1996

Androgen Receptors in the Hippocampus: Localization, Autoregulation, and Modulation of Gene Expression in the Adult Male Rat

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ANDROGEN RECEPTORS IN THE HIPPOCAMPUS: LOCALIZATION, AUTOREGULATION, AND MODULATION OF GENE EXPRESSION IN THE ADULT MALE RAT.

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

BY

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CHICAGO, ILLINOIS

JANUARY, 1996

ACKNOWLEDGEMENTS

I sincerely thank my co-advisors and mentors, Dr. Robert J. Handa and Dr. Sheryl G. Beck for their guidance, support, and drive to succeed against the odds. Together, they have taught me more about science, and myself, than I ever expected. I thank the members of my dissertation committee, Drs. Leonard C. Erickson, Nancy A. Muma, and Kathryn J. Jones, for their time and critical review of this work. I am indebted to many members of the Handa Lab, past and present, for their technical support and camaraderie. I especially thank George Hejna, Melanie Bollnow, Dr. Loyd Burgess, and Dr. Alan Nagahara for their help and participation in many aspects of my work. I also wish to thank all the staff, students and faculty of the Department of Cell Biology, Neurobiology, and Anatomy at Loyola for treating me as one of their own. I am particularly grateful to two great friends who have kept me sane through the ups and downs of graduate school -- Melinda Wilson and Dr. Susan Morgan. A very special thanks also goes to Danielle and Frank McGraw, not only for their friendship, but also for graciously letting me stay in their home time and time again during this past year.

Much appreciation and love goes to my family for their never ending emotional (and financial) support during this endeavor. Finally, I thank my husband, Jim Zilisch, for his patience and love -- that I now know can survive anything.

ABSTRACT

Androgens, testosterone (T) and 5α-dihydrotestosterone (DHT), have profound modulatory roles in the mammalian central nervous system by specifically binding to androgen receptors (ARs) in target cells. The studies contained in this dissertation were designed to characterize AR expression in the hippocampus, a central structure of the limbic system, and to determine if this area is a neural target for androgen's actions. In the first series of experiments, AR and AR messenger ribonucleic acid (mRNA) levels in the adult male rat hippocampus were found to compare closely to levels found in the hypothalamus, and AR mRNA expression was primarily concentrated in the CA1 pyramidal cell region of the hippocampus. Hippocampal AR and AR mRNA expression were uniquely autoregulated following the removal of circulating androgen in adult male rats, and in old male rats with reduced circulating levels of T. Next, the effect of selective AR activation on the constitutive expression of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) genes in the hippocampus were investigated. As compared to castrated control rats, DHT treatment of castrates decreased GR mRNA levels, but not MR mRNA levels, in the CA1 region of the hippocampus. Transcriptional cross-talk or interactions between AR and GR may mediate some aspects of androgen action on hippocampus-mediated behaviors. The final study in this dissertation investigated the influence of androgens

on the pattern and magnitude of inducible cellular immediate early gene (cIEG) expression in the rat hippocampus following exposure to a novel open field; a paradigm which stimulates the hippocampus. The induction of hippocampal *c-jun, jun-B* and *zif268* mRNA were not affected by androgen status, however, DHT treatment attenuated, and castration increased, novelty-induced *c-fos* mRNA expression in the CA1 region. These data suggest that AR activation changes the active properties of hippocampal neurons to incoming signals.

In summary, these studies have begun to define the sensitivity of the adult male rat hippocampus to androgens and provide a foundation for further investigation of androgen's roles in hippocampal function and hippocampally-mediated behaviors.

DEDICATION

To Jim and my parents

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ACTH	adrenocorticotropin hormone
ADX	adrenalectomy
AIDS	acquired immune deficiency syndrome
AP-1	activator protein-1
AR	androgen receptor
AR_c	cytosolic androgen receptor
AR_n	nuclear androgen receptor
bp	base pairs
CA1-CA3	Cornu Ammonis 1-3
Ca^{2+}	calcium
cAMP	cyclic 3',5'-adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cIEG	cellular immediate early gene
CNS	central nervous system
CORT	corticosterone
CREB	Ca^{2+}/cAMP-response-element-binding protein
DBD	DNA binding domain
DG	dentate gyrus
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DHTP	5α-dihydrotestosterone propionate

DNA	deoxyribonucleic acid
ER	estrogen receptor
FAAT	foot shock active avoidance training
Fra	Fos related antigen
FSH	follicle stimulating hormone
$GABA_A$	γ-aminobutyric $acid_A$
GDX	gonadectomy
GFAP	glial fibrillary acidic protein
GnRH	gonadotropin releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
h	hour
HPA	hypothalamic-pituitary-adrenal
HPT	hypothalamic-pituitary-testicular
HRE	hormone response element
HSP	heat shock protein
INAH	interstitial nucleus of the anterior hypothalamus
Kb	kilobase
kDa	kilodalton
LH	luteinizing hormone
LTP	long-term potentiation
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NMDA	N-methyl-D-aspartate
PCR	polymerase chain reaction
PR	progesterone receptor

RAF	receptor accessory factor
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
SBD	steroid binding domain
SGP-2	sulfated glycoprotein 2
SHBG	sex hormone binding globulin
SRF	serum response factor
T	testosterone
5'-UTR	5'-untranslated region
VP	ventral prostate

CHAPTER I

INTRODUCTION

Androgens have a wide range of biological effects in peripheral and central tissues that are exerted primarily through the activation of androgen receptors (AR) within target cells. For the most part, studies in the brain have concentrated on androgen action in hypothalamic regions, where testosterone (T) and dihydrotestosterone (DHT) have clear roles controlling aspects of reproductive behavior (Feder, 1984) and hormonal feedback mechanisms (Messi *et al.*, 1988). In recent years, the cloning of AR as well as the development of techniques that provide greater anatomical resolution have led the way to the discovery of abundant AR expression in many areas of the adult mammalian brain. Some of these areas include the amygdala, cortex, striatum and hippocampus (Sar and Stumpf, 1974; Handa *et al.*, 1987a; McLachlan *et al.*, 1991; Burgess and Handa, 1993a; Osada *et al.*, 1993). This widespread localization of AR in the central nervous system (CNS) suggests a much broader physiological importance for androgens than initially anticipated. Furthermore, since AR acts as a ligand-activated transcription factor, thereby increasing or decreasing the transcription of many target genes within a cell, the potential activational effects of androgens in neural tissue are many.

In mammals, gender differences exist not only in the levels of circulating androgen and sex behavior, but also in several non-reproductive behaviors. These

include aggressive tendencies, spatial ability, verbal ability, activity level, and certain cognitive functions. Because adult males produce much higher levels of the gonadal hormone, T, whereas the main circulating hormone in females is estrogen, it has been suggested that these gonadal hormones act in the brain to sexually differentiate behavior throughout life. However, currently there is little information regarding where in the brain or through what mechanism gonadal hormones exert these physiological effects.

Additional evidence implicating androgens as modulators of neural function comes from studies examining human subjects who abuse anabolic-androgenic steroids. Anabolic-androgenic steroids are synthetic variants of the endogenous male hormones, T and DHT. These steroids promote both androgenic (male sexual characteristics) and anabolic (muscle building) effects by specifically binding to intracellular ARs in target tissues. The use of supraphysiologic doses of anabolic-anabolic steroids to enhance athletic performance and physical appearance has become a serious social problem in recent years. In addition to the many peripheral side effects of these drugs, psychiatric evaluations of anabolic steroid abusers have revealed a wide range of adverse emotional and behavioral problems that are closely linked to steroid use or withdrawal (Katz and Pope, 1990; Uzych, 1992). The psychological ramifications of high level androgen use also suggest that some limbic areas of the brain may be sensitive to increasing levels of AR activation. Despite these many reports, little is known about the biological and cellular mechanisms of action of androgens, especially in neural tissues.

The hippocampus, a central structure of the limbic system, has been implicated in influencing a variety of behaviors including learning and memory formation (Teyler and DiScenna, 1985; Whishaw, 1987; Zola-Morgan and Squire, 1990; Eichenbaum and Otto, 1992), emotion (Derryberry and Tucker, 1992), spatial mapping (O'Keefe and Nadel, 1974; Olten, 1977; Olten *et al.*, 1979; Nadel and McDonald, 1980; Sutherland *et al.*, 1982; Bouffard and Jarrard, 1988; Best and Thompson, 1989), and cognition (O'Keefe and Dostrosvsky, 1971). Relatively high levels of AR expression have been detected in the mammalian hippocampal formation (Sar and Stumpf, 1973; McLachlan *et al.*, 1991; Burgess and Handa, 1993a; Kerr *et al.*, 1995), however, their physiological significance is unknown. Recently, androgenic compounds have been shown to influence hippocampus-mediated learning behavior (Flood *et al.*, 1992) and neuronal plasticity of hippocampal pyramidal cells (Pouliot *et al.*, 1995) in rodents. Although cellular mechanisms were not investigated in these studies, the authors suggested that such long-lasting neuronal events may result from AR-mediated modulation of cellular immediate early gene (cIEG) expression or alterations in membrane receptor-meidated actions.

Based on these observations, it was hypothesized that the adult hippocampus is a neural target for androgens. Furthermore, androgens act through the AR to change the basal and active properties of hippocampal pyramidal cells. Thus, either higher than normal levels of circulating androgen or the complete removal of circulating androgen by gonadectomy (GDX) may alter transcriptional activity in these neurons which may lead to changes in neuronal

plasticity or behavior.

The first series of experiments performed for this dissertation characterized AR and AR messenger ribonucleic acid (mRNA) expression in the adult male rat hippocampus using a multidisciplinary approach. AR and AR mRNA levels were quantitated in the hippocampus and compared to levels in other brain and peripheral tissues known to be sensitive to androgens. Saturation analysis of ^{3}H-DHT binding in various brain tissues was performed to determine receptor affinity and compare AR binding characteristics in the cortex, hypothalamus and hippocampus. In addition, the ability of hippocampal AR to regulate its own expression following the removal of circulating androgen in adult male rats and in old male rats who have reduced circulating levels of T was determined.

The second study in this dissertation was designed to investigate the effect of selective AR activation on the expression of the highly and constitutively expressed mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) genes in hippocampal pyramidal and granule cell layers. Both MR and GR are members of the steroid hormone receptor/transcription factor family and are known to mediate many important physiological effects in the hippocampus. Transcriptional cross-talk or interaction between AR and these co-localized, structurally related steroid hormone receptors may mediate some aspects of androgen's actions on hippocampal-mediated behaviors.

The third study in this dissertation investigated the influence of androgens on the pattern and magnitude of inducible cIEG expression in the rat hippocampus

following novel open field exposure, which stimulates the hippocampus. The induction of cIEGs serves as a marker for cellular activation. Thus, androgen modulation of cIEG induction following a stimulus would suggest that AR activation changes the active properties of hippocampal neurons to incoming signals.

Together, these studies have begun to define the sensitivity of the adult male rat hippocampus to androgens and provide the foundation for further investigation into androgen's roles in hippocampal physiology and hippocampal-mediated behaviors.

CHAPTER II

REVIEW OF RELATED LITERATURE

Mechanisms of Androgen Action

The Hypothalamic-Pituitary-Testicular Axis

Androgens have many biological effects on accessory sexual organs, a broad range of effects on metabolic processes, as well as important organizational and activational effects on behavior and cognition. In males, the secretion of androgens from the testes is under tight control by the brain via the hypothalamic-pituitary-testicular (HPT) axis. The closed feedback neuroendocrine loop of this axis consist of several anatomical structures including the central nervous system, the anterior pituitary gland, the testes, and the target organs where androgens ultimately exert their biological effects. As depicted in **figure 1**, the hypothalamus is under positive and negative influences by neurotransmitters from higher brain centers including the cortex and limbic system, as well as auditory, visual and olfactory centers. These signals coordinate the pulsatile release of gonadotropin releasing hormone (GnRH) from the medial basal hypothalamus into the hypophyseal-portal blood system (Belcheltz *et al.*, 1978). GnRH, in turn, regulates the pulsatile secretion of two anterior pituitary gonadotropic hormones, follicle stimulating hormone (FSH) and

luteinizing hormone (LH) (Clayton, 1987). Following secretion, these hormones act directly on the testes to stimulate the production of sex steroids that function locally to promote spermatogenesis or are released into the circulation where they act on many peripheral and central tissues.

The principal hormone of the testes, T, is a C_{19} steroid with a hydroxyl group at the 17 position. As shown in **figure 2**, T is synthesized from cholesterol in Leydig cells and, in humans, is also formed from androstenedione secreted from the adrenal cortex. In adult males, more than 95% of circulating T is of testicular origin and has a normal production rate of approximately 6-7 mg per day (Coffey, 1988). Females secrete very small amounts of T, probably originating from the ovary and adrenal gland (Botella-Llusia *et al.*, 1980; Higuchi and Espey, 1989). T circulates bound to albumin (~33%) and sex hormone binding globulin (SHBG, ~65%) as well as in a free form (~2%). T bound to albumin or in its free form are generally available for end target action, whereas the fraction bound to SHBG is less functionally active (Winters, 1990). In contrast, circulating T in rodents is primarily found in its free form.

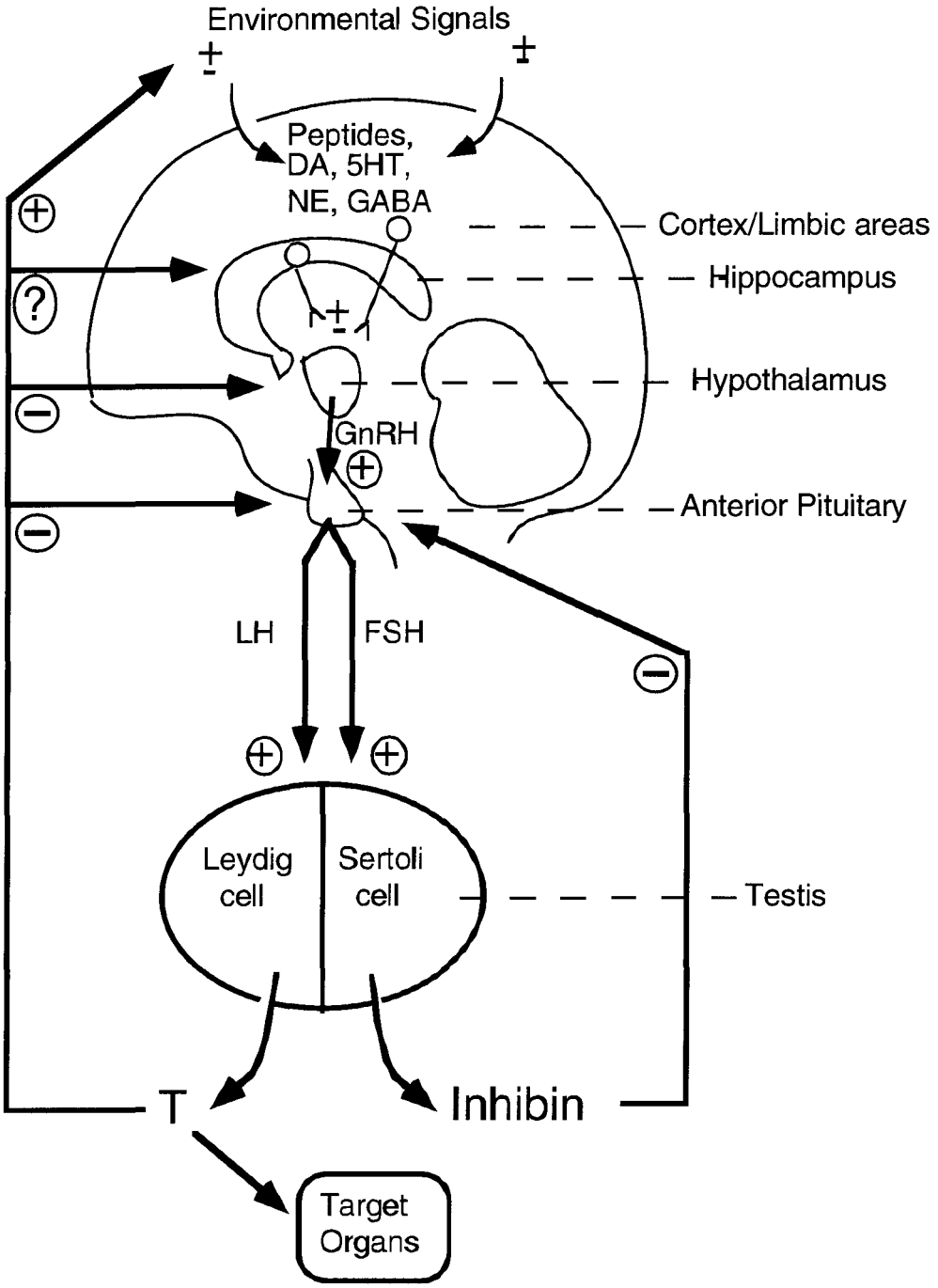

Figure 1. Schematic representation of the hypothalamic-pituitary-testicular (HPT) Axis. (+), stimulatory signals; (-), inhibitory signals; DA, dopamine; 5HT, 5-hydroxytriptamine; NE, norepinephrine; GABA, γ-aminobutyric acid; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone.

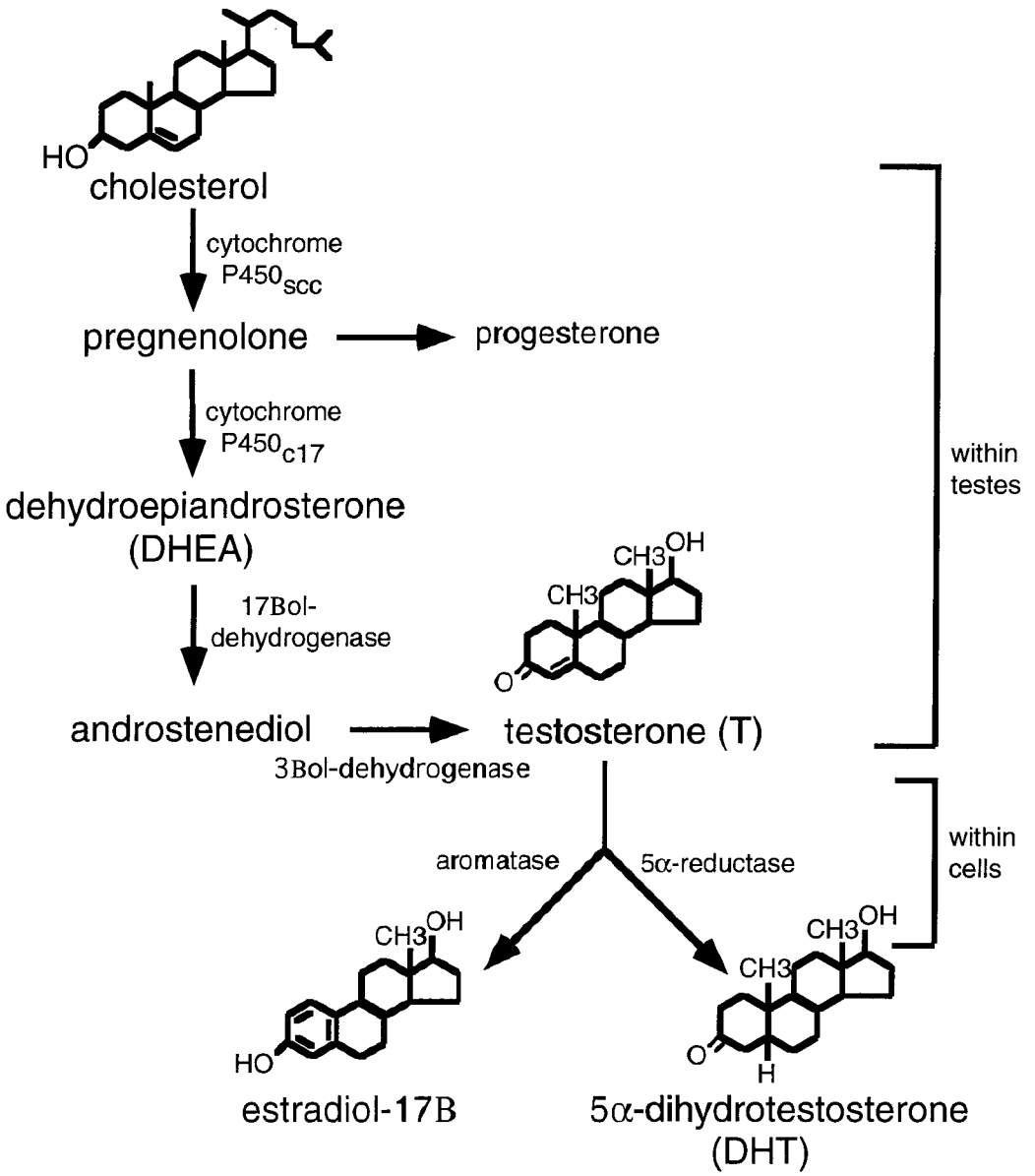

Figure 2. Biosynthetic pathway of testosterone in the testis and the potential active metabolites of testosterone within target cells.

Within target cells, T has several fates. It can directly bind to AR to exert its biological action, or it may be reduced by the intracellular enzyme, 5α-reductase, into DHT which specifically binds to the AR with higher affinity than T (Wilbert *et al.*, 1983). Thus, DHT formation is a way of locally amplifying the action of T in target tissues. DHT is found in the circulation of adult men in levels about one tenth those of T (30 to 80 ng/dl). Alternatively, in some cells where the enzyme aromatase is present, T can be converted into 17ß-estradiol (**see figure 2**). This locally produced estrogen can then interact with estrogen receptors (ER) if present in the cell.

To complete the HPT axis and tightly regulate its own production, T acts at the level of the pituitary (Sheckter *et al.*, 1989), hypothalamus (Messi *et al.*, 1988), and possibly higher brain centers such as the hippocampus to inhibit further production and release of GnRH and LH (**figure 1**). Although T negatively regulates LH secretion, it has little effect on plasma FSH. This differential secretion led to the search for inhibin, a glycoprotein produced by the testes that negatively regulates FSH secretion at the level of the anterior pituitary gland (Abeyawardene and Plant, 1989).

Intracellular Actions of Androgens: The Androgen Receptor

The magnitude of T action in target cells is determined by various factors including: the amount of diffusion of free hormone into the cells, the extent of metabolic conversions within the cells, the number of receptor proteins available for interaction with the steroids, and finally, receptor action at the transcriptional level. The transcriptional actions of androgens (T and DHT) in both peripheral and central

tissues have been linked in part to their ability to specifically bind and activate AR. The AR is a member of a superfamily of nuclear transcription factors which also includes other steroid hormone receptors such as the GR, MR, ER, thyroid hormone, and progesterone receptor (PR) (Evans, 1988). This structurally related superfamily also includes receptors for vitamin D, retinoic acid, as well as the newly described orphan receptors which share amino acid sequence homology of steroid hormone receptors but for which no known ligands have been found (Ribeiro *et al.*, 1995). All of these receptors when bound by ligand influence gene transcription via direct interactions with deoxyribonucleic acid (DNA) (Godowski and Picard, 1989). Protein chemistry (Wrange and Gustafsson, 1978; Carlstedt-Duke *et al.*, 1988) and complementary DNA (cDNA) cloning studies (Hollenberg *et al.*, 1985; Kumar *et al.*, 1986; Rusconi and Yamamoto, 1987; Lubahn *et al.*, 1988) have confirmed that each member of the steroid hormone receptor superfamily is structurally organized into at least three specific domains: a highly variable N-terminal region thought to be involved in transcriptional activation, a short and well-conserved cysteine-rich central domain responsible for DNA binding, and a high homology C-terminal end necessary for binding with a specific steroid hormone (Evans, 1988). The domain structure of AR will be discussed in more detail later in this review (see pp 36-42).

