 1996; Modell, Yassouridis et al. 1997; Maes, Lin et al. 1998; Scott and Dinan 1998; Holsboer 2000; Weber, Lewicka et al. 2000; Meynen, Unmehopa et al. 2006). One of the major side effects of systemic glucocorticoid treatment is depression (Mitchell and O'Keane 1998). Furthermore, GR antagonists and CRHR1 antagonists have shown to ameliorate the symptoms of depression (Keck and Holsboer 2001; O'Brien, Skelton et al. 2001; Belanoff, Rothschild et al. 2002; Gold, Drevets et al. 2002). Studies performed with high-risk patients showed abnormal HPA axis drive prior to onset of any clinical symptoms, suggesting a causative role for HPA axis dysregulation in depressive disorder (Holsboer 2000).

Glucocorticoids can modulate monoamine receptor synthesis, suggesting that corticosteroid alterations in monoaminergic signaling may underlie the etiology of depression and argues for the use of monaminergic therapies (De Kloet, Vreugdenhil et al. 1998; Bush, Middlemiss et al. 2003). Antidepressant treatment has positive effects on HPA axis markers of activity and sensitivity. Classical antidepressants reduce the hypercortisolaemia and restore the feedback sensitivity of the HPA axis in

line with remission of depressive symptoms (Nelson and Davis 1997; Hatzinger, Hemmeter et al. 2002). Benzodiazepines lead to a quick short-term relief of anxiety and depressive symptoms and have been shown to decrease plasma cortisol in depressed patients (Christensen, Lolk et al. 1989), and most likely act via reduction of CRH in the central nucleus of the amygdala (Skelton, Nemeroff et al. 2000). Animal models are in line with the human studies, where chronic administration of antidepressants will reduce HPA axis activity in normal animals (Shimoda, Yamada et al. 1988; Reul, Stec et al. 1993), and in animals genetically modified to have a hyperactive HPA axis (Pepin, Pothier et al. 1992; Montkowski, Barden et al. 1995; Barden 1999). The mechanism of this normalization is still unclear, however *in vitro* studies indicate an upregulation of GR levels in response to antidepressants (Pepin, Beaulieu et al. 1989; Peiffer, Veilleux et al. 1991; Holsboer and Barden 1996; Okugawa, Omori et al. 1999; Yau, Noble et al. 2001; Herr, Tsolakidou et al. 2003; Okuyama-Tamura, Mikuni et al. 2003). Similarly these effects are observed *in vivo* (Seckl and Fink 1992; Lopez, Chalmers et al. 1998). Alternatively, other studies have shown inhibition of GR target gene transcription following antidepressant treatment (Budziszewska, Jaworska-Feil et al. 2000).

AVP appears to have a critical role in the pathology of depression. For example, plasma AVP levels are elevated during depression, and normalize upon remission (van londen97 2003). The PVN of depressed patients also contains 3 times the number of CRH and AVP co-expressing neurons and overall AVP immunoreactivity as compared to normal patients (Raadsheer, Hoogendijk et al. 1994; Purba,

Hoogendijk et al. 1996). Furthermore, AVP administration to normal patients results in an abnormal cortisol suppression following a DEX/CRH test (a psychiatric test for depression that will be described in the next section). Interestingly, decreased depressive-like behaviors are observed in the vasopressin deficient animal model, the Brattleboro rat (Mlynarik, Zelena et al. 2007). From these data it may be proposed that the hyperactivity of the HPA axis seen in depression may be due to a loss of feedback constraint on the vasopressinergic system.

The dexamethasone suppression test

The dexamethasone suppression test (DST) is a well established neuroendocrine test and clinical predictor of depression (Carroll and Curtis 1976), and has been used in rodents as a model for the human test (Lurie, Kuhn et al. 1989). In the DST, the physiological response to the synthetic glucocorticoid is measured, where in normal patients a suppression of plasma ACTH and cortisol is observed. In a good portion of depressed patients this suppression is blunted and higher than normal plasma ACTH and cortisol is observed. Furthermore, normalization of this impaired DEX suppression is correlated with remission, while continued non-supression is correlated with a negative clinical outcome (Holsboer, Liebl et al. 1982; Greden, Gardner et al. 1983). However, the sensitivity of the DST is relatively poor, and identifies only 20-50% of clinically depressed patients depending on severity of depressive state, DEX dose, and individual differences (Arana, Baldessarini et al. 1985). More recently, the combined DEX/CRH test, where dexamethasone suppression in the presence of HPA axis drive (CRH) has

been proven to be 90% accurate in diagnosing depressed individuals (Heuser, Yassouridis et al. 1994; Varghese and Brown 2001). In the DEX/CRH test a dose of DEX is given at 2300 h followed by a dose of CRH (challenge) at 1500 h the following day. Blood is drawn periodically for up to two hours following the CRH challenge and analyzed for plasma cortisol levels. In DEX pre-treated depressed patients, a much higher plasma cortisol response to the CRH is observed than in normal patients. Furthermore, the DEX/CRH test has been used to accurately predict relapse and can be used as a biomarker to positively identify novel treatments for depression (Aubry, Gervasoni et al. 2007; Ising, Horstmann et al. 2007). The DST and DEX/CRH test measure common pathology, however the DEX/CRH has better diagnostic reliability(Watson, Gallagher et al. 2006).

The DST and the DEX/CRH test have been used in rodents as a model for the human test (Lurie, Kuhn et al. 1989; Hatzinger, Reul et al. 1996). For example, high anxiety-like behavior rats (HAB, an inbred rat strain) exhibit higher ACTH and CORT than low anxiety-like behavior rats following the DEX/CRH test (Keck, Wigger et al. 2002). Other studies have shown that transgenic mice expressing GR antisense in neuronal tissues have much higher CORT following a DEX/CRH test than their wild type counterparts (Stec, Barden et al. 1994), in addition to a myriad of behavioral imbalances (Marchetti, Morale et al. 2001). The DST may also be able to tease out individual strain differences in rats. For example, the Wistar Kyoto (WKY) rat is well known for its exaggerated responses to stressors as compared to other strains such as the Sprague-Dawley (SD) rat. Following the DST, the WKY strain exhibits higher ACTH levels than the

SD strain, and could explain the heightened despair in the forced swim test observed in WKY rats compared to SD rats (Rittenhouse, Lopez-Rubalcava et al. 2002).

Sex hormones and mood disorders

Estrogen is potent regulator of mood, with effects ranging from depressant to anti-depressant (Shors and Leuner 2003, Fink *et al.* 1998). Major depression is one of the most common psychiatric illnesses with a lifetime prevalence of greater than 17% in the general population (Ostlund *et al.* 2003, Varghese and Brown 2001). Several studies have consistently reported that major depressive episodes are twice as common in women as compared to men (Angold and Worthman 1993, Kornstein 1997, Llewellyn *et al.* 1997, Weissman *et al.* 1993), a difference that emerges at the time of puberty (Angold and Worthman 1993). Other psychological disorders more prevalent in women include eating disorders and insomnia (Ehlert *et al.* 2001, Buckley and Schatzberg 2005). In addition, women tend to suffer a more severe form of depression and greater functional impairment (Kornstein 1997, Thase *et al.* 1994). Importantly, numerous studies have confirmed the involvement of HPA axis dysregulation with depression, insomnia, and eating disorders (Barden 2004, Holsboer and Barden 1996, Modell *et al.* 1997, Varghese and Brown 2001, Ehlert *et al.* 2001, Buckley and Schatzberg 2005). Estradiol augments the activation of the HPA axis and, as the studies in this dissertation demonstrate, impairs HPA axis negative feedback. The link between these disorders and their inherent sex difference may be a result of the influence of estrogen on HPA axis function. Indeed, depressed women exhibit

higher diurnal amplitudes in serum estradiol levels than seen in normal patients (Bao, Ji et al. 2004). Interestingly, a recent study indicated an increase in ERα and a decrease in androgen receptor (AR) mRNA in laser-dissected PVN of post-mortem depressed patients (Wang, Kamphuis et al. 2008), further linking sex hormones with HPA axis activity and depression in humans.

Depressed males have significantly lower testosterone than their healthy counterparts (Heuser 2002), suggesting a role for androgens in the eitiology of depression in men. Indeed both testosterone levels and an AR polymorphism are linked to a higher risk of depression (Seidman, Araujo et al. 2001). It is not surprising then that testosterone levels are negatively correlated with depressive symptoms in bodybuilders (Dickerman and McConathy 1997). Furthermore, treatment of hypogonadal men with testosterone significantly improves overall mood (Wang, Alexander et al. 1996; Fink, Sumner et al. 1998; Fink, Sumner et al. 1999; Seidman and Walsh 1999; Wong, Kling et al. 2000). It is clear that sex hormones play an integral role in the molecular and behavioral markers of depression in both males and females.

CHAPTER THREE

Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus

Abstract

Numerous studies have established a link between individuals with affective disorders and a dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, most notably characterized by a reduced sensitivity to glucocorticoid negative (-) feedback. Furthermore there is a sex difference in the etiology of mood disorders with incidence in females being 2-3 times that of males, an association that may be a result of the influence of estradiol (E2) on HPA axis function. In these studies, we have examined the effect of E2 on glucocorticoid mediated HPA axis (-) feedback during both the diurnal peak and the stress-induced rise in corticosterone (CORT). Young female Sprague-Dawley (SD) rats were ovariectomized (OVX'd) and one week later treated subcutaneously (SQ) with oil or estradiol benzoate (EB) for four days. On the 4th day of treatment, animals were injected with a single dose of dexamethasone (DEX), or vehicle. EB treatment significantly increased the evening elevation in CORT and the stress-induced rise in CORT. In contrast, DEX treatment reduced the diurnal and stress induced rise in CORT and ACTH, and this reduction was not apparent following co-treatment with EB. To determine a potential site of E2's action, female SD rats were OVX'd and one week later, wax pellets containing E2, the estrogen receptor beta (ERβ) agonist

diarylpropionitrile (DPN), or the estrogen receptor alpha (ERα) agonist propylpyrazoletriol (PPT), were implanted bilaterally and dorsal to the paraventricular nucleus of the hypothalamus (PVN). Seven days later, animals were injected SQ with a single dose of DEX, or vehicle to test for glucocorticoid-dependent (-) feedback. Results show that E2 and PPT increased, while DPN decreased the diurnal peak and stress-induced CORT and ACTH levels as compared to controls. Furthermore, E2 and PPT impaired the ability of DEX to inhibit both the diurnal and the stress-induced rise in CORT and ACTH, whereas DPN had no effect. Neuronal activation was measured by *c-fos* mRNA expression within the PVN following restraint. E2 and PPT increased *c-fos* mRNA, and impaired the normal DEX suppression of neuronal activation in the PVN. Taken together, these data indicate that estradiol causes a dysregulation of HPA axis (-) feedback as evidenced by the inability of DEX to suppress diurnal and stress-induced CORT and ACTH secretion. Additionally, the ability of E2 to inhibit glucocorticoid (-) feedback occurs specifically via ERα acting at the level of the PVN.

Introduction

Major depressive disorder is a leading disability in the United States for young adults (Kessler, Chiu et al. 2005). Women are twice as likely as men to experience a major depressive episode, and they tend to suffer a more severe form of depression and greater functional impairment (Angold and Worthman 1993; Weissman, Bland et al. 1993; Thase, Reynolds et al. 1994; Kornstein 1997). Other psychological disorders more prevalent in women include eating disorders and insomnia (Ehlert, Gaab et al. 2001; Buckley and Schatzberg 2005). Abnormalities in the hypothalamic-pituitary-

adrenal (HPA) axis may underlie such pathologies as they are well documented in many mental disorders (Ehlert, Gaab et al. 2001; Varghese and Brown 2001; Barden 2004). For example, the combined dexamethasone suppression test (DST) / corticotropin releasing hormone (CRH) challenge shows a sensitivity of up to 90% in detecting major depressive disorder (Varghese and Brown 2001). Thus, the apparent dysregulation of the HPA axis in human affective disorders when put in context with a higher prevalence in women could indicate a role for estrogen in HPA axis dysfunction.

HPA axis activation is a homeostatic mechanism that is triggered by a physical or psychological stressor (see (de Kloet, Joels et al. 2005), for review). Stress-related neuronal inputs are integrated at the level of the paraventricular nucleus (PVN) of the hypothalamus to induce the secretion of CRH and arginine vasopression (AVP) into the hypophyseal portal vasculature. These neuropeptides stimulate the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which subsequently induces corticosteroid production by the adrenal cortex in humans (cortisol) and rodents (corticosterone; CORT). Importantly, HPA axis activation is terminated by negative feedback action of glucocorticoids. Elevations in circulating CORT inhibit the HPA axis by acting via the glucocorticoid receptor (GR) in a direct and indirect fashion through the anterior pituitary, hypothalamus and hippocampus (Reul and de Kloet 1985; Ratka, Sutanto et al. 1989). The main effect of elevated CORT is to decrease the synthesis and release of AVP, CRH and ACTH (Jingami, Matsukura et al. 1985).

In the brain, two distinct intracellular receptors have been shown to mediate the cellular responses to CORT (Reul and de Kloet 1985). The type-I, or

mineralocorticoid receptor (MR), is predominantly expressed in limbic structures such as the hippocampus (Reul, van den Bosch et al. 1987). The type-II, or glucocorticoid receptor (GR), is expressed throughout the brain with highest amounts in hypothalamic areas such as the PVN and supraoptic nucleus (SON), as well as in the hippocampus. Both receptors are also responsible for mediating the negative feedback effects of CORT on neuroendocrine systems. MR exhibits a 10-fold higher affinity for CORT than GR, thus MR is predominantly occupied by basal CORT levels and is important in regulating basal secretion of CORT (Reul, van den Bosch et al. 1987). The GR possesses a lower affinity for CORT and is thought to be the primary CORT receptor that modulates the negative feedback response to elevated CORT levels, although MR is also involved in this (Ratka, Sutanto et al. 1989; Spencer, Kim et al. 1998; Pace and Spencer 2005). Within the PVN, GR have been reported in both CRH and AVP containing neurons although there is much greater evidence for the presence of GR in AVP (Albeck, Hastings et al. 1994) rather than in CRH neurons (Uht, McKelvy et al. 1988). Nonetheless, CORT acts to inhibit CRH and AVP expression in PVN neurons (Kovacs and Mezey 1987; Swanson and Simmons 1989), but in a differential manner (Ma and Aguilera 1999; Kovacs, Foldes et al. 2000). Whether glucocorticoid mediated negative feedback on CRH and AVP neurons is truly direct or indirect is unknown.

Several studies have indicated that in the presence of estradiol there is an enhanced responsiveness of the HPA axis to a stressor which may be due in part to impairment of glucocorticoid negative feedback (Peiffer and Barden 1987; Turner 1990; Peiffer, Veilleux et al. 1991; Burgess and Handa 1992; Turner 1992; Burgess and Handa

1993; Carey, Deterd et al. 1995; Ferrini, Lima et al. 1995; Patchev, Hayashi et al. 1995). Furthermore, gonad intact females have higher, whereas ovariectomized (OVX'd) females have similar, peak diurnal plasma CORT as compared to gonad intact males (Atkinson and Waddell 1997; Seale, Wood et al. 2004). Potential mechanisms of action include competition between ERα and GR to regulate transcription at an AP-1 site (Uht, Anderson et al. 1997), and reduction of GR binding and protein levels in the hippocampus, hypothalamus, and anterior pituitary (Burgess and Handa 1992). In the preoptic area, a region with inhibitory PVN efferents, estradiol has been shown to alter GAD67 levels (Curran-Rauhut and Petersen 2002). In addition to indirect effects on HPA axis, estrogen may directly influence activity within neurosecretory neurons in the PVN. This is suggested by studies showing a greater induction of c-fos mRNA within PVN neurons in response to stress when estradiol is present either systemically or directly administered to the PVN (Yukhananov and Handa 1996; Rachman, Unnerstall et al. 1998; Lund, Munson et al. 2004; Lund, Hinds et al. 2006). Accordingly, studies have indicated that estradiol regulates several PVN neuropeptides (Levin and Sawchenko 1993), and upregulates restraint-induced CRH mRNA (Lund, Munson et al. 2004).

In brain, the actions of estradiol can be mediated by two distinct estrogen receptors, ERα and ERβ. Importantly, this laboratory has recently shown that the HPA enhancing effect of systemic estradiol administration can be mimicked by the local application of 17β-estradiol or a selective ERα agonist to the PVN (Lund, Hinds et al. 2006). ERα is not expressed within the PVN, yet is expressed in the peri-PVN, a region

implicated in HPA axis inhibition (Suzuki and Handa 2005). Within the PVN, ERβ is found predominantly in pre-autonomic neurons and thus is an unlikely site of a direct estradiol enhancement of HPA activity (Stern and Zhang 2003). Taken together, these studies suggest that estradiol might impair the glucocorticoid mediated negative feedback system directly or indirectly to modulate HPA axis activity. However, whether or not estradiol can alter glucocorticoid negative feedback has not been explicitly described.

In the studies described here, we have examined the possibility that estradiol impairs the sensitivity of the HPA axis to glucocorticoid negative feedback, by examining its effect on both the diurnal peak in CORT secretion and following a psychological stressor (restraint). Indeed, we have found that subtype-selective ER agonists when administered into the systemic circulation or locally to the peri-PVN region of the hypothalamus can impair glucocorticoid negative feedback. These data indicate that in the presence of estradiol there is a dysregulation of the HPA axis that is demonstrated by the inability of DEX to suppress diurnal and stress-induced CORT secretion. This impairment in negative feedback occurs specifically via estrogen receptor alpha acting at the level of the paraventricular nucleus.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (~250g) were obtained from Charles River Laboratories (Wilmington, MA). The rats were caged in pairs, housed in the Colorado State University vivarium and maintained on a 12:12 h light/dark cycle (lights on at 0700

h) with *ad libitum* access to rat chow and water. One week following arrival, rats were ovariectomized under isofluorane anesthesia. All animal protocols were previously approved by the Institutional Animal Care and Use Committee (IACUC) at Colorado State University.

Systemic hormone administration: diurnal experiment

One week following ovariectomy, animals were treated with oil or estradiol benzoate (EB, Sigma, 25ug/kg; subcutaneously (SQ)) daily for four days. On the fourth day of treatment, animals were injected SQ with a single dose of dexamethasone (DEX, Sigma) (30ug/kg) at 1200h. Controls received oil vehicle. Animals were sacrificed at 1800 h (lights out at 1900 h) on the same day; a time that is close to the diurnal peak in plasma CORT in our animal colony. Trunk blood was collected for CORT and ACTH analysis via radioimmunoassay (RIA).

Systemic hormone administration: stress experiment

One week following ovariectomy animals were treated with oil or EB (25ug/kg, SQ) daily for four days. On the fourth day of treatment, animals were injected SQ with a single dose of DEX (30ug/kg) at 0400 h, 6 hrs prior to restraint stress. Restraint was performed by removing the animal from its home cage and placing it into a plexiglass restraint tube (Plas-Laboratories, Lansing, MI). Animals were sacrificed directly from the tube 20 min later. Controls received oil vehicle. Control animals for restraint were killed immediately after removal from their home cage. Animals were sacrificed after 20

minutes of restraint at 1020 h and trunk blood samples collected for CORT and ACTH analysis via radioimmunoassay (RIA).

Stereotaxic implantation

One week following ovariectomy animals were fitted bilaterally with two 22 gauge stainless-steel cannulae (Small Parts, Miami Lakes, FL) with the aid of a small animal stereotaxic instrument (David Kopf Instruments, Kujunga, CA). The tips of the cannule were previously packed with one of the following compounds: EB, PPT (estrogen receptor alpha agonist, Tocris, Ellisville, MO), or DPN (estrogen receptor beta agonist, Tocris), dissolved into warmed beeswax to a final concentration of 0.5 uM, and packed to a height of 0.5 mm within the end of the cannulas. Controls received cannulae packed with wax alone. Stereotaxic coordinates to allow placement of the cannula tip to the region just above the PVN were: lateral 10 degree insertion angle to 1.8 mm posterior and 2.0 mm lateral to bregma, and 6.5mm below the skull surface. A 28 gauge stainless steel wire cut to extend 1.0 mm past the length of the cannulas was inserted into the cannulae and the pellet expelled. Following sacrifice of all animals, confirmation of pellet placement was confirmed in cresyl-violet stained brain sections. Animals where both pellets were >0.5mm away from the PVN were excluded based on previous studies using the same pellet hormone concentration and resulting spread (~0.5mm for estradiol) (Lund, Rovis et al. 2005).

Stereotaxic implantation: diurnal experiment

Seven days after stereotaxic surgery, animals were injected SQ with a single dose of dexamethasone (DEX, Sigma) (30ug/kg) at 1200 h. Controls received oil vehicle. Animals were sacrificed directly from home cage at 1800 h (lights out at 1900 h) the same day, near the diurnal peak in plasma CORT, and trunk blood was collected for CORT analysis using radioimmunoassay (RIA).

Local implantation: stress experiment

Seven days after stereotaxic surgery, animals were injected SQ (0400 h) with a single dose of DEX (30ug/kg) 6 hrs prior to restraint stress (at 1000 h). Controls received oil vehicle. Animals were sacrificed after 20 minutes of restraint at 1020 h and trunk blood samples collected for CORT and ACTH analysis via RIA.

Corticosterone radioimmunoassay

Trunk blood was centrifuged at 4 C and plasma removed and stored at -20 C until assayed. For assay, plasma samples were diluted in 0.01M PBS (1:25) and corticosteroid binding globulin was inactivated by incubation at 65°C for 1 hour. Samples (20ul) and standards (5-700 ng/ml) were incubated overnight at 4°C with antiserum (rabbit anticorticosterone; MP Biomedicals, Solon, OH) and [^{3}H] CORT (Perkin Elmer, Boston, MA) in 0.1% gelatin 0.01M PBS. Free CORT was separated from antibody-bound CORT with 1.0 ml dextran-coated charcoal. After centrifugation, the supernatant containing antibody-bound CORT was mixed with 4ml of scintillation fluid and counted with a

Packard 2900TR liquid scintillation counter (Meriden, CT). The intra-assay and inter-assay variance as measured by internal quality controls was 4.5% and 7.8% respectively.

ACTH radioimmunoassay

For assay, plasma samples were diluted in PBS-albumin (0.01M PBS, 0.9% NaCl, 0.1% Albumin, 100,000 KIU Aprotinin/L). Samples and standards (5-2000 pg per tube) were incubated overnight at 4°C with antiserum (rabbit anti-ACTH (Immunostar, Hudson, WI) and 2% normal rabbit serum. The following day, [^{125}I] ACTH (1-39) (Amersham, Piscataway, NJ) in PBS-albumin (100ul) was added to each tube as the tracer, and incubated overnight at 4°C. On the third day, goat anti-rabbit gamma globulin (Calbiochem, La Jolla, CA) was added to all tubes except totals, and tubes incubated overnight at 4°C. On the last day, 3 ml of PBS-albumin was added and tubes were centrifuged (>1,000 x g). Assay tubes were immediately decanted, blotted dry, and the resulting pellet counted with a Packard Cobra II gamma counter (Meriden, CT). The intra-assay and inter-assay variance as measured by internal quality controls was 3.8% and 6.1% respectively.

In situ hybridization

In situ hybridization for *c-fos* mRNA was performed as previously described (Lund *et al.* 2004) utilizing a 48-bp oligonucleotide probe targeted to the rat *c-fos* gene. The oligonucleotide probe was labeled using ^{35}S-dATP. Following hybridization slides were apposed to autoradiographic film (Kodak BioMax MR, Eastman Kodak Co., Rochester,

NY) and allowed to expose for 10 days. Autoradiograms were analyzed for density using a video camera (Sony XC-77, Tokyo, Japan) attached to a Nikon lens (Melville, NY). Scion Image (Frederick, MD) was utilized to count density per a fixed region encompassing the paraventricular nucleus. For each section, background density was obtained from a region adjacent to the PVN, and subtracted from the PVN measurement. Bilateral measurements were obtained from four separate sections and averaged to obtain the value for each individual animal.

Immunocytochemistry

To examine co-localization of ERα and GAD67, four young adult (300g) female SD rats were intracardially perfused with 4% buffered paraformaldehyde. Brains were postfixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, sectioned at 35uM on a Leitz cryostat, and stored in cryopreservative at -20 C until further processed for immunohystochemical detecton of ERα and GAD67. After standard washes, the free floating sections were incubated in 6% NGS in 0.1 M PBS with 0.1% TX to block nonspecific binding, then incubated for 72 hours at 4 C with the ERα antibody (C1355, obtained from Dr. M. Shupnik, UVA, 1:10,000 dilution) in 0.1 M PBS with TX in the presence of 2% NGS. Next, the tissue was washed three times for 10 minutes each in PBS with TX and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:500) in PBS with TX in the presence of 2% NGS for 2 hours at room temperature. Sections are subsequently washed and processed according to the avidin-biotin-peroxidase procedure (Vector Laboratories; 1:500). After standard

washes, the tissue was rinsed in 0.1 M Tris-buffered saline (pH 8.0) for 15 minutes and then developed with 3,3'-diaminobenzidine (DAB; 0.5 mg/ml; Sigma, St. Louis, MO) in 0.1 M Tris-buffered saline) containing 0.2% nickel sulfate and 0.01% hydrogen peroxide for 3–5 minutes to produce a blue-black reaction product. The reaction is stopped by several washes in 0.1 M PBS. Subsequently, the sections were incubated for 48 hours at 4 C with the mouse GAD67 antibody (Chemicon, Temecula, CA; 1:10,000 dilution) in PBS containing 0.01% TX and 2% NGS. The sections were then washed and processed according to the avidin-biotin-peroxidase procedure (Vector Laboratories; 1:500). After standard washes, the tissue is rinsed in 0.1 M Tris-buffered saline (pH 8.0) for 15 minutes and then developed with 3,3'-diaminobenzidine (DAB; 0.5 mg/ml; Sigma, St. Louis, MO) in 0.1 M Tris-buffered saline) containing 0.01% hydrogen peroxide for 3–5 minutes to produce a brown reaction product. Control sections, where primary antibody was omitted, were run in parallel. Complete loss of staining for the corresponding antigen was absent in these sections.

Data analysis

Analysis of variance was performed on data from each experiment using the Statview data analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). Fisher's protected least-squares difference (PLSD) analysis was used post hoc where appropriate. Differences were considered significant when $p < 0.05$. Data are expressed as group means ± SEM.

Results

Estradiol treatment augments the diurnal rise of CORT and ACTH and prevents DEX suppression of CORT

This experiment was designed to determine the effect of estrogen on glucocorticoid-mediated negative feedback during the diurnal rise in CORT secretion. EB alone significantly ($P < 0.01$) increased serum CORT and ACTH levels (Figure 3.1). DEX treatment also significantly ($P < 0.01$) suppressed serum CORT levels in vehicle treated animals. In contrast, in EB-treated animals, CORT and ACTH levels were not suppressed by DEX. Thus, in the presence of estradiol, the synthetic glucocorticoid DEX is unable to suppress the diurnal rise in CORT secretion.

Estradiol treatment augments restraint-induced CORT and ACTH secretion and prevents DEX suppression of restraint-induced CORT and ACTH

This experiment was designed to determine the effect of estradiol on glucocorticoid receptor mediated negative feedback on stress-induced activation of the HPA axis. Estrogen alone significantly ($P < 0.01$) increased stress-induced CORT and ACTH levels (Figure 3.2). EB + DEX animals showed significantly ($P < 0.01$) higher plasma CORT and ACTH compared to vehicle + DEX animals. In oil treated animals, DEX treatment suppressed CORT by an average of 289.6 ng/ml or 62.9% and ACTH by an average of 365.7 pg/ml or 74.9%. In contrast, when pretreated with EB, the DEX treated animals showed a 199.8 ng/ml or 34.4% on average suppression of CORT and a 281.9 pg/ml or 56.7% suppression of ACTH. Overall, CORT and ACTH values for EB +

DEX animals were similar to those of oil + vehicle controls. Thus, in the presence of estradiol, the stress-induced levels of CORT and ACTH are significantly increased and responsiveness to DEX is significantly decreased.

Estrogen acts via ERα near the PVN to augment the diurnal rise of CORT and ACTH and to prevent DEX suppression of CORT

This experiment was designed to determine whether estrogen impairs glucocorticoid negative feedback during the diurnal rise in CORT, whether this is through an ERα or ERβ mechanism and at which brain site this effect occurs. Animals were stereotaxically implanted with wax pellets containing ER agonists targeted to an area just superior to the PVN (Figure 3.3). Estradiol and PPT significantly increased ($P < 0.01$), whereas DPN significantly lowered ($P < 0.01$), CORT and ACTH when compared to controls given a wax-only pellet (Figure 4). DEX treatment significantly ($P < 0.01$) lowered CORT and ACTH levels in control and DPN implanted animals, however estradiol and PPT significantly ($P < 0.01$) impaired the suppression by DEX.

Estrogen acts via ERα near the PVN to augment restraint-induced CORT and ACTH secretion and to impair DEX suppression of restraint-induced CORT and ACTH

This experiment was designed to determine if estradiol acts via ERα at sites near the PVN to impair glucocorticoid dependent HPA negative feedback following a restraint stress activation of the axis. Animals were stereotaxically implanted with wax pellets containing ER agonists that were targeted to a site just superior to the PVN. 20 min after

the start of restraint, estradiol and PPT implanted animals had significantly higher ($P < 0.01$) CORT and ACTH than did controls (Figure 3.5). DPN implanted animals had significantly lower ($P < 0.01$) CORT and ACTH following restraint than did controls. Moreover, DEX treatment significantly lowered ($P < 0.01$) CORT and ACTH in both controls and DPN implanted animals, however estradiol and PPT treatment significantly impaired ($P < 0.01$) the ability of DEX to inhibit CORT and ACTH secretion.

Estrogen acts via ERα near the PVN to augment restraint-induced PVN c-fos mRNA expression and to impair DEX suppression of restraint-induced PVN c-fos mRNA expression

To examine neuronal activation following the restraint stress, *c-fos* mRNA expression was examined in the PVN. *c-fos* mRNA expression was significantly ($p<0.05$) higher in estradiol and PPT implanted animals and lower in DPN implanted animals as compared to controls (Figure 3.6). DEX treatment significantly reduced *c-fos* mRNA expression following restraint in controls and DPN treated animals, however estradiol and PPT treatment significantly impaired ($P < 0.01$) the ability of DEX to inhibit *c-fos* mRNA expression.

ERα is expressed in GABAergic neurons surrounding the PVN

To determine the neural sites of ERα expression in and around the PVN, we examined ERα immunoreactivity in OVX'd young adult female SD rats given replacement estradiol. Nuclear ERα immunoreactivity (ERα-IR) was sparse within the

periventricular PVN, but ERα-IR was seen surrounding the PVN proper especially in the dorsal region (Figure 3.7). Moreover, dual-immunocytochemistry revealed that ERα containing neurons in the peri-PVN also contained GAD (67.4 +/- 6.1% of ERα positive cells contained GAD67). Thus, ERα is expressed in the PVN surround, and is predominantly found in GABAergic neurons.

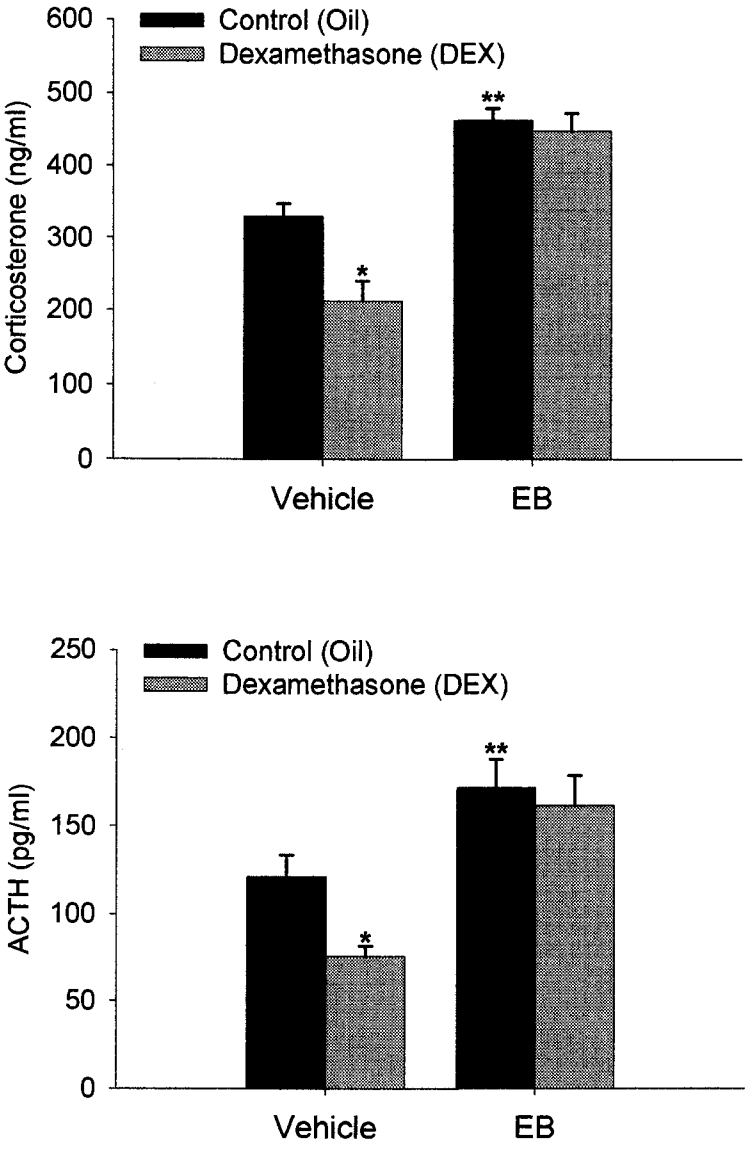


Figure 3.1. Estradiol impairs dexamethasone suppression of diurnal peak plasma CORT and ACTH. Data are expressed as mean ± SEM of 7 animals per group. *, ** indicates significant differences from control animals ($P < 0.01$). EB, estradiol benzoate.

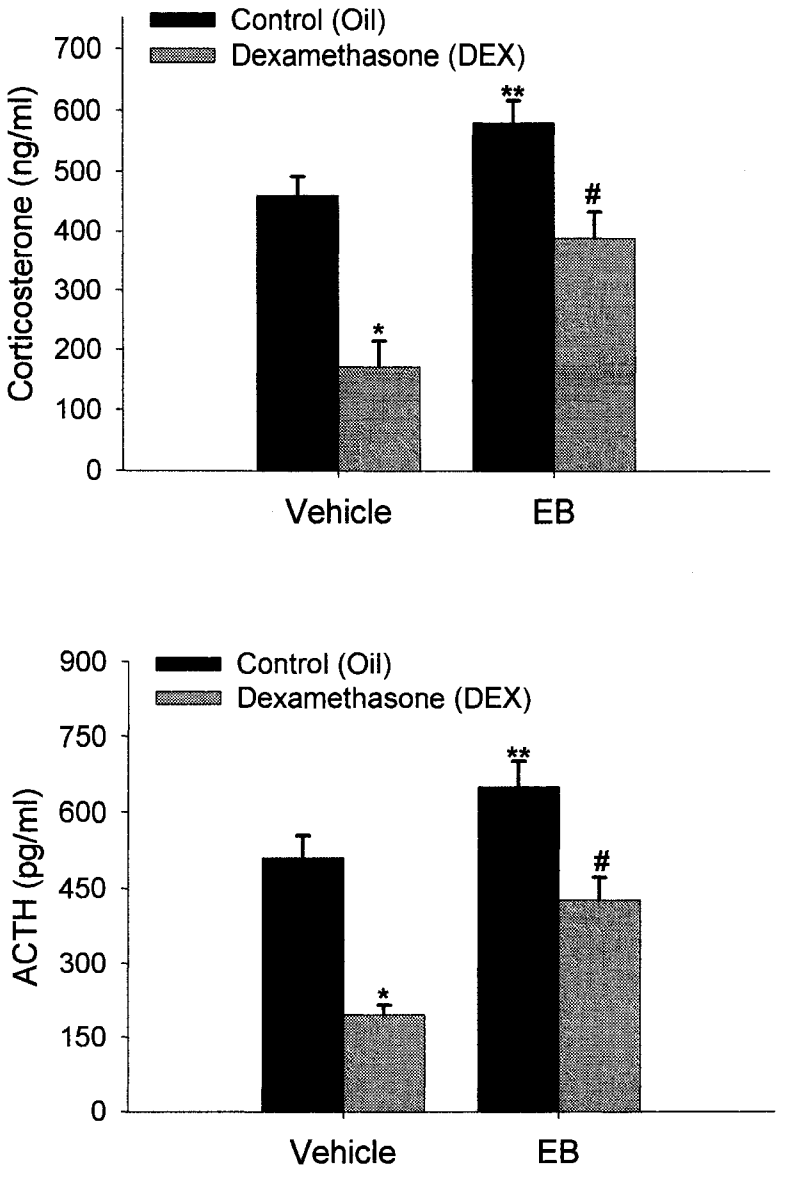


Figure 3.2. Estradiol impairs dexamethasone suppression of plasma CORT and ACTH following a 20 min restraint stress. Data are expressed as mean ± SEM of 7 animals per group. *, ** indicates significant differences from control animals ($P < 0.01$). EB, estradiol benzoate.

Figure 3.3. Representative photomicrograph of cresyl violet stained tissue indicating location of stereotaxically implanted wax pellet.

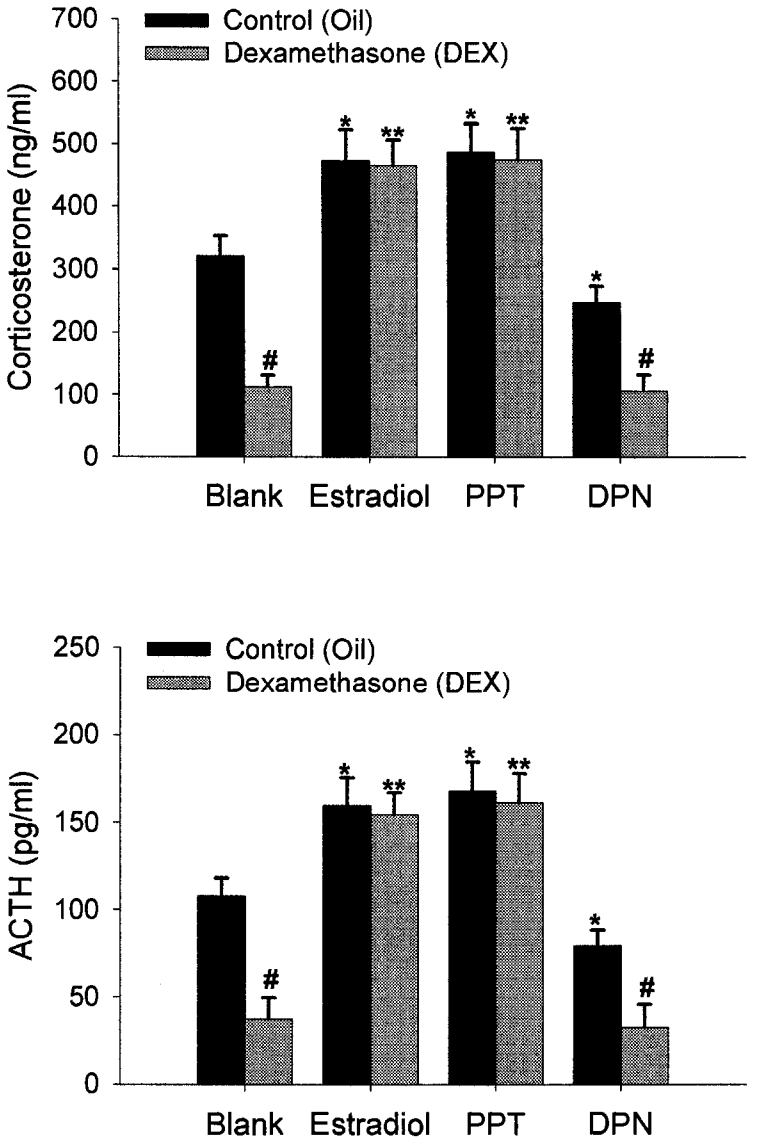


Figure 3.4. Estradiol impairs dexamethasone suppression of peak diurnal corticosterone and ACTH through activation of ERα near the paraventricular nucleus (PVN) of the hypothalamus. Wax pellets containing estradiol, the ERα agonist PPT, the ERβ agonist DPN, or no hormone (control, blank) were implanted to a region just above the PVN. Data are expressed as mean ± SEM of 10 animals per group. *, # indicates significant differences from oil-treated blank control animals ($P < 0.01$). ** indicates significant difference from dexamethasone-treated control animals ($P < 0.01$).

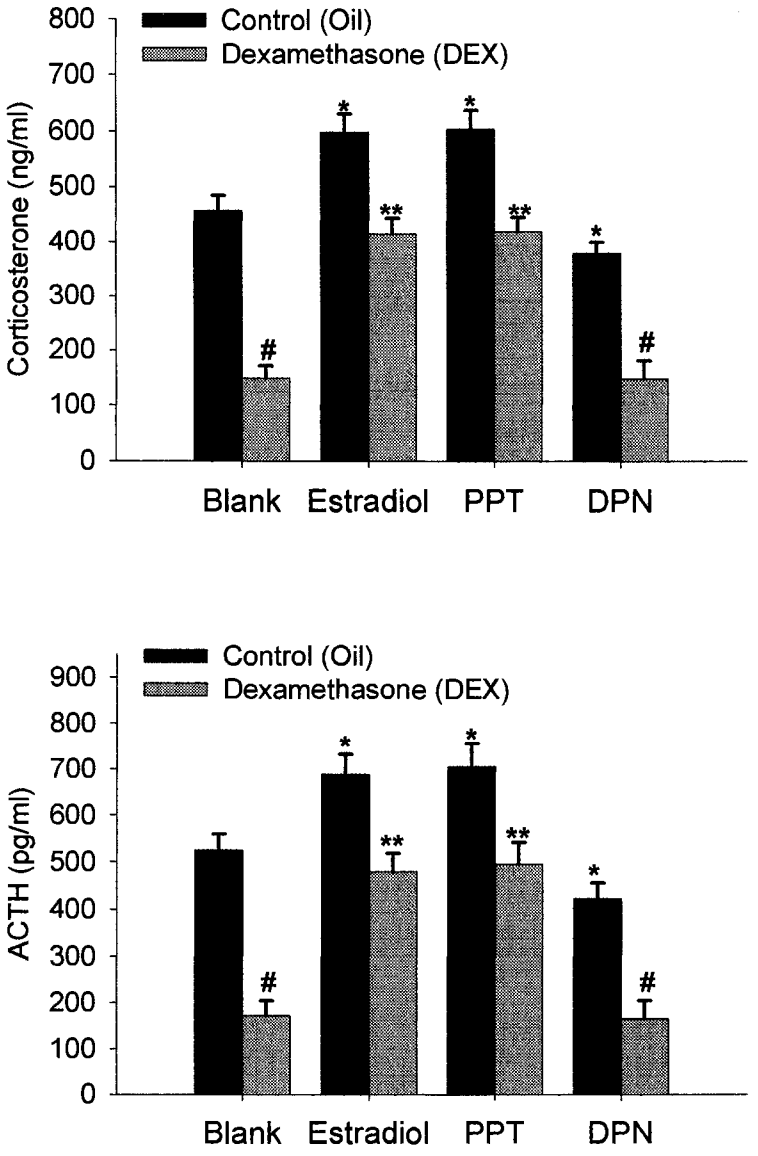


Figure 3.5. Estradiol impairs dexamethasone suppression of restraint-induced corticosterone and ACTH through activation of ERα near the paraventricular nucleus (PVN) of the hypothalamus. Wax pellets containing estradiol, the ERα agonist PPT, the ERβ agonist DPN, or no hormone (control, blank) were implanted to a region just above the PVN. Data are expressed as mean ± SEM of 6-7 animals per group. *, # indicates significant differences from oil-treated blank control animals ($P < 0.01$). ** indicates significant difference from dexamethasone-treated control animals ($P < 0.01$).

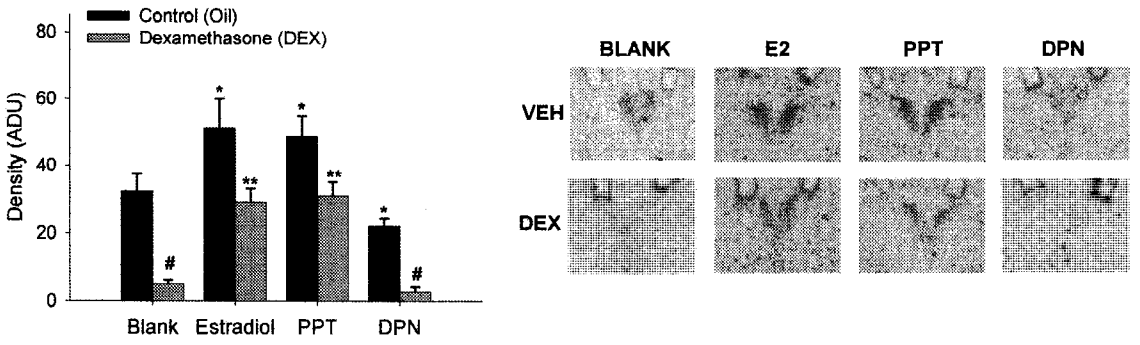


Figure 3.6. Estradiol impairs dexamethasone suppression of restraint-induced *c-fos* mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus through activation of ERα near the PVN. Wax pellets containing estradiol, the ERα agonist PPT, the ERβ agonist DPN, or no hormone (control, blank) were implanted to a region just above the PVN. *Panel A.* Relative levels of *c-fos* mRNA expression. Data are represented as mean arbitrary density units (ADU) ± SEM. n = 6-7 animals per treatment group. *, # indicates significant differences from oil-treated blank control animals ($P < 0.05$). ** indicates significant difference from dexamethasone-treated control animals ($P < 0.01$). *Panel B.* Representative film autoradiograms.

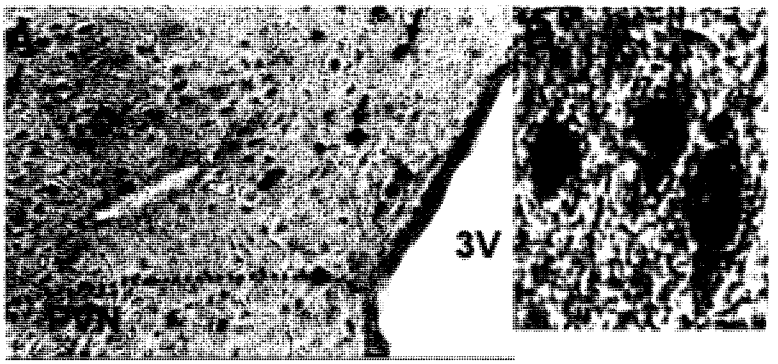


Figure 3.7. GAD67 is colocalized with ERα in the dorsal peri-PVN region (*panel A*). 67.4 +/- 6.1% of ERα-containing neurons also expressed GAD67 in this region. Cells expressing ERα (dark nuclear staining) and GAD67 (brown cytoplasmic staining) (*panel B*).