Despite the diversity of androgen target tissues, the basic sequence of events leading to androgen's effects on gene transcription are thought to be consistent from tissue to tissue. AR follows the traditional model of steroid action (O'Malley and Tsai, 1992; Tsai and O'Malley, 1994) as diagrammed in **figure 3**. This pathway

involves the passive diffusion of T into cells where it either binds directly to AR or is first enzymatically converted into DHT. Once T or DHT binds to the AR, the protein undergoes a conformational change and chaperone proteins, such as the 90 kDa heat shock protein (HSP90), dissociate from the receptor (Marivoet *et al.*, 1992). This transformation process exposes dimerization motifs and a zinc-finger DNA binding domain within the AR molecule. As a result, AR has the propensity for homodimerization with a second activated AR and it is this homodimer that has a high affinity for DNA (Forman and Samuels, 1990a; Truss and Beato, 1993; Wong *et al.*, 1993). Specifically, the activated AR complex binds to specific DNA sequences, termed hormone response elements (HREs), which flank target genes (Beato, 1989). Once anchored to the HRE, the complex is capable of modulating transcriptional activity either in a positive or negative fashion (Rundlett *et al.*, 1990). The activated DNA-bound receptor does not act alone to regulate transcription of a target gene, but rather secures a complex arrangement of specific stabilizing proteins, transcription factors and ribonucleic acid (RNA) polymerases which act together to ultimately increase or suppress the transcription process (Rundlett *et al.*, 1990; Adler *et al.*, 1993; Kupfer *et al.*, 1993). These events occur as quickly as 5 minutes after steroid injection into an animal, but measurable changes in steady state mRNA levels may take between 15 min and several hours (Spelsberg *et al.*, 1989).

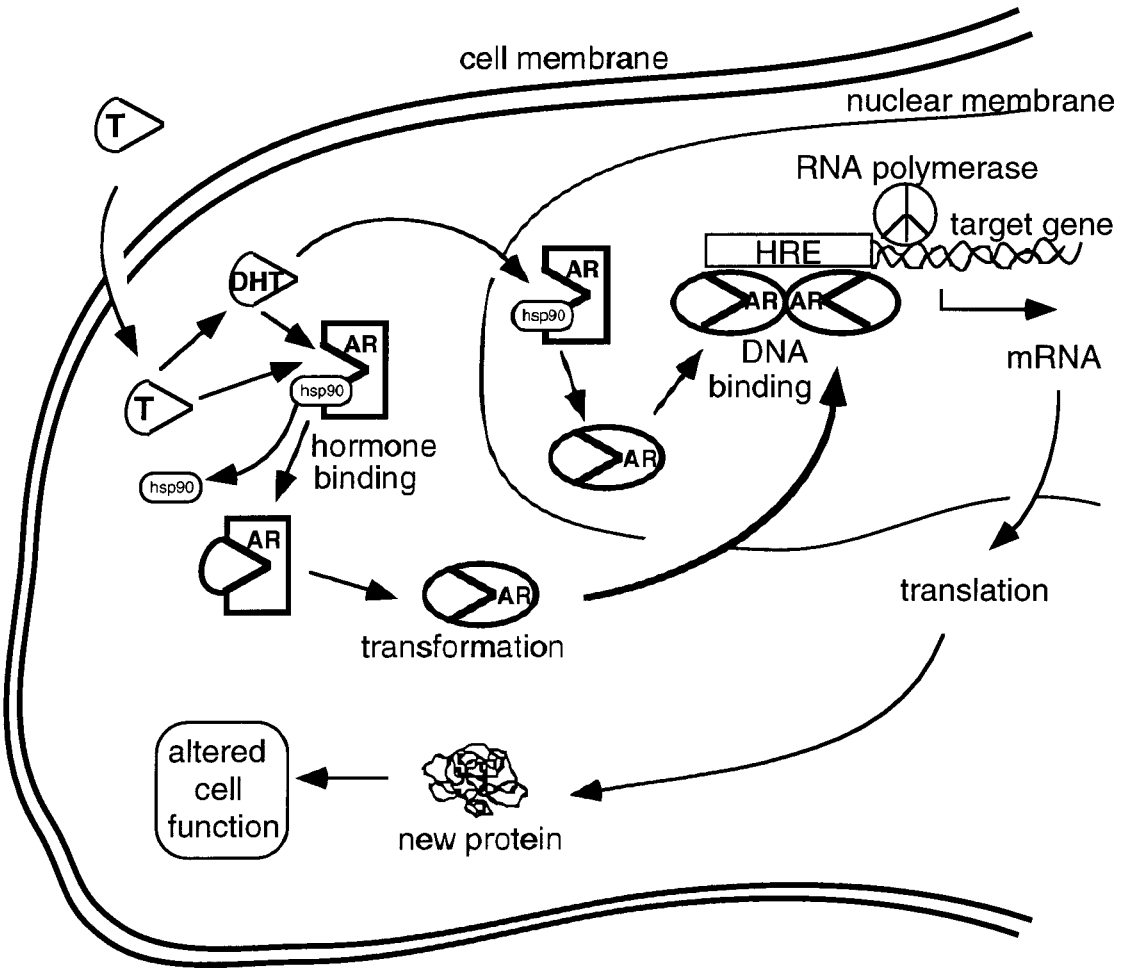

Figure 3. Schematic representation of the molecular pathway for androgen action in target cells. T, testosterone; DHT, dihydrotestosterone; AR, androgen receptor; HRE, hormone response element; hsp90, heat shock protein.

Non-Genomic Actions of Androgens

It has become apparent in recent years that not all actions of sex hormones involve "slow" gene transcription regulation. Rapid effects of steroid hormones and steroid precursor molecules on electrophysiological and neurochemical parameters have been reported (reviewed by McEwen, 1991). Although unique membrane receptors for steroid hormones have yet to be found, it is has been shown that some steroids allostericly interact with the γ-aminobutyric acid$_A$ ($GABA_A$) ligand-gated ion channel receptor and modulate its activity (Majewski, 1992). The most potent naturally occurring steroids with allosteric $GABA_A$-agonistic features are tetrahydroprogesterone, tetrahydrodeoxycorticosterone, and the T precursor, androsterone (Majewski *et al.*, 1986; Lambert *et al.*, 1987; Turner *et al.*, 1989). In contrast, some steroid molecules behave as noncompetitive antagonists at this receptor. Pregnenolone sulfate and the sulfate derivative of the T precursor, dehydroepiandrosterone (DHEA) belong to this latter category (Majewski and Schwartz, 1987; Mienville and Vicini, 1989; Majewski *et al.*, 1990). Interestingly, these latter compounds have been found to be synthesized *de novo* locally within the brain at concentrations much greater than those in plasma (LaCroix *et al.*, 1987). Thus, these neuroactive steroid metabolites and precursors have been termed "neurosteroids" (Baulieu and Robel, 1990).

Most recently, two anabolic-androgenic steroids, stanozolol and 17α-methyltestosterone, were found to modulate benzodiazepine binding to the $GABA_A$ receptor in the male and female rodent brain (Masonis and McCarthy, 1995). This

was the first report of direct membrane-bound receptor effects of androgens that are also known activators of intracellular ARs. The authors speculate that these membrane-bound receptor effects may account for some of the psychotropic responses following high doses of anabolic steroids. These findings certainly leave the door open for possible rapid membrane-bound receptor effects of T and DHT, however, to date, such reports are few (Teyler *et al.*, 1980).

Localization of Androgen Receptors

AR expression has been detected in a wide range of tissues by various methods including *in vivo* autoradiography with radioactive T or DHT, *in vitro* binding assays, *in situ* hybridization, polymerase chain reaction (PCR), and immunocytochemistry. In peripheral tissues, AR expression has been found in accessory male sex glands (e.g. ventral prostate (VP), seminal vesicles, and epididymis; Mangan *et al.*, 1968; Anderson and Liao, 1968; Sar *et al.*, 1970; Hansson and Tveter, 1971; Husmann *et al.*, 1990; Prins *et al.*, 1991; Blok *et al.*, 1992a), skeletal muscle (Saartok *et al.*, 1984), male external genitalia (e.g. penis and testes; Takane *et al.*, 1990; Blok *et al.*, 1991, 1992a), bone (Colvard *et al.*, 1989), adrenal gland (Osada *et al.*, 1993), uterus (Giannopoulos, 1973), as well as several other organs (e.g. kidney, lung, and liver; Roy *et al.*, 1974; Dubé and Tremblay, 1974) and glands (e.g. anterior pituitary, sweat, and sebaceous; Choudhry *et al.*, 1992; Osada *et al.*, 1993). This anatomical

distribution coincides with the regions known to mediate important peripheral androgen-dependent functions such as the development and maintenance of the male genitalia and secondary sex characteristics, hypertrophy of skeletal muscle, spermatogenesis, mineralization of bone and male-patterned hair growth (or loss) (Winters, 1990).

Many studies have also localized AR expression to specific areas of the CNS including the hypothalamus, medial preoptic area, cortex, amygdala, thalamus, bed nucleus of the stria terminalis, hippocampus, motor nuclei and brain stem (Sar and Stumpf, 1973, 1974; Barley *et al.*, 1975; Handa *et al.*, 1986, 1987a; Roselli *et al.*, 1989; Simerly *et al.*, 1990; Sarrieau *et al.*, 1990; Clancy *et al.*, 1992, 1994; Burgess and Handa, 1993a; Osada *et al.*, 1993). Several studies have confirmed that the distribution of AR mRNA in the brain and peripheral tissues match the distribution of the AR protein (Simerly *et al.*, 1990; Quarmby *et al.*, 1990; Takane *et al.*, 1991; Blok *et al.*, 1992a; Menard and Harlan, 1993). Most studies have focused on the areas of the brain involved in reproductive behaviors or endocrine feedback mechanisms. The reports of AR expression in extrahypothalamic regions such as the hippocampus and cortex have been meager. Interestingly, studies have found no dramatic sexual differences in AR mRNA distribution or AR binding levels in the adult rat brain (Simerly *et al.*, 1990; Handa *et al.*, 1986). Together, these findings suggest an important role of androgens in CNS function. An overview of androgen action in the brain will be covered in the following section.

As methods to detect AR have become more sensitive, it has become harder to

find tissues that fail to express AR at some level. However, a few tissues, including the spleen, are considered to be AR negative (Takada *et al.*, 1990; Osada *et al.*, 1993). Thus, it appears that sensitivity to androgens may be a function of the changing AR level in cells and the hormonal milieu, than strictly the presence or absence of AR expression.

Physiological Actions of Androgens in the CNS: Organizational Versus Activational Effects

Such widespread localization of AR in the brain suggests that androgens influence the action of most neurons (Mooradian *et al.*, 1987). Typically, the physiologic effects of gonadal steroids have been divided into those that are organizational, which occur during fetal development and the early neonatal period, and those that are activational, which occur later in life (Pheonix *et al.*, 1959, Young *et al.*, 1964). The former effects are considered relatively permanent changes in the size or connectivity of neural pathways, metabolism or steroid responsiveness of neurons and result in the development of sexually dimorphic brain structures and sex-typed behavior (Arnold and Breedlove, 1985). For example, in rodents, the amount and timing of gonadal steroid release in the perinatal period determines whether the male copulatory behavior, mounting, or the female behavior, lordosis, will manifest in adulthood (Sodersten, 1978). A possible correlate for this behavioral change

comes from studies demonstrating that androgen exposure in the late fetal or early neonatal period in the male leads to the enlargement of a sexually dimorphic preoptic-anterior hypothalamic nucleus (Gorski *et al.*, 1978). Additionally, castration of male fetuses or neonatal male rats results in a decrease in size of this nucleus and corresponding changes in sexual behavior (Raisman and Field, 1973; Arnold and Gorski, 1984). In humans, LeVay (1991) reported a sex difference in one of several interstitial nuclei of the anterior hypothalamus, termed INAH-3. His finding that INAH-3 was larger in healthy heterosexual men compared to healthy females or homosexual men with acquired immunodeficiency syndrome (AIDS) suggested that homosexual men may have brain organizational development closer to that of women than men. Some researchers have argued that these findings in homosexual men may have resulted from AIDS-related pathology, so studies are currently underway in homosexual men who have died from other causes. Several other studies have reported significant structural differences in male and female brain anatomy that may be the result of hormonal influences in early development and may account for some of the sexually dimorphic behaviors discussed below (Swaab and Fliers, 1985; Holloway and de Lascoste, 1986; Allen and Gorski, 1986, 1987; Allen *et al.*, 1989). Although the relative contribution of androgen binding to AR or the necessity of aromatization to estrogen and thus, ER action, to brain organizational processes is still a matter of debate (Feder, 1984; Breedlove, 1992), it appears that AR activation plays some part in the hard wiring of neuronal circuits during development (Goldfoot and van der Werff ten Bosch, 1975; Baum *et al.*, 1982; Meaney and McEwen, 1986).

Studies examining the activational effects of androgens on neural systems throughout puberty and adulthood have lagged behind the studies pinpointing hormonal influences during development. This is partially due to the ambiguity of measurable endpoints such as "motivation", "emotion", "spatial ability" and "learning" in animal models and also partially due to the difficulty in removing external environmental influences that may compensate for the effects of steroids. Additionally, some of the discrete functions of androgens cannot be assigned to one particular brain region, which makes studying the relationship between androgen action and behavior more difficult.

Hormonal effects in adulthood are termed "activational" because they activate neural pathways which are already present and presumably, relatively static in nature. In general, activational effects are considered transient and fluctuate in accordance with the level of circulating hormone. For example, in the rodent, the expression of male sexual behavior is partially dependent on the appropriate circulating levels of androgens, as castration of the adult male rat eliminates or reduces the frequency of male sexual behavior, and the administration of T can reinstate the sexual response to the appropriate sensory cues (Mitchell and Stewart, 1989; Baum, 1992). The link between circulating androgen levels and sex behavior in humans is more tenuous. Although castration has shown to reduce libido, this varies dramatically among individuals (Carter, 1992). Studies of sexual behavior in normal men is difficult as well. Certainly, sexual behavior is under the control of powerful external influences, such as partner preference and sexual partner availability. In every day life, these

influences may overcome individual variations in circulating hormone levels to control sexual behavior patterns.

Like sexual behavior, the effect of androgens on aggression, appears to have organizational and activational components (Swerdloff *et al.*, 1992). Male laboratory animals typically exhibit more aggressive behaviors than females (see review by Beatty, 1984). This sex difference is controlled by the presence or absence of T during certain critical developmental periods, as well as during puberty and adulthood. Edwards (1968) showed that male mice were relatively nonaggressive if castrated during early life and given androgen replacement therapy in adulthood. He also showed that genetically female mice would become as hostile as male mice if given T during fetal development and into adulthood. The development of aggression in male mice corresponds to the increases in circulating T at the time of puberty (McKinney and Desjardins, 1973; Gandelman, 1973). Adult castration reduces this behavior, while T administration restores it (Gandelman and vom Saal, 1975). Female rodents also display aggressive behaviors if given T in adulthood, however the administration of very high levels of androgen for prolonged periods was necessary to consistently elicit the response (Svare *et al.*, 1974; Barkley and Goldman, 1977). These studies suggest that the female rodent brain architecture is capable of responding to androgen but is generally less sensitive to the stimulus. Several reports indicate that both DHT and estrogen are important in stimulating intermale aggression and infanticide (Finney and Erpino, 1976; Svare, 1979) suggesting that both AR and ER activation are influential in the process.

Research examining the involvement of androgens in human aggressive behavior is somewhat more limited and, at best, correlative in nature. Studying healthy young males, Persky *et al.* (1971) showed a positive correlation between circulating levels of T and measures of dominance, hostility and aggressive behavior. Another study reported no such correlations (Brown and Davis, 1975). More recently, reports of violent behaviors associated with the abuse of anabolic-androgenic steroids (Strauss *et al.*, 1982; Haupt and Rovere, 1984; Lubell, 1989; Telander and Noden, 1989; Svare, 1990) also suggest some correlation between circulating androgen level and aggressive behavior in humans. It is still unclear as to the exact areas of the CNS most involved in the expression of aggressive behavior, however, the amygdala (Luiten *et al.*, 1985; Meaney and McEwen, 1986), hypothalamus and hippocampus (Siegal and Edinger, 1983) have been implicated.

Other behaviors that have been found to be modified by androgen action in the CNS include activity level (Broida and Svare, 1984), food intake (Bell and Zucker, 1971), sensation and perception (Pietras and Moulton, 1974), mood (Pope and Katz, 1988), and learning (Beatty and Beatty, 1970; Chambers, 1976; Flood *et al.*, 1992). As with aggressive behavior, the brain areas most associated with these functions have not been well defined. Despite this, such widespread effects of androgens on many defined behaviors implicate a physiological role for AR in higher brain centers such as the cortex and hippocampus.

The effects of sex steroids exclusively on hippocampal mediated behaviors and physiology are discussed below.

Hippocampal Structure and Function: Effects of Gonadal Hormones

Anatomy of the Hippocampus

The hippocampus is a centrally-located component of the limbic system and has been implicated in wide variety of behaviors. In the rodent, the hippocampus is a cashew-shaped structure situated along the curvature of the lateral ventricle (**figure 4**). Early neuroanatomists thought that the hippocampus resembled a seahorse, which is how it got its name (hippocampus is Greek for seahorse).

The cellular organization of the hippocampus is relatively simple in comparison to other brain regions which makes it uniquely suited for electrophysiologic study. The hippocampus proper is composed of three regions: the subiculum (adjacent to the entorhinal cortex), the Cornu Ammonis (CA1-CA3) pyramidal cell regions, and the dentate gyrus (DG) which contains granule cells. Pyramidal cells are found in a narrow layer, 3-5 cells thick, extending the length of the horn. The CA1 field is composed of densely-packed, medium-sized cells. The CA2 and CA3 regions contain larger, more loosely packed cells. The cells of the CA2 region differ from those of the CA3 field; they do not have dendritic spines on their apical dendrites. The DG contains one layer of very compacted granule cells stacked 4-10 cells deep (Amaral and Witter, 1989). Although this area has been best studied in the rodent model, the same basic pattern of organization is found in higher species.

Studies examining the connectivity of hippocampal neurons have revealed a

"trisynaptic circuit" with readily identifiable cell populations (reviewed by Amaral and Witter, 1989). The location, internal circuitry and defined regions of the rat hippocampus are depicted in **figure 4.** The main input to the hippocampus comes through the perforant path from the overlying region of the cortex, called the entorhinal cortex. Stimulation of the entorhinal inputs (perforant path) results in the activation of granule cells in the DG. These cells, in turn, activate the pyramidal cells of the CA3 region through the mossy fiber system. The axons of the CA3 pyramidal cells bifurcate, sending efferent stimuli out through the fimbria to the fornix as well as sending collateral branches (Schaffer collaterals) which synapse on the apical dendrites of the CA1 pyramidal cells. The efferents arising from CA1 pyramidal cells and exiting to the subiculum provide the major output for the hippocampal formation and complete the unidirectional open-loop circuit. These intrinsic connections have been verified electrophysiologically (Swanson *et al.*, 1982). As currently understood, this loop is important in receiving information and integrating the outgoing signals from the hippocampus. Therefore, interruption of this loop at any point might ultimately disrupt or alter function.

The extrinsic projections of the CA1 field are extensive and include the subiculum, lateral septal nucleus, olfactory bulb, nucleus accumbens, perirhinal cortex, prefrontal cortex, amygdala, hypothalamus, and the contralateral hippocampus (Swanson and Cowan, 1977; Van Groen and Wyss, 1990). In addition, the projections of the CA1 field are topographically organized with the septal third of CA1 projecting to different cortical regions than the temporal third of CA1 (Van

Groen and Wyss, 1990). Interestingly, many of these projections to the neocortex are reciprocal and enable this structure to ultimately compare and integrate incoming information with previously stored information (Squire and Zola-Morgan, 1988). Hippocampal afferents are as widespread as it's efferents. Neurons from every level of the diencephalon and brainstem project directly to some part of the hippocampus (Wyss *et al.*, 1979).

After examining the extensive connections of the hippocampus, it is not surprising that this structure has been implicated in a variety of behavioral functions. These include emotion (Derryberry and Tucker, 1992), motivation (Jarrard, 1973), memory and learning (Teyler and DiScenna, 1985; Whishaw, 1987; Zola-Morgan and Squire, 1990; Eichenbaum and Otto, 1992), spatial mapping (O'Keefe and Nadel, 1974; Olten, 1977; Olten *et al.*, 1979; Nadel and McDonald, 1980; Sutherland *et al.*, 1982; Bouffard and Jarrard, 1988; Best and Thompson, 1989), and cognition (O'Keefe and Dostrosvsky, 1971). The hippocampus has also been implicated as an important target for neuronal hormonal feedback regulation (reviewed by Jacobson and Sapolsky, 1991; Morano *et al.*, 1994).

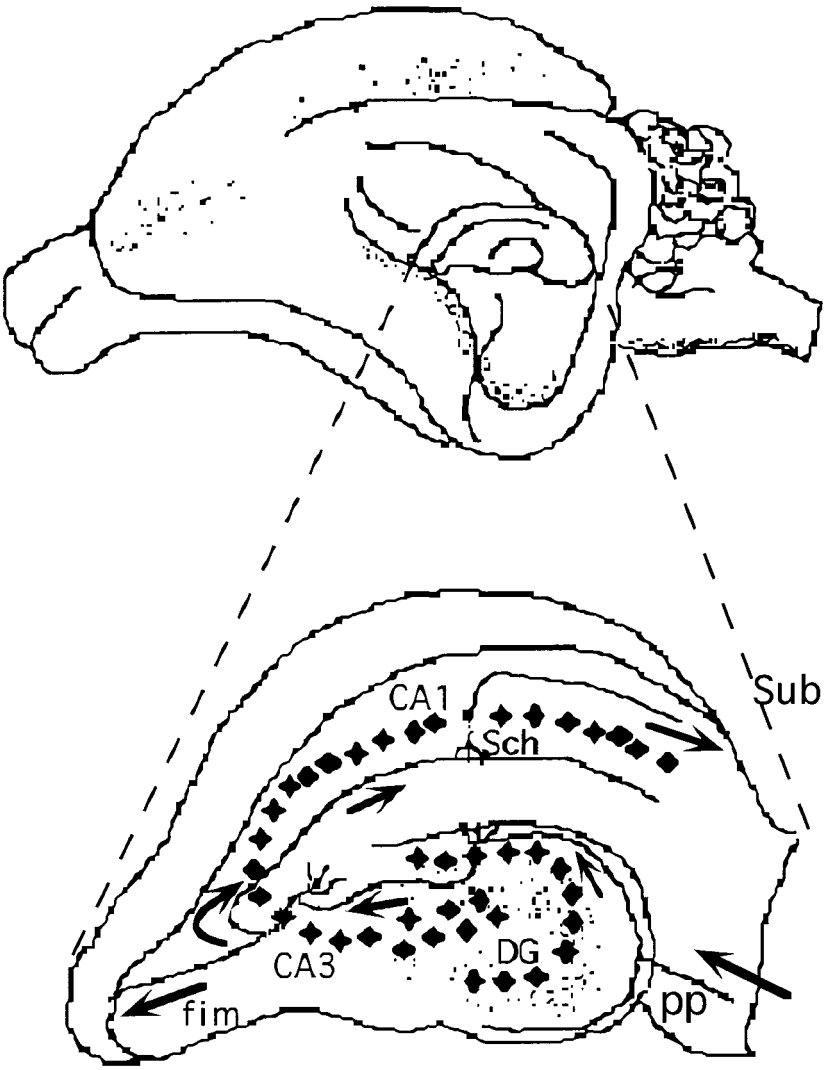

Figure 4. The hippocampus in the rat brain. The top illustration shows the general position of the hippocampus in the rat brain. A coronal section of one half of the hippocampus is enlarged to depict the cell body fields and trisynaptic circuit. The perforant pathway (pp) arrives from the overlying cortex and perforates the dentate gyrus (DG). The mossy fibers of the DG synapse on CA3 pyramidal cells which send Schaffer collaterals (Sch) that either exit through the fimbria (fim) or synapse onto the apical dendrites of CA1 pyramidal cells. Efferents from CA1 neurons exit to the subiculum (Sub) to complete the circuit.

Sexual Dimorphisms in Hippocampal Structure: Organizational Androgens

As earlier described for certain hypothalamic nuclei, many sex differences in the mammalian CNS are developmentally influenced by androgens. There is a growing body of literature demonstrating a relationship between gonadal hormones and gender differences in spatial ability (Beatty, 1984; Gaulin and Fitzgerald, 1986; Roof, 1993; Luine, 1994). Unfortunately, there are few studies describing the possible anatomical substrates through which gonadal hormones may produce this effect. The hippocampus is a likely candidate due to its proposed involvement in spatial navigation (O'Keefe and Nadel, 1974; Olten, 1977; Olten *et al.*, 1979; Nadel and McDonald, 1980; Sutherland *et al.*, 1982, 1983; Bouffard and Jarrard, 1988; Best and Thompson, 1989) as well as its sensitivity to hormones during development (Pfaff, 1966; O'Keefe and Handa, 1990). Studies have found that the levels of several neurotransmitters, and their receptors, in the hippocampal formation are sexually dimorphic (reviewed by Loy, 1986) and could contribute to sex differences in behavior later in life. Several other studies have demonstrated morphological differences in the hippocampi of male and female rodents. Unfortunately, the measurements used by the various investigators are somewhat convoluted and, thus, the data are difficult to interpret. Wilmer and Wilmer (1985) showed that, in certain strains of mice, females had fewer granule cells than males. Yanai (1979) did not observe this dimorphism in rats. Juraska and colleagues have made extensive size measurements of hippocampal granule neuron dendritic arbors that are believed to reflect the number of synapses (reviewed by Juraska, 1991). Subtle differences

between similarly treated, postpubertal male and female littermates were observed, but more interestingly, the change in the size of dendritic tree branching was in opposite directions following environmental enrichment of male and female rats (Juraska *et al.*, 1985). Castration of male rats at birth resulted in female-like dendritic branching patterns of granule cells and suggested that T, acting either during early life or at puberty alters dendritic tree plasticity (Juraska *et al.*, 1988). Recently, Roof (1993) also reported a sexual dimorphism in the DG of pre-pubescent rats. In this study, the granule cell layer of male and T-treated (at postnatal days 3 and 5) females was 8-9% greater in width and length and was asymmetrical as compared to untreated females. Additionally, the size of the DG correlated with performance on a spatial task (Morris water maze). Overall, males with the larger DG layer performed better. These anatomical and behavioral differences were still present in adult rats similarly treated soon after birth (Roof and Havens, 1992). Since little to no AR or ER expression have been found in the DG of the adult rat hippocampus (Stumpf and Sar, 1978; Loy *et al.*, 1988; Maggi *et al.*, 1989; Simerly *et al.*, 1990), it is not clear how the effects on granule cells manifest. Most speculate that they are a function of transsynaptic influences, however, there is little information on the distribution of AR and ER expression in the hippocampus during development.

Strangely, few studies have examined other cell body regions of the hippocampus for sexual dimorphisms. Meyer *et al.* (1978) demonstrated that pre-puberal castration of male rats resulted in an altered number of synaptic spines on CA1 pyramidal cells, but not on granule cells of the DG. Since hippocampal AR and

ER are highly expressed in the CA1 region (Simerly *et al.*, 1990; Maggi *et al.*, 1989), Meyer's findings could be attributed to a more direct effect of androgen or estrogen on hippocampal dendritic morphology.

Whether such differences in hippocampal structure occur in humans and play a part in the well documented sex differences in spatial abilities and verbal skills (Jarvik, 1975; Kimura, 1992) has yet to be elucidated and will be difficult to ascertain because of the inability to manipulate the hormonal milieu in the human fetus. Some clues have come from female fetuses exposed to high levels of adrenal androgens due to congenital adrenal hyperplasia and those unknowingly exposed to the synthetic estrogen, diethylstilbestrol. Later in life, these girls exhibited "masculinized" behavior patterns such as superior spatial skills and lower verbal I.Q. scores (Hines and Shipley, 1984; Resnick *et al.*, 1986; Nass and Baker, 1991). Although far from conclusive, these data do implicate gonadal hormones in the hard-wiring of higher neuronal systems, of which, the hippocampus is a likely candidate.

Hippocampal Neuronal Plasticity

No one has established conclusively how the brain forms new memories or generates such complex outcomes as emotion or cognition. However, it is known that neurons, especially those in the hippocampus, can change their pattern of dendritic synaptic connections and/or electrophysiological responses following a learning experience (Doyere *et al.*, 1993; Lisman and Harris, 1993) or damage (Onodera *et al.*, 1990; Levisohn and Isacson, 1991). These changes are referred to collectively as

neuronal plasticity. The role gonadal hormones play in neuronal plasticity in areas of the brain not associated with reproduction are just beginning to be explored.

The actions of estrogen, the primary circulating gonadal hormone in females, have been studied within the rat hippocampal formation to a greater extent than androgen. Striking examples of estrogen action in the adult rat hippocampus were demonstrated by Woolley and McEwen (1992, 1993) and Gould *et al.* (1990). These investigators found that estrogen increased the dendritic spine density of CA1 neurons and that these changes fluctuated in accordance to the 4-5 day menstrual cycle of the adult female rat. In addition, the removal of circulating estrogen by ovariectomy resulted in dramatic decreases in dendritic spine density. These effects were specific for CA1 pyramidal cells, as ovariectomy or steroid replacement did not affect spine density in CA3 pyramidal cells or granule cells of the DG. Similar changes in spine density in response to estrogen have been described in ventromedial hypothalamic neurons (Frankfurt *et al.*, 1990); an area where estrogen likely acts to control some aspects of reproductive behavior and hormone secretion. Since changes in the number or size of hippocampal dendritic spines appears to be correlated with changes in the synapses that they receive, as well as with altered neuronal electrophysiology (Chang and Greenough, 1984), and possibly the modification of behavior or learning (Purpura, 1974; DeVoogd *et al.*, 1985; Popov and Bocharova, 1992), these studies provide an exciting anatomical correlate to fluctuating behavior patterns and sexually dimorphic behavior in adulthood. Interestingly, women did not perform as well on certain spatial tasks during the preovulatory estrogen surge as compared to other times

of the menstrual cycle characterized by lower estradiol levels (Komenich *et al.*, 1978; Hampson and Kimura, 1988, 1992; Hampson, 1990). Although the morphological alterations in hippocampal neurons in response to estrogen have only been demonstrated in a female rat model, these data provide a strong basis for establishing the relationship between circulating gonadal hormones and behavior.

In addition to the modulation of dendritic spine density, estrogens have been found to rapidly (within 20 minutes) alter CA1 cell neuronal excitability in response to the stimulation of glutaminergic Schaffer collaterals (Teyler *et al.*, 1980; Wong and Moss, 1992). Additionally, two days following subcutaneous estrogen injections, both glutamate and GABA receptor binding were upregulated in this area (Schumacher *et al.*, 1989; Weiland, 1992). These effects may contribute to estrogens known part in the lowering of the threshold for seizures originating in the hippocampus (Terasawa and Timiras, 1968). There is also a considerable amount of evidence to suggest that estrogen enhances mood in women (Ditkoff *et al.*, 1991; Palinkas and Barrett-Conner, 1992; Sherwin, 1994). Although the mechanisms underlying this effect have not been investigated, areas in the limbic system, including the hippocampus, are likely targets for estrogen action. Taken together, these studies further indicate that estrogen has long term activational effects on hippocampal physiology.

Studies examining androgen effects on hippocampal plasticity have not been as plentiful as those performed with estrogen, even though it appears that the hippocampus contains a higher concentration of AR mRNA than ER mRNA (Simerly *et al.*, 1990). Flood and Roberts (1988) demonstrated that a single subcutaneous

injection of the largely adrenal-derived, androgenic precursor steroid, DHEA (see **figure 2**), as well as its sulfated derivative, DHEAS, strikingly improved T-maze footshock active avoidance training (FAAT) in middle-aged and old mice. The same group later showed that immediate post-training intracerebroventricular administration of various androgenic compounds including pregnenolone, DHEA, androstenedione, T and DHT all improved retention for FAAT (Flood *et al.*, 1992). The authors have speculated that the memory-enhancing effects of steroids lasting long after fast neural events have ceased may have been through their modulation of the rate of transcription of cIEGs. In contrast, Goudsmit *et al.* (1990) found that T administration did not reverse age-related spatial memory deficits in rats and may actually impair retention in middle-aged rats. Clark *et al.* (1995) also did not observe any improvements or impairments in the acquisition or retention of the Morris water maze when male rats were given high levels of anabolic-androgenic steroids for 12 weeks. These conflicting data are difficult to resolve since there is very little consistency in the length and mode of androgen administration, as well as in the behavioral "learning" tasks employed.

The underlying cellular mechanisms of androgen action on hippocampal physiology are just beginning to be explored. For example, Kus *et al.* (1995) have found that treatment of castrated adult male rats with the AR-selective androgen, dihydrotestosterone propionate (DHTP), decreased the binding of the N-methyl-D-aspartate (NMDA) receptor antagonist, [^{3}H]MK-801, in CA1 pyramidal cells. NMDA receptors are known to mediate the actions of glutamate, the major excitatory

neurotransmitter in the hippocampus and, in particular, the Schaffer collaterals synapsing on CA1 pyramidal cells. Although far from being well understood, these data suggest that *in vivo* AR activation can alter normal adult hippocampal physiology and may affect learning.

The most direct evidence for androgenic effects on hippocampal neurons has been demonstrated using an *in vitro* hippocampal slice preparation. Gonadal steroids have been shown to increase neuronal excitability in the female rodent hippocampal CA1 pyramidal cells (Teyler *et al.*, 1980). However, in this study no consistent effects were found in males. In conjunction with studies being performed in our laboratory, Pouliot *et al.* (1995) demonstrated that DHTP prevents NMDA's excitotoxic electrophysiologic effects in CA1 pyramidal cells. These events are likely AR mediated since the effects were only observed after several hours of androgen treatment. These findings are consistent with the NMDA receptor binding studies of Kus *et al.* (1995) and may be an important underlying mechanism for behavior since it has been shown that the activation of hippocampal glutamate receptors mediate processes involved in the synaptic plasticity associated with learning and memory (Morris *et al.*, 1986; Tonkiss *et al.*, 1988) epileptogenesis (Gilbert, 1988) and schizophrenia (Collinge and Curtis, 1991).

The effects of androgens on hippocampal physiology are also seen following damage. When the hippocampus is deafferented, the surviving neurons rapidly form new branches and new connections to compensate for the loss. This process is called sprouting and is considered another form of neuronal plasticity. Since learning is also

believed to utilize this type of plasticity, it is a convenient model to employ when examining the role of gonadal hormones in cognition. As had been found with dendritic branching in the undamaged rat hippocampus, estrogen was found to be critical for maintaining the sprouting response to differentiation in females (Morse *et al.*, 1992; Scheff *et al.*, 1988a). Androgens did not appear to act similarly in males. Neither castration nor adrenalectomy alone had any effect on the sprouting response (Scheff *et al.*, 1988b; Scheff and Dekosky, 1989). However, if males were both castrated and adrenalectomized, sprouting was decreased. In this instance, it appears that the hormones secreted from the gonads and adrenal gland serve complementary functions which maintain sprouting. The interaction of gonadal and adrenal derived hormones should likely be taken into greater consideration when examining effects in the hippocampus since this area is rich in several types of steroid receptors (Van Eekelen *et al.*, 1988; Simerly *et al.*, 1990; Kerr *et al.*, 1995a).

Few studies have examined the direct effects of androgens on human adult hippocampal function probably due to the fact that men do not show large natural fluctuations in circulating T levels over a set period of time (unlike women whose estrogen levels fluctuate throughout the menstrual cycle). Thus, correlating androgen levels and behavior is much harder in men. In spite of these limitations, Hampson and Kimura (1988) found that spatial reasoning in men varied in relation to small yearly fluctuations of their circulating T levels. Additionally, studies in men have demonstrated a positive correlation between circulating T levels and spatial ability, cheerfulness, and some mood traits (Adler *et al.*, 1986; Hubert, 1990). A negative

correlation between T and DHT levels and verbal ability (O'Carroll *et al.*, 1985; Christianse and Krussman, 1987) and anxiety (Altschuler *et al.*, 1990) in men has also been reported. Interestingly, women having above average plasma levels of free T scored lower on a visual memory task; this task is typically performed better by women than men (Phillips and Sherwin, 1991). These data fit well with a comparison of spatial ability, mathematical reasoning, and perceptual speed in both men and women revealing that women with higher than normal T levels, and men with lower than average T levels, performed best on tests which are hippocampally mediated (Shute *et al.*, 1983; Gouchie and Kimura, 1991; Kimura, 1992). These data suggest that an undefined "optimum" level of T is required for superior cognitive function and that either too little or too much is detrimental to performance. In support of these studies, T supplementation to older men, who naturally have up to a 40% decline in free circulating T levels (Davidson *et al.*, 1983; Vermeulen, 1991), has proved beneficial for spatial cognition, but was not effective in the improvement of verbal or visual memory, motor speed, cognitive flexibility, or mood (Janowski *et al.*, 1994). This latter study also implies that the hippocampus remains sensitive to androgens during aging.

Many of the behavioral studies mentioned in this section suggest subtle activational effects of androgen on hippocampal function throughout life, however, few actually pinpoint the exact location of the androgenic effect and do not fully elucidate the cellular mechanisms involved. Further studies are needed to elucidate the processes involved in androgen modulation of hippocampal synaptic events and

neuronal plasticity. The use of molecular tools to study the transcriptional effects of steroid receptors and their interactions with various second messenger systems and other intracellular pathways should allow progress in this complex area of study.

Structure of the Androgen Receptor

Although both the human and rat androgen receptors have been studied using biochemical methods for many years, the androgen receptor has been cloned only recently (Chang *et al.*, 1988; Lubahn *et al.*, 1988; Tan *et al.*, 1988; Trapman *et al.*, 1988; Brinkmann *et al.*, 1989; Faber *et al.*, 1989; Tilly *et al.*, 1989; Gaspar *et al.*, 1990; He *et al.*, 1990). DNA sequence analysis confirmed that the androgen receptor has the same functional domain structure as other steroid hormone receptors (discussed in detail below) and both the rat and human androgen receptors share complete amino acid sequence identity in their DNA-binding and steroid-binding domains (Tan *et al.*, 1988). The rat androgen receptor, has a cDNA sequence 4191 base pairs in length, and encoding for 902 amino acids which results in a protein of approximately 98 kilodalton molecular weight (Tan *et al.*, 1988). The complete androgen receptor gene encompasses at least 90 kilobases of DNA in the q11-12 region of the X chromosome (Lubahn *et al.*, 1988; Brinkmann *et al.*, 1989) and includes eight exons and seven intervening introns (Jänne and Shan, 1991).

Domain Structure of the AR

As with the other members of the steroid hormone receptor superfamily, the AR can be divided into four distinct functional regions. Starting from the N-terminal the regions are as follows: the transactivation domain, the DNA-binding region, the hinge region and the ligand binding sequence (reviewed by Godowski and Picard, 1989 and Jänne *et al.*, 1993). This characteristic domain organization of AR is depicted in **figure 5**. Although the AR gene and protein may appear modular in nature, each part works in concert with the others such that disruption of one activity can severely cripple the normal action of AR. A brief description of each of the functional domains follows.

The Transactivation Domain

The transactivation domain, also termed the A/B region or hypervariable domain, is the least understood region as its function has not been delineated in great detail for any of the intracellular receptors. This domain has the least conserved amino acid sequence among the superfamily of intracellular receptors. The hypervariability renders this area the most immunogenic part of the protein and it is likely to play a role in the specificity of receptor action. Data from studies examining the two distinct PR forms that differ solely in the length of their A/B domain suggest that this area optimizes the transactivation process of the receptor as well as determines target gene specificity (Tora *et al.*, 1988; Kastner *et al.*, 1990). The entire coding sequence for the 559 amino acid-long AR N-terminal domain, along

with a 1-Kb-long 5'-untranslated sequence (Tilley *et al.*, 1990a) was found to be present in the large first exon of the AR gene (Faber *et al.*, 1989; Kuiper *et al.*, 1989). Interestingly, the N-terminal domains of AR, MR, GR and PR make up approximately half the size of each of the receptors. This is exceptionally large as compared to other nuclear receptors and coincides with the observation that AR, MR, GR and PR all share the same HRE sequence on DNA (Forman and Samuels, 1990b; Freedman and Luisi, 1993). Several studies have demonstrated that particular stretches of the transactivational region of the AR are critical for cell- and receptor-specific regulation of target genes presumably by interacting with components of core transcriptional machinery, coactivators, or other transactivators (Simental *et al.*, 1991; Adler *et al.*, 1992; Palvimo *et al.*, 1993; Kupfer *et al.*, 1993; Jenster *et al.*, 1995). This may help to explain the large size of this region and how four receptors that have the potential to bind to the same HRE can elicit different effects through the use of their divergent N-terminal domains. It has also become apparent from the analysis of AR deletion mutants and AR/GR chimeras that sequences within the long transactivation domain also have specific roles in stabilizing the AR by slowing the rate of ligand dissociation and preventing AR degradation (Zhou *et al.*, 1995), in modulating nuclear import of the receptor complex (Simental *et al.*, 1991; Wilson *et al.*, 1991; Zhou *et al.*, 1994a) and AR dimerization (Wong *et al.*, 1993).

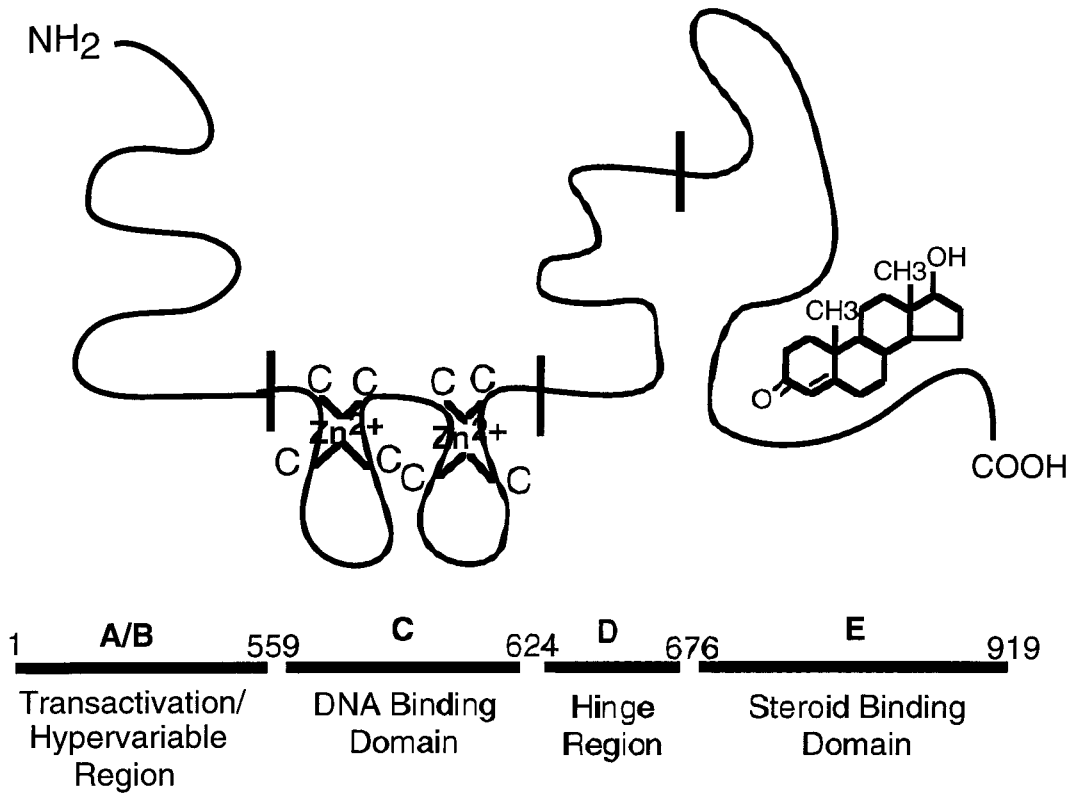

Figure 5. Schematic representation of the domain structure of the androgen receptor. Amino acid length is based on the human receptor sequence published by Lubahn *et al.* (1988).

DNA Binding Domain

All steroid receptors, including the AR, recognize specific DNA sequences with a well-conserved functional domain encompassing 66-68 amino acid residues termed the DNA binding domain (DBD). This cysteine-rich region folds into two motifs that are variations of "zinc fingers" found in other nucleic acid binding proteins (Miller *et al.*, 1985). Each finger is comprised of two pairs of cysteine residues that coordinate in a tetrahedral fashion around a single zinc atom (Freedman *et al.*, 1988). The N-terminal zinc finger is largely responsible for DNA recognition, whereas the second finger appears to modulate the dimerization of the two receptor molecules during its association with DNA (Green *et al.*, 1988; Danielsen *et al.*, 1989; Umesono and Evans, 1989; Luisi *et al.*, 1991). The AR DBD displays tremendous amino acid homology with that of the MR, GR and PR. As a result, all four receptors recognize the same 15 basepair palindromic-like nucleic acid sequence flanking target genes. This sequence (5'-GGTACANNNTGTTCT-3') was first described as the consensus glucocorticoid response element (GRE) (Beato, 1989; Roche *et al.*, 1992; Zilliacus *et al.*, 1995), but now has been more generally termed an HRE. Research is currently underway to determine how four receptors with obviously different functions can distinguish a common HRE upstream of target genes (Adler *et al.*, 1993; Robins *et al.*, 1994). This distinction would be especially critical in areas like the hippocampus where AR, MR and GR are all highly expressed and are likely co-localized within certain neurons (Kerr *et al.*, 1995b). As discussed above, findings indicate that the divergent N-terminal domain likely makes protein-

protein interactions that specifies transcriptional regulation to some degree. It has also become apparent that HRE orientation within the enhancer region of target genes affects hormone receptor stringency (Adler *et al.*, 1993). Additionally, it can not be ruled out that some overlap of target gene expression may occur in cells containing more than one of these receptors.

The Hinge Region

Next to the DBD, a variable hinge region exists (region D) in the AR protein. This area may allow the AR protein to bend or alter its conformation and has also been shown to contain part of a nuclear targeting signal (Zhou *et al.*, 1994a). Although not yet well studied specifically for the AR, the analysis of the action of the highly homologous GR has demonstrated that the hinge region also affects the affinity of the receptor for DNA (Rusconi and Yamamoto, 1987).

Steroid Binding Domain

The C-terminal region of the AR spans about 250 amino acid residues (653-910) and is primarily involved in ligand binding. This region, termed the steroid binding domain (SBD), forms a hydrophobic pocket that exhibits high affinity for androgens. Surprisingly, the SBD of AR displays a 50-55% homology with similar domains in GR and PR (Trapman *et al.*, 1988; Hollenberg *et al.*, 1985; Mishari *et al.*, 1987). This homology may account for a few reports of promiscuous binding of androgens, progestins and glucocorticoids with more than one receptor type (Mayer

and Rosen, 1975; Jänne and Bardin, 1984a, 1984b; Ahima and Harlan, 1992; Kemppainen *et al.*, 1992). In addition to hormone binding, a 54 amino acid stretch of AR's SBD is required for the interaction of the large docking heat shock protein, HSP90 (Marivoet *et al.*, 1992).

AR Messenger Ribonucleic Acid

The exact size and number of the androgen receptor mRNA isoforms have been controversial and vary depending on the species and tissue analyzed. A major form, approximately 11 kb in length, has consistently been reported in peripheral tissues including prostate, skeletal muscle, liver, kidney, seminal vesicle, epididymis, anterior pituitary gland and coagulating gland (Tan *et al.*, 1988; Trapman *et al.*, 1988; McLachlan *et al.*, 1991; Burgess and Handa, 1993a). In addition to this, a novel 9.3 kb transcript has been detected in rat neural tissues (McLachlan *et al.*, 1991; Burgess and Handa, 1993a). The smaller form was prominent in the cortex, cerebellum, and brain stem; while in the hippocampus and hypothalamus, both the larger and smaller transcripts were expressed to a similar degree. It is thought that the two mRNA species differ in part in the length of their 5'-untranslated region (5'-UTR), but complete sequence analysis is still necessary. The significance of the smaller form found predominantly in the CNS is not known. The 5'-UTR of the human AR mRNA, that spans about 1100 bp, has been shown to play an essential role in the induction of AR translation (Mizokami and Chang, 1994). This 5'-UTR is one of the longest reported 5'-UTR in mammalian systems (Trapman *et al.*, 1988).