Discussion

In healthy individuals the physiological product of the stress axis, corticosteroids (cortisol in humans, corticosterone in rodents), normalize the activity of the axis via negative feedback at multiple sites including the hypothalamus, pituitary gland, and adrenal cortex. Impairment of tonic or phasic HPA axis inhibition results in abnormal endogenous glucocorticoid secretory patterns and levels, which are correlated with several disease states such as hypertension, inflammation, obesity, heart disease and diabetes (Baid and Nieman 2004; Whitworth, Williamson et al. 2005; Pasquali, Vicennati et al. 2006; Tomlinson and Stewart 2007; Tait, Butts et al. 2008). Estrogen is a well-known regulator of HPA axis sensitivity. Females tend to react stronger hormonally to a stressor than males, and are more susceptible to diseases linked to abnormalities in HPA axis function. However, at present the mechanism whereby estrogen alters HPA axis function is unknown.

In the studies described here, we investigated the effect of estradiol on glucocorticoid-mediated negative feedback. Basal HPA axis activity is circadian in nature, where a diurnal peak in secretion occurs just prior to onset of activity (early morning for humans, and early evening for rodents). Therefore, we examined basal glucocorticoid negative feedback at the diurnal peak in CORT in addition to stress-activated CORT secretion. Furthermore, we determined which estrogen receptor subtype is responsible for this effect and the brain site mediating estradiol's effects on glucocorticoid-mediated negative feedback. Based on our results, we believe that

estradiol exerts its action at the level of the PVN, through an ERα mediated disruption of glucocorticoid receptor mediated negative feedback.

Glucocorticoid-mediated HPA axis negative feedback is dependent upon the dose and the duration of exposure (Abe and Critchlow 1980). Normal function of the HPA axis is dependent upon permissive and reactive actions of glucocorticoids (for review see (Sapolsky, Romero et al. 2000)). For example, adrenalectomy (ADX) results in higher PVN neuropeptide synthesis and secretion both at the basal and stressed states. Additionally, feedback can occur either directly at the level of the CRH and AVP neurons of the PVN as well as the corticotrophs of the anterior pituitary, or indirectly though GR and MR containing brain regions that project to the PVN such as the hippocampus and amygdala (Davidson and Feldman 1967; Bohus and Strashimirov 1970; Dallman, Akana et al. 1987; Sawchenko 1987). Glucocorticoid effects vary according to duration of exposure. Fast feedback occurs within seconds to minutes, whereas delayed feedback occurs within minutes to hours (Dallman, Akana et al. 1987). Fast feedback occurs in a timeframe that is too short for de novo protein synthesis, and must be attributable to non-genomic actions of glucocortioids. These actions have been shown to involve the classical GR and MR as well as endocannabinoids (Widmaier and Dallman 1984; Hinz and Hirschelmann 2000; Di, Malcher-Lopes et al. 2003; Patel, Roelke et al. 2004; Di, Malcher-Lopes et al. 2005). Delayed feedback occurs in a timeframe that likely involves influences on gene transcription and de novo protein synthesis and is attributable to activation of GR and/or MR, and may be direct or indirect in nature. For

the purpose of this study we examined delayed glucocorticoid-mediated HPA axis negative feedback.

In order to more closely relate our findings to the function of the HPA axis in the human condition of depression, we utilized a dexamethasone suppression test (DST). The DST is a well established clinical predictor of depression (Carroll and Curtis 1976), and has been used in rodents as a model for the human test (Lurie, Kuhn et al. 1989). More recently, the combined DEX/CRH test, where dexamethasone suppression in the presence of HPA axis drive (CRH) has been proven to be 90% accurate in diagnosing depressed individuals (Varghese and Brown 2001). Furthermore, the DEX/CRH test has been used to accurately predict relapse and can be used as a biomarker to positively identify novel treatments for depression (Aubry, Gervasoni et al. 2007; Ising, Horstmann et al. 2007). The DST and DEX/CRH test measure common pathology, however the DEX/CRH has better diagnostic reliability(Watson, Gallagher et al. 2006). Therefore, in this study, we examined the DST under the basal state as well as under HPA axis drive (restraint stress) to better match the parameters of the DEX/CRH test utilized in humans.

In our initial set of experiments, we found that the synthetic glucocorticoid dexamethasone, suppressed peak diurnal and restraint-induced CORT in OVX'd females. The degree of suppression was slightly higher following restraint than during the diurnal peak. This may be in part due to the higher overall activity of the HPA axis following a stressor, or due to decreased glucocorticoid sensitivity during diurnal peak secretion. This decreased sensitivity is thought to be partly responsible for the normal diurnal

elevation in CORT (Bradbury, Akana et al. 1994). Interestingly, following estradiol replacement to OVX'd females, the diurnal increase in CORT was significantly increased and dexamethasone suppression of the diurnal peak was completely inhibited. Therefore, estradiol acts to inhibit glucocorticoid negative feedback during the diurnal rise in CORT. Moreover, estradiol treatment increased CORT following a restraint stress and impaired dexamethasone suppression of stress-induced CORT. In both cases, the impairment was not due to estradiol's actions at the adrenal cortex, as plasma CORT closely matched plasma ACTH in all animals. This provides strong evidence that estradiol directly impairs glucocorticoid-mediated negative feedback. These results also suggests that the increased responsiveness to a stressor that is seen in females is likely due to decreased sensitivity to glucocorticoid feedback.

Results from these experiments indicated that the impairment in negative feedback must occur either in the brain or at the level of the pituitary gland. Previous data from this laboratory indicated that estradiol placed centrally near the paraventricular nucleus (PVN) of the hypothalamus of OVX'd female rats resulted in a stronger hormonal response to a restraint stress than controls (Lund, Hinds et al. 2006). Additionally, in a separate earlier study, placement of DEX near the PVN blunted CRH hnRNA transcription following a stressor (Kovacs, Kiss et al. 1986; Sawchenko 1987). Therefore, we designed studies to determine the responsible estrogen receptor subtype and the location of estradiol's effect on glucocorticoid negative feedback. Our data implicate the peri-PVN region as a critical brain site integrating estradiol influence on HPA axis output and glucocorticoid mediated negative feedback. Our studies utilized

the same procedure used previously where wax pellets containing subtype selective agonists PPT (ERα) and DPN (ERβ) in addition to the endogenous ER ligand estradiol were placed billaterally to a region just above the PVN. This provides for continuous local release confined to a 0.5 mm radius around the wax pellet that allows for the examination of local ER activation independent of peripheral effects (Lund, Hinds et al. 2006).

We found that activation of ERα in the region of the PVN increased diurnal gain of the HPA axis and was sufficient to block all suppressive effects of DEX during the diurnal peak in CORT. Following a restraint stress, peri-PVN ERα activation significantly impaired DEX suppression and resulted in CORT and ACTH levels similar to untreated, stressed controls. As expected, *c-fos* mRNA expression following the restraint stress closely matched the hormonal output of the axis. Animals implanted with estradiol or PPT pellets had stronger activation of PVN neurons following a stressor than control or DPN implanted animals, and this activation was not diminished by DEX to the same degree as seen in control animals. From these findings we conclude that estradiol impairs glucocorticoid-mediated HPA axis negative feedback during the diurnal peak and following a restraint stress specifically thorough activation of ERα-containing neurons in the region of the PVN.

The actions of estradiol can be mediated by both ERα and ERβ. Whereas ERβ is found in PVN neurons, ERα is not expressed in CRH or AVP neurons in the PVN (Suzuki and Handa 2005), thus ruling out a direct action of estradiol through ERα on the function of these neurons. However, ERα is found in stress-responsive areas that

provide inhibitory γ-aminobutyric acid (GABA) input into the PVN including the bed nucleus of the stria terminalis (BnST) and medial preoptic area (MPOA) (Shughrue, Lane et al. 1997) and the periPVN region (Herman, Tasker et al. 2002). In support of a GABAergic mechanism, our studies show co-localization of ERα immunoreactivity (IR) with glutamic acid decarboxylase 67 IR (GAD67-IR). GAD67 is the rate-limiting enzyme in the production of GABA. Although we found that ERα-IR was largely absent from the PVN, it was present in neurons immediately surrounding the PVN (peri-PVN), especially dorsally. Additionally, a majority of these ERα-IR neurons in the dorsal peri-PVN region also contained GAD67-IR indicating that they are inhibitory interneurons. This region of the peri-PVN contains GAD-IR neurons that project to the parvocellular PVN (Roland and Sawchenko 1993). Application of a GABA agonist locally to this region can enhance the CORT response to a stressor (Cullinan 1998), presumably by inhibiting GABAergic transmission to hypophysiotropic CRH neurons . Therefore, it is plausible that estradiol may work via ERα to inhibit GABAergic transmission from these peri-PVN neurons.

Our findings are consistent with several other estrogen-sensitive systems that indicate a role for estrogen modulation of GABAergic neurotransmission. These include the actions of estrogens on dendritic spine growth (Murphy, Cole et al. 1998) and the positive-feedback response of gonadotropin-releasing hormone neurons (Herbison 2008). Given that the direct administration of estradiol or an ERα agonist to the PVN results in impaired glucocorticoid mediated negative feedback, and the presence of ERα in GAD 67 containing neurons surrounding the PVN, these results

suggests that ERα might inhibit glucocorticoid negative feedback by reducing an inhibitory GABAergic input into the PVN.

Stress-integration at the level of the PVN involves both inhibitory γ-aminobutyric acid (GABA) and excitatory glutamate circuits (see (Herman, Tasker et al. 2002; Herman, Mueller et al. 2004; Kovacs, Miklos et al. 2004), for review). Recent studies have suggested that a substantial part of PVN excitation and inhibition is gated by local circuit neurons in the vicinity of the PVN. Studies have shown that micro-injection of glutamate elicits, while local application of a glutamate receptor antagonist inhibits ACTH and CORT secretion (Darlington, Miyamoto et al. 1989; Ziegler and Herman 2000). Inhibitory GABA containing neurons synapse on parvicellular PVN neurons and are present in the immediate surround of the PVN, BnST, MPOA, lateral hypothalamic area (LHA), and anterior hypothalamic area (AHA) (Boudaba, Szabo et al. 1996; Miklos and Kovacs 2002). These regions also provide stress responsive afferents to the PVN (Cullinan, Helmreich et al. 1996; Bali, Erdelyi et al. 2005). In addition, microinjection of muscimol (GABA-A receptor agonist) into the PVN attenuates, while bicuculline (GABA-A receptor antagonist) augments stress-induced ACTH secretion (DiMicco, Stotz-Potter et al. 1996; Cole and Sawchenko 2002). Furthermore, studies have shown stress-induced increases in the GABA-synthesizing enzyme GAD 67 mRNA in PVN-projecting areas including peri-PVN, MPOA, BnST, ARC, and hippocampus (Bowers, Cullinan et al. 1998). The presence of both ionotropic glutamate receptors and GABA-A receptors in CRH and AVP neurons indicates their ability to integrate inhibitory and excitatory inputs (Aubry, Bartanusz et al. 1996;

Cullinan, Helmreich et al. 1996). This is corroborated by studies showing local GABA-A receptor blockade increases CRH and AVP expression (Bali and Kovacs 2003). Recent studies by Tasker *et al.* have shown glucocorticoids secreted in response to a stressor act in a rapid feedback fashion to decrease glutamate and increase GABA release onto PVN neurons (Di, Malcher-Lopes et al. 2005). Taken together, these studies suggest that local GABA circuits are key to stress integration at the PVN and play a vital role in glucocorticoid dependent negative feedback although other mechanisms of action for estrogen interference of glucocorticoid-mediated negative feedback must also be considered.

It is possible that our results reflect estradiol's ability to impair either GR or MR autoregulation (Burgess and Handa 1992), and thus impair glucocorticoid-mediated feedback. Earlier molecular studies have shown that GR and ER can interact in control of a reporter gene regulated through simple hormone response elements (Uht, Anderson et al. 1997). How this relates to neuronal function is not entirely clear since studies have not definitively colocalized ERα or ERβ with GR or MR in brain regions known to be important in CORT mediated negative feedback, and ERα has been shown to be absent in GR containing CRH and AVP neurosecretory neurons of the PVN. Until future studies indicate the existence of ERα and GR within the same cell population, it must be postulated that estradiol works indirectly to influence glucocorticoid negative feedback.

Although the effects of estradiol observed here may also be peripheral, causing inhibition of negative feedback at the level of the pituitary or adrenal glands, our

results seem to rule out this possibility. The changes in CORT secretion that we observed were mimicked by ACTH secretion, and the local administration of hormone to the peri-PVN region rules out potential actions at peripheral sites. Alternatively, effects could involve alteration of corticosteroid-binding globulin (CBG) levels or binding capacity. Increased CBG levels might lead to decreased sensitivity to endogenous and exogenous corticosteroids, and thus lower glucocorticoid-mediated negative feedback. Circulating CBG levels are 2.5 times higher in females than in males, however this sex difference appears to be due to the organizational actions of sex hormones (Mataradze, Kurabekova et al. 1992). Furthermore, estradiol treatment does not influence CBG levels in females (Mataradze, Kurabekova et al. 1992; McCormick, Linkroum et al. 2002), and has been shown to increase or have no effect on CBG levels in males (McCormick, Linkroum et al. 2002; Lund, Munson et al. 2004). To circumvent this potential confound, we utilized a synthetic glucocorticoid (DEX), which does not bind CBG (Kolanowski and Pizarro 1969; Koblinsky, Beato et al. 1972). DEX has been shown to inhibit HPA axis activity when administered directly to the PVN (Kovacs and Makara 1988), and an intraperitoneal injection of femtomolar ^{3}H-DEX produces binding in glucocorticoid-concentrating brain regions within 1 h (Hassan, Patchev et al. 1999).

An unlikely alternative possibility is that hormone or agonist could have diffused far enough from the pellet to enter the third ventricle, and thus enter cerebral spinal fluid (CSF) circulation. In this case, a di- or tri-synaptic mode of inhibition may be plausible, where estrogen could induce changes in GABA synthesis

or release, in areas with indirect connections with the PVN like the hippocampus, lateral septum, or amygdala. We do not think that this is a viable alternative since the diffusion of hormone would result in CSF concentrations too low to appreciably effect ER signaling in distant brain regions.

Yet another possibility is that estradiol could increase GABA receptor levels in brain regions that provide inhibitory input to the PVN, thus increasing potential inhibition of these inhibitory PVN inputs. Indeed, some studies have shown that estrogen can increase GABAA receptor subunit expression in the MPA and BnST (Herbison and Fenelon 1995). Whether these changes can affect glucocorticoid mediated HPA negative feedback is uncertain. Such a mechanism is unlikely except at the level of the PVN unless there is a widespread diffusion of hormone away from the pellet, which we have not observed and would likely result in very low levels of hormone in distant brain sites.

Our data indicates that ERα activation in the peri-PVN induces an increase in neuronal activation within the PVN following stress with DEX pretreatment as measured by the induction of *c-fos* mRNA expression. This argues for a site of action contained to the immediate surround of the PVN. Unfortunately, we currently do not know if there are changes in GAD mRNA and protein levels in ERα-expressing cells within the peri-pvn region following ER agonist treatment in the presence or absence of DEX and/or a stressor. Nor do we know if there are direct projections of these neuronal populations to the PVN. Unfortunately, such studies are technically difficult given the close association of peri-PVN neurons to the PVN proper.

While it appears from these studies that the inhibitory effects of estradiol on glucocorticoid negative feedback are mediated through ERα, we did observe that the activation of ERβ in and around the PVN results in a slight but significant suppression in HPA responsivity. DEX suppression was not impaired by ERβ activation. Therefore, it can be proposed that ERβ acts in an opposing fashion to increase glucocorticoid-mediated negative feedback. Further experiments would need to be done to establish a dose-response curve for DEX in the presence or absence of ER agonists. ERβ is expressed in a small number of CRH and AVP containing parvocellular PVN neurons, suggesting a potential direct effect on CRH and AVP transcription (Laflamme, Nappi et al. 1998; Hrabovszky, Kallo et al. 2004; Suzuki and Handa 2005). Furthermore, ERβ has been shown to drive CRH and AVP promoter activity *in vitro* (Shapiro, Xu et al. 2000; Miller, Suzuki et al. 2004; Pak, Chung et al. 2007). Alternatively, ERβ may act peripherally as it is expressed within corticotrophs of the anterior pituitary and within the adrenal cortex (Mitchner, Garlick et al. 1998; de Cremoux, Rosenberg et al. 2008). However, our studies report decreased stress responsivity with local peri-PVN administration of an ERβ agonist, which argues against this possibility.

Impairment of glucocorticoid negative feedback is a key characteristic of major depressive disorder in humans (for reviews see (Swaab, Bao et al. 2005; Bao, Meynen et al. 2008)). Depressed patients have a marked reduction in ability to respond and adapt to a stressor, and exhibit increased neuropeptide levels in the PVN. Furthermore, in these individuals, DEX is unable to suppress the stimulatory actions of CRH, in much the same fashion as those animals treated with estradiol and PPT and subjected to a

restraint stress. These data may provide a link between the higher incidence of depression and stress sensitivity observed in women. Interestingly, a recent study indicated an increase in ERα and a decrease in androgen receptor (AR) mRNA in laser-dissected PVN of post-mortem depressed patients (Wang, Kamphuis et al. 2008), further linking sex hormones with HPA axis activity and depression in humans.

In summary, estradiol directly impairs glucocorticoid-mediated negative feedback through a peri-PVN ERα dependent mechanism. These data suggest that the increased stress sensitivity seen in females might be due to decreased sensitivity to glucocorticoid-mediated HPA axis negative feedback.

CHAPTER FOUR

Estrogen receptor beta (ERβ) agonist diarylpropionitrile (DPN): biological activities of *R*- and *S*-DPN

Abstract

Estrogen has been shown to have positive and negative effects on anxiety and depressive-like behaviors. These opposing actions of estrogen could be explained by the existence of two distinct estrogen receptor (ER) systems, ER alpha (ERα) and ER beta (ERβ). Recent studies have implicated ERb in regulating mood-related behaviors including anxiety. The ERβ agonist, diarylpropionitrile (DPN) has been shown to have anxiolytic properties in rats. DPN exists as a racemic mixture of two enantiomers, *R*-DPN and *S*-DPN. Molecular modeling predicts the *S*-enantiomer to be the biologically active form. In this study, we compared *R*-DPN and *S*-DPN for their *in vitro* binding affinity, ability to activate transcription *in vitro* at an estrogen response element (ERE), and *in vivo* endocrine and behavioral responses. *In vitro* binding studies utilizing recombinant rat ERβ revealed that *S*-DPN has a several fold greater relative binding affinity (RBA) for ERβ than does *R*-DPN. Furthermore, co-transfection of N-38 immortalized hypothalamic cells with an ERE-luc reporter and ERb revealed that *S*-DPN is a potent activator of transcription *in vitro*, whereas *R*-DPN is not. Subsequently, we examined anxiety-like behaviors using the open field (OF) test and elevated plus maze (EPM), or depressive-like behaviors, using the forced swim test (FST). Young adult

female Sprague-Dawley rats were ovariectomized and one week later administered (s.c.) one of several selective ERβ agonists: racemic DPN, *S*-DPN, *R*-DPN, WAY-200070 (Wyeth, Princeton, NJ), or the ERα agonist propylpyrazoletriol (PPT), or vehicle daily for seven days. On the forth and fifth day of treatment, animals were tested on the OF and EPM, respectively. After seven days of treatment, animals were tested in the FST. Rats treated with racemic DPN, *S*-DPN, and the ERβ agonist, WAY-200070, showed significantly decreased anxiety-like behaviors in both the open field and elevated plus maze and significantly less depressive-like behaviors in the FST. In concordance with the calculated RBA and transcriptional activity, these results demonstrate that the *S*-enantiomer is the biologically active form of DPN. These studies also indicate that estrogen's positive effects on mood, including its anxiolytic and anti-depressive actions, are likely due to its actions at ERβ and raise the possibility that selective ERβ agonists can be used in the treatment of anxiety or mood disorders such as anxiety.

Introduction

Estrogens have diverse and powerful biological actions due in part to the existence of two estrogen receptor (ER) subtypes, ERα and ERβ (Green, Walter et al. 1986; Kuiper, Enmark et al. 1996). Adding to this diversity is the existence of multiple splice variants [for review see (Zhao, Toresson et al. 2005; Weiser, Foradori et al. 2008)], and differential expression of each receptor subtype in various tissues (Shughrue, Lane et al. 1997; Shughrue, Lane et al. 1998). ERα predominates, while ERβ plays a minor role, in classical estrogen-sensitive tissues such as uterus, mammary glands, pituitary, and bone (Gustafsson 2000). However, a role for ERβ has been found in the ovary,

prostate, cardiovascular system, and central nervous system (CNS) (Gustafsson 2000; Zhao, Dahlman-Wright et al. 2008).

The development of ERα and ERβ knockout animals (αERKO and βERKO respectively) as well as the discovery of subtype selective ligands has provided initial insight into the biological function of each receptor. Importantly, subtype selective high affinity agonists and antagonists allow for the pharmacological examination of receptor-mediated functions in a normal wild-type animal [for review see (Veeneman 2005; Harris 2007)]. For example, propylpyrazoletriol (PPT) has a relative binding affinity (RBA, as compared to estradiol = 100) for ERα of 49 and binding is 410-fold more selective for ERα than ERβ. In contrast, diarylpropionitrile (DPN) has a RBA for ERβ of 18 and binding is 72-fold more selective for ERβ than ERα (Stauffer, Coletta et al. 2000; Meyers, Sun et al. 2001). Transcriptional selectivity of these compounds are greater than that of binding, with DPN having a 170-fold greater relative potency in transcription assays for ERβ (Meyers, Sun et al. 2001). Other ligands, including MPP (ERα antagonist), WAY-200070 (ERβ agonist), PHTPP (ERβ antagonist), and plant-derived phytoestrogens (genistein, coumesterol, and equol) all exhibit some selectivity for ERβ. Interestingly, the dihydrotestosterone metabolite, 5α-androstane, 3β, 17β-diol (3β-Diol), is a somewhat selective ERβ agonist and can activate ERE-mediated transcription in the presence of ERβ at a much greater selectivity than binding would predict (Handa, Pak et al. 2008).

Studies utilizing knockout animals, as well as subtype selective agonists and antagonists have been critical in determining the biological actions specific to ERα or

ERβ. These studies have implicated ERβ signaling in alleviating several pathologies including inflammation, inflammatory bowel syndrome, endometriosis, and heart disease [for reviews see (Harris 2006; Harris 2006; Harris 2007)]. Important to the studies described here, these studies have also indicated a role for ERβ signaling in anxiety- and depressive-like behaviors. For instance, estradiol's anti-depressant actions in the forced swim test (FST) are absent in βERKO animals (Rocha, Fleischer et al. 2005), and these animals exhibit increased anxiety-type behaviors in the elevated plus maze (EPM) relative to wild-type animals (Krezel, Dupont et al. 2001; Imwalle, Gustafsson et al. 2005). Studies utilizing receptor subtype selective agonists are in concordance with the experiments using knockout animals. Treatment of gonadectomized males and female rats with the ERβ agonist DPN results in less depressive-like behavior in the FST and horizontal crossing task, and decreased anxiety-type behaviors in the EPM (Walf, Rhodes et al. 2004; Lund, Rovis et al. 2005; Walf and Frye 2005).

The ERβ selective ligand DPN (Meyers, Sun et al. 2001), exists as a racemic mixture of two enantiomers, *S*-DPN and *R*-DPN (Figure 1). Furthermore, examination of the ligand-receptor interaction by molecular modeling predicts the *S*- enantiomer to be responsible for DPN's selectivity for ERβ (Sun, Baudry et al. 2003). The stereochemistry of *S*-DPN allows the nitrile group of DPN to positively interact with Met-336 in the ligand binding pocket of ERβ. The nitrile group in *R*-DPN points away from this residue, and thus does not form as stable of a complex with ERβ. Additionally, the modeling indicates that *R*-DPN forms a lower energy (more favorable) complex with ERα than ERβ. Thus, the *S*- enantiomer likely has higher affinity and selectivity for ERβ than the *R*-

enantiomer. To date, asymmetric synthesis and/or chromatographic separation of the two enantiomers have not been reported. Studies utilizing DPN employ a racemic mixture of two stereoisomers, which likely have differing pharmacological effects. To this end, in the studies described here, we have separated *S*-DPN and *R*-DPN by chiral high-pressure liquid chromatography (HPLC) (Figure 1), and compared their action in estrogen receptor binding, transcriptional activation of an estrogen responsive reporter gene, and anxiety-type and learned helplessness behaviors. Our studies indicate that *S*-DPN is indeed the biologically active enantiomer of DPN. Furthermore, R-DPN is nonselective and has little activity at ERβ. Therefore future studies utilizing DPN should carefully consider using an enantiomeric-pure preparation.

Materials and Methods

Animals

60 day old female Sprague Dawley rats were obtained from Harlan Laboratories (San Diego, CA), housed individually, and maintained on a 12h:12h light schedule (lights on at 0600h) in temperature and humidity controlled rooms at the Laboratory Animal Research Facility at Colorado State University. Animals had *ad libitum* access to a soy free diet (modified AIN-93G, DYET#101591 from DYETS, Inc., Allentown, PA, with corn oil substituted for soy oil). One week after arrival, animals were ovariectomized under isofluorane anesthesia. Following ovariectomy, animals were handled daily (5 minutes each animal) by the same experimenter. All animal studies were previously approved by the Animal Care and Use Committee at Colorado State University.

Hormone / Drug treatments

Beginning one week following ovariectomy, animals were given a single daily s.c. injection of either hydroxypropyl betacyclodextran (vehicle; 27% w/w in saline; CTD Inc., High Springs, FL), DPN (2.0 mg/kg), *S*-DPN (2.0 mg/kg), *R*-DPN (2.0 mg/kg), WAY-200070-3 (2.0 mg/kg), or PPT (1.0 mg/kg) in a total volume of 0.2 ml. Injections occurred at 0800 h for 7 days. DPN was synthesized *de* novo as previously described (Lund et al., 2005), WAY-200070-3 was provided by Wyeth Discovery Neuroscience (Princeton, NJ), and PPT and PHTPP were purchased from Tocris Inc. (Ellisville, MO). *R*-DPN and *S*-DPN were obtained in >99% enantiomeric excess (ee) by separation of racemic DPN via reverse-phase chiral HPLC (Chiralpack IC column; hexanes:isopropanol 70:30 mobile phase; UV detection 250 nm). Purity of the resulting compounds was confirmed by NMR. Three hours after the daily treatment injection on days 4-7, animals underwent behavioral testing consisted of two paradigms established as indicators of anxiety (elevated plus maze, open field), and one paradigm established as an indicator of helplessness (forced swim test), as described below.

Saturation isotherms

In vitro ligand binding assays were performed as previously described (Handa, Reid et al. 1986; Handa, Stadelman et al. 1987), with a minimum of four assays per ER and saturation isotherms constructed as follows. Full-length rat ERβ1 and ERα were synthesized *in vitro* using the TnT-coupled reticulocyte lystate system (Promega, Madison, WI; according to manufacturer's protocol) with T7-RNA polymerase from their

respective plasmid expression vectors (pcDNA3.0-ERβ; T. T. Brown, Pfizer, Groton, CT) (PSG5-ERα; R. Price, UCSF, San Francisco, CA). Aliquots (100 ul) of the translation reaction mixture were incubated 90 min at room temperature (ERβ1), or 18 h at 4 C (ERα) with increasing (0.01–1000 nM) concentrations of E2, DPN, *S*-DPN, or *R*-DPN and 1.0 nM [^{3}H]-E2 in duplicate. These incubation conditions were previously shown to be optimal for these receptor types (Lund, Rovis et al. 2005). Non-specific binding was determined using 200-fold excess of the ER agonist, diethylstilbestrol. After incubation, bound and unbound [^{3}H]-E2 were separated by passing the incubation reaction through a 1 ml lipophilic Sephadex LH-20 (Sigma) column. IC50 was calculated from a non-linear regression of the log transformed data using GraphPad Prism (v. 3.0, San Diego, CA), and Ki was determined by using the Cheng and Prusoff equation [Ki=IC50/(1+([radioligand]/Kd of radioligand))].

Transcriptional activation assay

The mouse derived hypothalamic cell line N-38 (Titolo, Cai et al. 2006) (CELLutions Biosystems, Inc; Toronto, CA) was used for all transcriptional assays. Cells were maintained in 1X DMEM (Cellgro, Manassas, VA) containing 1x non-essential amino acids, L-glutamine, 4.5% glucose, and 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA) at 37 C with 5% CO_2. Cells were plated at 100,000 cells per well in 24-well plates, grown to 60-70% confluency prior to transfection, and used prior to passage 25. The ERE-tk-luciferase reporter construct contained two ERE sequences coupled to a minimal tk promoter subcloned into pGL2-Basic plasmid (Promega Corp.,

Madison, WI). The β-galactosidase expressing SV40-LacZ reporter (PJ3, generously provided by Dr. Rosalie Uht, Univeristy of Virginia, VA) was used as an internal control for plasmid transfer efficiency. Plasmid expression vectors for ERα and ERβ were the same as used in the saturation isotherms. Empty vector controls were used with each plasmid. Constructs were transfected in triplicate, and each assay repeated 4-6 times. Transfections were carried out using a lipid-mediated reagent (Fugene 6; Roche Diagnostics, Indianapolis, IN) according to manufacturer's protocol using a total DNA concentration of 0.33 ug/well and 1 ul/well Fugene 6. Optimal total DNA amounts and Fugene concentrations were determined empirically. Cells were incubated with the transfection complex for 16 h, media was replaced with fresh media containing 10% charcoal-stripped FBS (to ensure estrogen-free culture conditions) for 8 h, then media was replaced with treatment media for 16 h. Hormone treatments were made by diluting 17-β-estradiol (E2; Sigma, St. Louis, MO), DPN, *S*-DPN, and *R*-DPN in ethanol and used at a final concentration of 1nM, 10nM, or 100 nM (ethanol ≤ 0.001%). Vehicle contained the equivalent amount of ethanol. After 16 h of treatment, cells were washed with 1X PBS and lysed. To measure luciferase activity 20 ul lysate was added to 100 ul luciferin substrate (Promega Corp., Madison, WI), and to measure β-galactosidase activity 40 ul lysate was added to 200 ul galacton substrate (Tropix-GalactoLight; Applied Biosystems, Foster City, CA). Relative light units (RLU) were measured using as 20/20 TD luminometer (Turner Designs, Sunnyvale, CA). RLU for each treatment were normalized to the respective empty expression vector control, and data are expressed as percent change compared with vehicle-treated, empty expression vector controls.

Open field

The open field test was performed on the fourth day of treatment and was conducted as previously described (Handa, Cross et al. 1993). Behavioral measures included activity (total square crossings), latency to exit middle squares, interactions with novel objects, rearing, head dips, total time spent grooming, and number of fecal boli.

Elevated plus maze

Maze performance was evaluated on the fifth day of treatment and was run as previously described (Handley and McBlane 1993). Behaviors measured included number of entries into open and closed arms, total time spent in open and closed arms, rearing, head dips, total time spent grooming, and the number of fecal boli.

Forced swim test

The forced swim test (FST) was evaluated on the seventh day of treatment and was a modified version of the Porsolt Swim Test (Porsolt, Le Pichon et al. 1977). Total time spent swimming, struggling, and immobile in a glass cylindrical container (40 cm x 27 cm) filled with 30 cm of 25C fresh water was recorded for 5 minutes. Animals were acclimated to the test one day prior (sixth day of treatment) via a 10 minute swim test. Swimming was defined as movement of the forelimbs and hind limbs that does not break the surface of the water, struggling was defined as movement of the forelimbs

rapidly to break the surface of the water and/or attempting to climb against the wall of the container, and immobility was defined as absence of any movement except for slight movements necessary for the animal to keep its head above water.

Plasma corticosterone analysis

20 min following the FST, animals were rapidly decapitated and trunk blood collected into ice-chilled tubes containing 0.5M EDTA and aprotinin (4 mg/ml, Sigma, St. Louis, MO). Plasma levels of corticosterone were determined by radioimmunoassay as previously described (Lund, Rovis et al. 2005). Intra-assay and inter-assay variance were 4.3% and 8.9% respectively.

In situ hybridization

20 min following the FST, brains were harvested and frozen in cold 2-methylbutane (-40C) and stored at -80 C until processed for *in situ* hybridization (ISH). ISH for *c-fos* mRNA was performed as previously described (Lund, Munson et al. 2004) utilizing a 48-bp oligonucleotide probe (5'-GCAGCGGGAGGATGACGCCTCGTAGTCCGCGTTGAAACCCGAGAACAT-3') targeted to the rat *c-fos* gene.

Statistics

Analysis of variance was performed on data from each experiment using the Statview data analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). Fisher's PLSD

analysis was used post hoc where appropriate. Differences were considered significant when $p < 0.05$. Data are expressed as group means ± SEM. Transfection data are expressed as percent change compared with vehicle-treated, empty vector controls.

Results

Relative binding affinities of S-DPN and R-DPN for ERs

Relative binding affinities for the enantiomers of diarylpropionitrile for *in vitro* transcribed ERα and ERβ were determined via competitive binding assays. Displacement of [^{3}H]-E2 from each receptor was quantitated to determine the affinity of the two enantiomers for each receptor subtype (Table 4.1). At optimal binding conditions with respect to each receptor, the *S*- enantiomer had greater affinity for ERβ ($K_i = 0.27 \pm 0.05$) than did the *R*- enantiomer ($K_i = 1.82 \pm 0.21$). Selectivity for ERβ was similar for the two enantiomers (approx. 80-fold). *S*-DPN exhibited an affinity for ERβ that was close to that of the cognate ligand, E2 ($K_i = 0.13 \pm 0.02$), and had good binding selectivity for ERβ over ERα. Thus, by binding parameters, *S*-DPN is a high affinity, partially selective ERβ agonist.

Comparison of S-DPN and R-DPN on ERβ-dependent ERE-luciferase activity

To determine if DPN exhibits enantiomer-specific activation of ERE-dependent transcription through ERβ or ERα, we cotransfected an ERE-luciferase reporter plasmid with an expression vector for ERβ into the mouse hypothalamic cell line N-38. These cells were chosen since the hypothalamus is a brain region rich in ER expression and is important in reproduction and homeostasis (both targets of estrogen action in the

brain). N-38 cells do express small amounts of ERα and ERβ (Belsham et al., 2004), however when transfected with an ERE-luciferase promoter construct, estradiol did not stimulate luciferase expression (data not shown). Thus, in these experiments the cells were co-transfected with ERα or ERβ expression vectors. In cells co-transfected with ERβ, there was a significant ($p < 0.01$) ligand-independent (vehicle) effect (~300% of empty vector control) (Figure 4.2A). The racemic mixture of DPN significantly ($p < 0.01$) activated transcription above the observed constitutive level in a dose-dependent manner where 10nM and 100nM DPN were statistically identical to the effect seen with 1nM estradiol. *S*-DPN stimulated ERE-luciferase activity in a similar fashion to estradiol at all doses, and was significantly higher than racemic DPN at the 1nM dose ($p = 0.022$). In contrast, *R*-DPN did not stimulate luciferase expression to levels greater than that seen in the absence of ligand.

Comparison of S-DPN and R-DPN on ERα-dependent ERE-luciferase activity

In cells co-transfected with ERα, there was a smaller but significant constitutive activity observed (~185% of empty vector control) (Figure 4.2B). The racemic mixture of DPN did not stimulate transcription at the 1nM or 10nM concentrations, however, a small but significant increase ($p = 0.009$) in activity was observed at the 100nM concentration. However, this activation was at a much lower level than any dose of estradiol (278% of empty vector control for 100nM DPN, versus 560% for 1nM estradiol). The same effect was observed with *R*-DPN, where a significant increase in ERE-luciferase activity was only seen at the 100nM concentration ($p < 0.05$).

Interestingly, *S*-DPN did not stimulate luciferase activity at any of the doses. This suggests that the ERα-dependent activity of the racemate is due to the *R*-enantiomer of DPN.

Comparison of S-DPN and R-DPN on ERβ-dependent ERE-luciferase activity in the presence of an ERβ antagonist

In order to inhibit ERβ-dependent transcription we used an ERβ antagonist (PHTPP) in N-38 cells co-transfected with an ERE-luciferase reporter construct and an ERβ expression vector. All treatments were given at 1nM concentration, with or without 10μM PHTPP (concentration required to inhibit the effect of 1nM estradiol, (Compton, Sheng et al. 2004)). Interestingly, the antagonist significantly ($p = 0.02$) decreased the ligand-independent activation of ERβ, but not to the levels seen in the empty vector controls (297% to 181% of empty vector control) (Figure 4.2C). Racemic DPN significantly increased transcriptional activity, but E2 and *S*-DPN activated transcription to a much greater level than racemic DPN. *R*-DPN was similar to the vehicle control. When the agonists were given in conjunction with the antagonist, PHTPP, ERE-luciferase levels were similar to treatment with the antagonist alone. Thus, *S*-DPN is a potent and selective activator of ERE-dependent transcription in N-38 cells, and this activation can be abolished with concomitant treatment with an ERβ antagonist.

Anxiety-type behaviors and learned helplessness in S-DPN and R-DPN treated animals

To determine whether DPN's anxiolytic and anti-depressant effects are via the *S* enantiomer, we examined the effect of each enantiomer on anxiety-like behaviors using the elevated plus maze (EPM) and open field (OF) test. We used the forced swim test (FST) to evaluate depressive-like behaviors. Rats treated with racemic DPN, *S*-DPN, and WAY-200070 showed significantly decreased anxiety-like behaviors in both the open field and elevated plus maze. In the OF, these animals made more rears, interacted more with a novel object, and spent more time in the middle squares of the OF arena than did vehicle, PPT, or *R*-DPN treated animals ($p<0.01$; figure 4.3A). In the EPM, racemic DPN, *S*-DPN, and WAY-200070 treated females had significantly higher open arm entries, open arm time, rearing and head dips than did control, PPT, or *R*-DPN treated animals ($p<0.01$; Figure 4.3B). Rats treated with racemic DPN, *S*-DPN, and WAY-200070 showed significantly less depressive-like behaviors in the FST (Figure 4.3C). These animals spent significantly more time struggling, and less time immobile than did control, PPT, or *R*-DPN treated animals ($p<0.01$). Thus, *S*-DPN is the behaviorally active enantiomer of DPN with respect to its anxiolytic and anti-depressant activity.

Comparison of the hypothalamic-pituitary-adrenal axis response following the forced swim test in S-DPN and R-DPN treated animals

To determine the effect of *S*-DPN and *R*-DPN on HPA axis activation, serum corticosterone was measured from blood samples taken 20 minutes after the start of the swim test. Animals treated with DPN, *S*-DPN, and WAY-200070 had significantly lower serum CORT than vehicle treated animals, whereas the *R*-enantiomer had no

effect compared to controls (Figure 4.4). Conversely, treatment with the ERα agonist PPT significantly ($p < 0.05$) increased serum CORT.

Activation of PVN neurons by stress is inhibited by S-DPN

c-fos mRNA expression was examined in the paraventricular nucleus of the hypothalamus (PVN) in brains taken from animals subjected to the forced swim test. *c-fos* mRNA was expressed in PVN neurons but expression was significantly ($p<0.05$) lower in DPN, *S*-DPN, and WAY-200070 treated animals as compared to treatment with vehicle, *R*-DPN, or PPT (Figure 4.5). Thus, the effects of racemic DPN on HPA axis activation can be attributed to the *S*- enantiomer and not the *R*- enantiomer. Furthermore, the comparable effect seen with WAY-200070 supports an ERβ-dependent mechanism.

Table 4.1. Affinities of estrogen receptor subtypes for the enantiomers of DPN in comparison to estradiol. Data shown as mean ± SD Ki (nM).

	ERα	ERβ
Estradiol	0.10 ± 0.02	0.13 ± 0.02
DPN	48.1 ± 3.5	0.61 ± 0.07
S-DPN	20.8 ± 2.9	0.27 ± 0.05
R-DPN	147 ± 12.3	1.82 ± 0.21

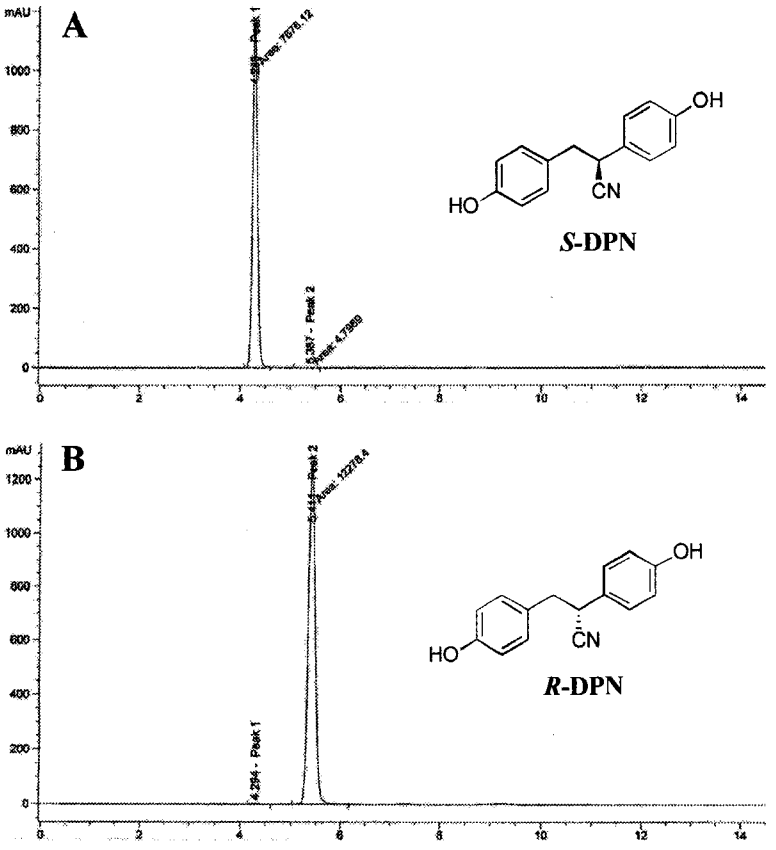


Figure 4.1. Separation of diarylpropionitrile enantiomers by reverse phase chiral high-pressure liquid chromatography. *Panel A.* Chromatogram of *S*-DPN. *Panel B.* Chromatogram of *R*-DPN.

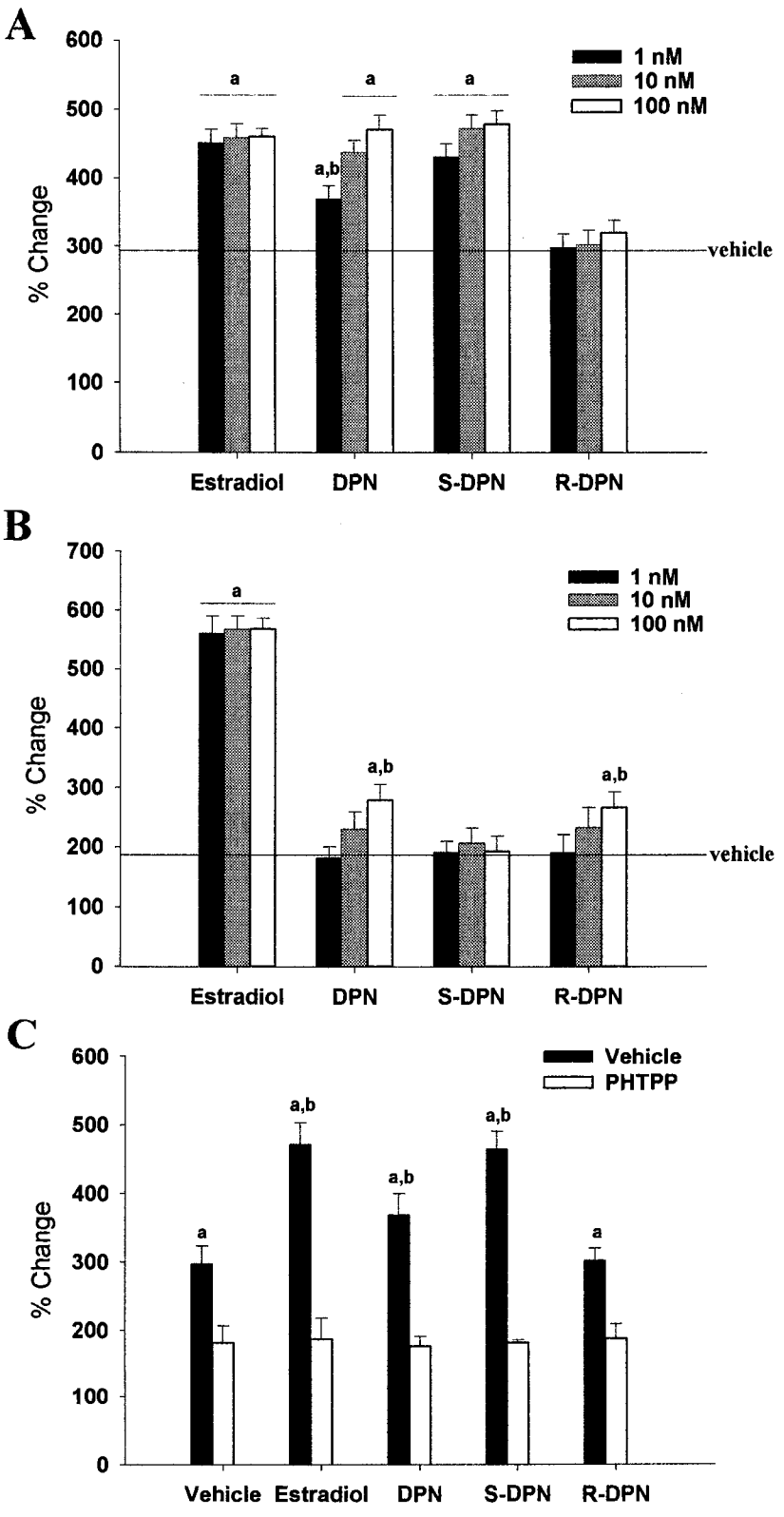

A
600
500
400
300
200
100
0
% Change
a
a
a
a,b
1 nM
10 nM
100 nM
vehicle
Estradiol
DPN
S-DPN
R-DPN
B
700
600
500
400
300
200
100
0
% Change
a
a,b
a,b
1 nM
10 nM
100 nM
vehicle
Estradiol
DPN
S-DPN
R-DPN
C
600
500
400
300
200
100
0
% Change
a
a,b
a,b
a,b
a
Vehicle
PHTPP
Vehicle
Estradiol
DPN
S-DPN
R-DPN

Figure 4.2. Effects of estrogen receptor ligands on ERE-luc promoter activity mediated by ERβ (*Panel A and C*) or ERα (*Panel B*). N-38 cells were cotransfected with an ERE-tk-luc reporter construct and an expression vector containing ERα or ERβ. Following transfection, cells were treated with vehicle (0.001% EtOH), estradiol, DPN, *S*-DPN, or *R*-DPN for 16 h. Data are represented as percent change in relative light units (RLU) from vehicle-treated empty vector controls (set at 100%). [a] indicates significant differences ($P < 0.05$) from vehicle-treated controls, [b] indicates significant differences ($P < 0.05$) within treatment groups. Solid horizontal lines indicate constitutive activity of receptor in absence of ligand. *Panel A.* Effect of 1nM, 10nM, and 100nM concentrations of ligands on ERE-luc promoter activity in cells cotransfected with ERβ. *Panel B.* Effect of 1nM, 10nM, and 100nM concentrations of ligands on ERE-luc promoter activity in cells co-transfected with ERα. *Panel C.* Effect of ligands (1nM) on ERE-luc promoter activity in cells co-transfected with ERβ in the presence or absence of the ERβ antagonist PHTPP (10μM).