Whether both transcripts found in the rat CNS encode for fully functional androgen receptor proteins and are regulated or translated similarly are questions remaining to be answered. Although some earlier studies suggested a two receptor system for the binding of T and DHT (Sheridan, 1981, 1991), it appears unlikely that two unique ARs are translated from each of the mRNA transcripts as virtually all well-controlled biochemical studies have found a single androgen binding site in both peripheral tissues and the brain (Wilson and French, 1976; Tilley *et al.*, 1990b).

The AR Protein: Steroid Binding, Receptor Recycling and Metabolism

The AR is a large phosphoprotein that is found within peripheral target cells in relatively low abundance (2000-6000 receptors/cell and less than 100 femtomoles DHT binding sites per milligram protein) as compared to most membrane-bound receptors (Fang and Liao, 1971). In brain tissue, AR concentration is generally an entire order of magnitude less than that found in peripheral reproducive tissues. A striking feature of AR is its extreme lability and rapid degradation ($t_{1/2}$ = 1-1.5 h) in the absence of agonist ligand binding (Kemppainen *et al.*, 1992; Zhou *et al.*, 1995). In the presence of androgen, AR is degraded at a somewhat slower rate ($t_{1/2}$ = 6 h). In comparison, ligand-free GR degrades with a half-time of 4-6 h, about 5-fold slower than AR, and in the presence of dexamethasone, degradation can be slowed to 16 h (Zhou *et al.*, 1995). This instability has made AR exceptionally difficult to study *in vitro* and potent proteolytic inhibitors were required to stabilize AR in its intact 100-120 kDa form (Wilson and French, 1979). As a result, studies on AR binding

properties, metabolism and recycling still lag behind those exploring the other steroid hormone receptors.

The study of AR binding kinetics of T and DHT, as well as of antiandrogens such as hydroxyflutamide, have been followed with great interest with hopes of elucidating how these hormones sometimes exert differential physiological effects. It has been well established that DHT is 2.5 to 10 times more potent in bioassays and, in broken cell preparations, DHT binds to the AR with a several-fold higher affinity than T (approximately 0.25 - 2 nM versus 0.4 - 5 nM) (Wilbert *et al.*, 1983; Winters, 1990). Wilson and French (1976) demonstrated that despite relative affinity constants that are nearly equivalent, T binds and dissociates from AR about three times faster than DHT. Presently, it is unclear how altered binding kinetics translate to differences in transcriptional activity; however, the authors speculated that AR nuclear retention time may be longer with DHT binding. Interestingly, at ten-fold higher concentrations than DHT, T was able to overcome this rapid dissociation rate by simple mass action (Grino *et al.*, 1990). This finding suggests that when localized T concentrations are undiluted (i.e. paracrine actions of T within testes) it can be as physiologically potent as DHT. AR degradation was also differentially affected by T and DHT binding (Kemppainen *et al.*, 1992; Zhou *et al.*, 1995). These observations suggest that DHT initiates a slightly different conformational change in the receptor complex that promotes its stabilization, and possibly, its transcriptional efficiency. Interestingly, antagonists of the AR, which compete with agonists for AR binding, but do not permit the receptor to assume a transcriptionally active form, initiated distinct

conformations of the AR complex as detected by proteolytic digestion (Wong *et al.*, 1993; Kallio *et al.*, 1994; Kuil and Mulder, 1994). Additionally, AR antagonists including hydroxyflutamide and cyproterone acetate were not able to stabilize AR and prevented agonist-induced stabilization even at a 100-fold molar excess (Kemppainen *et al.*, 1992). Thus, it appears that a precise conformation is required for maximal receptor stabilization and the induction of transcriptional activation. DHT appears to be the ligand most likely to generate this conformation.

In the last ten years controversy has abounded in endocrinology over whether steroid receptors are found exclusively in the cytoplasm in the unoccupied form and translocate to the nucleus only following ligand binding. This had been the original hypothesis following discoveries using *in vitro* binding techniques on broken cell preparations and high speed centrifugation to separate cell fractions (Jensen *et al.*, 1968). The recent development of specific antibodies for each of the hormone receptors has spawned most of this controversy; as it is now possible to clearly identify the intracellular localization of steroid hormone receptor labelling both in the presence and absence of circulating hormone, without disrupting membrane integrity. Using such histological studies, several groups determined that both bound and unbound ER, PR and GR were primarily confined to the nucleus (King and Greene, 1984; Welshons *et al.*, 1984, 1985; Gasc *et al.*, 1989). Since this initial observation, several reports have described cytoplasmic staining of unbound ER (Fox *et al.*, 1991; Blaustein *et al.*, 1992), PR (Blaustein *et al.*, 1992) and GR (Ahima and Harlan, 1991), adding further confusion. Some of the discrepancies may be the result of non-

specific antibodies, or it has been proposed that some of these antibodies may not reliably recognize both the bound and unbound conformational states of the receptors. The recent characterization of AR immunohistochemical localization in transfected COS cells indicate that in the absence of androgens, AR immunoreactivity (ARIR) is located predominately in the cytoplas. The addition of androgen shifts ARIR to the nucleus (Simental *et al.*, 1991; Jenster *et al.*, 1993). This latter finding has also been observed *in vivo* in the male hamster brain (Wood and Newman, 1993). Taken together, these data indicate that androgen is one factor that regulates the partitioning of the AR to the nucleus, however, it can not be ruled out that the equilibrium of AR intracellular distribution can vary with cell or tissue type (Husmann *et al.*, 1990). The physiologic significance of AR intracellular partitioning is not known, however, it could potentially affect the ease by which AR "sees" its ligand within the cell. Regardless of the cytoplasmic or nuclear localization of unbound AR, hormone binding serves to anchor the AR receptor complex in nuclei.

Earlier studies examining skeletal muscle suggested that physiological levels of T were sufficient to fully occupy and transform all available AR (Wilson, 1988). In contrast, studies done in neural tissue have demonstrated that only a fraction (30-50%) of total AR in the cell are transformed to the nuclear, DNA-bound form under physiological conditions (Handa *et al.*, 1987a; Roselli *et al.*, 1989). These observations suggest that circulating androgen levels are an important component regulating the magnitude of androgen action in the CNS and suggests that very high levels of circulating androgen can transform a greater proportion of neural cytosolic

AR and possibly elicit a greater transcriptional response.

Regulation of Androgen Receptor Gene Expression

As discussed earlier, a functional AR gene is essential for normal male development. However, the distribution, timing and magnitude of AR expression during development and throughout adulthood are also important determinants of androgen sensitivity. Thus, it has become imperative to analyze how the AR gene is regulated in concert with androgen regulation of target genes. The 5' flanking regions of the rat, mouse and human AR gene have been cloned (Baarends *et al.*, 1990; Tilley *et al.*, 1990a; Faber *et al.*, 1991a,b; Kumar *et al.*, 1992; Song *et al.*, 1993; Mizokami *et al.*, 1994), allowing for the detailed examination of molecular mechanisms controlling AR gene expression. In this section, current understanding of the AR gene promotor region as well as various endocrine and non-endocrine factors that act to regulate the AR gene are reviewed.

The AR Gene Promoter

The major site of transcription initiation is approximately 1.1 kb upstream of the initiation codon in the human AR mRNA (Tilley *et al.*, 1990a; Mizokami *et al.*, 1994) and this appears to be similar for the rat AR gene (Song *et al.*, 1990). Sequence analysis of rat, human and mouse AR promoter regions have confirmed that

all three lack typical "TATA" or "CAAT" sequence motifs, but instead, each of the promoter regions lies in a GC-rich region and contains a putative SP1 binding site that is characteristic of a "housekeeping" promoter (Baarends *et al.*, 1990; Tilley *et al.*, 1990; Faber *et al.*, 1991a,b; Kumar *et al.*, 1992; Song *et al.*, 1993; Mizokami *et al.*, 1994).

The complete sequence analysis of 2656 bp of the rat AR upstream region has revealed consensus DNA-binding sequences for numerous known transcription factors (Song *et al.*, 1993). Several half-palindrome sites for AR/PR/GR (TGTTCT) and one half-site for the ER (AGGTCA) were detected. Although steroid receptors could potentially bind to these half-sites and confer steroidal regulation of AR expression, their true physiological significance has not been investigated. Also identified were the potential binding sites for the transcription factors SP1, C/EBP, Pu.1, *Zeste* (a *Drosophila* homeobox protein), *zif268* (a zinc finger motif cIEG protein), PEA3 (an enhancer protein), NFκB and for the Fos/Jun heterodimer. The presence of these binding regions strongly suggests that multiple factors, including AR itself, have the potential to modulate AR expression. Further delineation of the regulatory regions of the AR gene will prove to be beneficial in the understanding of the interplay of various transcription factors in the tissue-specific expression and regulation of the AR gene.

Autologous Regulation of AR Expression

Several studies have examined the regulation of AR synthesis in response to hormonal manipulation in both neural and non-neural tissues. In the majority of peripheral tissues studied, AR expression (as measured by steady state mRNA levels) was increased by short term castration (1-4 days) and decreased by androgen treatment (Tan *et al.*, 1988; Shan *et al.*, 1990; Takane et a., 1990; Quarmby *et al.*, 1990; Blok *et al.*, 1991, 1992a; Abdelgadir *et al.*, 1993). AR is not autoregulated in in peripheral tissues in testicular feminized rats; a genetically engineered animal strain where the AR gene is mutated so that the resulting AR protein is unable to bind androgen (Quarmby *et al.*, 1990). Similarly, AR was not regulated in skin fibroblasts from patients with androgen insensitivity syndrome (Kaufman *et al.*, 1981). This syndrome also involves a genetic mutation of the AR gene that renders the AR protein unable to bind ligand. The absence of AR autoregulation in individuals who do not have functional AR, but normal levels of circulating androgen, supports a receptor-mediated process. In the human prostate carcinoma cell line LNCaP, nuclear run-on analysis demonstrated that androgen treatments prompted a 75% reduction in AR transcription initiation (Blok *et al.*, 1992b; Wolf *et al.*, 1993). In accordance with these findings, recent studies by Prins and Woodham (1995) have shown castration-induced increases in AR mRNA levels in rat VP, however, this regulation was lobe specific and was shortlived in some areas. In addition, nuclear run-on assays demonstrated that these increases were due to an increase in the rate of AR transcription. Thus, in most peripheral tissues, it appears that the AR gene is

autologously regulated, at least on an acute basis, such that the activated androgen receptor-hormone complex primarily acts on the genome to prevent the transcription of new AR mRNA. The binding of activated AR complexes to the half-site HREs found in the AR promoter region (Song *et al.*, 1993) could potentially mediate this response. Conversely, positive regulation of AR and AR mRNA levels by androgens have been reported in isolated smooth-muscle cells from the rat penis (González-Cadavid *et al.*, 1993) and in human genital skin fibroblasts (Gad *et al.*, 1988) suggesting that particular cell types may be genetically programmed to respond differently to androgen at certain developmental stages. Additionally, the aromatization of T to estradiol also appears to affect AR mRNA levels and could account for this tissue-specific regulatory pattern (Lin *et al.*, 1993).

Immmunoblot analysis of corresponding AR protein changes in the rat VP and several cell lines under similar experimental conditions that had caused several-fold increases in AR mRNA revealed that androgen withdrawal by castration elicited modest or no increases in immunoreactive receptor protein content (Shan *et al.*, 1990; Wolf *et al.*, 1993). Additionally, Krongrad *et al.* (1991) have shown that androgen-mediated down-regulation of AR mRNA is associated with a transient up-regulation of AR protein in the human prostate cancer cell line, LNCaP. These data support the recent finding that androgens stabilize the AR protein (Zhou *et al.*, 1995). Ultimately, AR concentrations are likely controlled through multiple mechanisms including the rate of transcription, mRNA stability, mRNA translational efficiency, and the turnover rate of the protein.

Studies of autologous AR regulation in brain tissue have been sparse and much more difficult to interpret. Using northern blot analysis, Quarmby *et al.* (1990) demonstrated a three-fold increase in AR mRNA in whole rat brain four days after castration as compared to the intact animal. The administration of T propionate one day before sacrifice prevented this increase. Consistent with these data, Burgess and Handa (1993a) demonstrated significant increases in hypothalamic-preoptic area AR mRNA content four days after castration when measured by ribonuclease (RNase) protection assay. This effect was reversed by DHT treatment one day prior to sacrifice. In contrast, McLachlan *et al.* (1991) did not observe any changes in the amount of either the 9.3 or 11 kb AR mRNA forms in rat cortex one and three days following castration, however, their densitometric analysis of northern blots may not have been sensitive enough to detect small changes. Using a more sensitive RNase protection assay, Abdelgabir *et al.* (1993) reported no effects of 2, 4 or 7 day treatment with T, DHT or estrogen on AR mRNA levels in the rat hypothalamus, preoptic area, cortex, hippocampus or amygdala. Unfortunately, these data must be interpreted cautiously as they are based on one or two animals per group. In contrast, Handa *et al.* (1995) have demonstrated acute increases in AR mRNA in the medial preoptic area of the hypothalamus following castration. However, after two month castration, these increases in AR mRNA levels were dramatically reduced or absent. These effects of castration were reversed by DHT and estrogen. Conversely, rats treated with 14 daily injections of high-dose anabolic-androgenic steroids showed increases in AR immunoreactivity in most AR-positive brain regions, including the

CA1 region of the hippocampus (Menard and Harlan, 1993). Whether the androgen treatment truly upregulated AR numbers or simply translocated more AR to the bound conformational state of the receptor that could have been preferentially recognized by their antibody was not determined.

The inconsistent findings in brain tissue suggest that a unique, tissue-specific AR regulatory process may be occurring in the CNS as compared to most non-neural reproductive tissues. Taken together, it appears doubtful that circulating androgen levels are the sole determinant of AR mRNA levels in neural tissue. Other factors such as the length of androgen treatment, the mode of steroid administration, and the presence of tissue-specific regulatory proteins may play important roles in determining neural AR mRNA expression. Whether changes in brain AR mRNA translate into similar changes in the receptor protein have not been determined and may be complicated by the fact that two AR mRNA transcripts are found in neural tissue. A much clearer understanding of AR regulation in the brain is necessary to predict the responsiveness of neural tissue to androgens.

AR Regulation by Other Factors

Recently, it has become apparent that the AR gene is influenced by several other regulatory signals, including peptide hormones, growth factors, neurotransmitters and other steroid hormone receptors. Additional data suggest that the AR gene is regulated by membrane associated second messengers commonly stimulated by neurotransmitters or peptide hormones. For example, FSH, a hormone

whose actions are mediated via cyclic 3',5'-adenosine monophosphate (cAMP) and activates the protein kinase A pathway, or the addition of cAMP analogs alone, increased both AR protein and AR mRNA in Sertoli cells (Verhoeven and Cailleau 1988; Blok *et al.*, 1989, 1992b, 1992c). Additionally, cAMP stimulated a mouse AR 5'-chloramphenicol acetyltransferase construct in mouse and rat pituitary cell lines (Lindzey *et al.*, 1993). Similarly, epidermal growth factor, which activates the protein kinase C second messenger pathway, decreased AR mRNA levels in LNCaP cells (Mizokami *et al.*, 1992). Some of these effects could potentially be mediated directly via the calcium (Ca^{2+})/cAMP-response-element-binding protein (CREB), the activating transcription factor, AP2 (Imagawa *et al.*, 1987; Montminy *et al.*, 1990), or indirectly via induction of other transcription factors such as the activator protein (AP1) components, Fos and Jun. The localization of several of these transcription factor binding sites within the 5' promoter regions of the human, rat and mouse AR genes support such mechanisms (Baarends *et al.*, 1990; Tilley *et al.*, 1990a; Faber *et al.*, 1991a,b; Kumar *et al.*, 1992; Song *et al.*, 1993; Mizokami *et al.*, 1994). Additionally, cellular Ca^{2+} levels may also play a part in AR expression. The progressive lowering of Ca^{2+} concentrations significantly decreased AR protein levels in rat Leydig cell culture (Nakhla *et al.*, 1989), and incubation of LNCaP cells with the Ca^{2+} ionophore, A23187, or the intracellular endoplasmic reticulum Ca^{2+} adenosine triphosphatase inhibitor, thapsigargin, down-regulated AR mRNA and AR protein levels in a time- and dose-dependent manner (Gong *et al.*, 1995).

Several studies have suggested that the expression of one steroid hormone

receptor may interfere with or alter the transcriptional activity of another steroid hormone receptor expressed in the same cell line (Meyer *et al.*, 1989; Bansal and Latchman, 1990; Kumar *et al.*, 1994). In particular, the overexpression of ER significantly inhibited AR transcriptional activity with the addition of androgen and estrogen to the cell culture (Kumar *et al.*, 1994). The authors proposed that high levels of DNA-bound ER may compete for some unknown factor also necessary for transcriptional activation to occur through AR. Whether such an interaction could result in decreased transcription of the AR gene has yet to be determined. Estrogen-induced down-regulation of the AR in the adult rat VP has been demonstrated *in vivo* (Rennie *et al.*, 1988; Prins, 1992), however, estrogen induced upregulation of AR has been repeatedly demonstrated in other tissues (Handa *et al.*, 1987a, 1987b, 1995; Handa and Rodriguez, 1991). In some circumstances, estrogen may directly regulate AR expression through the estrogen response element half-site found in the promoter region of the rat AR gene (Song *et al.*, 1993). Although no studies have looked at alternate factors regulating AR in neural tissue, interactions between AR and other ligand-activated transcriptions factors or second messenger pathways could be particularly important in brain areas like the hippocampus that express high levels of certain membrane receptors and multiple types of intracellular steroid receptors.

Androgen-Regulated Gene Networks

It is now widely accepted that steroid receptors initiate their diverse biological responses through selective regulation of cell-specific gene networks (Yamamoto, 1985; Meisfield, 1989). In order to understand the function and mechanism of action of androgens, androgen-responsive genes from a variety of cell types need to be identified. However, despite androgen's many physiological effects in peripheral and central tissues and the estimation that almost every tissue or organ possesses an androgen-regulated gene (Mooradian *et al.*, 1987), surprisingly few androgen-regulated genes have been characterized.

In the periphery, the rat prostate gland has served as an important target tissue for the study of androgen dependent gene expression. Natural growth and maintenance of the rat VP is dependent upon androgen, and castration initiates epithelial cell apoptosis (Isaacs, 1984; Kyprianou and Isaacs, 1988; Rennie *et al.*, 1988). The study of androgen action in this tissue is clinically relevant for the potential improvements in the diagnosis and treatment of prostate cancer -- the second leading cause of cancer-related death in American men (Coffey, 1993). Several prostate-specific androgen-regulated genes have been characterized, including prostatic steroid binding protein (the principle secretory protein of the rat VP) (Page and Parker, 1982; Allison *et al.*, 1989), probasin (a single-polypeptide protein that may be a ligand carrier) (Spence *et al.*, 1989; Rennie *et al.*, 1993), human glandular kallikrein-1 (a serine protease) (Morris, 1989; Murtha *et al.*, 1993), and prostate

specific antigen (a serine protease that is an important marker for prostate cancer) (Lilja, 1985; Riegman *et al.*, 1991). All these genes appear to be regulated by AR complexes through HRE sequences present in their promoter regions (Riegman *et al.*, 1991; Murtha *et al.*, 1993; Rennie *et al.*, 1993).

The expression of a more ubiquitous glycoprotein, termed sulfated glycoprotein 2 (SGP-2), has also been demonstrated to be under the control of androgen in variety of tissues (Bettuzzi *et al.*, 1989). In the rat VP, SGP-2 mRNA levels increased 16-fold 4 days after castration (Bettuzzi *et al.*, 1989) and also increased in association with programmed cell death (Buttyan *et al.*, 1989). Subtraction hybridization analysis determined that the transcription of this gene accounts for the majority (92%) of castration-induced mRNAs in the rat VP (Briehl *et al.*, 1990) and suggests that the androgen gene network in this tissue is relatively small. SGP-2 is also the major glycoprotein secreted by Sertoli cells (Collard and Griswold, 1987) and, at least in male reproductive tissues, appears to have a role in sperm function. Interestingly, SGP-2 was also found to be produced in the rat brain (Bettuzzi *et al.*, 1989; Day *et al.*, 1990), and the homologous human RNA species was increased in the hippocampus during Alzheimer's disease (May *et al.*, 1990). SGP-2 was first shown to increase in the rat hippocampus following entorhinal cortex lesions suggesting a role for this protein in either the cell death process or in the regenerative phase involving synaptogenesis or axonal reorganization. More recently, Day *et al.* (1990, 1993) demonstrated that 3 weeks after castration, there was increased SGP-2 expression [along with glial fibrillary acidic protein (GFAP)]

specifically in astrocytes within the molecular layer of the rat hippocampus. Since no studies have detected AR expression in this area of the hippocampus, the mechanism of androgens' actions in astrocytes is unclear; but the authors speculated that androgen-mediated changes in pyramidal cell neural activity could account for the results. Androgens have also been found to upregulate the expression of two major neuronal cytoskeletal elements, β-tubulin and β-actin, in androgen-sensitive spinal motorneurons (Matsumoto *et al.*, 1992, 1993). As androgens appear to play a role in hippocampal synaptic reorganization and sprouting (Morse *et al.*, 1988; Scheff *et al.*, 1988) as well as promote axonal regeneration and synaptic input in other CNS loci (Matsumoto *et al.*, 1988; Jones, 1993), androgen-regulated SGP-2, GFAP and cytoskeletal protein expression may prove to be important markers for such processes.

Few other studies have examined androgen regulated genes in the brain, however, androgens have been shown to positively regulate GnRH mRNA (Park *et al.*, 1988) and aromatase cytochrome P450 mRNA levels (Abdelgadir *et al.*, 1994) in the rat hypothalamus, as well as negatively regulate D-2 dopamine receptor content in the rat striatum (Watanabe *et al.*, 1989) and NMDA receptor levels in the hippocampus (Kus *et al.*, 1995). The continued identification of androgen-regulated genes will provide additional clues to the cell-specific events initiated by AR and will help to elucidate androgen's ultimate function in target tissues.

Hippocampal Glucocorticoid Receptors: Action, Location, and Regulation

Glucocorticoids are adrenal steroid hormones typically secreted in response to stress (Munck *et al.*, 1984). This secretion is controlled by the brain via the hypothalamic-pituitary-adrenal (HPA) axis. This axis is a closed-loop endocrine system in which the end product, the adrenal glucocorticoids, feedback onto various brain regions including the hippocampus, hypothalamus and pituitary to inhibit the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary gland.

In the rat, corticosterone (CORT) is the major circulating glucocorticoid hormone. It's effects on the body are widespread and, for the most part, are beneficial. CORT is a potent anti-inflammatory agent and also acts to mobilize energy stores and maintain osmotic balance in time of need (Baxter and Forsham, 1972; Axelrod and Reisine, 1984; Munck *et al.*, 1984). In the central nervous system, CORT has been found to induce changes in the levels of several neurotransmitter receptors (Jhanwar-Uniyal and Leibowitz, 1986; Martire *et al.*, 1989; Clark and Cotman, 1992) and in the regulation of second messenger pathways (Harrelson and McEwen, 1987). These changes may be the mechanism by which CORT influences certain aspects of behavior including mood, attention, learning and adaptation (reviewed by McEwen *et al.*, 1986).

The actions of CORT are mediated in the brain and periphery through its binding to specific intracellular receptors (McEwen *et al.*, 1986). Radioligand binding studies have demonstrated that CORT acts through two types of receptors (De

Kloet *et al.*, 1975; Reul and De Kloet, 1985). The first, termed the Type I receptor, or MR, has a very high affinity for CORT (K_d = 0.5nM) as well as aldosterone (K_d = 1.5 - 2.0nM) and dexamethasone (K_d = 0.8 - 2.6nM). The second receptor, termed the Type II, or GR, is distinguishable by its much lower affinity for CORT (K_d = 2.5 - 5.0nM) and aldosterone (K_d > 25nM), yet much higher affinity than the Type I receptor for the synthetic glucocorticoid RU 28362. MR, having a high affinity and low capacity for endogenous glucocorticoids, is thought to be occupied at low basal levels of CORT and thus mediate the effects of glucocorticoids on ongoing neural activity. In contrast, GR is thought to occupied only after increases in CORT occur, such as following stress.

More recently, MR and GR have been distinguished by their molecular characteristics (Arriza *et al.*, 1987; Hollenberg *et al.*, 1987; Patel *et al.*, 1989). Both receptors are members of the superfamily of steroid hormone receptors (along with AR), which when bound to ligand, are able to act as transcription factors as discussed earlier in this review. The rat MR and GR share considerable amino acid sequence homology which likely contributes to some of the overlap in ligand binding and transcriptional activity between them, yet both are products of distinct MR and GR genes.

In addition to their structural and binding characteristics, GR and MR differ in their neuroanatomical distribution (Fuxe *et al.*, 1985; Reul and De Kloet, 1985; Van Eekelen *et al.*, 1987; Sarrieau *et al.*, 1988). MR is predominantly localized in septum and hippocampus. In contrast, the distribution of GRs over the brain is much

more widespread. GR is found in brain regions including the hippocampus, septum, paraventricular nucleus, supraoptic nucleus, and the medial amygdala. Recent *in situ* hybridization analysis has revealed distinct patterns of expression of GR and MR mRNA within the various regions of the rat hippocampus (Van Eekelen *et al.*, 1988). MR mRNA was demonstrated in all pyramidal cell fields (CA1-4) of the hippocampal formation and the granule cells of the DG. In contrast, GR mRNA was mainly restricted to CA1 and CA2 pyramidal cells and the DG. GR-like immunoreactivity mapping has demonstrated similar hippocampal distribution of the GR protein in male and female intact rats (Ahima *et al.*, 1992). These high levels of corticosteroid receptors expressed in the hippocampus are thought to mediate glucocorticoid effects on neuronal proliferation and differentiation, neuronal death, membrane potential, and neuroendocrine feedback mechanisms (McEwen *et al.*, 1986). Interestingly, the distribution of MR, GR and AR mRNA in the hippocampus overlap, with especially high levels of all three receptors in almost all pyramidal cells of CA1. Such cellular overlap in expression may suggest some interactive function or cooperativity of AR and GR in hippocampally-mediated behaviors.

Corticosteroids are known to modulate the expression of their own receptors as evidenced by numerous *in vitro* studies demonstrating GR autoregulation in several different types of cell culture systems (Cidlowski and Cidlowski, 1981; Svec and Rudis, 1981; McIntyre and Samuels, 1985; Berkovitz *et al.*, 1988). More recently, the *in vivo* regulation of GR by glucocorticoids has been characterized in the hippocampus. In most cases, adrenalectomy (ADX) caused an increased level of GR

mRNA in the hippocampus within one day (Reul *et al.*, 1989; Sheppard *et al.*, 1990). These increases were returned to intact levels by dexamethasone administration (Sheppard *et al.*, 1990). Using *in situ* hybridization, anatomical specificity of this regulation has been demonstrated. Eight days following ADX, elevated levels of GR and MR mRNA are found in the CA1-2 subfields of the hippocampus (Herman *et al.*, 1989). In contrast, a similar treatment has been found to decrease GR-like immunoreactivity in these areas (Ahima *et al.*, 1992). The exact reasons for such discrepancies between protein and mRNA levels is unclear, however, several studies have found a role of glucocorticoids in modulating the stability of the receptor protein (McIntyre and Samuels, 1985; Dong *et al.*, 1988; Hoeck *et al.*, 1989). Autologous regulation of GR also appears to be exerted at the level of GR mRNA synthesis (see review by Burnstein and Cidlowski, 1992). Several experiments have found that the GR cDNA contains intragenic signals that activated GR complexes can bind to and subsequently act by repressing transcription initiation or blocking elongation (Burnstein *et al.*, 1990, 1991; Okret *et al.*, 1986). The exact nature of these intragenic sequences has not been investigated.

The actions of other steroid hormones on hippocampal GR regulation have been investigated recently. Estrogen, the prominent circulating sex steroid in females, has been found to alter the regulation of CORT receptor mRNAs in the female hippocampus (Burgess and Handa, 1993b). In this study, estrogen treatment resulted in a loss of the GR's ability to down-regulate its mRNA.

Sex differences have recently been observed in the regulation of the

intracellular location of hippocampal GR-like immunoreactivity of ADX rats by CORT and progesterone (Ahima *et al.*, 1992). In this study, estradiol treatment of ADX male or female rats did not significantly alter staining intensities in any area of the hippocampusas compared to the untreated ADX male or female rats. Similarly, recent evidence has demonstrated upregulation of rat GR immunoreactivity in the pyramidal cell layer of CA1 and granular layer of the DG of the rat hippocampus after a one week treatment with anabolic-androgenic steroids (Ahima and Harlan, 1992). These data suggest a link between AR activation and GR regulation in the areas of the hippocampus which contain high levels of both of these receptors. It is not known if these anabolic steroids are transactivating ARs which in turn alter the transcriptional rate of the GR gene or are acting through some other mechanism in hippocampal cells. It is possible, however, that the behavioral changes observed during anabolic-androgenic steroid abuse may be mediated in part through its effects on GR regulation and resulting changes in hormonal feedback mechanisms.

Cellular Immediate Early Genes

Despite accumulating molecular data on steroid hormone-receptor complex action on individual HREs, the steps leading from hormonal signals to the modulation of neuronal activity remain poorly defined. New avenues to approach such questions have resulted from the observation that neuronal stimulation rapidly activates the transcription of several cIEGs. Most of the cIEGs encode for proteins which act as

transcription factors and regulate, in a hierarchial fashion, the transcription of target genes that determine the overall behavior or phenotype of the cell (reviewed by Morgan and Curran, 1991). In essence, cIEG protein products are the "third messengers" of the stimulus-transcription coupling cascade that produce the long-term or "hard-wired" changes in neurons (reviewed by Morgan and Curran, 1989).

In general, cIEGs share several characteristics. First, they are expressed in very low or undetectable amounts in quiescent cells, but are rapidly transcribed within minutes of cellular activation. Second, their transcriptional activation is short-lived and does not require new protein synthesis, however protein synthesis is necessary to shut-off the transcriptional process. Last, cIEG mRNAs and proteins have short half-lives (minutes to a few hours), and thus, are characteristic of an early signalling system that triggers further regulation of gene expression (Sheng and Greenberg, 1990).

cIEG Forms and Mechanisms of Induction

To date, the best studied cIEG is *c-fos*, but others, including several *c-fos* family members (*fosB* and Fos related antigen, *fra*), several *jun* family members (*c-jun, junB, junD*), *zif268* (also known as *NGFI-A, krox24, TIS-8* and *Erg-1*), *c-myc*, and *c-Ha-ras* are also expressed in neuronal tissue and are currently being examined. The *c-fos* gene encodes a nuclear protein, Fos, that has an apparent molecular weight of 62 kDa and is subject to extensive post-translational modifications (Schilling *et al.*, 1991). Using a leucine-zipper motif and surrounding basic regions, the Fos and Jun

family member proteins bind to DNA regulatory regions either as homodimers (Jun-Jun dimers) or heterodimers (Fos-Jun dimers) to form the transcription factor known as AP-1 (reviewed by Curran and Franza Jr., 1988; Cohen and Curran, 1989). In this case, additional regulation of gene transcription occurs depending on the relative amounts of Fos and Jun expressed in the cell after stimulation (Chiu *et al.*, 1989; Schütte *et al.*, 1989; Diamond *et al.*, 1990).

The three known Jun proteins (Jun, JunB and JunD) differ from each other in their transactivation properties, binding affinities, and cellular function (Chiu *et al.*, 1989; Ryseck and Bravo, 1991). *c-jun* and *junB* are considered classical cIEGs in that they are rapidly and transiently expressed in cells following various stimuli (reviewed by Sheng and Greenberg, 1990). In contrast, *junD* is constitutively expressed in considerable amounts in many tissues and exhibits delayed and prolonged induction following certain stimuli (Gass *et al.*, 1992; Demmer *et al.*, 1993; Herdegen *et al.*, 1995). Functionally, Jun has been linked to the promotion of cell growth, whereas JunB and JunD act to inhibit cell proliferation (Schlingensiepen *et al.*, 1994). The *zif268* gene, which encodes for a lone-acting, zinc-finger-containing transcription factor, was initially found to be rapidly induced in mammalian neurons following seizures (Saffen *et al.*, 1988), although, it was also found to be constitutively expressed in some areas of the brain (Schlingensiepen *et al.*, 1991; Hughes *et al.*, 1992; Herdegen *et al.*, 1995).

The expression of *c-fos* was initially studied in PC12 pheochromocytoma cells and was found to be induced by neurotrophic factors (Greenberg *et al.*, 1985), agents

that activate classical neurotransmitter receptors (Greenberg *et al.*, 1986), depolarizing conditions (Morgan and Curran, 1986), and a variety of agents that provoke Ca^{2+} influx through voltage-gated channels (Morgan and Curran, 1986). These findings prompted researchers to look for inducible *c-fos* (as well as its closely related cIEGs) in the nervous system. Although a precise function for Fos and other cIEG proteins has yet to be established, they have been implicated in diverse processes such as neuronal differentiation, proliferation, cell death, and signal transduction (Muller *et al.*, 1985, Schlingensiepen *et al.*, 1994).

cIEG Expression in Neural Tissue

Recently, many studies have examined cIEG induction in neuronal tissue of intact animals. In summary, cIEG expression, as measured by immunocytochemistry or mRNA content in neuronal tissue, has been shown to increase by pharmacological (Morgan *et al.*, 1987, Sonnenberg *et al.*, 1989), electrical (Dragunow and Robertson, 1987), surgical (White and Gall, 1987), and physiological stimuli (Bullitt, 1990, Senba *et al.*, 1994). Related findings have been comprehensively reviewed by Morgan and Curran (1989, 1991).

Although it may appear that the cIEG induction is a non-specific, ubiquitous phenomenon in response to cellular activation, in fact, the pattern of cIEG expression in the brain, and the specificity in which cIEGs are induced, are very dependent on the given stimulus. For example, sexual behavior increased Fos immunoreactivity selectively in the male rat medial preoptic area of the hypothalamus and nucleus

accumbens (Robertson *et al.*, 1991). Coincidentally, neurotransmitter release in both of these areas have been implicated in the control of male sexual behavior (Mas *et al.*, 1990; Warner *et al.*, 1991). Taken together, the examination of these gene products serve as a useful tool for mapping specific neuronal populations which are activated following a stimulus (Sagar *et al.*, 1988) and distinct combinations of cIEGs could confer specificity in the cellular response to different stimuli.

The study of cIEG expression in the hippocampus following physiological stimuli has been particularly useful in identifying specific roles for these protein products. One popular model of neuronal plasticity in the mammalian CNS is long-term potentiation (LTP). LTP is a lasting enhancement of synaptic efficacy in hippocampal neurons following brief high-frequency perforant pathway stimulation (Bliss and Gardner-Medwin, 1973). LTP has been shown to persist from days to months in the absence of any further stimulation which makes it an attractive model to study the mechanisms responsible for long-term memory (Teyler and Discenna, 1984). Such a prolonged time course of LTP decay has led researchers to implicate transcriptional changes in the maintenance of this phenomenon; cIEGs being among the first genes to be examined. Several cIEGs have been found to be induced in DG granule cells following LTP induction (Abraham *et al.*, 1991; Richardson *et al.*, 1992). In these studies, the most consistently induced cIEG, *zif268*, correlated best with LTP persistence (Richardson *et al.*, 1992; Williams *et al.*, 1995). Members of the *c-fos* and *c-jun* gene families were also induced in the hippocampus under these conditions, but did not correlate with LTP induction or stabilization (Demmer *et al.*,

1993). The induction of LTP, as well as the corresponding cIEG induction, both appeared to be dependent on hippocampal NMDA receptor activation (Demmer *et al.*, 1993). Interestingly, *zif268* was also basally expressed in CA1 pyramidal cells (Hughes *et al.*, 1992) and this basal expression in CA1 neurons was largely NMDA-receptor mediated (Worley *et al.*, 1991). *zif268* expression may be involved with new learning, inasmuch as destruction of CA1 neurons (Kubo *et al.*, 1993) and NMDA antagonists injected into the hippocampus (Ohino *et al.*, 1992) impaired learning. Consistent with these findings linking cIEGs induction with the learning process, brightness discrimination training, learning a bar-pressing task, as well as two-way active-avoidance behavioral training elevated cIEG mRNA levels, namely *c-fos, c-jun* and *zif268* mRNA, in the rodent hippocampus (Tischmeyer *et al.*, 1990; Nikolaev *et al.*, 1992; Heurteaux *et al.*, 1993).

In addition to learning and memory, other forms of hippocampal plasticity have been correlated with cIEG induction. Distinct induction patterns for *c-fos, c-jun, junB* and *NGFI-B* were demonstrated in each cell body region of the rat hippocampus following transient forebrain ischemia, which may relate to the delayed neuronal death of CA1 neurons following anoxia as compared to other hippocampal cell body regions (Neumann-Haefelin *et al.*, 1994). Fos protein expression also immediately preceded the appearance of ribosomes and structural remodeling of dendritic spines of partially deafferented dentate granule cells (Chen and Hillman, 1992). In primary rat hippocampal cultures, the selective inhibition of *c-jun* expression using antisense oligonucleotides prevented neuronal cell death and promoted neuronal survival

suggesting a role of Jun in programmed cell death in this brain area (Schlingensiepen *et al.*, 1994). Additionally, several studies have demonstrated that the induction of *c-fos* mRNA and protein in the hippocampus is prominent in the CA1 pyramidal cell layer following exposure to a novel environment (Handa *et al.*, 1993; Papa *et al.*, 1995). Thus, *c-fos* mRNA induction may be a good marker for CA1 pyramidal cell activity.

Interaction of cIEGs and Steroid Hormone Receptors

Although the majority of evidence to date relates cIEG induction through neural excitation via membrane receptors for glutamate (Sonnenberg *et al.*, 1989; Lerea and McNamara, 1993; Wan *et al.*, 1994; Papa *et al.*, 1995), adrenergic compounds (Gubits *et al.*, 1989), opiates (Chang and Harlan, 1990) or acetylcholine (Greenberg *et al.*, 1986), the possibility of direct and/or indirect hormonal modulation of cIEGs is now emerging (see reviews by Landers and Spelsberg, 1992; Schuchard *et al.*, 1993; Hyder *et al.*, 1994). Estrogen treatment has been shown to cause a rapid and transient increase in *c-fos* mRNA in the uterus (Loose-Mitchell *et al.*, 1988) and hypothalamus (Insel, 1990) of ovariectomized rodents. This very rapid induction appears to be a direct effect of the transformed estrogen receptor complex acting on estrogen response elements that flank the *c-fos* gene (Weisz and Rosales, 1990; Hyder *et al.*, 1991a, 1991b). To date, response elements for the androgen receptor have not been identified upstream of cIEG genes, however, studies in prostate and prostatic cell lines have demonstrated androgen-induced changes in several cIEGs including *c-*

myc and *c-fos* (Quarmby *et al.*, 1987; Buttyan *et al.*, 1988; Rennie *et al.*, 1989; Wolf *et al.*, 1992). One recent study found no effects of androgen treatment on mating-induced Fos immunoreactivity in hypothalamic brain regions of castrated male rats (Baum and Wersinger, 1993). Whether androgens can affect cIEG induction in other cell types or brain areas is not presently known. Potentiation of *c-fos* and *c-jun* mRNA content in the hippocampus (Li *et al.*, 1992) and hypothalamus (Jacobson *et al.*, 1990) have been demonstrated following ADX; an effect the authors attribute to the removal of circulating glucocorticoid hormone. It is likely safe to assume that a mechanism of androgen modulation of cIEG expression is available in the CNS, especially in areas of the hippocampus where there is an anatomical overlap of AR synthesizing cells with those cells where cIEGs are induced following various physiologic stimuli. Androgen modulation of cIEG expression would implicate androgens in the long term alteration of hippocampal function and would suggest that the hormonal status of the animal affects the active response of hippocampal cells to incoming information.

Clinical Implications

The study of androgen action in the hippocampus has the potential to impact several areas of clinical medicine. There is growing concern over the health risks and psychological problems associated with the long term abuse of anabolic-androgenic

steroids. Few studies on how high-doses of androgen affect brain tissue have been performed. Essentially, anabolic-androgenic steroids are synthetic derivatives of T and DHT which act through the AR to elicit many of their effects. When taken in high doses and combined with rigorous training and a high protein diet, anabolic steroids can produce large increases in muscle mass in a relatively short period of time (Haupt and Rovere, 1984). Such results have led to a dramatic surge in anabolic steroid abuse by professional, college, high school and recreational athletes in order to enhance their performance or body appearance. Currently, both males and females use anabolic steroids and it is estimated that there are at least one million users in the United States alone (Marshall, 1988). Typically, steroid abusers take multiple forms of hormone at once and thus provide circulating androgen 10-200 times physiological levels (Narducci *et al.*, 1990). Common peripheral side effects of such steroid abuse include testicular atrophy, virilization (females), increased risk of heart disease, acne, and hepatotoxicity (Narducci *et al.*, 1990). In addition, recent clinical evidence suggests various psychotropic effects of high dose anabolic steroids. These include violent behavior, hyperactivity, psychoses, hallucinations, depression, suicide ideation, antisocial behavior, and panic disorders (Lubell, 1989; Katz and Pope, 1990; Uzych, 1992). These psychological changes appear to be the result of chronically high levels of androgen reaching the brain, however, the underlying mechanisms are unknown. Limbic areas of the brain that control aggression and emotion, including the amygdala and hippocampus, are likely targets for androgen action. Although the clear answer to these problems is the prevention of anabolic-androgenic steroid abuse,

an understanding of the cellular actions of long term, high dose androgens in brain tissue may aide in the treatment of those individuals who still choose to illegally use these drugs.

The increasing number of studies touting the beneficial androgenic effects on hippocampal plasticity, memory and overall well-being throughout adulthood have recently prompted studies examining T supplementation in older men. Typically, circulating androgen levels decline with age in both men (Davidson *et al.*, 1983; Vermeulen, 1991; Vermeulen and Kaufman, 1995) and women (Zumoff *et al.*, 1995). Whether T replacement to levels found in younger individuals can improve certain memory skills, mood and libido are just beginning to be explored (Goudsmit *et al.*, 1990; Janowski *et al.*, 1994; Tenover, 1994). Currently, not much information exists on androgen sensitivity in brain or peripheral tissues during the aging process (Goudsmit *et al.*, 1988; 1990b). Such research would certainly shed light on the validity and safety of such treatments in older men and women.

Summary

The increasing number of reports of psychological side effects of anabolic-androgenic steroid abuse, as well as the possible beneficial effects of physiological levels of androgens on neuronal plasticity, have prompted a heightened research interest into the intracellular mechanisms of androgens in brain tissue. The presence of relatively high levels of androgen receptors and their mRNAs in the CA1

pyramidal cells of the hippocampus suggests that this area is a major neural target for androgens. Subsequently, changes in androgen sensitivity in hippocampal pyramidal neurons, which form a major output of the hippocampus to limbic and cortical areas, may underlie some of the behavioral effects of anabolic-androgenic steroids. The quantification and regulation of AR and its mRNA in the hippocampus following various androgen treatments were determined to begin to examine the responsiveness of the hippocampus to circulating androgen. The action of the AR at the transcriptional level is also not well understood. Changes in the expression of various constitutively expressed or inducible genes are possible mechanisms that could alter the way in which hippocampal pyramidal cells respond to incoming signals. Therefore, a multidisciplinary approach was used to characterize the hippocampal AR, its regulation and its effects on constitutive and inducible gene expression following androgen removal and replacement. Together, these studies have begun to define the sensitivity of the adult hippocampus to androgens and serve as a basis for further investigation of activational androgenic effects on hippocampally-mediated behaviors, such as cognition, memory formation and spatial ability.

CHAPTER III

DISTRIBUTION AND HORMONAL REGULATION OF ANDROGEN RECEPTOR (AR) AND AR MESSENGER RNA IN THE RAT HIPPOCAMPUS

Abstract

The action of androgens in both peripheral and central tissues are linked in part to their ability to specifically bind and activate ARs. ARs have been well studied in the rat hypothalamus and peripheral reproductive tissues, where they are directly involved in endocrine feedback mechanisms and reproduction. Previous studies have revealed relatively high levels of AR and AR mRNA in the rat hippocampus; however, the action of androgen in this brain region remains unclear. To begin to address this issue, a multidisciplinary approach was used to quantitate hippocampal AR and AR mRNA levels and to investigate AR autoregulation following various hormonal manipulations. *In vitro* binding assays revealed a single, saturable, high affinity binding site for androgen in hippocampal cytosols. Western immunoblot analysis of hippocampal, hypothalamic, cortical and ventral prostate cytosol preparations using an AR specific antibody showed a primary signal at approximately 110-140 kilodaltons suggesting a single AR species in both brain and peripheral

tissues. The expression of AR mRNA in the intact adult male rat hypothalamus and hippocampus was quantified using a RNase protection assay. Comparable levels of AR mRNA were found in the hippocampus and hypothalamus. In addition, *in situ* hybridization analysis revealed a unique distribution of AR mRNA in the hippocampus. AR mRNA was found predominately in the CA1 pyramidal cells which form the major signal output of the hippocampal trisynaptic circuit. RNase protection assay demonstrated a significant decrease in AR mRNA content in the hippocampus of animals killed four days following castration, or in intact rats after four daily injections of the AR antagonist, flutamide (15 mg/animal), as compared to mRNA levels in intact controls ($P < 0.01$). In contrast, a 35% increase ($P < 0.05$) in the hippocampal AR mRNA content was found in old (22 month-old) male rats as compared to young (5 month-old) male rats. In both cases, [^{3}H]-DHT binding to the cytosolic preparation did not parallel the changes observed in the AR mRNA content. In summary, these data demonstrated that hippocampal cells containing AR can respond to circulating androgen to alter AR gene expression. Furthermore, AR mRNA autoregulation was be both age and tissue specific and did not directly follow the regulatory patterns previously described for other steroid hormone receptors found in the hippocampus.

Introduction

T and its 5α-reduced metabolite, DHT, are the major circulating androgenic hormones in males. Androgen action is linked in part to its ability to specifically bind and activate ARs. In neural tissue, AR are distributed in a pattern consistent with androgenic effects on the regulation of gonadotropin secretion and reproductive behaviors (Sar and Stumpf, 1973; Lieberburg *et al.*, 1977; Handa *et al.*, 1986; Roselli, 1991).

Studies revealing relatively high levels of AR and its mRNA in extrahypothalamic brain areas such as the cortex, lateral septum and the hippocampus of the rat (Sar and Stumpf, 1974, 1977; Handa *et al.*, 1987a; Roselli *et al.*, 1989; Simerly *et al.*, 1990; McLachlan *et al.*, 1991; Zhou *et al.*, 1994b) present the possibility that androgen action in the brain is not limited to the expression of some reproductive behaviors and endocrine feedback mechanisms. Recently, androgenic compounds have been shown to influence some hippocampal-mediated learning and memory tasks in rats (Roofs and Havens, 1992; Flood *et al.*, 1992) as well as modulate NMDA receptor levels (Kus *et al.*, 1995) and NMDA receptor-mediated electrophysiological properties (Pouliot *et al.*, 1995) in hippocampal pyramidal cells. In humans, sex-related differences in certain memory skills as well as other cognitive functions (Kimura, 1992) implicate gonadal hormones as important organizational modulators of hippocampal physiology. Fluctuations in gonadal hormone levels during the normal monthly cycle in women or the seasonal cycle in men (Hampson

and Kimura, 1992) as well as T supplementation in older men (Janowsky *et al.*, 1994) have been shown to significantly alter cognitive ability. These studies suggest an active role of gonadal hormones on hippocampal function throughout life; however, their mechanism of action is not understood.