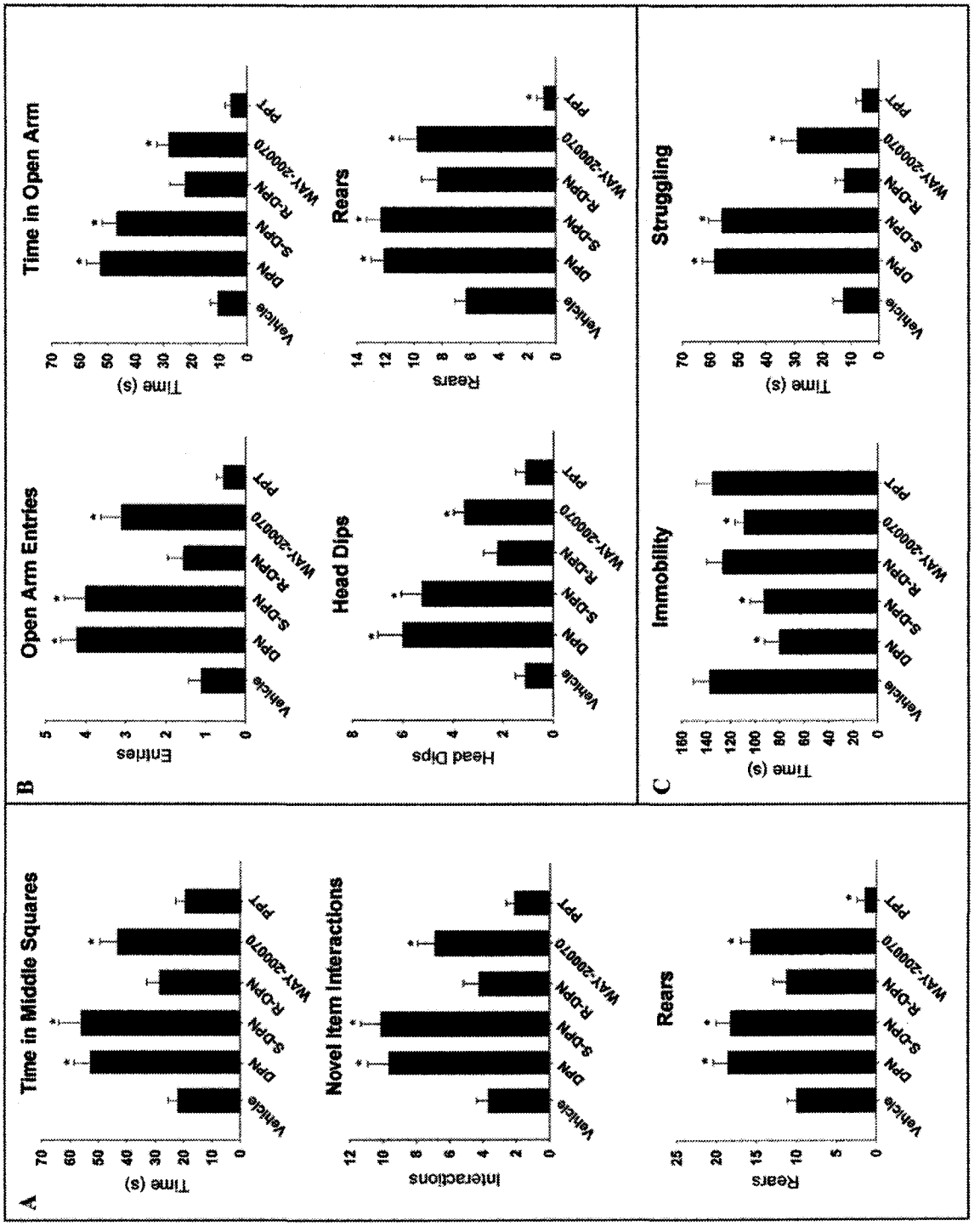

A
Time in Middle Squares
Time (s)
Novel Item Interactions
Interactions
Rears
B
Time in Open Arm
Time (s)
Rears
Open Arm Entries
Entries
Head Dips
C
Struggling
Time (s)
Immobility
Time (s)
Vehicle
DPN
S-DPN
R-DPN
WAY-200070
PPT

Figure 4.3. Effects of estrogen receptor ligands on anxiety-related behavior in the open field (OF) (*Panel A*) and elevated plus maze (EPM) (*Panels B*), and learned helplessness in the forced swim test (FST) (*Panel C*). Behaviors measured include: time spent in the middle squares, novel item interactions, and rearing in the OF; open arm entries, time spent in open arm, and rearing in the EPM; head dips, time spent immobile, and time spent struggling in the FST. Data are represented as mean ± SEM. n = 9 animals per treatment group. * indicates significant difference ($P < 0.05$) compared with vehicle treatment.

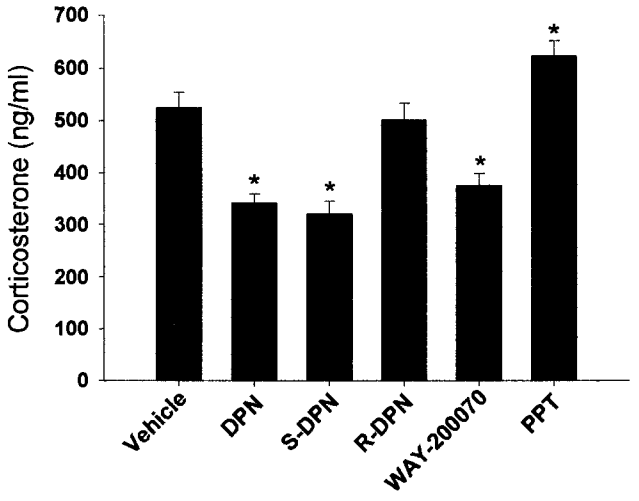


Figure 4.4. Effects of estrogen receptor ligands on plasma corticosterone following the forced swim test. Plasma samples were obtained 20 minutes after the start of the swim test. Data are represented as mean plasma corticosterone ± SEM. n = 6 animals per treatment group. * indicates significant difference ($P < 0.05$) compared with vehicle treatment.

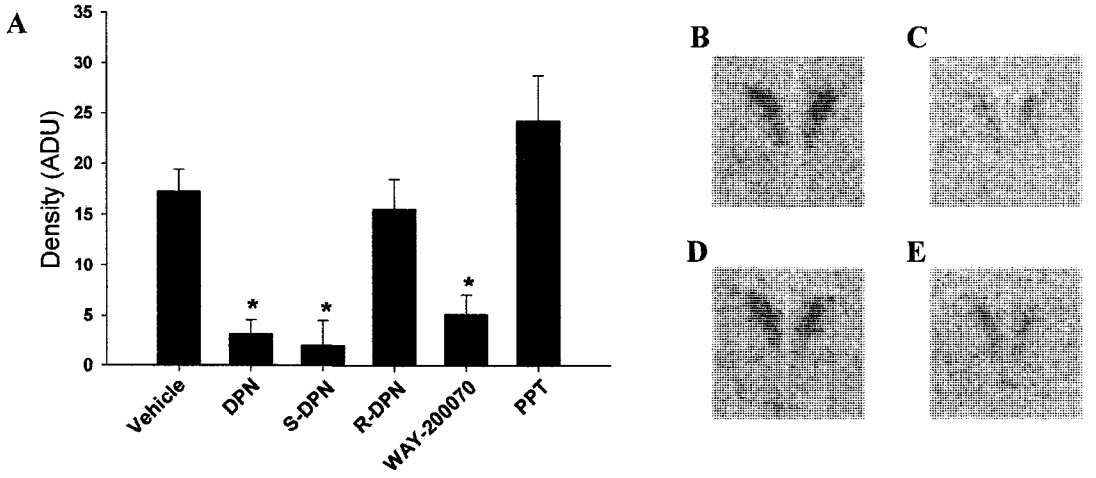


Figure 4.5. Effects of estrogen receptor beta ligands on paraventricular nucleus of the hypothalamus (PVN) *c-fos* mRNA levels following the forced swim test as measured by *in situ* hybridization. *Panel A.* Relative levels of *c-fos* mRNA expression. Data are represented as mean arbitrary density units (ADU) ± SEM. n = 6 animals per treatment group. * indicates significant difference ($P < 0.05$) compared with vehicle treatment. *Panel B-E.* Representative film autoradiograms for vehicle (*Panel B*), *S*-DPN (*Panel C*), *R*-DPN (*Panel D*), and WAY-200070 (*Panel E*) treated animals.

Discussion

Since the discovery and characterization of diarylpropionitrile (DPN) (Meyers, Sun et al. 2001) as a potent and highly selective estrogen receptor beta (ERβ) agonist, it has been utilized in numerous *in vitro* and *in vivo* studies aimed at determining ERβ's biological function. DPN is a chiral molecule and thus is synthesized as two enantiomeric forms, *S*-DPN and *R*-DPN, which may have different binding and transactivational properties. Molecular modeling predicts that *S*-DPN associates with the ligand binding pocket of ERβ in a much more favorable fashion as compared to *R*-DPN (Sun, Baudry et al. 2003). The proximal phenol ring (β-ring) of *S*-DPN mimics the A-ring of E2, and it likely engages in hydrogen-bonding with the same residues as E2 (Glu-305, Arg-346, and His-475). Furthermore, the positioning of the nitrile group in *S*-DPN is more favorable for engaging in stabilizing interactions with the sulfur of Met-336, and *S*-DPN may be able to engage in H-bonding with Thr-299 that cannot occur with *R*-DPN.

In order to better interpret results of studies utilizing the racemic mixture of DPN, we sought to determine the biological profile for each enantiomer, and determine whether one form was the preferred ERβ agonist. The results described here indicate that the *S*- enantiomer of DPN has a higher affinity for ERβ than the *R*- enantiomer, and is solely responsible for DPN's ERβ-dependent transcriptional activation of an estrogen responsive reporter gene *in vitro*. Furthermore, *S*-DPN, when administered to animals, is anxiolytic and anti-depressive in behavior paradigms designed to test anxiety- and depressive-like behaviors. *R*-DPN has no effect in these behavioral models. Therefore,

S-DPN is the pharmacologically active enantiomer of DPN, and it's use for future studies investigating ERβ biology should be considered.

S-DPN is a high affinity ERβ agonist

In competitive binding assays using recombinant receptor, *S*-DPN had a 6.7-fold higher affinity for ERβ compared to *R*-DPN. Like the endogenous ligand for ERβ, 17β-estradiol (E2), *S*-DPN has a sub-nanomolar affinity for ERβ. Additionally, it has greater affinity for ERα compared to *R*-DPN, but it is over 200-fold less potent at ERα than E2, and it retains similar selectivity for ERβ as the racemic mixture (80-fold selective). It is important to note that binding to a receptor does not necessarily lead to functional activation of the receptor and thus transcriptional activity was tested in further studies. DPN binds ERα with sub-micromolar affinity, and it can cross the blood-brain-barrier, yet when peripherally administered (1mg/kg, s.c.) it does not activate ERα in the brain (Lund, Rovis et al. 2005). These binding results do not directly conform to the modeling predictions that *R*-DPN has a lower energy state conformation (stronger affiliation) with ERα than ERβ. However, we do find that *S*-DPN is a more potent ERβ ligand as predicted by the modeling.

S-DPN is a potent activator of ERβ-dependent transcription at an ERE

To determine whether the binding profiles correlated to functional activity, we compared the ability of the two enantiomers to stimulate transcription through an ERE-luciferase promoter reporter construct. Rather than use a neuronal cell type that does

not normally express ERs, we chose the immortalized cell line N-38 that expresses both ER subtypes (Titolo, Cai et al. 2006). Since these cells are derived from the mouse hypothalamus and express several neuropeptides like oxytocin and neuropeptide Y, they provide a excellent model to study neuroendocrine systems *in vitro* (Belsham, Cai et al. 2004). Additionally, they most likely have all the transcriptional machinery present to respond genomically to hormonal stimulation, whether it is through ER or another hormone receptor. Interestingly, when transfected with an ERE-luciferase reporter gene and treated with 100nM E2 or 100nM DPN, there is no stimulation seen above the empty vector control. One possible explanation for this lack of response may be that the estrogen receptors expressed are non-functional, or somehow inhibited by other proteins and/or endogenous factors. However, when either ERα or ERβ are coexpressed with the reporter gene there are significant ligand-independent and ligand-dependent increases in luciferase activity seen in these cells. Therefore, we were able to separately determine the ERα- and ERβ-dependent transcriptional activities for both enantiomers in a neuroendocrine cell model.

Our results confirm results of Pak et al (2005), that when ERβ is cotransfected with an ERE-luciferase reporter gene there is a significant ligand-independent effect of ERβ (~300%) versus empty vector controls (Pak, Chung et al. 2005). *S*-DPN further stimulates luciferase activity above the constitutive level for ERβ and to a level statistically identical to E2 at all doses tested (1nM, 10nM, 100nM). At the 1nM dose, *S*-DPN significantly increased promoter activity to a level greater than racemic DPN. By contrast, *R*-DPN did not stimulate promoter activity above the constitutive level at any

dose tested. Therefore, *S*-DPN appears to be similar to estradiol at inducing ERβ induced transcription mediated by an ERE promoter site, whereas *R*-DPN is ineffective.

When ERα is cotransfected with an ERE-luciferase promoter reporter construct we also found a significant ligand-independent effect of ERα (~185%) versus empty vector controls, albeit not to the levels seen with ERβ. S-DPN was not effective at stimulating ERα mediated transcription at any dose, thus demonstrating its selectivity for ERβ. Interestingly, we found that *R*-DPN did increase luciferase expression at the 100nM dose, as did the racemic mixture. Racemic DPN has been previously shown to induce ERα-dependent transcription through an ERE promoter at higher doses (Meyers, Sun et al. 2001). Thus, our data demonstrate that it is the *R*- enantiomer that provides loss of selective activity to the racemic compound.

Because N-38 cells also have been reported to contain ERα and ERβ (Titolo, Cai et al. 2006), albeit they appear to be non-functional in our hands, we further confirmed the ERβ specificity of *S*-DPN, by employing a selective ERβ antagonist, PHTPP, which has been shown to fully antagonize the effect of E2 on ERβ initiated transcription through an ERE promoter site. PHTPP completely blocked the effect of E2, *S*-DPN, and racemic DPN, suggesting that the induced transcriptional activity is indeed ERβ dependent. Interestingly, PHTPP did not block the ligand-independent effect of ERβ. This is in contrast to the non-selective ER antagonist 4-hydroxy tamoxifen, which has been shown to fully inhibit the constitutive effect of ERβ in HT-22 cells (Pak, Chung et al. 2005). It is possible that ERβ undergoes a conformational change upon binding PHTPP that still allows it to engage DNA with a low-level constitutive activity whereas tamoxifen cannot.

In this state the ligand-receptor complex may not be able to bind DPN or E2, or it blocks the ability of agonists to alter the conformation of the receptor to one that is more transcriptionally active.

S-DPN treatment decreases anxiety-type behaviors and learned helplessness

Previous work from our lab has shown that peripherally administered racemic DPN can cross the blood-brain-barrier, bind ER, and decrease anxiety and fear in both female and male adult rats in the elevated plus maze and open field test (Lund, Rovis et al. 2005). These behavioral paradigms examine the natural conflict of exploration of a novel environment versus avoidance of a brightly lit and exposed environment (Handley and McBlane 1993). The anxiolytic effect of DPN seen in these tests is abolished by co-treatment with the ER antagonist tamoxifen, indicating an ERβ-dependent mechanism. Other studies have also indicated an anti-depressant effect of DPN in the forced swim test (Walf, Rhodes et al. 2004; Walf and Frye 2007), and that this effect is lost on βERKO animals (Walf, Koonce et al. 2008). The forced swim test provides a measure of despair as measured by the propensity to remain immobile, which can be decreased with anti-depressant treatment (Porsolt, Le Pichon et al. 1977).

In this study we confirmed the findings that racemic DPN exhibits anxiolytic and anti-depressive activity. We now show that *S*-DPN is the enantiomer solely responsible for the behavioral effects seen with DPN. Furthermore, treatment with the selective ERβ agonist WAY-200070 mimicked the effects seen with DPN and *S*-DPN. WAY-200070 has a RBA of 133 and is 68-fold selective for ERβ (Malamas, Manas et al. 2004). Recent

studies have shown WAY-200070 to be anxiolytic in the four-plate test, and anti-depressant in the tail suspension test when peripherally administered to male mice (Hughes, Liu et al. 2008). Therefore, the similarities seen across the three behavioral paradigms (open field, elevated plus maze, and forced swim test), and amongst the different ERβ agonists, all point to an ERβ dependent mechanism.

S-DPN treatment attenuates the stress response

Estradiol has been shown to potentiate CORT secretion following a stressor whether administered peripherally or locally within the hypothalamus (Lund, Munson et al. 2004; Lund, Hinds et al. 2006). This appears to be largely due to the activation of ERα, as PPT treatment mimics this effect. Peripheral and central administration of the ERβ agonist DPN has an opposing, inhibitory effect on stress-induced CORT secretion. Furthermore, these same effects on CORT secretion are observed following the elevated plus maze (Lund, Rovis et al. 2005). In agreement with these previous studies, our results show that DPN decreased plasma CORT levels following the forced swim test. Further, *S*-DPN seems to be the enantiomer responsible for reducing stress induced activation of the HPA axis since S-DPN inhibited CORT secretion, whereas *R*-DPN treatment had no effect. Selectivity for ERβ in this test was further demonstrated by the administration of another ERβ agonist, WAY-200070, which mimicked the effect seen with DPN and *S*-DPN further confirming an ERβ dependent mode of action. In all of these studies the effect of WAY 200070 was less than that of S-DPN. This is likely due to the lower affinity of WAY 200070 for ERβ as compared to S-DPN.

S-DPN acts to inhibit activation of PVN neurons

The expression of the immediate early gene *c-fos* is a well-described marker for neuronal activation. Estradiol has been shown to increase the expression of *c-fos* mRNA in the paraventricular nucleus of the hypothalamus following a stressor (Lund, Munson et al. 2004). This increase appears to be due to activation of ERα, as PPT mimics this effect, whereas DPN decreases stress-induced *c-fos* mRNA in the PVN (Lund, Hinds et al. 2006). Our results are in accordance with these studies as PPT increased, while DPN decreased PVN *c-fos* expression 20 minutes after the start of the forced swim test. Additionally, the effect of DPN is due to the *S*-enantiomer as *S*-DPN treated animals had significantly less, whereas *R*-DPN treated animals had similar levels of *c-fos* mRNA in the PVN as compared to controls. The ERβ agonist WAY-20070 mimicked DPN and *S*-DPN, supporting an ERβ-specific mechanism. Whether this is a direct or indirect effect of ERβ remains to be determined. There are populations, albeit small, of ERβ-containing CRH neurons in the medial parvocellular division of the PVN (Laflamme, Nappi et al. 1998; Suzuki and Handa 2005), providing a potential direct mode of action. However, ERβ is also expressed in other cell types in the PVN and in brain regions like the amygdala, hippocampus, dorsal raphe nucleus, suprachiasmatic nucleus, and the bed nucleus of the stria terminalis that send glutamatergic and GABAergic projections to the PVN, providing potential indirect modes of action (Shughrue, Lane et al. 1997; Shughrue and Merchenthaler 2001; Herman, Tasker et al. 2002).

In summary, the ERβ agonist diarylpropionitrile exists as two enantiomers with different biological activities. *S*-DPN provides the ERβ potency and selectivity in both receptor binding and transcriptional activation. Furthermore, *S*-DPN is the active enantiomer in ERβ-dependent effects seen in anxiety and learned helplessness behavioral paradigms. Currently, DPN is the standard ERβ agonist of choice for *in vivo* and *in vitro* studies. Our results indicate that an enantiomeric-pure preparation should be considered in future studies utilizing DPN.

CHAPTER FIVE

Activation of Estrogen receptor beta prevents the anxiogenic actions of glucocorticoid receptor agonist delivery to the central nucleus of the amygdala

Abstract

Anxiety and depression have tremendous and often immeasurable impacts on society and the economy. Often associated with these disorders is a chronically elevated level of endogenous glucocorticoids. The central nucleus of the amygdala (CeA) is a glucocorticoid responsive brain region that coordinates the behavioral, physiological, and neuroendocrine responses to threatening stimuli. Chronically high glucocorticoid levels may act upon the CeA to alter these adaptive responses into maladaptive and detrimental reactions. The underlying sex difference in depression and anxiety may be in part due to a higher propensity for these maladaptive reactions in women than in men. In these experiments we examined the effect of direct administration of a glucocorticoid receptor (GR) agonist to the CeA on anxiety behaviors and hypothalamic-pituitary-adrenal axis output. Furthermore, we determined whether peripheral administration of an ERβ agonist known to be anxiolytic could alter the effect of GR activation in the CeA. Young adult female Sprauge-Dawley rats were ovariectomized and bilaterally implanted via stereotax with a beeswax pellet containing the GR agonist RU28362 to a region just dorsal to the CeA. Controls received a blank pellet. Four days following implantation animals were administered the ERβ agonist *S*-DPN or vehicle

subcutaneously daily for four days. On the fourth day of treatment animals were examined for anxiety-type behaviors on the elevated plus maze (EPM). Vehicle treated animals with RU28362 implants displayed significantly higher anxiety-type behaviors in the EPM and plasma CORT than vehicle treated controls. *S*-DPN treated animals regardless of implant displayed significantly lower anxiety-type behaviors and plasma CORT than vehicle treated controls or vehicle treated animals implanted with RU28362. These results indicate that delivery of a GR agonist to the CeA is anxiogenic, and peripheral administration of an ERβ agonist can block this effect. These data suggest a role for estradiol signaling via ERβ in modulating glucocorticoid-dependent effects of the CeA on behavior and neuroendocrine function.

Introduction

Anxiety has an overwhelming and immense impact on society both economically and socially. Approximately 25% of adults will suffer from one or more forms of anxiety disorder in their lifetime, and the yearly economic cost is estimated to be at least $40 billion per year (Gordon and Hen 2004). Anxiety disorders are more prevalent in females than males (Bekker and van Mens-Verhulst 2007), suggesting a potential role for sex hormones in the etiology of anxiety. Furthermore, patients with anxiety disorder exhibit impaired hypothalamic-pituitary-adrenal (HPA) axis function (Ehlert, Gaab et al. 2001; McEwen 2005). The amygdala mediates anxiety in humans and rodents (Chapman, Schroeder et al. 1954; Kopchia, Altman et al. 1992; Davis 1997), potentiates HPA axis activity (Prewitt and Herman 1994; Bhatnagar and Dallman 1998), and contains androgen and estrogen receptors (Gray, Piechowski et al. 1993; Laflamme, Nappi et al.

1998; Shughrue and Merchenthaler 2001). Therefore the amygdala may be an important modulator of sex-specific effects seen in anxiety disorders.

The amygdala coordinates behavioral, autonomic, and endocrine responses to threats or danger to an animal (Davis 1992; Davis 1997; LeDoux 1998). The central nucleus of the amygdala (CeA) in particular, controls major efferent pathways from the amygdalar complex (for review see (Sah, Faber et al. 2003)). The CeA has decending projections to regions involved in autonomic, endocrine, and behavioral responses to emotional stimuli (Veening, Swanson et al. 1984; Gray, Carney et al. 1989; Marcilhac and Siaud 1997). Injection of the GABA receptor agonist muscimol into the CeA of rats decreases anxiety-type behaviors in the elevated plus maze, suggesting a facilitative role for the CeA in anxiety behaviors (Moreira, Masson et al. 2007). Accordingly, CeA lesions lead to decreased anxiety and decreased neuroendocrine responses to a spectrum of stressors (Beaulieu, Di Paolo et al. 1986; Kopchia, Altman et al. 1992; Feldman, Conforti et al. 1994).