In aging male rats, a gradual decline in circulating levels of T has consistently been reported (Ghanadian *et al.*, 1975; Bethea and Walker, 1979; Kaler and Neaves, 1981). Androgen-mediated behaviors decline similarly with age in the male rat; however, restoration of circulating T levels equivalent to the young male will not fully restore behavior, suggesting that age-related deficits in behavior are probably due to changes in androgen responsiveness in certain brain areas (Chambers and Phoenix, 1984; Goudsmit *et al.*, 1990; Chambers *et al.*, 1991). Studies examining other steroid hormone receptors have shown significant decreases in hippocampal GR and MR density in aged rats (Sapolsky *et al.*, 1983; Van Eekelen *et al.*, 1991). How the aging process and its associated decline in circulating androgen levels affects AR expression in the hippocampus has not been explored.

Based on these data, it was hypothesized that the hippocampus is a major neural target for androgens. In the studies reported here, a multidisiplinary approach was used to characterize, quantify and localize AR and AR mRNA in the rat hippocampus. Furthermore, the responsiveness of the hippocampal AR and AR mRNA expression to removal of circulating androgen by castration as well as to naturally occurring deficits in circulating androgens such as those found in the aging male rat were examined.

Materials and Methods

Animals and Tissue

Young (3- to 5-month-old) and old (22- to 24-month-old) male Fischer 344 rats (Harlan Inc, Indianapolis, IN) were maintained on a 12-h light, 12-h dark cycle (lights on at 0700 h) and given free access to food and water. Bilateral GDX was performed under ether anesthesia and all animals were sacrificed by decapitation. Brain dissections of the hypothalamus, hippocampus and cortex were performed as previously described by Handa *et al.* (1986).

Experiment 1: Characterization, Quantification and Localization of AR and AR mRNA in the Hippocampus of Young Male Rats

To confirm the presence of AR in the rat neural tissues hippocampal AR were characterized using *in vitro* binding of [^{3}H]-DHT to hippocampal, cortical and hypothalamic cytosols obtained from rats castrated 24 h before death. Prior castration was necessary to ensure that all available AR were free of ligand and unbound to DNA. In addition, western immunoblot analysis was performed on cytosolic protein extracts from intact rat hippocampus, hypothalamus and cortex to determine the approximate size of the AR protein found in these neural tissues. To determine whether the location of the expression of AR mRNA in neural tissue mimicked that of its protein, total RNA isolated from the cortex, hypothalamus, and hippocampus of intact young rats was assayed by RNase protection assay. The distribution of AR

mRNA in the hippocampus of the intact rat was further analyzed using *in situ* hybridization.

Experiment 2: Regulation of Hippocampal AR and AR mRNA

In this experiment the regulation of AR and AR mRNA after androgen removal or AR antagonism was examined. Young animals were left intact, gonadectomized for 4 days, or subcutaneously injected daily with 15 mg of the AR antagonist, flutamide (30 mg/ml; dissolved in sesame oil), for 4 days. Total RNA was isolated from each hippocampus and assayed for steady state levels of AR mRNA using the RNase protection assay. *In vitro* binding of [^{3}H]DHT to hippocampal cytosols from animals gonadectomized for 12 h, 24 h, or 4 days was used to determine whether changes in AR protein levels mimic the changes in mRNA levels under similar conditions. To estimate total receptor numbers in intact rats, castration 12 h prior to sacrifice was performed to ensure that all AR were free of ligand and not bound to DNA and, thus, could be obtained in the cytosolic fraction.

Experiment 3: Hippocampal AR Levels in Aged Rats

To investigate the effect of naturally occurring reductions in T on hippocampal AR and mRNA content, I compared steady state levels of AR and AR mRNA in the hippocampus of young *vs.* old intact rats. *In vitro* binding and RNase protection assay were used for the quantification of AR and AR mRNA levels, respectively. Saturation analysis of [^{3}H]DHT binding were also performed to analyze possible age-

related changes in AR binding affinity.

In vitro Binding Assay

Tissue was analyzed for concentration of cytosolic AR as previously described (Handa *et al.*, 1986). Briefly, brains were rapidly removed from the skull and placed on crushed ice for dissection. Each brain region was homogenized in 600 μl of TEGMD buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, 25 mM molybdate, 1 mM dithiothreitol; pH 7.4); for saturation analysis, six hippocampi were pooled and homogenized in 1.5 ml of TEGMD buffer. The homogenate was centrifuged at 100,000 x g for 15 min in a TFT 80.4 rotor in a Sorval OTD55B ultracentrifuge (Sorval, Norwalk, CT) at 4°C to obtain a pure cytosolic fraction. For single point assay, 100 μl of the cytosolic fraction was incubated with 2 nM of [^{3}H]DHT (1,2,4,5,6,7-^{3}H(N)-5α-Androstan-17ß-ol-3-one, 110-150 Ci/mmol, New England Nuclear (NEN) Research Products, Boston, MA) for 20-24 h at 0-4°C for determination of total AR binding (total incubation, 150 μl). A 400 nM concentration (200-fold excess) of radioinert AR specific agonist, methyltrienolone (R1881, NEN Research Products), was incubated in parallel tubes with [^{3}H]DHT to determine nonspecific binding. For saturation curves, purified cytosolic fractions were aliquoted (100 μl) into 1.5 ml conical tubes containing [^{3}H]DHT (0.05 nM to 2 nM). A parallel set of incubation tubes containing an additional 200-fold excess of unlabelled R1881 were used to determine nonspecific binding. Following the overnight incubation at 4°C, all samples were passed through Sephadex LH-20 columns to

separate bound from free ligand. Six hundred microliters of eluate containing bound radioactivity were collected. Four milliliters of UltimaGold scintillation fluid (Packard, Downers Grove, IL) was added to the eluate, and the radioactivity was counted in a Packard 1900 LA liquid scintillation counter at 37% efficiency. Specific binding was calculated by subtracting nonspecific binding from total binding. Ten microliters of the remaining cytosol was used for measurement of protein levels by the method of Lowry *et al.* (1951). All receptor data are expressed as femtomoles (fmol) per mg protein. Scatchard transformations were generated by computer using The LIGAND program (version 3.0, Elsevier North Holland, Amsterdam, The Netherlands).

RNA Isolation

Dissected brain regions were homogenized separately in 4 M guanidinium isothiocyanate (Boehringer Mannheim, Indianapolis, IN) buffer containing 50 mM sodium citrate, pH 7.0, 0.5% sarkosyl and 0.1 M ß-mercaptoethanol. Total RNA was isolated as previously described by Chirgwin *et al.* (1979), by pelleting through a 5.7 M CsCl cushion for 14-16 h at 147,000 x g at 15°C. The resuspended RNA pellet was phenol-chloroform-isoamyl alcohol (24:24:1) extracted, and the aqueous phase was then further purified by ethanol precipitation. The resultant pellets were washed with 70% ethanol, resuspended in diethylpyrocarbonate-treated H_2O and stored at -70°C until used for the RNase protection assay. RNA content was determined by UV absorbance at 260 nm.

RNase Protection Assay

In vitro solution hybridization of AR mRNA was performed as previously described by Burgess and Handa (1993a). To generate antisense radiolabelled RNA probes, a 141 basepair fragment of the rat AR2 cDNA (Tan *et al.*, 1988) was subcloned into a pGEM 3Z plasmid vector (Promega), as depicted in **figure 6A**. A radiolabelled antisense RNA probe was transcribed following linearization of these vectors with *Eco*R1 and transcription with SP6 DNA-dependent RNA polymerase in the presence of α-^{32}P-labeled CTP (800 Ci/mmol; Amersham, Arlington Heights, IL). This procedure and subsequent RNase protection assay are outlined in **figure 6B**. The resulting antisense probe had a specific activity of more than 10^9 cpm/μg. Aliquots of the transcribed RNAs were analyzed on denaturing 5% acrylamide, 7.5 M urea gels to confirm their integrity. Only ^{32}P-labeled cRNA transcripts that were more than 90% full length were used in subsequent assays. Sense strand RNAs were transcribed from the same construct, using the T7 polymerase, following linearization with Pst I. Dilutions of *in vitro* synthesized sense strand RNA (>99% full length) were used to generate the standard curves performed in each assay.

Either 10 μg sample RNA or dilutions of *in vitro* transcribed sense strand RNA [50, 25, 12.5, 5 and 2.5 attomoles(amol)] were hybridized in solution to a molar excess (100,000 cpm) of ^{32}P-labeled antisense RNA (total incubation volume, 30 μl). The standard curves generated were linear, with correlation coefficients consistently greater than 0.99. Ten micrograms of transfer RNA were used as a

negative control. Following hybridization overnight and digestion of unprotected fragments with RNases A and T1 (40 μg/ml and 2 μg/ml, respectively), the protected fragments were phenol-chloroform-isoamyl (24:24:1) extracted, ethanol precipitated and resuspended in 10 μl formamide load buffer (80% formamide, 10 mM EDTA, 1 mg/ml Bromophenol blue, 1 mg/ml xylene cyanole). Resuspended fragments were electrophoresed through 5% acrylamide-7.5 M urea gels at 300 V. Gels were fixed in 7% acetic acid and dried. Radioactivity in the gels was counted directly by a Betascope 6000 analyzer (Betagen, Waltham, MA). Values are expressed as fmol protected probe per mg input RNA. Each sample was run in duplicate in each assay, and the resulting values were averaged to obtain a final value for each animal. Autoradiograms were obtained by exposing the dried gels to Hyperfilm (Amersham, Lake Forest, IL) at -70°C for 4-7 days. Validation of the assay and a typical standard curve (cpm in the protected band *vs.* amol of sense stand added) are shown in **figures 7A** and **7B**.

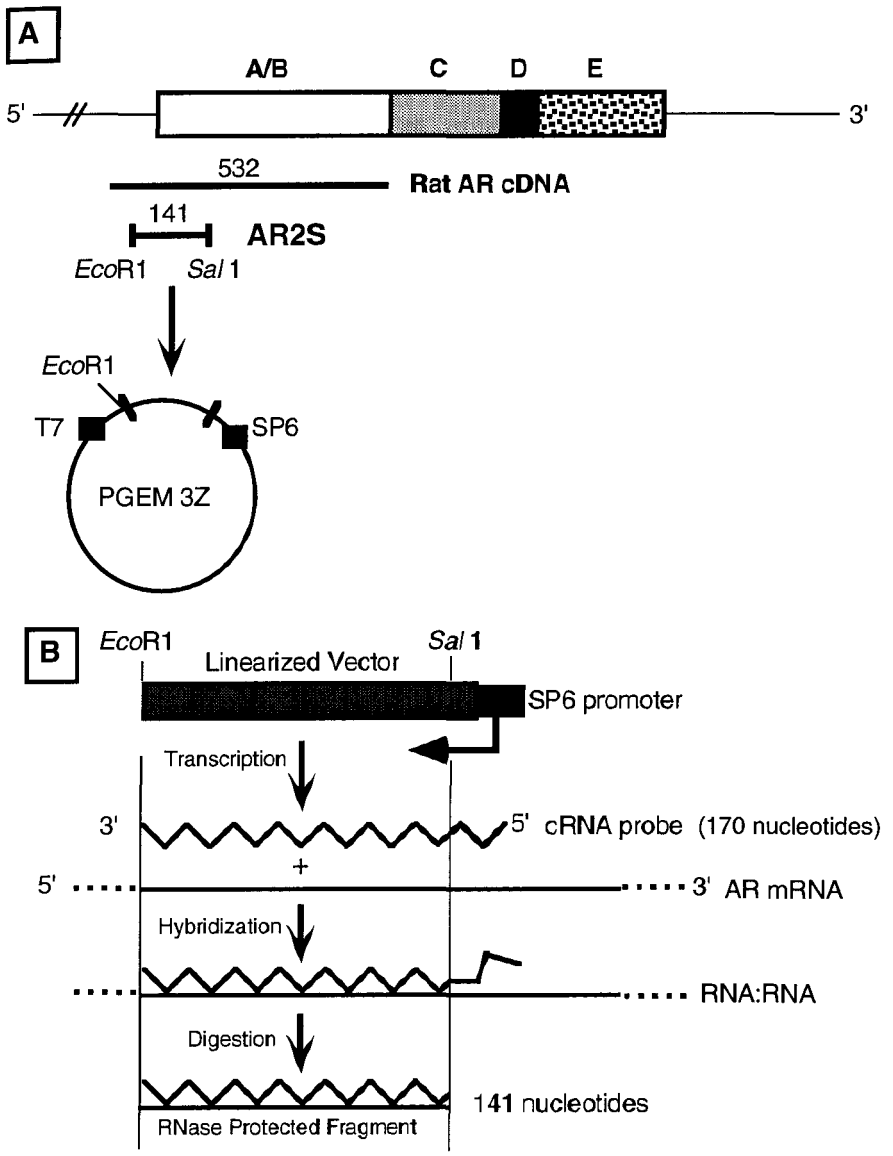

Figure 6. (A) Schematic representation of the AR2S cDNA construct prepared by subcloning a 141 nucleotide fragment of the rat AR cDNA, corresponding to the 5'-translated region. **(B) The RNase protection assay process.** The plasmid is linearized with *Eco*R1 and *in vitro* transcribed with SP6 RNA polymerase to produce a uniformly labelled antisense cRNA transcript of 170 bases. This probe hybridizes to AR mRNA and following digestion of all single stranded RNA and purification, the resulting 141 nucleotide protected fragment was left.

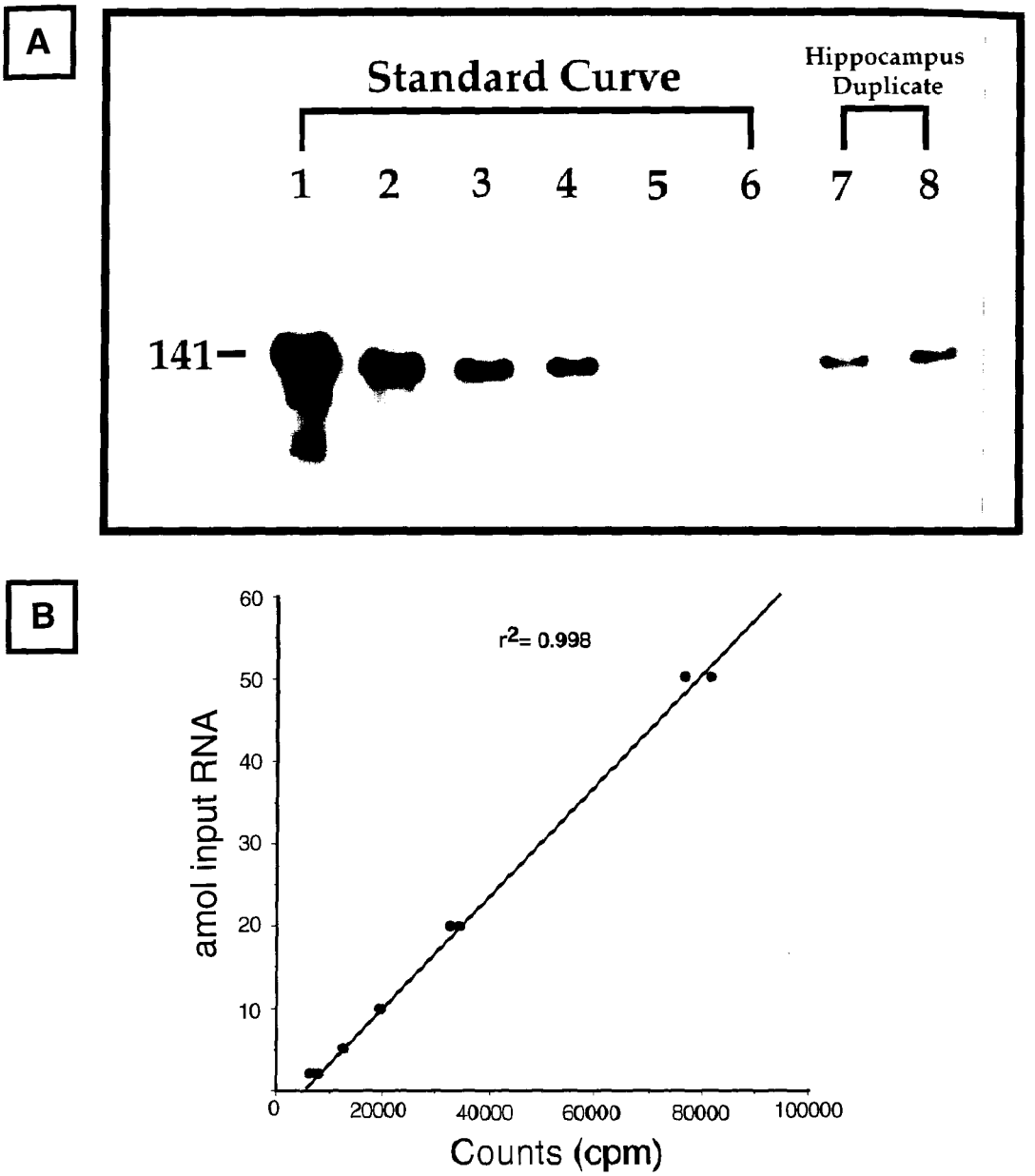

Figure 7. Verification of the AR RNase protection assay. Sense strand RNA were hybridized to excess ^{32}P-labelled antisense RNA probe and digested with RNase as described in Methods. **(A) Respresentitive autoradiogram of gel electrophoresis.** Lanes 1 to 5 represent decreasing amounts of added sense strand RNA [50, 25, 12.5, 5 and 2.5 attomoles (10^{-18})]. Lane 6 is the transfer RNA control, and lanes 7 and 8 demonstrate representitive duplicate bands from 10μg added hippocampal RNA. **(B) AR RNase protection assay standard curve.** Protected counts (as measured by the Betascope) plotted versus the amount of added sense strand RNA.

In Situ Hybridization

In situ hybridization was performed using the *in vitro* transcribed AR cRNA probe as described above, but labeled with [^{35}S]UTP (800 Ci/mMol, Amersham, Arlington Heights, IL). Completeness of transcription was determined by 5% acrylamide-7.5 M urea gel electrophoresis. The specific activity of the probes averaged 1 x 10^9 cpm/μg. Only probes greater than 90% full length were used for *in situ* hybridization.

Whole brains were rapidly removed from skull and immersed in cold isopentane (-30°C). Brains were stored frozen at -80°C until sectioned. Brains were sectioned at 16 μm in a Leitz 1600 cryostat and mounted onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). *In situ* hybridization using the ^{35}S-labelled cRNA probe was performed according to the method previously described by Handa *et al.* (1993). Approximately 85 μl of a 20 x 10^6 cpm/ml hybridization solution (50% formamide, 20% dextran sulfate, 1.2 M NaCl, 20 mM Tris, 0.04% Denharts solution, 2 mM EDTA, 0.02% salmon sperm DNA, 0.1% yeast RNA, 0.1 % sodium thiosulfate, 100 mM dithiothreitol (DTT), 0.1% sodium dodecyl sulfate (SDS)) were added to each slide, coverslipped and allowed to hybridize in a humidified incubator for 16 h at 65°C. Slides were rinsed in 2 x saline sodium citrate (SSC) and nonhybridized RNA was digested with RNase A (20 μg/ml: 37°C for 30 min). Slides were washed to a final stringency of 0.1 x SSC at 60°C. Autoradiograms were obtained by exposing slides to X-ray film (Hyperfilm B-max, Amersham, Arlington Heights, IL) for 21 days. Following film exposure, slides were dipped in nuclear

tract emulsion (Kodak NTB-3) and exposed for 35 days before development and cresyl violet staining. These sections were examined under bright- and darkfield illumination using a Zeiss Axioplan microscope (Zeiss, New York, NY). Resulting images were digitized from photographic slides. Composite figures were made using Adobe Photoshop software.

Western Immunoblot Analysis

Freshly dissected tissues were homogenized in 300 - 600 μl of Tris-EDTA buffer, pH 7.4, supplemented with 0.2 mg/ml Aprotinin, 1 mM DTT and 0.1% SDS. Cytosolic extracts were made by centrifuging at 100,000 x g for 30 min in an TFT 80.4 rotor in a Sorval OTD55B ultracentrifuge at 4°C. Protein levels in the cytosol were determined by the method of Lowry *et al.* (1951). After boiling for 5 min, 50 μg of the denatured cytosolic extracts were electrophoresed on 1.5 mm SDS-polyacrylamide gels consisting of a 5% stacking gel and an 8% resolving gel. Protein was electrophoretically transferred to polyvinylidene difluoride membrane (Polyscreen™, NEN Research Products, Boston, MA) at room temperature for 1 h at 200 amps in a buffer containing 0.048 M Tris, 0.039 M glycine, 0.037% SDS and 20% methanol. Membranes were incubated overnight at room temperature in blocking buffer (5% Carnation nonfat dry milk in 1 X TBS, 0.05% Tween-20, and 0.02% sodium azide) and then incubated for 1 h with purified PG-21 antisera (1 μg/ml). This is a rabbit antiserum raised against a synthetic peptide corresponding to the first 21 amino acids of the rat and human AR (generously supplied by Dr. Gail

Prins, University of Illinois College of Medicine). A preabsorption control consisting of 1 μg/ml PG-21 and a 10-fold molar excess of the antigenic peptide AR_{21} (0.2 μg/ml) was incubated on corresponding membranes to demonstrate specificity. All membranes were incubated at room temperature in biotinylated goat antibody to rabbit IgG (2 μg/ml) in 5% dry milk and 0.05% Tween-20 (TBST) for 1 h. After each incubation, membranes were washed with TBST (2 X 15 min) at room temperature. Immunoreactive bands were visualized using Renaissance™ western blot chemiluminescence reagent (NEN Research Products, Boston, MA; 0.125 ml/cm^2 membrane for 2 min) and exposed to autoradiographic film (Reflection™, DuPont, Boston, MA) for 5 - 10 min.

Statistical Analysis

All data were analyzed by a one-way analysis of variance followed by the Student-Newman-Keuls multiple comparisons test. $P < 0.05$ was considered significant.

Results

Characterization, Quantification and Localization of AR and AR mRNA in the Rat Hippocampus

To determine whether the binding characteristics of AR in the hippocampus were similar to that previously reported in other neural tissues, such as the hypothalamus, we examined the *in vitro* binding of [^{3}H]DHT to hippocampal, cortical and hypothalamic cytosols obtained from young male rats castrated one day prior to sacrifice. Scatchard analysis of [^{3}H]DHT binding to AR in each of the three cytosols (**figure 8**) demonstrated a saturable, high affinity binding site which was best fit by a single site model and had an apparent K_d of 0.2 nM. The highest concentration of AR binding was found in the hypothalamus with an approximate binding capacity (Bmax) of 4.5 fmol/mg protein, followed closely by hippocampal binding with an approximate Bmax of 3.9 fmol/mg protein. Cortical tissue had the lowest AR concentration of the three tissues with a Bmax of approximately 1.4 fmol/mg protein.

Western immunoblots were performed to characterize and compare rat AR immunostaining in neural and peripheral tissues believed to express relatively high levels of AR. A prominent specific AR protein approximately 110-140 kilodalton (kDa) in size was detected in ventral prostate, hippocampus, hypothalamus, cortex and pituitary gland using the PG-21 antibody (**figure 9**, lanes 1-5, respectively). This corresponds well to the known molecular weight of the rat AR. In ventral prostate and hypothalamus, smaller immunoreactive bands approximately 45-85 kDa in size

were visible and are thought to be a cleavage products of AR (**figure 9**, lanes 1 and 3). All bands were completely competed by excess antigenic AR_{21} peptide (**figure 9**, lanes 6 and 7, ventral prostate and hippocampus shown). Quantification of the resulting autoradiograph bands would not be meaningful due to potential differences in degradation or cleavage rates of AR in the tissues studied. Efforts to minimize degradation through the addition of molybdate, multiple protease inhibitors, and increased SDS concentrations were unsuccessful in eliminating all of the degradation products. The extreme labile nature of AR protein, especially in the absense of ligand, has been reported by others (Kemppainen *et al.*, 1992; Zhou *et al.*, 1995). Additionally, studies in rat peripheral tissues have detected multiple bands upon AR immunoblot analysis and these authors cited region-specific degradation as the probable cause of multiple smaller bands (Shan *et al.*, 1990; Prins *et al.*, 1991). The addition of excess ligand, as well as the believed relative stability of the steroid binding region of the AR protein, makes AR binding analysis more suitable for the measurement of AR concentrations in neural and peripheral tissues.