The CeA is a major extrahypothalamic site of corticotropin-releasing hormone (CRH), and CRH signaling is implicated in fear and anxiety (Heilig, Koob et al. 1994; Heinrichs, Menzaghi et al. 1995). Therefore, the CeA may modulate anxiety-type behaviors by altering CRH signaling in the central nervous system (CNS). The CRH neurons of the CeA send their axons the bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN), lateral hypothalamus, midbrain central gray, parabrachial region, raphe nuclei, and nucleus of the solitary tract (Gray, Piechowski et al. 1993). The direct and indirect CRH-containing projections to the medial parvocellular PVN are likely

responsible for the stimulatory effect of the CeA on HPA axis activity (Gray, Carney et al. 1989; Marcilhac and Siaud 1997). For example, lesions of the CeA inhibit the corticosterone (CORT) response to a wide variety of stressors (Allen and Allen 1974; Beaulieu, Di Paolo et al. 1986; Beaulieu, Pelletier et al. 1989; Van de Kar, Piechowski et al. 1991; Feldman, Conforti et al. 1994; Prewitt and Herman 1994).

Anxiety disorder is often accompanied with HPA axis dysfunction that results in chronically elevated glucocorticoids. Glucocorticoids may act through high affinity mineralocorticoid receptors (MR) or low affinity glucocorticoid receptors (GR) (reul de kloet 85). Basal state levels of glucocorticoids occupy primarily MR, whereas elevated glucocortiocoid levels result in MR and GR occupation. The CeA has a robust expression of GR, and accordingly can respond to acute or chronically elevated CORT (Reul and de Kloet 1985; Morimoto, Morita et al. 1996). Indeed, elevated CORT causes an increase in CRH mRNA, whereas adrenalectomy decreases CRH mRNA within the CeA (Swanson and Simmons 1989; Makino, Gold et al. 1994; Watts and Sanchez-Watts 1995; Palkovits, Young et al. 1998; Viau, Soriano et al. 2001). Furthermore, direct administration of a corticosterone pellet just dorsal to the CeA increases CRH mRNA in the CeA (Shepard, Barron et al. 2000). These studies also show that CORT delivery to the CeA causes increased anxiety-type behaviors, AVP mRNA with in the PVN, and plasma CORT following exposure to the elevated plus maze (Shepard, Barron et al. 2000; Shepard, Barron et al. 2003; Myers, Gibson et al. 2005). Taken together, the CeA likely plays an integral role in responding to acutely or chronically elevated glucocorticoids and in the subsequent precipitation of the maladaptive behaviors associated with anxiety.

The sex hormone estrogen has also been shown to augment HPA axis activity and alter anxiety-type behaviors (Burgess and Handa 1992; Handa, Burgess et al. 1994; Lund, Munson et al. 2004; Lund, Rovis et al. 2005). Estrogen can act via two receptors, estrogen receptor beta (ERβ) and estrogen receptor alpha (ERα) (Green, Walter et al. 1986; Kuiper, Enmark et al. 1996). Treatment of ovariectomized female rats with the ERβ agonist diarylpropionitrile (DPN) results in decreased anxiety-type behaviors and plasma CORT following exposure to the elevated plus maze (Lund, Rovis et al. 2005). The CeA is integral in the behavioral responses of fear and anxiety and responsiveness of the HPA axis, and expresses ERβ (Laflamme, Nappi et al. 1998; Osterlund, Kuiper et al. 1998; Shughrue and Merchenthaler 2001). Therefore, in these studies we sought to determine if local administration of a GR agonist to the CeA induces anxiety-type behaviors on the elevated plus maze, and whether peripheral administration of an ERβ agonist could alter this effect.

Materials and Methods

Animals

Young adult female Sprague Dawley rats (~225g) were obtained from Harlan Laboratories (San Diego, CA), housed individually, and maintained on a 12h:12h light schedule (lights on at 0600h) in temperature and humidity controlled rooms at the Laboratory Animal Research Facility at Colorado State University. Animals had *ad libitum* access to a soy free diet (modified AIN-93G, DYET#101591 from DYETS, Inc., Allentown, PA, with corn oil substituted for soy oil). One week after arrival, animals were ovariectomized under isofluorane anesthesia. Following ovariectomy, animals

were handled daily (5 minutes each animal) by the same experimenter. All animal studies were previously approved by the Animal Care and Use Committee at Colorado State University.

Stereotaxic implantation

One week following ovariectomy animals were fitted bilaterally with two 22 gauge stainless-steel cannulae (Small Parts, Miami Lakes, FL) with the aid of a small animal stereotaxic instrument (David Kopf Instruments, Kujunga, CA). The tips of the cannule were previously packed with the glucocorticoid receptor agoinist RU28362 (Roussel-Uclaf, Romainville, France) dissolved into warmed beeswax to a final concentration of 0.5 uM, and packed to a height of 0.5 mm within the end of the cannulas. Controls received cannulae packed with wax alone. Stereotaxic coordinates to allow placement of the cannula tip to the region just above the central nucleus of the amygdala were: 2.3 mm posterior and 3.8 mm lateral to bregma, and 6.3 mm below the skull surface. A 28 gauge stainless steel wire cut to extend 1.0 mm past the length of the cannulas was inserted into the cannulae and the pellet expelled.

Hormone / Drug treatments

Beginning four days following stereotaxic implant, animals were given a single daily subcutaneous injection of either hydroxypropyl betacyclodextran (vehicle; 27% w/w in saline; CTD Inc., High Springs, FL), or *S*-DPN (1.0 mg/kg) in a total volume of 0.2 ml. Injections occurred at 0800 h for 4 days. *S*-DPN was synthesized *de* novo as previously

described (Lund et al., 2005, Weiser et al. unpublished). Three hours after the daily treatment injection on day 4, animals underwent behavioral testing on the elevated plus maze (EPM), a paradigm established as an indicator of anxiety.

Elevated plus maze

Maze performance was evaluated on the fourth day of treatment (seventh day post-implantation) and was run as previously described (Handley and McBlane 1993). Behaviors measured included number of entries into open and closed arms, total time spent in open and closed arms, rearing, head dips, total time spent grooming, and the number of fecal boli.

Plasma corticosterone analysis

20 min following the EPM, animals were rapidly decapitated and trunk blood collected into ice-chilled tubes containing 0.5M EDTA and aprotinin (4 mg/ml, Sigma, St. Louis, MO). Plasma levels of corticosterone were determined by radioimmunoassay as previously described (Lund, Rovis et al. 2005). Intra-assay and Inter-assay coefficient of variance (COV) were 3.9% and 5.1% respectively.

Immunocytochemistry

To examine extent of RU28362 spread from implanted pellets, two animals from each group were intracardially perfused with 4% buffered paraformaldehyde. Brains were postfixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, sectioned at

35uM on a Leitz cryostat, and stored in cryopreservative at -20 C until processed for immunocytochemistry. After standard washes, the free floating sections were incubated in 1.5% NGS in 0.01 M PBS with 0.03% TX to block nonspecific binding, then incubated for 24 hours at 4 C with the GR antibody (PA-511, Affinity Bioreagents, 1:400) in 0.01 M PBS with TX in the presence of 1.5% NGS. Next, the tissue was washed three times for 10 minutes each in PBS with TX and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA; 1:400) in PBS with TX in the presence of 1.5% NGS for 2 hours at room temperature. Sections are subsequently washed and processed according to the avidin-biotin-peroxidase procedure (Vector Laboratories; 1:400). After standard washes, the tissue was rinsed in 0.1 M Tris-buffered saline (TBS, pH 8.0) for 15 minutes and then developed with 3,3'-diaminobenzidine (DAB; 0.5 mg/ml; Sigma, St. Louis, MO) in 0.1 M Tris-buffered saline) and 0.01% hydrogen peroxide in 0.1M TBS for 3–5 minutes to produce a brown reaction product. The reaction is stopped by several washes in 0.01 M PBS. Control sections, where primary antibody was omitted, were run in parallel. Complete loss of staining for the corresponding antigen was absent in these sections.

Statistics

Analysis of variance was performed on data from each experiment using the Statview data analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). Fisher's PLSD analysis was used post hoc where appropriate. Differences were considered significant

when $p < 0.05$. Data are expressed as group means ± SEM. Transfection data are expressed as percent change compared with vehicle-treated, empty vector controls.

Results

Bilateral implantation of the GR agonist RU28362 in beeswax via stereotax results in nuclear localization of GR within the central nucleus of the amygdala

GR immunoreactivity (GR-IR) was examined seven days following bilateral implantation of the GR agonist RU28362 via stereotax to a region dorsal to the CeA (Figure 5.1). RU28362 implants resulted in nuclear GR-IR within the CeA (dark brown staining within cell body) (Figure 5.2). Blank implants resulted in largely cytoplasmic GR-IR within the CeA (light brown staining in cytoplasm) (Figure 5.2). Nuclear GR-IR was primarily contained to the area of the CeA, and observed no further than 1.0mm from pellet.

The GR agonist RU28362 administered to the central nucleus of the amygdala decreases anxiolytic behavior

We examined whether GR activation in the CeA alters behavior on the elevated plus maze (EPM). Animals were bilaterally implanted with beeswax pellets containing the GR agonist RU28362 to an area just dorsal to the CeA (Figure 5.1). Seven days following implantation the animals were examined on the EPM for anxiety-type behaviors. Administration of RU28362 to the CeA caused a significant ($P < 0.05$) decrease in the percentage of open arm entries (Figure 5.3A), percentage open arm

time (Figure 5.3B), and head dips (Figure 5.3C) as compared to animals with blank (beeswax) implants.

Peripheral administration of the ERβ agonist S-DPN increases anxiolytic behavior and blocks the anxiogenic effect of the GR agonist RU28362 administered to the central nucleus of the amygdala

We determined whether administration of an ERβ agonist alters the effect of GR activation in the CeA on behavior in the EPM. Animals were bilaterally implanted with beeswax pellets containing the GR agonist RU28362 to an area just dorsal to the CeA (Figure 5.1). Four days following implantation the animals were administered *S*-DPN, a selective ERβ agonist, or vehicle daily and examined on the EPM for anxiety-type behaviors seven days post-implantation. *S*-DPN treatment significantly ($P < 0.05$) increased the percentage of open arm entries (Figure 5.3A), percentage open arm time (Figure 5.3B), and head dips (Figure 5.3C) as compared to animals with vehicle treatment regardless of implant type. Peripheral administration of *S*-DPN effectively blocked the decrease in anxiolytic behaviors seen with RU28362 implanted animals given vehicle injections.

The GR agonist RU28362 administered to the central nucleus of the amygdala increases plasma corticosterone, an effect blocked by peripheral administration of the ERβ agonist S-DPN

To examine the corresponding hormonal response to the EPM, plasma corticosterone (CORT) was measured in plasma obtained from trunk blood collected 20 minutes after exposure to the elevated plus maze. Animals with RU28362 implants just dorsal to the CeA had significantly ($P < 0.05$) higher plasma CORT following the EPM than animals with blank implants (Figure 5.4). Furthermore, peripheral *S*-DPN treatment significantly ($P < 0.05$) decreased plasma CORT following the EPM regardless of implant type. Therefore, peripheral administration of *S*-DPN effectively blocked the increase in plasma CORT seen with RU28362 implanted animals given vehicle injections.

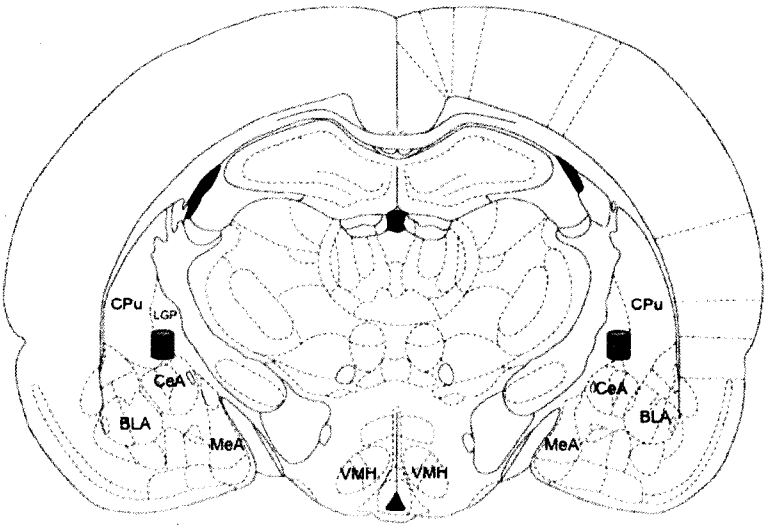


Figure 5.1. Representative sketch of bilateral pellet implant location. Illustration adapted from Paxinos and Watson (Paxinos and Watson 1998). BLA, basolateral amygdala; CeA, central nucleus of the amygdala; CPu, caudate putamen; LGP, lateral globus pallidus; VMH, ventral medial hypothalamus.

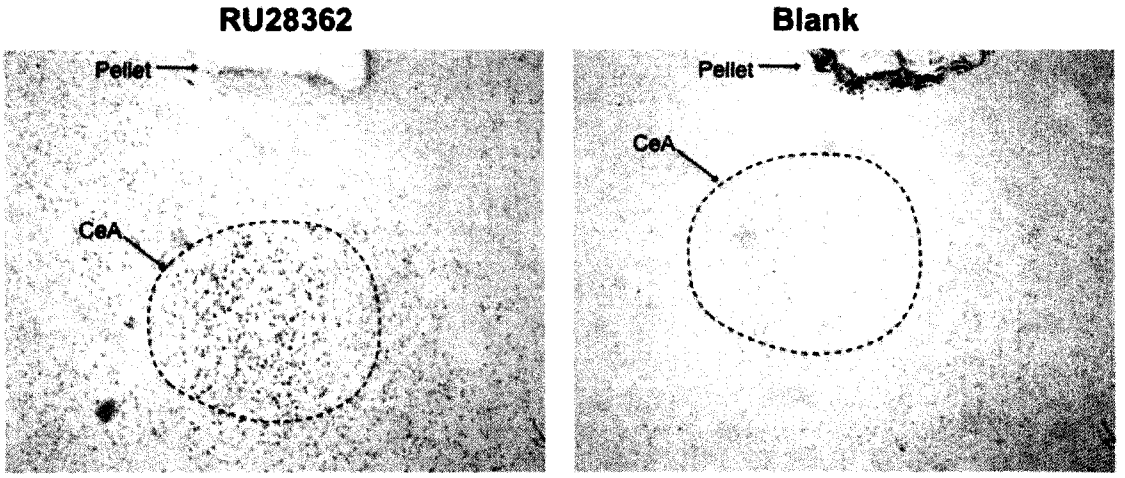
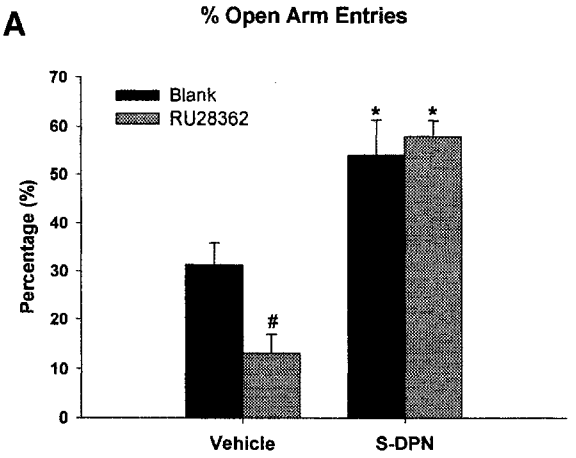
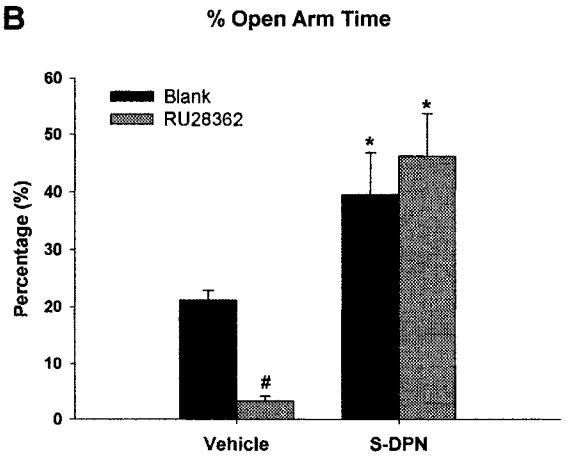


Figure 5.2. Glucocorticoid receptor immunoreactivity (GR-IR) in the central nucleus of the amygdala (CeA). Bilateral implants of the GR agonist RU28362 to a region dorsal to the CeA resulted in nuclear GR-IR within the CeA (dark brown staining within cell body). Blank implants resulted in largely cytoplasmic GR-IR within the CeA (light brown staining in cytoplasm). Nuclear GR-IR was observed no further than 1.0mm from pellet.

A


% Open Arm Entries
Blank
RU28362
Percentage (%)
70
60
50
40
30
20
10
0
*
*
#
Vehicle
S-DPN

B


% Open Arm Time
Blank
RU28362
Percentage (%)
60
50
40
30
20
10
0
*
*
#
Vehicle
S-DPN

C

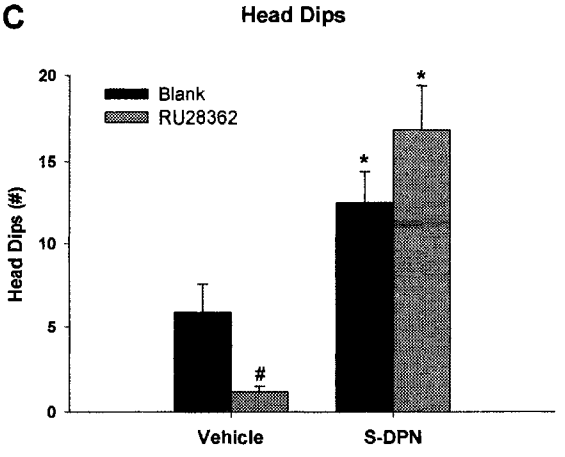

Head Dips
Blank
RU28362
Head Dips (#)
20
15
10
5
0
*
*
#
Vehicle
S-DPN

Figure 5.3. The GR agonist RU28362 delivered bilaterally to the central nucleus of the amygdala (CeA) decreases anxiolytic behaviors on the elevated plus maze (#). Peripheral treatment with the ERβ agonist *S*-DPN increases anxiolytic behaviors and blocks the anxiogenic effect of RU28362 administered to the CeA (*). *Panel A*: Open arm entries expressed as percentage of total arm entries (closed + open). *Panel B*: Open arm time expressed as percentage of total time on maze. *Panel C*: Number of head dips over edge of open arm. Data are represented as mean ± SEM; n = 7-8 animals per treatment group. *, # indicates significant difference ($P < 0.05$) compared with animals implanted with blank pellets and vehicle treated (controls).

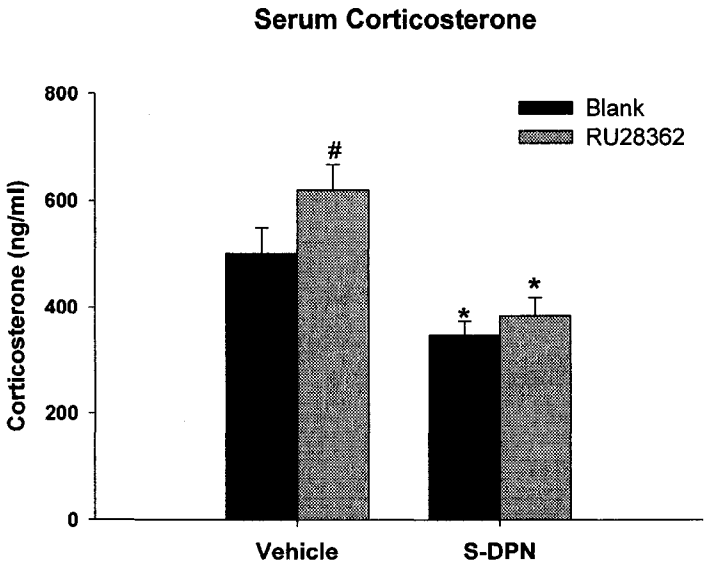


Figure 5.4. The GR agonist RU28362 delivered bilaterally to the central nucleus of the amygdala (CeA) increases plasma corticosterone 20 minutes following the elevated plus maze (#). Peripheral treatment with the ERβ agonist *S*-DPN decreases plasma corticosterone following the elevated plus maze and blocks the augmentation of plasma corticosterone seen with RU28362 administration to the CeA (*). Data are represented as mean ± SEM; n = 7-8 animals per treatment group. *, # indicates significant difference ($P < 0.05$) compared with animals implanted with blank pellets and vehicle treated (controls).

Discussion

The CeA is well poised to integrate glucocorticoid and sex hormone signaling during time of perceived or actual danger. The presence of glucocorticoid receptors, androgen receptors, and estrogen receptors enable the CeA to respond to fearful situations in a manner dependent upon the internal hormone milieu (Reul and de Kloet 1985; Bingaman, Baeckman et al. 1994; Morimoto, Morita et al. 1996; Laflamme, Nappi et al. 1998; Osterlund, Kuiper et al. 1998; Shughrue and Merchenthaler 2001). The results of this study indicate that activation of GR in the CeA results in increased anxiety and CORT secretion. Furthermore, peripheral treatment of an ERβ agonist can decrease anxiety and CORT secretion despite GR activation in the CeA.

The CeA contains the highest density of GR expression in the amygdala, and is responsive to elevated levels of glucocortiocoids (Swanson and Simmons 1989; Makino, Gold et al. 1994; Watts and Sanchez-Watts 1995; Morimoto, Morita et al. 1996). Therefore, we delivered the GR agonist RU28362 bilaterally to an area dorsal to the CeA via stereotaxic implantation. This results in chronic activation of GR within the CeA independent of exogenous CORT levels. The placement of the pellet insures sustained GR occupancy without mechanical damage to the CeA. Upon binding ligand, GR translocates to the nucleus and therefore GR immunoreactivity (GR-IR) provides for a good marker of agonist spread. We observed nuclear GR-IR within a radius of 1.0mm from the RU28362 pellet, which included the CeA. Predominantly cytoplasmic GR-IR was observed around blank pellets. Previous studies where CORT pellets (30 μg) are placed in this same region result in a CORT diffusion radius of 0.5 to 1.0 mm, which

includes the CeA, but excludes other glucocorticoid sensitive regions such as the BNST, PVN, and hippocampus (Shepard, Barron et al. 2003).

Our results indicate that administration of a GR agonist to the CeA results in increased anxiety-type behaviors in the elevated plus maze (EPM) and plasma CORT following maze exposure. Generally, treatments that reduce open arm exploration (time spent on open arm and open arm entries) are considered anxiogenic (Pellow, Chopin et al. 1985; Handley and McBlane 1993). These results implicate GR activation in the CeA in the generation of anxiety-type behaviors in the rat. This is in accordance with previous studies where delivery of the mixed GR and MR agonist CORT to the CeA is shown to be anxiogenic and augment CeA CRH mRNA expression and plasma CORT secretion (Shepard, Barron et al. 2000; Shepard, Barron et al. 2003; Myers, Gibson et al. 2005). Furthermore, this effect is blocked via peripheral administration of a CRHR1 antagonist, suggesting that the anxiogenic effect of CORT delivered to the CeA is mediated through CRH signaling (Myers, Gibson et al. 2005). Indeed, elevated glucocorticoid levels increase CRH levels in the CeA, and this in turn may result in increased CRH transmission from the CeA to other brain regions integral in the behavioral and endocrine responses to stressors. The CeA does have direct stimulatory projections to the medial parvocellular region of the PVN (Gray, Carney et al. 1989; Marcilhac and Siaud 1997). This region contains the CRH and AVP neurons that comprise the main effector arm of the HPA axis. Alternatively, the CeA could access the PVN though an indirect pathway involving the lateral BNST. The CeA sends CRH-positive axons to the lateral BNST, which in turn has direct stimulatory projections to the medial

parvocellular PVN (Gray 1993; Herman, Cullinan et al. 1994; Herman, Figueiredo et al. 2003). Correspondingly, stimulation of the lateral BNST increases CRH mRNA expression in the medial parvocellular PVN and plasma CORT levels (Dunn 1987; Herman, Cullinan et al. 1994). Interestingly, the hippocampus also accesses the PVN via an indirect path through the BNST (Herman, Dolgas et al. 1998). In this regard, the CeA may counteract the inhibitory influence of the hippocampus on HPA axis output, especially during times of chronically elevated glucocorticoids.

Gonadal steroids play an integral role in modulating mood and HPA axis function (Handa, Burgess et al. 1994; Fink, Sumner et al. 1998; McCormick, Linkroum et al. 2002; Shors and Leuner 2003; Weiser, Foradori et al. 2008). Estrogen in particular has been reported to have opposing effects on both mood and HPA axis activity. This could be explained, in part, by the existence of two main receptors for estrogen, ERα and ERβ (Green, Walter et al. 1986; Kuiper, Enmark et al. 1996). Animal studies have implicated estrogen signaling via ERα in generating anxiety-type behaviors and in augmentation of HPA axis activity (Lund, Rovis et al. 2005). Alternatively, studies have shown an opposite role for estrogen signaling though ERβ (Krezel, Dupont et al. 2001; Imwalle, Gustafsson et al. 2005; Lund, Rovis et al. 2005; Walf and Frye 2006). Specifically, treatment of OVX females with the ERβ agonist *S*-DPN decreases anxiety-type behaviors in the EPM, despair in the forced swim test, and plasma CORT secretion (Weiser et al. unpublished data). However the mechanism of ERβ-dependent effects on behavior and HPA axis function remain to be determined. Therefore, in this study we sought to determine whether systemic treatment of *S*-DPN could alter glucocorticoid specific actions on the

CeA. Our results indicate that peripheral administration of *S*-DPN blocks the anxiogenic effect local administration of a GR agonist to the CeA. In fact, *S*-DPN treatment proved to be anxiolytic to the same extent regardless of GR activation in the CeA. Therefore, glucocorticoid-sensitive modulation of anxiety-type behaviors through the CeA can be inhibited by activation of ERβ.

In this study *S*-DPN was administered peripherally, and consequently several different modes of action are possible. DPN has been shown to pass the blood-brain barrier, and thus could exert its effect centrally (Lund, Rovis et al. 2005). The CeA does express ERβ (Laflamme, Nappi et al. 1998; Osterlund, Kuiper et al. 1998; Shughrue and Merchenthaler 2001), indicating a potential direct effect of ERβ. Accordingly, *S*-DPN treatment causes an increase in c-Fos immunoreactive cells in the CeA following the forced swim test. Furthermore, these c-Fos positive cells are not immunoreactive for CRH and are generally localized to the lateral CeA (Weiser et al. unpublished observations), a region rich in GABA expression (Sun and Cassell 1993). The phenotype of ERβ-expressing neurons in the CeA is unknown, but the possibility exists that ERβ is expressed in GABAergic interneurons of the CeA and upon ligand-activation acts to inhibit nearby CRH neurons. Interestingly, infusion of a benzodiazepine (positive GABA receptor modulator) into the CeA produces anxiolytic effects that can be blocked by injection of a GABA antagonist into the CeA (Davis, Rainnie et al. 1994; McKernan, Wafford et al. 1995). Furthermore, local application of the GABA agonist muscimol to the CeA results in decreased anxiety in the elevated plus maze (Moreira, Masson et al.

2007). This highlights the importance of GABA signaling for CeA-specific modulation of behaviors.

Alternatively, *S*-DPN may act via ERβ-expressing areas that project to the CeA such as the medial amygdala, BNST, arcuate nucleus, hippocampus, and dorsal raphe nucleus (Ottersen 1980; Ottersen 1981; Canteras and Swanson 1992; Dong and Swanson 2004; Dong and Swanson 2006). Another potential mechanism could be independent of the CeA. Activation of ERβ in brain regions that have direct and indirect projections to the parvocellular PVN (BNST, hippocampus, arcuate nucleus) may act to override the CeA-dependent augmentation of HPA axis drive. Additionally, ERβ is expressed in a small percentage of CRH-expressing neurons of the PVN (Laflamme, Nappi et al. 1998; Suzuki and Handa 2005), suggesting a potential direct effect on HPA axis output. However, the majority of ERβ-expressing neurons in the PVN are brainstem- and spinal cord- projecting neurons and not neurosecretory neurons (Bingham, Williamson et al. 2006).

Anxiety and depression are psychiatric disorders that are often accompanied by chronically elevated glucocorticoid levels. These elevated glucocorticoids can act upon the CeA to change normal behavioral and neuroendocrine responses to threatening stimuli into maladaptive and pathological responses. A higher propensity for these maladaptive responses in females may be responsible for the underlying sex difference in depression and anxiety. For example, functional imaging studies show increased activity in the amygdala in depressed patients (Drevets 2000; Drevets 2001), and females respond to fearful faces with a more robust activation of the amygdala than do

men (Dickie and Armony 2008). Sex steroid may act at the level of the CeA to alter behavioral and physiological consequences of elevated glucocorticoids.

In summary, the data presented here indicate that GR activation in the CeA can induce anxiety-type behaviors and an elevated hormonal (CORT) response to a psychological stressor. Additionally, treatment with an ERβ agonist can override the effect of GR in the CeA and further attenuates anxiety-type behaviors and dampens CORT secretion following a stressor. These data suggest a role for estradiol signaling via ERβ in modulating glucocorticoid-dependent effects of the CeA on behavior and neuroendocrine function.

CHAPTER SIX

Discussion

Stress-related psychiatric disorders like depression, anxiety, insomnia, and anorexia occur more often in women than in men, and women respond stronger hormonally to a stressor than men. The increased stress sensitivity observed in women may contribute to the etiology of these disorders. Interestingly, these sex differences do not appear until after puberty, suggesting a role for sex hormones. Additionally, disturbances in mood and stress reactivity are reported during periods of estrogen fluctuation in women including during puberty, the menstrual cycle (premenstrual dysphoric disorder), pregnancy (postpartum depression) and menopause (postmenopausal depression). Therefore, in addition to genetic predispositions and environmental influences, fluctuating estrogen levels likely play an integral role in contributing to or exacerbating the pathophysiology of stress-related psychiatric disorders. Estrogen replacement therapy to improve mood especially in postmenopausal women can have potentially harmful side effects due to its trophic actions, especially with respect to estrogen sensitive tumors. The anatomical locations, cellular actions, and molecular mechanisms of estrogen's influence on stress sensitivity and mood are poorly understood. The data presented in this dissertation lays the foundation for understanding the influence of estrogen signaling on HPA axis function and behavior, particularly though the classic intracellular estrogen receptors, ERα and

ERβ. Dissecting the individual contributions of ERα and ERβ with respect to HPA axis activity and anxiety- or depressive-type behaviors will provide insight into how fluctuating levels of estrogen, or an abnormal receptor balance, may contribute to stress-related pathologies. Due to the inherent difficulty of performing human studies, and relative lack of data in humans, inferences and putative mechanisms mentioned herein will be based primarily on data from animals.

Estrogen may exert its actions directly through a multitude of receptors (ERα, ERβ, GPR30, membrane ER, ER-X) and indirectly via the proteins, second messenger pathways, peptides, and neurotransmitters activated or inhibited by these receptors. A majority of the biological actions of estradiol are mediated through one or both of the classical intracellular receptors, ERα and ERβ. These actions are influenced by the relative tissue distribution of the receptors, receptor affinities for estradiol, availability of the receptors (expression, cellular localization, degradation), and expression of associated proteins (cofactors, enzymes, chaperone proteins, etc). Furthermore, sex differences (as seen with mood disorders) may be due to the organizational or activational effects of sex hormones. However, in the case of stress reactivity, removal of the gonads in adult rats leads to similar stress responses in males and females (Handa, Burgess et al. 1994). Additionally, treatment of young men with a one or two day estradiol treatment regiment results in significantly elevated salivary ACTH and cortisol following a social stress test (Kirschbaum, Schommer et al. 1996), suggesting a key activational component to sex differences in HPA axis function. Taking into account these factors in designing the experiments described in this dissertation, I focused on

the classical estrogen receptors (ERα and ERβ) and examined their role in the activational effects of estradiol on HPA axis function and behavior.

The first set of experiments described in this dissertation is focused on determining the mechanisms of estrogen influence on the HPA axis. Previous studies have shown a sex difference in the HPA axis response to a stressor, where females respond with higher neuronal activation and CRH expression in the PVN, higher ACTH release from the anterior pituitary, and higher CORT secretion from the adrenal cortex (Handa, Burgess et al. 1994; McCormick, Linkroum et al. 2002; Viau, Bingham et al. 2005). The HPA axis is regulated by a tight negative feedback system though the actions of glucocorticoids at the pituitary and in the brain (Dallman, Akana et al. 1987; de Kloet, Joels et al. 2005). Therefore, I determined if estradiol impairs glucocorticoid negative feedback, and if so by which receptor and in what location (Chapter 3). In these studies I have shown evidence that estradiol impairs glucocorticoid-dependent negative feedback both at the diurnal surge in CORT and during a stress response. Additionally, the effect of systemic estradiol is mimicked by local administration of an ERα agonist to the region of the PVN. Local administration of an ERβ agonist decreases the hormonal response to a stressor and does not impair glucocorticoid negative feedback.

The next set of experiments sought to determine whether these receptor subtype specific effects on HPA axis activity translated into behavioral changes. Chronically elevated glucocorticoid levels and a hyperactive HPA axis are associated with psychiatric disorders such as depression and anxiety (Dinan 1994; McEwen 2005; Swaab, Bao et al. 2005). Therefore, I examined the effect of various ERα- and ERβ-specific

agonists on anxiety-type and despair behaviors in a battery of behavioral platforms (Chapter 4). The results indicate that selective activation of ERα increases, whereas ERβ decreases anxiety-type behaviors and despair in ovariectomized female rats. Following a psychological stressor such as the forced swim test, pretreatment with an ERα agonist increases plasma ACTH and CORT and neuronal activation in the PVN, whereas pretreatment with an ERβ agonist has the opposite effect. Whether the behavioral effect is secondary to influences on HPA axis, or vice versa, is currently unknown and will be discussed in this chapter.

The last set of experiments described in this dissertation was aimed at tying together the effects of estrogen signaling via ERβ on glucocorticoid secretion and anxiety-type behaviors. In the studies described in Chapter 5, I found that local administration of a GR agonist to the CeA results in increased anxiety-type behaviors. The CeA is a glucocorticoid responsive brain region that modulates the behavioral as well as physiological response to danger (Kopchia, Altman et al. 1992; Davis 1997; Shepard, Barron et al. 2000). The anxiogenic effect of GR activation in the CeA is blocked by peripheral administration of an ERβ agonist. This suggests that ERβ may act via modulation of CeA-specific signaling thus altering both behavior and neuroendocrine (HPA axis) outcomes of psychological stressors. Furthermore, ERβ may act to block the maladaptive effects of elevated glucocorticoids on fear and anxiety.

In this chapter, I will first discuss the putative mechanisms of ERα- and ERβ-specific effects on HPA axis activity. Second, I will address putative mechanisms of ERα- and ERβ-specific effects on behavior. Thirdly, I will discuss the integration of this

dueling antagonistic receptor system, and propose an overall model derived from the studies described in this dissertation and in the literature. Finally, I will discuss the clinical relevance of my findings.

Putative mechanisms of ERα- and ERβ-specific effects on HPA axis activity

In the experiments described in this dissertation I have established opposing effects of ERα and ERβ activation on the drive of the HPA axis. Initial clues into potential mechanisms of action can be first determined by examining relative distribution of the receptors. Areas of ERα and ERβ co-expression include BNST, amygdala, preoptic area, arcuate, VMH and hippocampus (Shughrue, Lane et al. 1997; Osterlund, Kuiper et al. 1998; Shughrue, Scrimo et al. 1998; Shughrue and Merchenthaler 2001). Only ERβ is found in the PVN, SON, and SCN. This relative distribution in combination with subtype-specific molecular actions likely contributes to the opposing effects on HPA axis activity.

Estradiol acts to augment basal and stress-induced glucocorticoid secretion. The HPA axis is tightly regulated by glucocorticoid feedback at the basal state (permissive actions) and at the activated state (reactive actions). This feedback occurs at the pituitary, PVN, and other glucocorticoid-sensitive brain regions (de Kloet, Joels et al. 2005). The studies described in Chapter 1 show that systemic estradiol treatment interferes with glucocorticoid negative feedback at the basal and activated state. Therefore, estradiol interference of glucocorticoid negative feedback is a clear systems-level mechanism for the increased HPA axis activity observed in females.

To get at a cellular mechanism, I chose to examine the PVN as a potential site of action. ERα is expressed in the adrenal cortex, in the corticotrophs of the anterior pituitary, and to a lesser extent in the hippocampus; all of these regions are critical in glucocorticoid-mediated negative feedback. Similarly, ERβ is expressed in the adrenal cortex, PVN, hippocampus, and to a lesser extent in the corticotrophs of the anterior pituitary. However, previous studies performed by Lund and colleagues showed that local administration of estradiol to the PVN resulted in an augmented CORT response to restraint stress, similar to that seen with systemic treatment of estradiol (Lund, Hinds et al. 2006). Previous studies have indicated a critical role for the PVN in glucocorticoid negative feedback, and a perhaps a secondary role for the hippocampus and anterior pituitary (Dallman, Akana et al. 1987; Kovacs and Mezey 1987; Sawchenko 1987; Kovacs, Foldes et al. 2000). Lund's experiments led me to believe that the effects of estradiol on glucocorticoid negative feedback were indeed localized to the PVN area. Correspondingly, I found that estradiol does impair basal and stress-induced glucocorticoid negative feedback when administered to the area of the PVN. Furthermore, this effect is mimicked by local administration of an ERα agonist, whereas an ERβ agonist does not impair negative feedback and conversely reduces ACTH and CORT secretion following a stressor. Interestingly, ERα is not expressed within the PVN, but ERβ is (Suzuki and Handa 2005). Thus, the actions of ERα must be through an indirect pathway not intrinsic to the CRH and AVP effector neurons of the HPA axis. Indeed, ERα is expressed in the surrounding region of the PVN (Simerly and Young 1991; Suzuki and Handa 2005). Therefore, the ERα-dependent impairment of glucocorticoid

negative feedback and increased HPA axis drive is likely through peri-PVN neurons, while the ERβ-dependent decrease in HPA axis drive is likely through PVN neurons themselves.

There are several potential cellular mechanisms for ERα-dependent effects. The peri-PVN provides substantial GABAergic input to the parvocellular PVN (Roland and Sawchenko 1993; Boudaba, Szabo et al. 1996). Nearly half of all synapses in the PVN are GABAergic (Decavel and Van den Pol 1990; Decavel and van den Pol 1992), and the PVN is under tonic inhibition by GABA (Bali and Kovacs 2003). Excitation of peri-PVN neurons with glutamate elicits GABA-dependent inhibition of PVN neurons (Boudaba, Szabo et al. 1996), and GABA application enhances the CORT response to a stressor (Cullinan 1998). Furthermore, many stress-responsive brain regions such as the hippocampus, lateral septum, and prefrontal cortex, access the PVN by way of projections to the peri-PVN region. These neurons show robust c-fos expression following a stressor (Cullinan, Helmreich et al. 1996; Cole and Sawchenko 2002) and express GR (Weiser and Handa, unpublished data). Consequently, they are able to weigh stress-related neuronal input with the status of endogenous glucocorticoid levels. I found that a good majority of ERα immunoreactive peri-PVN neurons also expressed GAD67, the rate-limiting enzyme for GABA synthesis. Therefore, ERα may act to counter GR-dependent activation of peri-PVN neurons and effectively release the PVN from this inhibition. In this regard, the inhibition of the peri-PVN neurons would effectively block the inhibitory influence from the hippocampus, which sends glutamatergic projections to the peri-PVN and is

essential in glucocorticoid-dependent inhibition of the PVN (Herman, Mueller et al. 2004).

ERα might be regulating one or more of several important molecular players in the peri-PVN. One obvious candidate is GABA. Since GAD is upregulated in the peri-PVN following a stressor (Bowers, Cullinan et al. 1998), it is plausible that ERα interferes with the expression, translation, or stability of GAD67. Estradiol has been shown to alter GAD67 expression in the AVPV and MPN, and this mechanism would be in accordance with several other estrogen-sensitive systems that indicate a role for estrogen in modulation of GABAergic neurotransmission (Herbison and Fenelon 1995; Murphy, Cole et al. 1998; Herbison 2008). Another candidate might be glutamate receptors. ERα could impair the ability of peri-PVN neurons to respond to glutamatergic input. ERα has been shown to associate with glutamate receptors in the plasma membrane and alter glutamate signaling (Boulware, Kordasiewicz et al. 2007; Dewing, Boulware et al. 2007; Micevych and Sinchak 2008). Lastly, ERα may also interrupt GR signaling and/or expression. Estrogen has been shown to impair GR autoregulation (Burgess and Handa 1992), and impair GR-dependent gene transcription (Uht, Anderson et al. 1997).

Whereas activation of ERα potentiates the stress response, peripheral or local administration of an ERβ agonist to the PVN region dampens the response to a stressor. Cellular and molecular mechanisms for this ERβ-dependent effect most likely involve PVN parvocellular or magnocellular neurons. ERβ is colocalized with CRH, AVP, OT and PRL within PVN (Laflamme, Nappi et al. 1998; Suzuki and Handa 2005). Therefore,

mechanisms involving one or more of these neuropeptides must be considered. The first obvious mechanism is a direct effect on the neurosecretory CRH and AVP neurons of the parvocellular PVN. ERβ has been shown to drive ligand-dependent and ligand-independent transcriptional activity of CRH and AVP promoters in vivo (Shapiro, Xu et al. 2000; Miller, Suzuki et al. 2004; Pak, Chung et al. 2007; Ogura, Kageyama et al. 2008). Stimulation of CRH and AVP transcription (both powerful ACTH secretagogues) would be counter to the inhibitory effects on stress-induced ACTH and CORT secretion that I observed. However, a good majority of ERβ-expressing cells in the PVN do not project to the median eminence (Stern and Zhang 2003; Bingham, Williamson et al. 2006), suggesting an indirect-effect of ERβ activation. Interestingly, electron microscopy studies have shown the existence of parvocellular axon collaterals within the PVN proper (van den Pol 1982; Liposits, Paull et al. 1985; Rho and Swanson 1989; Silverman, Hou-Yu et al. 1989), and additional studies have demonstrated dendritic release of AVP within the PVN (Ludwig and Leng 1998). Furthermore, AVP release within the PVN imparts a negative tone on HPA activity (Landgraf, Wotjak et al. 1998). Chronic estradiol replacement in gonadectomized mice results in decreased AVP mRNA in the PVN, an effect that is lost in βERKO mice (Nomura, McKenna et al. 2002). The decreased AVP in the PVN may be a result of the inhibitory paracrine action of magnocellular or parvocellular ERβ-containing AVP neurons. Thus, ERβ may act to alter AVP paracrine input to median eminence-projecting CRH and AVP neurons.

ERβ is expressed in 84% of OT neurons in the medial parvocellular PVN and may also influence the expression of oxytocin in the PVN, which has been shown to dampen

the HPA response to a variety of stressors (Windle, Shanks et al. 1997; Neumann, Kromer et al. 2000; Neumann, Wigger et al. 2000; Mantella, Vollmer et al. 2004; Windle, Kershaw et al. 2004). Furthermore, estradiol has been shown to increase OT mRNA in the PVN, an effect that is lost in βERKO animals (Nomura, McKenna et al. 2002). Accordingly, central administration of an OT receptor antagonist to ovariectomized female rats abolishes the effect of peripheral ERβ treatment on stress-induced ACTH and CORT release (Kudwa et al., unpublished). Additionally, the effect of ERβ activation on HPA axis activity is lost on OT receptor knockout (OTRKO) animals (Kudwa et al., unpublished). Since a portion of ERβ-expressing neurons in the PVN are spinal cord projecting pre-autonomic neurons, ERβ may work via modulation of OT expression to alter autonomic responses to homeostatic threats. Thereby throttling autonomic innervation of the adrenal gland. However, ACTH secretion mimics CORT secretion in the effects observed with the central OT antagonist, suggesting that OT-dependent effects of ERβ activation on HPA axis activity must be central. Indeed, microdialysis studies have shown that OT is released in the PVN following a stressor (Nishioka, Anselmo-Franci et al. 1998), and that swim stress-induced ACTH release can be inhibited by reverse dialysis of an OT antagonist into PVN (Neumann, Torner et al. 2006). Oxytocin receptor (OTR) mRNA is expressed in the PVN, however OTRs have not yet been definitively colocalized with parvocellular CRH neurons (Yoshimura, Kiyama et al. 1993; Vaccari, Lolait et al. 1998). Taken together, ERβ activation may lead to an increase in systemic (via the posterior pituitary) and/or local PVN OT release, and in turn inhibit the activity of parvocellular CRH and AVP neurons.

Putative mechanisms of ERα- and ERβ-specific effects on behavior

In studies described in this dissertation I found that ERα activation increases, whereas ERβ activation decreases, anxiety-type and depressive-type behaviors. Unlike the experiments on HPA axis activity and glucocortiocoid-dependent negative feedback, these studies did not have the advantage of localized administration of agonist, and thus putative mechanisms must be derived from analysis of data from peripherally treated animals. This opens up a breadth of brain regions that are directly or indirectly tied to behavior. Studies utilizing microinjection, selective lesions, electrophysiology, and imaging techniques have identified several brain regions involved in anxiety and depression including the cortex, hippocampus, amygdala, and hypothalamus (Gordon and Hen 2004; Szily and Keri 2008).

First, to address prospective mechanisms for the anxiogenic actions of ERα one must examine its expression profile in brain. ERα is expressed in the amygdala and certain areas of the hypothalamus (POA, ARC, VMH), and to a lower extent in hippocampus and cortex. The role of ERα in the brain is largely associated with the control of reproductive behaviors (VMH) and GnRH secretion (POA). Its role in anxiety and depressive-type behaviors has not been extensively studied. One ERα-rich candidate region is the CeA. The CeA processes neuronal input following a perceived danger or threat and responds appropriately through often-reciprocal connections to the brainstem, limbic system, hypothalamus, and cortex (Veening, Swanson et al. 1984; Gray, Carney et al. 1989; Gray 1993; Marcilhac and Siaud 1997). Furthermore, the CeA

is a major extrahypothalamic source of CRH(Makino, Gold et al. 1994; Watts and Sanchez-Watts 1995), and impaired or dysfunctional CRH signaling has been implicated in the eitiology of depression (Arborelius, Owens et al. 1999; Keck and Holsboer 2001). ERα has been shown to stimulate transcriptional activity of the CRH promoter (Miller, Suzuki et al. 2004), albeit weakly, and could theoretically stimulate CRH signaling from the CeA. Stimulation of the CeA induces CRH expression in the CeA and PVN (Gray 1993), and increases anxiety-type behaviors (Kopchia, Altman et al. 1992; Davis 1997). However, ERα has not been definitely colocalized with CRH in the CeA as of yet.

ERα-dependent effects on behavior may be secondary to its effects on HPA axis activity and glucocorticoid-dependent negative feedback. Release of the PVN from GABAergic constraints through the actions of ERα may result in chronically high levels of glucocorticoids. The beneficial actions of acute glucocortioids can become maladaptive at persistently elevated levels, and contribute to the etiology of mood disorders. For instance, administration of a continuous high dose of CORT to the CeA induces anxiety-type behaviors and elevated plasma CORT (Shepard, Barron et al. 2000; Myers, Gibson et al. 2005). Moreover, elevated glucocorticoids may cause neuronal cell death in brain regions important for mood and glucocorticoid negative feedback like the cortex and limbic system (Lee, Ogle et al. 2002; Swaab, Bao et al. 2005). Taken together, the anxiogenic effect of ERα activation could be as a result of the collective modifications of CRH signaling and/or GABA signaling that perpetuates elevated glucocorticoid secretion and ultimately maladaptive behaviors in response to threatening situations.