Quantification of AR mRNA levels in neural tissue using the RNase protection assay paralleled our findings of AR binding levels. Similar steady state levels of AR mRNA were found in young male hypothalamus and hippocampus with values of 557 $\pm$ 56 and 539 $\pm$ 54 amol/mg input RNA, respectively. AR mRNA levels in the cortex were significantly lower than in both hippocampus and hypothalamus (310 $\pm$ 32 amol/mg input RNA, $P<0.01$).

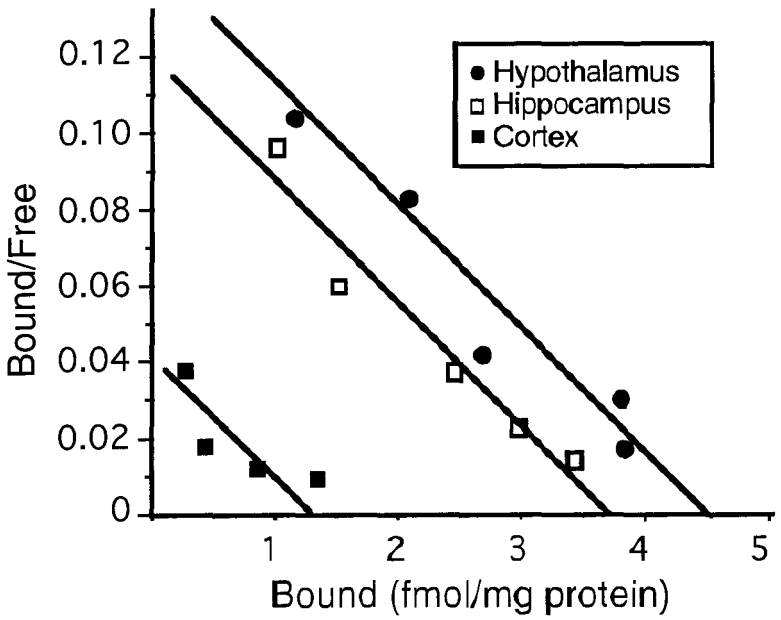

Figure 8. Scatchard analysis of specific [^{3}H]DHT binding in rat brain. Cytosolic preparations were analyzed from the hypothalamus (●), hippocampus (□), and cortex (■) of young male rats castrated 24 hours prior to sacrifice. Tissues from 6 rats were pooled to obtain cytosolic preparations. Cytosols were incubated with 0.05 nM - 2 nM [^{3}H]DHT with and without a 200-fold excess of unlabelled R1881 to obtain saturation isotherms. Scatchard transformations and dissociation constants (K_d) were generated by computer using the LIGAND program. A K_d value of 0.22 nM were obtained for all three tissues studied. Reprinted, by permission, from J.E. Kerr, R.J. Allore, S.G. Beck, R.J. Handa. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger RNA in the rat hippocampus. Endocrinology, 136(8):3213-3221, 1995. © **The Endocrine Society.**

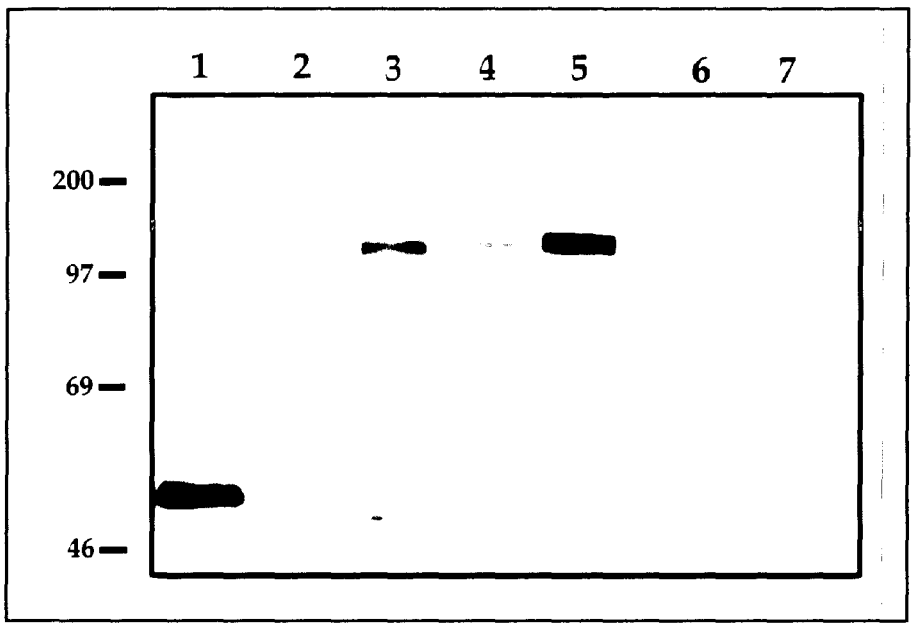

Figure 9. Western immunoblot analysis of AR using the PG-21 antisera in cytosolic preparations from young adult male rat ventral prostate (lanes 1 and 6), hippocampus (lanes 2 and 7), hypothalamus (lane 3), cortex (lane 4) and pituitary gland (lane 5). Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 8% gen and transferred to polyvinylidene difluoride membrane. Strips were incubated with PG-21 antisera (1μg/ml) in the absence (lanes 1-5) or presence (lanes 6 and 7) of a 10-fold molar excess of the antigenic peptide AR_{21}. Bands were visualized using chemiluminescence. The position of the molecular weight markers (kDa) are shown on the left. The major immunoreactive band is at ~110-140 kDa.

Further investigation of AR mRNA in the hippocampus demonstrated that it is not expressed equally in all cellular regions. The examination of emulsion-coated tissue sections following *in situ* hybridization revealed that AR mRNA is predominately expressed in the CA1 pyramidal cell region of the intact male rat hippocampus. For comparison, AR mRNA was expressed in near equivalent levels in the ventromedial nucleus and arcuate nucleus of the hypothalamus where AR is known to play a role in hormonal feedback and sexual behavior (**figure 10**). The examination of the hippocampus at high magnification revealed that virtually all CA1 neurons expressed AR mRNA (**figure 11A**). Much lower expression of AR mRNA was detected in the CA3 region (**figure 11B**) and expression was absent in the DG (**figure 11C**). The level of exposed silver grains over the CA1 pyramidal cells is comparable to levels found over the cells of the ventromedial nucleus of the hypothalamus (**figure 11D**).

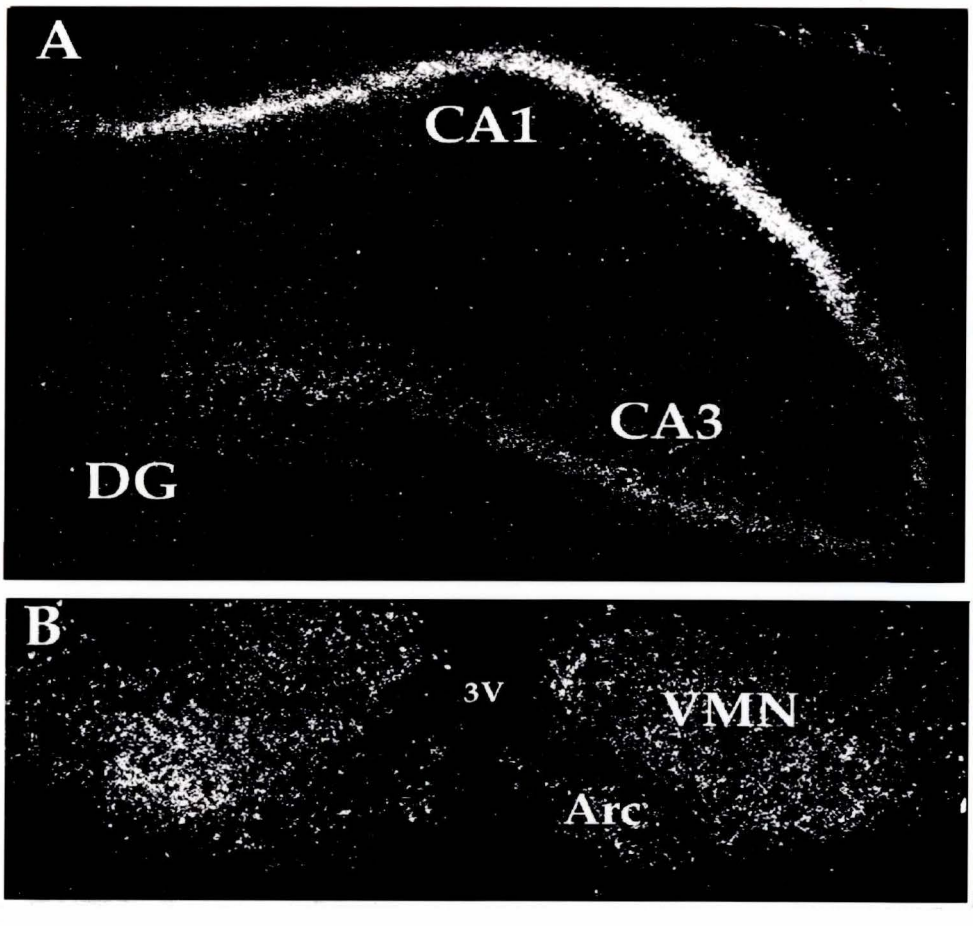

Figure 10. Localization of AR mRNA in rat brain using in situ hybridization. Darkfield photomicrographs (magnification = 100X) illustrating the distribution of AR mRNA in the hippocampus (A) and the ventromedial nucleus (VMN)/arcuate nucleus (Arc) of the hypothalamus (B) in the young male rat. AR mRNA expression is highest in the CA1 pyramidal cell region of the hippocampus and comparable levels are found in the VMN/Arc. Images were digitized from photographic slides and composite figures were generated using Adobe Photoshop software. 3V, Third ventricle. Reprinted, by permission, from J.E. Kerr, R.J. Allore, S.G. Beck, R.J. Handa. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger RNA in the rat hippocampus. Endocrinology, 136(8):3213-3221, 1995.

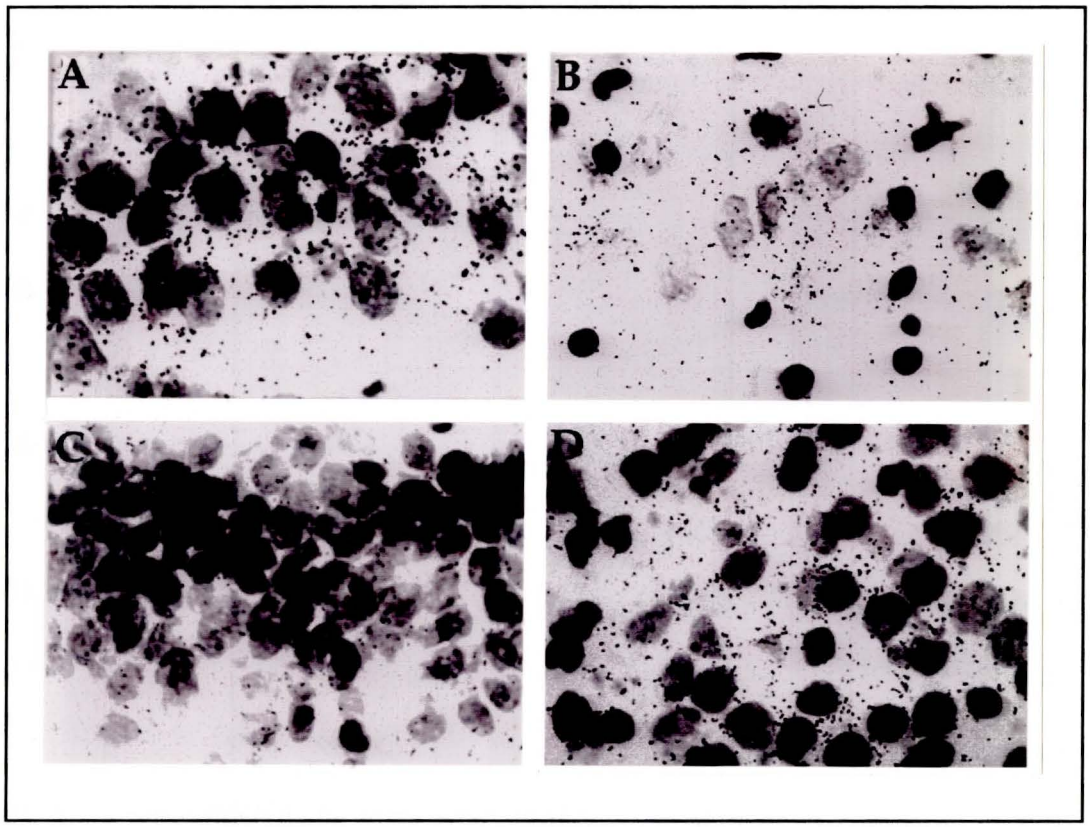

Figure 11. Localization of AR mRNA in hippocampal and hypothalamic neurons of young intact male rats. Digitized brightfield photomicrographs (magnification = 1000X) show exposed silver grains over tissue following *in situ* hybridization of ^{35}S-labelled cRNA probe to AR mRNA. Cresyl violet darkly stains cell nuclei, whereas perikarya are pale to invisible due to RNase treatment of the tissue during *in situ* hybridization. Dense labelling is evident over cells of the CA1 region of the hippocampus (A) and ventromedial nucleus of the hypothalamus (D). Little to no labelling is found over the CA3 pyramidal cell region (B) and dentate gyrus (C) of the hippocampus. Reprinted, by permission, from J.E. Kerr, R.J. Allore, S.G. Beck, R.J. Handa. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger RNA in the rat hippocampus. Endocrinology, 136(8):3213-3221, 1995.

Short Term Regulation of Hippocampal AR

As measured by RNase protection assay, animals castrated 4 days previously and animals injected for 4 days with the androgen receptor antagonist, flutamide, had decreased hippocampal AR mRNA concentrations as compared to intact animals ($P<0.02$, **figure 12**). Castration 1 day prior to death did not alter AR mRNA levels in the hippocampus as compared to that in intact controls. In contrast, [^{3}H]DHT binding to hippocampal cytosols was increased in 1- and 4-day castrates compared to that in control animals castrated 12 h prior to sacrifice ($P<0.05$, **figure 13**).

Age-Related Changes in Hippocampal AR Expression

To determine whether hippocampal AR levels are altered in the old rat with physiologically relevant reductions in circulating androgen, AR mRNA content as well as AR binding levels and kinetics were examined in young and old intact male rats. Using the RNase protection assay, hippocampal AR mRNA concentration was 539 $\pm$ 54 amol mRNA/mg input RNA in the young animals as compared to 729 $\pm$ 46 amol mRNA/mg input RNA in the old rats (**figure 14**). This represents a 35% age-related increase ($P<0.05$). Age-related differences were not found in the cortex or hypothalamus (**figure 14**). In contrast, *in vitro* binding studies revealed no significant changes between total cytosolic [^{3}H]DHT binding in the hippocampi of young and old animals (4.47 $\pm$ 0.25 and 5.19 $\pm$ 0.3 fmol bound/mg protein, respectively; **figure 15**), and no alterations in AR binding affinity (K_d = 0.24 and 0.26 nM, respectively; data not shown) were detected.

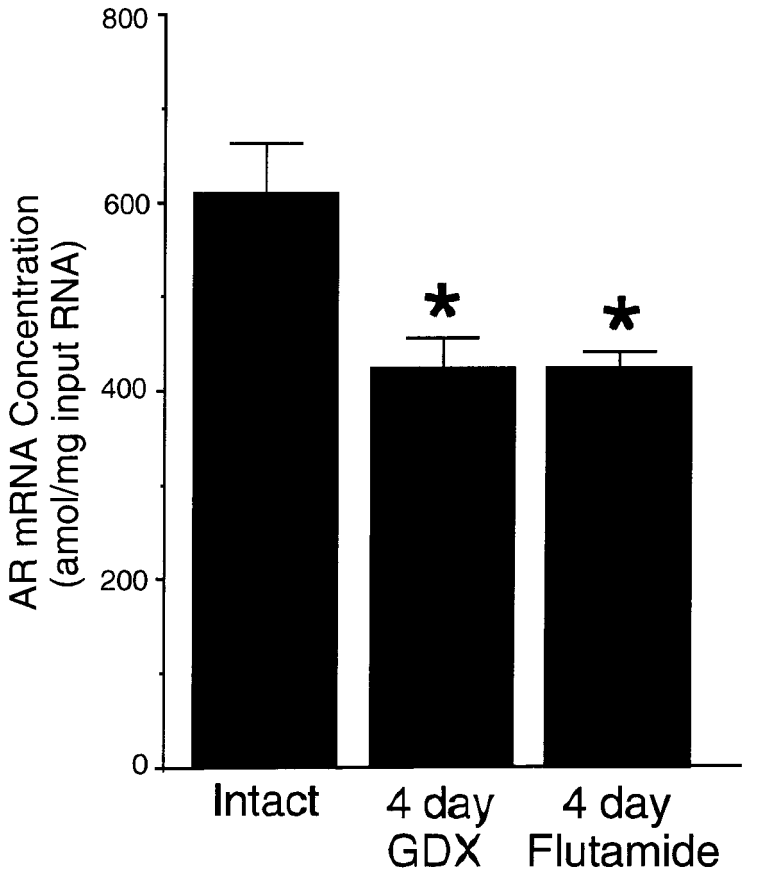

Figure 12. Hippocampal AR mRNA regulation. Effect of castration and AR blockade on the concentration of AR mRNA in the hippocampus of young male Fischer 344 rats. Animals were left intact, bilaterally gonadectomized for 4 days (4 day GDX), or injected daily with the AR antagonist, flutamide (15mg/day), for 4 days (4 day Flutamide). One-way ANOVA revealed a significant effect of treatment ($F=8.0$, $df=2$; $P < 0.004$). * Indicates significantly different ($P < 0.01$) from intact value, as determined by post-hoc analysis. Data are expressed as attomoles of protected probe (cAR mRNA) per mg input RNA. Each bar represents the mean $\pm$ SEM of 6-7 determinants. Reprinted, by permission, from J.E. Kerr, R.J. Allore, S.G. Beck, R.J. Handa. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger RNA in the rat hippocampus. Endocrinology, 136(8):3213-3221, 1995. © **The Endocrine Society**.

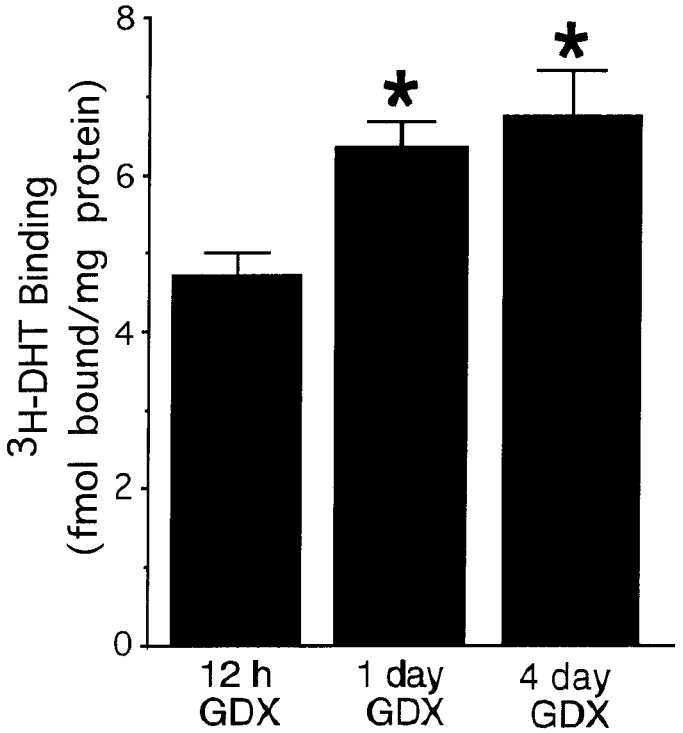

Figure 13. [^{3}H]DHT binding to cytosolic preparations of the hippocampus from young male Fischer 344 rats killed 12 hours (12 h GDX), 1 day (1 day GDX), or 4 days (4 day GDX) after castration. One-way analysis of variance revealed a significant effect of treatment ($F=6.5$, $df=2$; $P < 0.01$). *, Significantly different ($P < 0.05$) from 12 hour castrates, as determined by post-hoc analysis. Each bar represents the mean $\pm$ SEM of 8 determinants. Reprinted, by permission, from J.E. Kerr, R.J. Allore, S.G. Beck, R.J. Handa. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger RNA in the rat hippocampus. Endocrinology, 136(8):3213-3221, 1995. © **The Endocrine Society.**

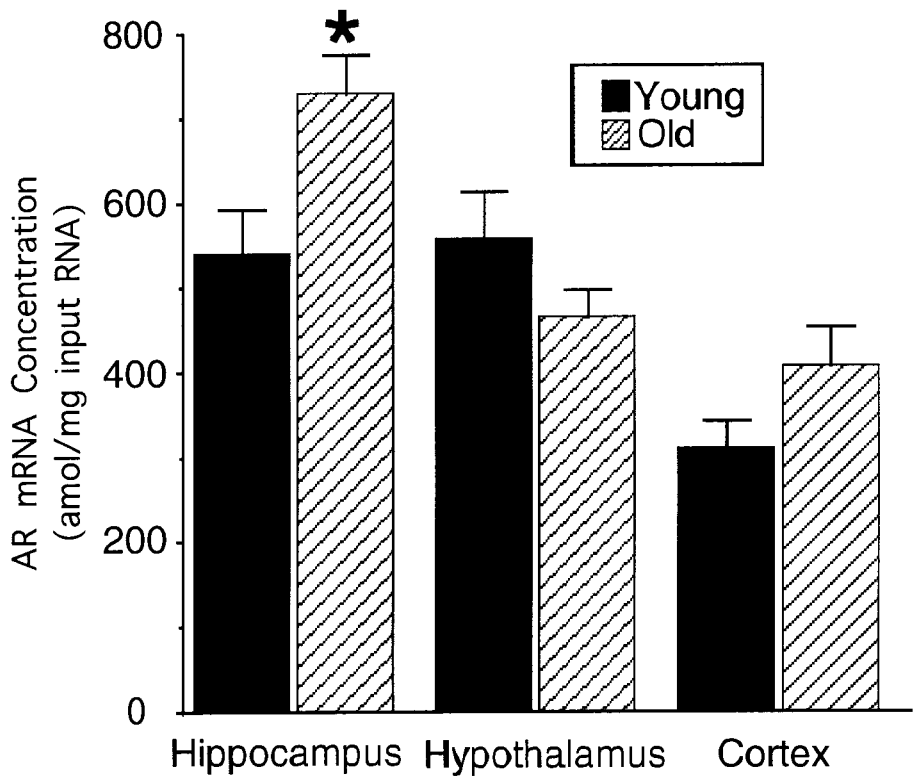

Figure 14. AR mRNA concentration in various tissues of intact young (3- to 5-month old) and old (22- to 24-month old) intact male Fischer 344 rats. A two-tailed *t* test revealed a significant effect of age in the hippocampus (*, $P < 0.05$). Data are expressed as attomoles of protected probe (cAR mRNA) per mg input RNA. Each bar represents the mean ± SEM of 5-7 determinants. Reprinted, by permission, from J.E. Kerr, R.J. Allore, S.G. Beck, R.J. Handa. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger RNA in the rat hippocampus. Endocrinology, 136(8):3213-3221, 1995. © **The Endocrine Society.**

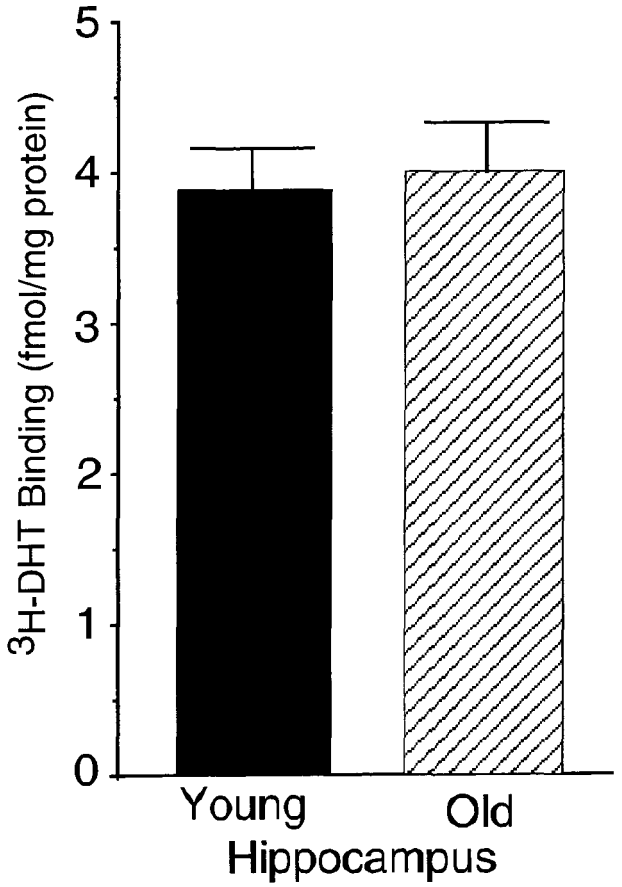

Figure 15. [^{3}H]DHT binding to hippocampal cytosolic preparations from young (3- to 5- month old) and old (22- to 24-month old) male Fischer 344 rats castrated 24 hours prior to sacrifice. Each bar represents mean ± SEM of 16 determinants. There were no significant differences. Reprinted, by permission, from J.E. Kerr, R.J. Allore, S.G. Beck, R.J. Handa. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger RNA in the rat hippocampus. Endocrinology, 136(8):3213-3221, 1995. © **The Endocrine Society.**

Discussion

In these studies a multidisciplinary approach was used to characterize and quantify AR in the rat hippocampus. The presence of high levels of AR and AR mRNA in the hippocampus was demonstrated by RNase protection assay, *in situ* hybridization, western immunoblot and *in vitro* binding analysis suggesting that this area is a major neural target for androgen.