Contrary to the effects of ERα, peripheral administration of an ERβ agonist is anxiolytic. The results presented in Chapter 4 indicate that the ERβ agonists S-DPN and WAY-200070 are anxiolytic when administered to ovariectomized female rats. These results are in accordance with experimental data from transgenic animals in which βERKO animals display increased anxiety and learned helplessness (Krezel, Dupont et al. 2001; Imwalle, Gustafsson et al. 2005; Rocha, Fleischer et al. 2005). In addition, the anxiolytic and antidepressant effect of DPN is lost in βERKO animals (Walf, Koonce et al. 2008; Walf, Koonce et al. 2008). The anxiolytic effect of ERβ activation is likely centrally derived given that ICV injection of ERβ antisense oligodeoxynucleotides increases anxiety-type behaviors in the elevated plus maze and open field in addition to increasing despair in the forced swim test (Walf, Ciriza et al. 2008). As previously detailed, ERβ is expressed in the PVN, SON, SCN, BNST, amygdala, preoptic area, hippocampus, and to a lesser extent in the arcuate and VMH. Of these regions, the PVN, BNST, amygdala, and hippocampus play particularly important direct and indirect roles in emotionality.

The data presented in Chapters 3 and 5 implicate the PVN and the CeA as potential sites of action. As discussed in the previous section, ERβ application to the region of the PVN decreases the ACTH and CORT response to a stressor, possibly though the inhibitory actions of intra-PVN OT release. Thus, the anxiolytic actions of ERβ may be secondary to effects on HPA axis activity. During times of chronic stress, which may precipitate depressive episodes or anxiety, ERβ might effectively attenuate the output of the HPA axis and reign in glucocorticoid secretion. This would effectively prevent the potentially damaging effect of continuously elevated levels of glucocorticoids.

In an alternative mechanism, ERβ activation may influence excitation of the amygdala, especially during times of high glucocorticoid secretion. As previously mentioned, the CeA is a glucocorticoid responsive brain region that plays an essential role in coordinating the systems level responses to threatening stimuli. These adaptive responses may become pathological with repetitive or high chronic levels of endogenous glucocorticoids and precipitate anxiety-type behaviors. Interestingly, a high degree of binding is observed in the CeA of animals peripherally injected with radiolabeled estradiol (Pfaff and Keiner 1973). This advocates for the possibility of direct estradiol effects on CeA function. In the studies described in Chapter 5, I found that administration of a GR agonist to the CeA increases anxiety, and that this effect can be blocked by peripheral administration of an ERβ agonist. This suggests that ERβ activation can ameliorate glucocorticoid-dependent effects of the CeA on anxiety, perhaps due to direct actions of ERβ within the CeA. However expression of ERβ is relatively modest in the CeA, and the phenotype of these neurons is currently unknown. ERβ activation could directly reduce the expression of CRH in the CeA, however this would be counter to the effects on CRH promoter activity observed in vitro. Alternatively, there is a subpopulation of GABAergic neurons in the lateral portion of the CeA in which activation of ERβ could potentially increase GABA neurotransmission. These neurons influence CRH neurotransmission from the CeA in addition to contributing to the GABAergic efferent projections of the CeA (Sun and Cassell 1993). An increase in GABA signaling in the CeA has been shown to decrease anxiety-type behaviors (Davis, Rainnie et al. 1994; Moreira, Masson et al. 2007). Interestingly, S-DPN

treatment does increase c-Fos immunoreactivity in the CeA following the forced swim test (Weiser and Handa, unpublished data). Whether or not these are inhibitory GABAergic neurons remains to be determined.

In yet another mechanism, ERβ activation may alter serotonergic neurotransmission to influence anxiety- and depression-like behaviors. ERβ is the predominant estrogen receptor in the midbrain/brainstem raphe nucleus, particularly in the dorsal and ventral divisions of the dorsal raphe nucleus (DRN) (Nomura, Akama et al. 2005). Greater than 90% of these ERβ-IR neurons also exhibit tryptophan hydroxylase (TPH, rate limiting enzyme in the synthesis of serotonin) immunoreactivity, suggesting a potential role for estrogen in regulation of TPH in the DRN (Nomura, Akama et al. 2005). Accordingly, recent studies by Hiroi et al. show that estradiol treatment of ovariectomized female rats significantly increases TPH2, the predominant brain isoform of TPH, in the mid-ventromedial and caudal subregions of the DRN (Hiroi, McDevitt et al. 2006). These studies are corroborated by findings in ERβKO mice where serotonin content in the DRN, preoptic area, BNST, and hippocampus, areas of high ERβ expression, is significantly decreased compared to wildtype animals (Imwalle, Gustafsson et al. 2005). Furthermore, 5-HT_{1A} receptor levels are significantly increased in the amygdala of ERβKO mice, and 5-HT_{1A} receptor KO mice exhibit significantly increased anxiety in comparison to their wild type counterparts (Heisler, Chu et al. 1998; Parks, Robinson et al. 1998; Ramboz, Oosting et al. 1998). These data suggest that ERβ activation may influence behavior through serotonin signaling, perhaps by altering

serotonin neurotransmission from the brainstem (i.e. DRN) to the amygdala (McQueen, Wilson et al. 1997; McQueen, Wilson et al. 1999; Krezel, Dupont et al. 2001).

Finally, an intriguing and potentially encompassing mechanism involves OT signaling and might explain ERβ-dependent effects on both HPA axis output and behavior. As mentioned in the previous section, central administration of an OTA blocks the effect of ERβ activation on stress-induced ACTH and CORT. In addition to these effects, the centrally administered OTA also blocks the anxiolytic effect of ERβ activation (Kudwa et al., unpublished). The anxiolytic effect of ERβ activation is also lost on OTRKO animals. These data delineate a clear involvement of OT signaling in ERβ-dependent effects on anxiety and HPA axis function. Indeed, studies have shown a clear role for OT in anxiety. OT knockout mice display increased anxiety (Mantella, Vollmer et al. 2003; Amico, Mantella et al. 2004), and centrally administered OT is anxiolytic (Windle, Shanks et al. 1997; Ring, Malberg et al. 2006). Furthermore, the anxiolytic effects of OT are attributable to its actions in the CeA (Bale, Davis et al. 2001; Neumann 2002) and PVN (Neumann, Wigger et al. 2000; Blume, Bosch et al. 2008). Studies have shown that OT infused into the CeA is anxiolytic (Bale, Davis et al. 2001), and adenoviral-mediated over-expression of OTR in the CeA decreases anxiety (Bosch, Waldherr et al. 2006). Interestingly, the PVN and CeA have a reciprocal direct connection (Palkovits, Young et al. 1998). Bilateral lesions of the PVN increase CRH mRNA expression in the CeA independent of adrenal status (ADX or non-ADX), suggesting that the PVN has direct inhibitory influence on the CeA (Palkovits, Young et al. 1998). Whether or not this direct projection is oxytocinergic or not is unknown, and it remains to be determined whether

a subset of ERβ-containing OT neurons of the PVN project to the CeA. Taken together, ERβ activation may increase OT inhibition of CeA neurons either through an augmentation of systemic OT release from magnocellular PVN neurons, or via direct release of OT from the PVN onto neurons of the CeA.

Integrating ERα and ERβ-dependent effects: the dueling estrogen receptor system and the "set point" ratio

Thus far, this chapter has focused on putative mechanisms of exclusively ERα- or ERβ-dependent signaling in controlling HPA axis activity and anxiety- and depressive-type behaviors. However, *in vivo* the cognate ligand for these receptors, estradiol, is promiscuous and subsequently signals through both receptors. Therefore, these putative mechanisms must be weighed with sex hormone secretion patterns and levels in addition to predispositions for signaling through one receptor or the other; a set point which may be derived from the influences of circulating hormones and/or genetic factors.

Fluctuating endogenous levels of estradiol associated with puberty, cyclicity (estrous, rodents; menstrual, humans), pregnancy and menopause may alter this set point. Evidence suggests that estradiol decreases the brain expression of ERβ globally (Patisaul, Whitten et al. 1999; Shima, Yamaguchi et al. 2003), whereas it appears to have varying effects on ERα expression depending on dose and brain region (Simerly and Young 1991; Osterlund, Kuiper et al. 1998; Prange-Kiel, Wehrenberg et al. 2003; Rose'Meyer, Mellick et al. 2003). Interestingly, the effect on ERβ expression is lost in

ERαKO animals, implicating estradiol signaling via ERα in the downregulation of central ERβ expression (Nomura, Korach et al. 2003). Estradiol may contribute to the sex differences observed in stress sensitivity and mood disorders by tipping the set point towards ERα-dominated signaling.

The ERβ/ERα set point may also be influenced by age. Associated with increasing age is a global decline in ERβ expression (Yamaguchi-Shima and Yuri 2007). ERα expression remains relatively constant with age (Wilson, Rosewell et al. 2002), thereby shifting the set point towards ERα dominance. In addition, the expression of ERβ in the SCN fluctuates diurnally, an effect that is blunted with age (Wilson, Rosewell et al. 2002). This may in turn influence the diurnal activity of the HPA axis, and perhaps increase the gain of the HPA axis thereby inducing chronically high levels of glucocorticoids.

The antagonism of estradiol signaling through ERα and ERβ is likely important for maintaining normal adaptive responses to stressors. Imbalances in this ratio might shift the balance of signaling towards dominance in the favor of signaling through one receptor over the other. In depressed patients there is a significant increase in ERα expression in the PVN (Wang, Kamphuis et al. 2008). ERα-dependent signaling may lead to the impaired glucocorticoid negative feedback observed in depressed patients. Taken together, the predisposition for stress-related disorders may be related to the set point ratio of ERα-to-ERβ signaling. This set point is likely governed by collective influences of sex hormones, genetics, and age.

This dueling antagonistic relationship between ERα and ERβ in regulation of the HPA axis and behaviors might be a biological mechanism to initiate appropriate neuroendocrine and behavioral reactions to stressors with respect to internal hormonal milieu. For example, during pregnancy when endogenous levels of estrogen are high, a robust stress response may elicit the proper mechanisms to protect the mother and fetus from potential harm. On the other hand, when endogenous estrogen levels are low, such as during starvation, it may be more advantageous to tone down the stress response in order to conserve resources. This check-and-balance system when chronically activated in one direction or the other may lead to stress-related pathologies.

Overall putative mechanism

Figure 6.1 depicts a schematic representation of the opposite effects of ERα- and ERβ-dependent signaling on HPA axis activity and anxiety state. Stress-sensitive neuronal circuits originating from the brain stem, cortex and limbic regions provide threat-dependent input to the peri-PVN, PVN and CeA. These brain regions in turn stimulate the appropriate neuroendocrine and behavioral responses. Selective activation of ERα might hinder GABAergic inhibition of CRH and AVP neurons, and consequently drive CRH production and glucocorticoid secretion. Additionally, activation of ERα could potentiate CRH production in the CeA, which in turn precipitates anxiety-type behaviors and further stimulates CRH expression in the PVN. Alternatively, activation of ERβ induces OT expression in the PVN, consequently inhibiting CRH

expression in the PVN in a paracrine fashion and dampening glucocorticoid secretion. Activation of ERβ might also inhibit CRH expression in the CeA by way of an oxytocinergic PVN-CeA connection, or direct modulation of GABAergic neurotransmission within the CeA, thereby reducing PVN CRH expression, glucocorticoid secretion, and anxiety-type behaviors. The ratio of ERα-to-ERβ signaling likely determines the degree of glucocorticoid secretion and anxiety-type behaviors displayed. A preponderance towards ERα-dominant signaling will impair glucocorticoid negative feedback, heighten glucocorticoid secretion, induce excessive levels of fear and distress, which in turn leads to pathological anxiety.

Clinical significance

There exists a significantly higher propensity for stress-related psychiatric disorders in women than in men. Women are more likely than men to suffer from major depression, anxiety, eating disorders, and insomnia (Angold and Worthman 1993, Kornstein 1997, Llewellyn et al. 1997, Weissman et al. 1993, Ehlert et al. 2001, Buckley and Schatzberg 2005). Anxiety and depression in particular have significant social and economic impacts. Approximately one in four adults will suffer from some form of anxiety disorder and nearly one in six adults will suffer from depression at some point in their lifetime (Kessler, McGonagle et al. 1994; Kessler, Chiu et al. 2005). Anxiety and depression are complex and incapacitating psychiatric illnesses that share analogous symptoms and have extensive comorbidity (Freeman, Freeman et al. 2002; Nutt, Ballenger et al. 2002; Nutt and Stein 2006). The etiology of these disorders appears to

be quite complicated, however both are correlated with abnormalities in HPA axis activity and glucocorticoid negative feedback.

Much of the preclinical work on depression has focused on the relationship between stress and depression (Bao, Meynen et al. 2008). An underlying physiological and psychological loss of control over stress sensitivity and responsiveness appear to be important factors in the development of depression. Abnormalities in the HPA axis are now well documented in many mental disorders including anxiety and depression (Ehlert, Gaab et al. 2001; Varghese and Brown 2001; Barden 2004). It is well known that stressors are key instigators for the onset of depressive episodes (Kendler, Karkowski et al. 1999; Paykel 2001), and the HPA axis is regarded as the 'final common pathway' for the numerous symptoms of major depressive disorder (Bao, Meynen et al. 2008). Patients with depression often exhibit elevated plasma cortisol, impaired dexamethasone suppression, decreased GR expression and function, increased hypothalamic CRH and AVP, augmented adrenal sensitivity to ACTH, and/or enlargement of the pituitary and adrenal glands (Krishnan, Doraiswamy et al. 1991; Dinan 1994; Raadsheer, van Heerikhuize et al. 1995; Rubin, Phillips et al. 1996; Modell, Yassouridis et al. 1997; Maes, Lin et al. 1998; Scott and Dinan 1998; Holsboer 2000; Weber, Lewicka et al. 2000; Meynen, Unmehopa et al. 2006). Studies performed with high-risk patients showed abnormal HPA axis drive prior to onset of any clinical symptoms, suggesting a causative role for HPA axis dysregulation in depressive disorder (Holsboer 2000). Accordingly, antidepressant treatment has positive effects on HPA axis markers of activity and sensitivity. Classical antidepressants reduce

hypercortisolism and restore the feedback sensitivity of the HPA axis in line with remission of depressive symptoms (Nelson and Davis 1997; Hatzinger, Hemmeter et al. 2002). In addition, benzodiazepines lead to a quick short-term relief of anxiety and depressive symptoms and have been shown to decrease plasma cortisol in depressed patients (Christensen, Lolk et al. 1989). Animal models are in line with the human studies, where chronic administration of antidepressants reduces HPA axis activity in normal animals (Shimoda, Yamada et al. 1988; Reul, Stec et al. 1993), and in animals genetically modified to have a hyperactive HPA axis (Pepin, Pothier et al. 1992; Montkowski, Barden et al. 1995; Barden 1999).

Conclusions

There exists a large body of evidence demonstrating that estradiol increases basal and stress-induced HPA axis activity. Furthermore, experiments detailed in this dissertation indicate that estradiol impairs glucocorticoid negative feedback, and that this may be responsible for estradiol's effects on the HPA axis. A dysregulation in the feedback sensitivity of the HPA axis through the actions of estradiol may contribute to the sex difference observed in stress-related psychiatric diseases. According to the working model presented in this chapter, a shift towards ERα-dependent signaling would act to impair glucocorticoid negative feedback and elevate glucocorticoid levels. These elevated glucocorticoids may act upon certain brain regions such as the CeA to change normal behavioral and neuroendocrine responses to threatening situations into pathological responses. The actions of ERβ are largely antagonistic to those of ERα, and therefore a natural balance of ERα-to-ERβ signaling must be met in order to maintain normal adaptive responses to stressors.

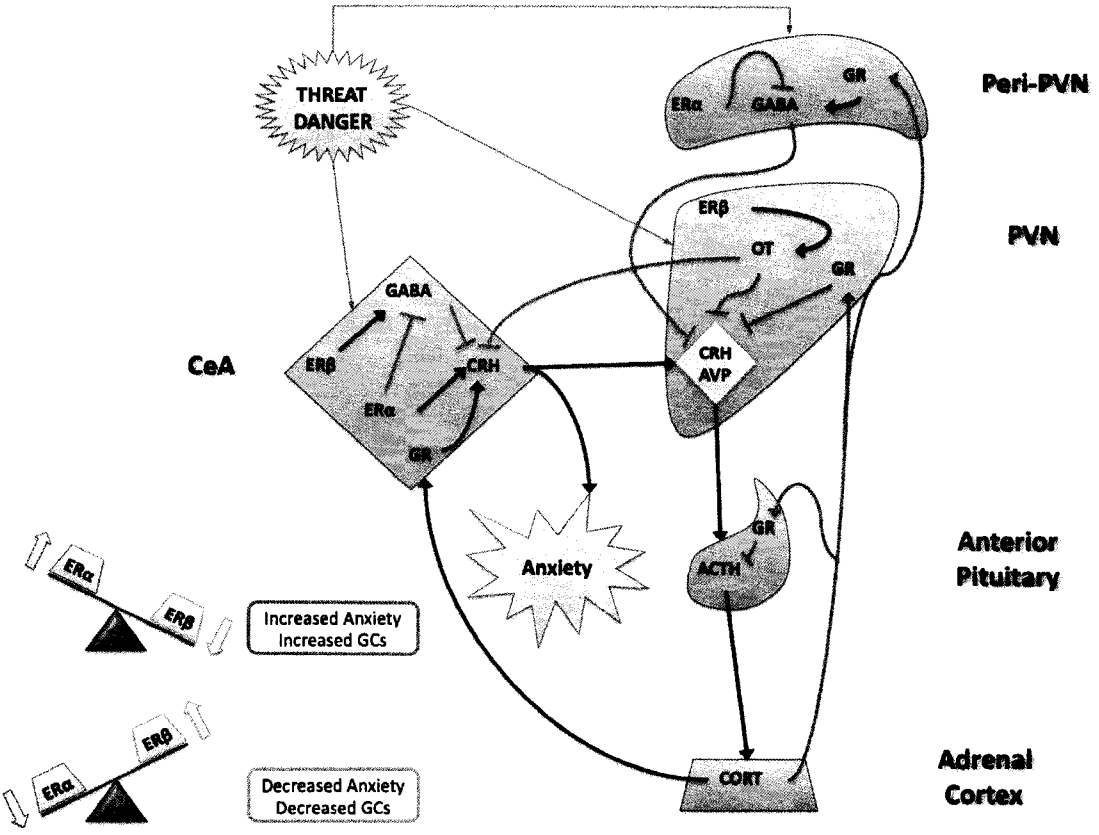


Figure 6.1. Schematic diagram of putative ERα- and ERβ-dependent effects on HPA axis activity and anxiety-type behaviors. A threatening or dangerous situation stimulates neural input originating from the brainstem, cortex, and limbic regions to the peri-PVN, PVN and CeA. These regions respond by altering glucocorticoid secretion and behavior. Elevated glucocorticoids feedback to inhibit CRH expression in the PVN, and ACTH synthesis and secretion from the anterior pituitary. Conversely, glucocorticoids stimulate CRH expression in the CeA. Activation of ERα releases the PVN from GABAergic inhibition originating from the peri-PVN region resulting in enhanced glucocorticoid secretion. Additionally, ERα activation may potentiate CRH expression in the CeA directly or indirectly via inhibition of GABA, and induce anxiety-type behaviors and further stimulate glucocorticoid secretion. Activation of ERβ induces OT expression in the PVN, which inhibits CRH expression within the PVN in a paracrine fashion, and inhibits CRH expression in the CeA by way of a PVN-CeA oxytocinergic projection. This subsequently results in decreased glucocorticoid secretion and decreased anxiety. Overall, increased ERα-to-ERβ signaling ratio will augment glucocorticoid secretion and anxiety-type behaviors, whereas increased ERβ-to-ERα signaling ratio will dampen glucocorticoid secretion and anxiety-type behaviors.

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LIST OF ABBREVIATIONS

(-)	negative
3β-Diol	5α-androstane-3β, 17β-diol
5-HT	serotonin
AC	anterior commissure
ACTH	adrenocorticotropic hormone
ADU	arbitrary density unit
ADX	adrenalectomy
AHA	anterior hypothalamic area
AMP	adenosine monophosphate
AMY	amygdala
AP-1	activator protein 1
AR	androgen receptor
ARE	androgen response element
ARC	arcuate nucleus
AVP	arginine vasopressin
AVPV	anteroventral periventricular nucleus
BDNF	brain-derived neurotropic factor
βERKO	estrogen receptor beta knockout
BnST	bed nucleus of the stria terminalis

BSA	bovine serum albumin
BZD	benzodiazepine
cAMP	cyclic adenosine monophosphate
CBG	corticosterone binding globulin
CBP	CREB binding protein
CeA	central nucleus of the amygdala
CNS	central nervous system
CORT	corticosterone
CRE	cAMP response element
CREB	CRE binding protein
CRH	corticotropin releasing hormone
CRHR	CRH receptor
CSF	cerebrospinal fluid
DAB	3,3'-diaminobenzadine
DBD	DNA binding domain
DEX	dexamethasone
DHT	dihydrotestosterone
DMEM	dulbecco's modified eagle medium
DMH	dorsomedial hypothalamus
DNA	deoxyribonucleic acid
DPN	diarylpropionitrile
DRN	dorsal raphe nucleus

DST	dexamethasone suppression test
E2	estradiol
EB	estradiol benzoate
EDTA	ethylenediaminetetraacetic acid
EPM	elevated plus maze
ERα	estrogen receptor alpha
ERαKO	estrogen receptor alpha knockout
ERβ	estrogen receptor beta
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FSH	follicle stimulating hormone
FSL	flinder's sensitive line
FST	forced swim test
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAS	general adaptation syndrome
GDX	gonadectomy
GnRH	gonadotropin-releasing hormone
GPR	g protein coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element

HAT	histone acetyltransferase
HDAC	histone deacetylase
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
HPLC	high pressure liquid chromatography
HRE	hormone response element
HSD	hydroxysteroid dehydrogenase
HSP	heat shock protein
IC50	50% inhibitory concentration
ICC	immunocytochemistry
IR	immunoreactivity
ISH	in situ hybridization
LBD	ligand binding domain
LH	luteinizing hormone
LHA	lateral hypothalamic area
LS	lateral septum
LUC	luciferase
MAOI	monoamine oxidase inhibitor
MAPK	mitogen-activated protein kinase
MeA	medial amygdala
MPOA	medial preoptic area
MR	mineralocorticoid receptor

mRNA	messenger ribonucleic acid
NGS	normal goat serum
NOS	nitric oxide synthase
NTS	nucleus of the solitary tract
OCD	obsessive compulsive disorder
OFT	open field test
OT	oxytocin
OTR	oxytocin receptor
OTRKO	OTR knockout
OVX	ovariectomy
Pa	paraventricular nucleus
PaAP	Pa anterior parvocellular
PaDC	Pa dorsomedial cap
PaLM	Pa lateral magnocellular
PaMM	Pa medial magnocellular
PaMP	Pa medial parvocellular
PaP	posterior Pa
PaV	ventral Pa
PeM	periventricular magnocellular
PFC	prefrontal cortex
PKA	protein kinase A
PKC	protein kinase C

POA	preoptic area
POMC	pro-opiomelanocortin
PPT	propylpyrazoletriol
PR	progesterone receptor
PRL	prolactin
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus
RBA	relative binding affinity
RIA	radioimmunoassay
RLU	relative light unit
RNA	ribonucleic acid
SCN	suprachiasmatic nucleus
SD	sprauge-dawley
SEM	standard error of the mean
SHR	steroid hormone receptor
SON	supraoptic nucleus
SQ	subcutaneous
SS	somatostatin
SSRI	serotonin reuptake inhibitor
T	testosterone
TPH	tryptophan hydroxylase
TRH	thyrotropin releasing hormone

TrkB	neurotrophic tyrosine kinase receptor type 2
TX	triton X
VMH	ventromedial hypothalamus
WKY	wistar kyoto