The significance of the finding that the majority of AR mRNA is found in the hippocampal CA1 region is unclear. However, as these neurons complete the unidirectional trisynaptic circuit and provide the major output for the hippocampal formation to other cortical and limbic structures (Van Groen and Wyss, 1990), the high density of AR mRNA in practically every cell in this region suggests a role for androgens in the modulation of hippocampal output. Recent electrophysiologic and binding studies have found androgen-mediated changes in NMDA sensitivity (Pouliot *et al.*, 1995) and NMDA receptor number (Kus *et al.*, 1995) in hippocampal CA1 pyramidal cells. This modulation of NMDA receptors may be one mechanism by which androgens could phenotypically alter the response of hippocampal CA1 neurons to incoming signals.

The distribution of AR mRNA overlaps the distribution of ER, GR and MR mRNA in the hippocampus, in that all mRNAs are found in the CA1 region (Simerly *et al.*, 1990; Van Eekelen *et al.*, 1988). Consequently, AR may synergize with these receptors in regulating hippocampal functions known to be sensitive to adrenal

hormones (see review, de Kloet *et al.*, 1993a) or estrogen (see review, Becker, 1992). Consistent with this notion, androgen has been shown to inhibit ACTH and corticosterone responses to stress in a fashion similar to corticosterone (Handa *et al.*, 1994a).

Western immunoblot analysis was performed to further characterize AR in central tissues. The specific AR signal observed at approximately 110-140 kDa in rat central tissues, as well as in the rat VP, corresponds well to the known mol wt of the rat AR. This mol wt parallels the findings of other published AR western immunoblots of protein samples obtained from a variety of species or cell lines, various peripheral tissues and using a multitude of antibodies (Zhou *et al.*, 1994b; Young *et al.*, 1988; Prins *et al.*, 1991; Wolf *et al.*, 1993). This single band in rat brain cytosols confirms previous studies (Barley *et al.*, 1975; Roselli, 1991) suggesting a single AR despite the presence of two AR mRNA forms in neural tissues. We believe that the smaller bands ranging from 45-85 kDa that were observed in ventral prostate and hypothalamus are degradation or cleavage products of the intact AR protein for two reasons. First, all the bands were completely competed by excess antigenic AR_{21} peptide suggesting that these are AR protein fragments and are not the result of non-specific antibody binding. Secondly, when the prepared protein samples were left for any length of time, or frozen prior to electrophoresis we observed a greater proportion of the lower molecular weight bands and a dramatic decrease of the large 110-140 kDa band. Other studies in rat peripheral tissues have also detected these degradation products (Zhou *et al.*, 1994b; Prins *et al.*, 1991).

Our finding that AR mRNA levels in the hippocampus were decreased after 4 days of castration or AR antagonism is unusual. Most previous studies examining AR mRNA regulation in brain (Quarmby *et al.*, 1990; Burgess and Handa, 1993a) and peripheral tissues (Tan *et al.*, 1988; Quarmby *et al.*, 1990; Takane *et al.*, 1990; Blok *et al.*, 1991, 1992a) have found that steady state AR mRNA levels increase following castration; however, discrepancies do exist (McLachlan *et al.*, 1991; González-Cadavid *et al.*, 1993; Abdelgadir *et al.*, 1993). Earlier studies revealed an increase in AR mRNA in the medial preoptic area of the hypothalamus shortly after castration, but AR mRNA levels were significantly decreased in the same area in rats castrated 8 weeks prior to sacrifice (Handa *et al.*, 1993b). Burgess and Handa (1993a) reported apparent increases in hippocampal AR mRNA expression, as measured by Northern blot analysis, in rats castrated for 7 weeks before death. This latter study, along with our present findings, suggest a unique biphasic regulatory pattern of AR mRNA that appears to be both time- and tissue-specific. Unfortunately, the measurement of steady state levels of AR mRNA gives us little information as to where AR may confer its transcriptional control. Evidence for steroid receptor modulation at transcriptional (King, 1992) and post-transcriptional (Nielsen and Shapiro, 1990) stages have been reported, and changes in AR mRNA synthesis as well as changes in mRNA stability or turnover in response to androgen removal could account for our results.

The fact that changes in AR binding do not parallel changes in AR mRNA levels can be interpreted in several ways. First, due to the nature of the cytosolic *in*

vitro binding assay, and the necessity to castrate the control animals 12 h prior to sacrifice, it is possible that this time frame was not sufficient enough to allow for previously bound AR to cycle out of the nucleus and be measured in the cytosolic fraction. This would result in a false low control level and would not compare correctly to the AR binding levels in the 1- and 4-day castrates. Although no detailed studies of the rate of AR recycling following androgen removal have been done in hippocampus, the studies of Krey and McGinnis (1990) in rat hypothalamus suggest that the time it takes for AR to cycle out of the nucleus following T removal is relatively rapid (within 4 h) and renders this explanation for our results unlikely. Alternatively, androgen removal may enhance hippocampal AR protein stability to alter androgen sensitivity during fluctuations in circulating hormone. A rapid increase in AR stability 1 day after androgen removal may trigger the down-regulation of AR mRNA that we observed after 4 days of hormonal depletion. Although this mechanism could be occurring locally within the hippocampal neurons, recent evidence points to the enhanced stability of AR by ligand (Kemppainen *et al.*, 1992; Zhou *et al.*, 1995).

Discrepancies between steroid hormone receptor mRNA and protein levels following hormone manipulations have been shown in human breast and prostate tumor cell lines (Krongrad *et al.*, 1991; Wolf *et al.*, 1993). These studies suggest that neither the measurement of steady state mRNA, nor protein levels alone, can adequately determine hormonal sensitivity. In the hippocampus, where AR expression is predominantly found in the CA1 pyramidal cell region, it may be necessary to

measure AR and AR mRNA changes with much greater anatomical acuity using immunocytochemical and *in situ* hybridization analysis rather than from extracts of whole hippocampal homogenates. The possibility of differential regulation of AR in individual pyramidal cell regions exists. Furthermore, the finding that both the neural-specific 9.3-kb AR mRNA and the more widely distributed 11-kb AR mRNA are expressed in approximately equal amounts in the hippocampus (McLachlan *et al.*, 1991; Burgess and Handa, 1993a) allows for the possible differential regulation of these forms following hormonal manipulations. A recent study describing the differential regulation of three variants of the MR mRNA within the hippocampus after ADX (Kwak *et al.*, 1993) supports this possibility. Presently, methods to accurately quantitate and localize AR mRNA forms independently have not been developed, and the use of northern blot hybridization to detect subtle changes in AR mRNA levels in brain tissue, where expression is relatively low, is difficult. Complete sequence analysis of the 9.3-kb transcript, and the generation of probes directed at detecting this form, would prove useful to elucidate hippocampal AR regulatory mechanisms.

The physiologic significance of the relatively small changes ($\sim$35%) in hippocampal AR and AR mRNA levels following short-term castration remains to be elucidated. The changes in AR expression that were observed do not parallel the reported 2- to 10-fold induction of AR mRNA in rat whole brain and peripheral tissues following similar treatment (Quarmby *et al.*, 1990). However, these reported increases are based entirely on film density and do not accurately represent molar

amounts as does the RNase protection assay. Additionally, few studies have quantitatively investigated AR and AR mRNA regulatory mechanisms in discrete brain nuclei that contain relatively low levels of AR as compared to the accessory sexual organs. In brain areas such as the hippocampus where moment-to-moment fine-tuning of hormonal feedback may be necessary, small and rapid changes in AR expression could have great functional significance.

To further investigate the regulatory actions of AR, we used old intact male rats as a physiologically relevant model of long term deficits in circulating androgen. The upregulation of hippocampal AR mRNA levels in intact old rats compared to their young counterparts was an intriguing finding; however, subsequent changes in AR binding were not detected. Serum T levels in old male Fischer 344 rats are less than half that in young rats (Chambers *et al.*, 1991; Gruenewald *et al.*, 1992). This deficit alone could have triggered the autologous up-regulation of AR mRNA that was observed. Other hormonal changes in aging rats, including increased serum CORT (Landfield *et al.*, 1978), progesterone, and estrogen (Gruenewald *et al.*, 1992), have been reported and may be responsible for altered AR mRNA levels in the old hippocampus. Alternatively, low levels of aromatase, the enzyme responsible for the intracellular conversion of T to estrogen, have been found in the rat hippocampus (Abdelgadir *et al.*, 1994). Age-related decreases in aromatase activity have been shown in the preoptic area of the male rat (Chambers *et al.*, 1991). Although yet unexplored, alterations in hippocampal aromatase activity leading to changes in the availability of T to bind to AR, could contribute to altered AR autoregulation and our

observed increases in AR mRNA. Unfortunately, at present, little is known about the interactions between the steroid receptors and aromatase activity in hippocampal neurons.

Despite AR mRNA increases, it appears that AR remains constant in the hippocampus during long-term deficits in circulating androgen, and that androgen sensitivity is maintained in this region. These data differ from those of previous studies that have shown dramatic losses of hippocampal GR and GR mRNA as well as MR and MR mRNA expression in the aged male rat (McEwen, 1992). These GR and MR losses appear to be related to cell death and occur mainly in the CA3 pyramidal cell region (Sapolsky *et al.*, 1990). It is possible that the age-related maintenance of AR content that was observed may be related to the sparing of CA1 neurons. This sparing of CA1 neurons with the concomitant age-related loss of other hippocampal cells could explain the increases were observed in AR mRNA concentrations, because data from the RNase protection assay are expressed as AR mRNA per μg of total hippocampal RNA. Without the use of individual cell counts and techniques with greater cellular resolution, it is premature to speculate as to whether androgens have a protective role in the hippocampus with aging. Unfortunately, the extreme density of CA1 neurons in the rat hippocampus makes individual cell counting virtually impossible in this region. Additionally, the use of thinner slices to try to overcome the density problem would likely push AR mRNA levels too low to be detected reliably with *in situ* hybridization. Regardless, studies using other models have implicated androgens as important modulators of axon regeneration following injury

(Jones, 1993) or of hippocampal neuron survival after stress (Mizogushi *et al.*, 1992). The maintenance of hippocampal AR, and perhaps androgen sensitivity, may prove to be beneficial in maintaining cognitive ability during the aging process.

In summary, these studies have demonstrated high levels of functional AR in the hippocampus and argue strongly for a direct transcriptional effect of androgens in hippocampally mediated behaviors. Consequently, changes in the levels of AR in this area due to hormonal manipulation or normal aging would have a profound influence on the expression of these behaviors. The regulation of AR expression in the hippocampus did not appear to follow the well described regulatory pattern of other steroid hormone receptors either after short term hormone removal or during the aging process. These studies point to the importance of maintaining AR numbers regardless of hormone status and suggest a reliance on the action of androgen in the hippocampus throughout life.

CHAPTER IV

ANDROGENS MODULATE GLUCOCORTICOID RECEPTOR mRNA, BUT NOT MINERALOCORTICOID RECEPTOR mRNA LEVELS, IN THE RAT HIPPOCAMPUS

Abstract

AR, MR and GR are ligand-activated transcription factors that alter gene expression and have a wide variety of effects in the CNS. High levels of AR, MR and GR mRNA have been found in the CA1 pyramidal cell region of the rat hippocampus and all three of these proteins bind to a similar HRE in DNA suggesting the possibility of common receptor function or cross-talk between these receptors at the level of transcription. To begin to investigate this hypothesis, we examined the regulation of AR, MR and GR mRNA expression in the rat hippocampus following treatment with androgens in combination with GDX and/or ADX. Three month old male Sprague-Dawley rats were either castrated for three weeks, castrated and immediately implanted with two Silastic capsules filled with the non-aromatizable androgen, DHTP, or left gonadally intact. Four days prior to sacrifice, these animals were either adrenalectomized or sham operated. GR, MR and AR mRNA were measured in the hippocampal subfields using *in situ* hybridization. In the CA1 region, DHTP treatment of castrates decreased GR mRNA levels to 69 percent of

levels found in gonadally intact rats and prevented the ADX-induced increases in GR mRNA observed in the gonadally intact and castrated animals. No changes in GR mRNA were observed in the CA3 region or DG, where AR expression is low or absent. There was no effect of androgen treatment on MR mRNA levels nor did GDX or androgen replacement alter the increases in MR mRNA following ADX. AR mRNA levels in the CA1 region were unchanged across all treatment groups. *In vitro* binding studies revealed almost complete nuclear occupancy of hippocampal AR in DHTP-treated castrates. No appreciable *in vitro* binding of DHT to hippocampal MR or GR ($K_i \approx 1500$ nM) was observed which suggests that androgen regulation of GR mRNA in the hippocampus is occurring through AR binding. These data demonstrate a functional similarity of androgens and glucocorticoids in the regulation of GR mRNA levels in an area where AR and GR are colocalized. Androgen-mediated downregulation of GR expression may prove to be an important event in the adaptive responses of CA1 pyramidal cells to hormonal stimuli.

Introduction

Adrenal corticosteroids and gonadally-derived androgenic steroids have profound effects on stress responses, memory, mood and hormonal homeostasis (Roof and Havens, 1992; De Kloet *et al.*, 1993a; Dubrovski *et al.*, 1993; Handa *et al.*, 1994b). These hormones exert their effects by specifically binding to intracellular receptors, which, following transformation and interaction with HREs of target genes,

either activate or repress transcription (Beato, 1989). The receptors for these hormones have been mapped throughout the mammalian CNS. Two types of corticosteroid receptors have been identified based on affinity and distribution (Reul and De Kloet, 1985). The type I or MR is characterized by its high affinity for CORT and is selectively localized in the hippocampal formation and other limbic regions (Beaumont and Fanestil, 1983). The type II receptor, or GR, has a ten-fold lower affinity for corticosteroid, but is present in nearly all tissues (Veldhuis *et al.*, 1982). A single form of AR has been reported in neural tissues including the hypothalamus, cortex, amygdala and hippocampus (Sar and Stumpf, 1977; Kerr *et al.*, 1995a).

Although GR, MR and AR are all expressed in the hippocampus, each shows a unique pattern in relative density across hippocampal subfields (Reul and De Kloet, 1985; Kerr *et al.*, 1995a). Particularly high levels of GR, MR and AR mRNA and protein have been found in the CA1 pyramidal cell region (Van Eekelen *et al.*, 1988; Kerr *et al.*, 1995a). These neurons complete the hippocampal trisynaptic circuit and form the major efferents to cortical and limbic areas of the brain (Van Groen and Wyss, 1990). The overlapping expression of these three receptors in the CA1 area is interesting because all three presumably bind and activate the same HRE (Chandler *et al.*, 1983; Beato, 1989). This suggests the possibility of common receptor functions within cells or cross-talk at the transcriptional level.

Regarding the functional aspects of hippocampal MR, GR, and AR, numerous studies point to an involvement of MR and GR in glucocorticoid feedback inhibition

of the HPA axis (Ratka *et al.*, 1989; Jacobson and Sapolsky, 1991). Presently, little is known about the functional role of AR in the hippocampus; however, androgens have also been shown to inhibit HPA axis function (Handa *et al.*, 1994a) and to modulate several hippocampal-mediated behaviors including emotionality (Hubert, 1990), memory formation (Roof and Havens, 1992) and the response to novelty (see Chapter V and Kerr *et al.*, 1995c).

In many rat tissues, levels of AR, GR and MR are autologously regulated by their respective ligand. For example, depletion of endogenous glucocorticoids by ADX elicits an increase in GR and MR (Herman, 1993); whereas prolonged elevation of circulating glucocorticoids, such as following chronic stress, results in downregulation of brain corticosteroid receptors (Sapolsky *et al.*, 1984). Similarly, in peripheral tissues and whole brain, AR expression is increased following GDX and these increases are reversed by androgen treatment (Quarmby *et al.*, 1990; Blok *et al.*, 1992a). However, exceptions to these rules have been reported (Sheppard *et al.*, 1990; Peiffer *et al.*, 1991; Abdelgadir *et al.*, 1993; Herman, 1993; Kerr *et al.*, 1995a) and it appears that the regulation of AR, GR and MR expression differs depending on the tissue, as well as length of time following treatment and mode of steroid administration. Several studies have demonstrated heterologous regulation of brain GR levels by other hormones including insulin (Tornello *et al.*, 1982), vasopressin (Veldhuis and De Kloet, 1982a), ACTH (Veldhuis and De Kloet, 1982b), thyroid hormone (Meaney *et al.*, 1987) and estrogen (Ferrini and DeNicola, 1991; Burgess and Handa, 1993b) which suggest that many factors may ultimately determine

steroid receptor levels in a given tissue. Compelling evidence for the involvement of hippocampal adrenal steroid receptors in the treatment of affective disorders (Seckl and Fink, 1992), hippocampal cell death (Sapolsky *et al.*, 1988) and altered regulation of the HPA axis (De Kloet *et al.*, 1991) renders mechanisms that modulate hippocampal MR and GR concentrations of great clinical relevance.

Recently, studies have demonstrated sex differences in hippocampal ^{3}H-CORT binding (Turner and Weaver, 1985) and GR mRNA concentrations (Bohn *et al.*, 1994), as well as androgen-mediated changes in nuclear GR immunoreactivity in selected regions of the rat hippocampus (Ahima and Harlan, 1992). Collectively, these data suggest that androgen status may influence adrenocorticoid receptor expression in the hippocampus. To examine this possibility, we tested the hypothesis that androgen treatment could alter GR or MR mRNA levels in a fashion similar to previously described autoregulatory mechanisms. This was accomplished using *in situ* hybridization histochemistry to quantitate steroid hormone receptor mRNA levels in each hippocampal subfield under conditions of selective or combined occupation of AR, GR and MR. This methodology circumvents the pitfalls of *in vitro* radioligand binding studies which require prior ADX or GDX to clear steroids from already occupied binding sites. These studies also begin to explore possible mechanisms mediating cross-talk between steroid hormone receptors coexpressed in the hippocampus.

Materials and Methods

Animals

Three month old male Sprague-Dawley rats (Charles River, Inc., Portage, MI) were housed in environmentally controlled quarters and maintained on a 12:12 h light dark schedule (lights on at 0700 h) with food and water available *ad libitum*. Bilateral GDX and ADX, or sham ADX, were performed under ether anesthesia. At the time of GDX, some rats received hormone replacement by the subcutaneous implantation of two Silastic capsules (2.5 cm long, 0.07" i.d., 0.125" o.d.) filled with the non-aromatizable androgen, DHTP, (Steraloids, Inc., Wilton, NH). Previous studies in our laboratory have shown that these capsules provide a constant level of circulating DHT that is 2-5 fold higher than DHT levels found in intact male rats (Pouliot *et al.*, 1995), but is similar to total circulating androgen levels (Bingamen *et al.*, 1994). Following ADX, rats maintained with 0.9% NaCl in their drinking water. All rats were sacrificed by decapitation between 09:00 and 11:00 h.

Experimental Procedures

In the first series of experiments, we examined the effects of androgen removal or replacement on the steady-state levels of hippocampal GR, MR and AR mRNAs in ADX and sham ADX male rats. Androgen treatments (intact, GDX, and GDX + DHTP) lasted for three weeks, and each rat was either ADX or sham operated in the morning four days prior to sacrifice. At the time of sacrifice, trunk

blood was collected and brains were rapidly removed from the skull, frozen in pre-chilled isopentane (-30°C), and stored at -70°C until sectioned and processed for *in situ* hybridization. Brains were sectioned in the coronal plane and three series of brain sections from the same animals were used for determining GR, MR and AR mRNA. Serum CORT was measured using radioimmunoassay (RIA) as previously described (Burgess and Handa, 1992). The completeness of the ADX procedure was determined by the absence of CORT and any presumably ADX animal that showed detectable levels of serum CORT were removed from the study.

To evaluate the levels of circulating androgen reaching the hippocampus in the intact, GDX and GDX + DHTP groups, we determined the level of hippocampal AR occupancy obtained following these androgen treatments. Animals were left intact, GDX or GDX and implanted with two Silastic capsules of DHTP at the time of surgery as described earlier. Rats were sacrificed three weeks after the onset of treatment and their brains were rapidly removed and placed on ice. The hippocampus was dissected out of each brain and homogenized for *in vitro* binding analysis with ^{3}H-DHT. Anterior pituitary glands from selected animals were also taken for binding analysis because this tissue contains a very high concentration of AR and thus served as an inter-assay control.

To assess the selectivity of binding in hippocampal cytosols, we examined the ability of DHT, CORT, RU 28362 (a GR specific agonist), and dexamethasone to compete for ^{3}H-dexamethasone labelled MR and GR sites (Burgess and Handa, 1992) in hippocampal cytosolic fractions using an *in vitro* binding assay. Rats were ADX'd

24 h prior to sacrifice to allow for glucocorticoids to clear from the circulation and leave MR and GR binding sites unoccupied. Following sacrifice, whole hippocampi were dissected out of the brain, homogenized and cytosolic extracts were purified for *in vitro* competition binding analysis.

In Situ Hybridization

For the *in situ* hybridization procedure, antisense ^{35}S-labelled riboprobes were used to detect GR, MR and AR mRNA. The GR and MR riboprobes were reverse transcribed as previously outlined by Burgess and Handa (1993b) using ^{35}S-UTP as the radioactive nucleotide (800 Ci/mmol, Amersham, Arlington Heights, IL). Briefly, the original rat GR cDNA construct (Meisfield *et al.*, 1986) was kindly provided by Dr. K. Yamamoto, UC San Francisco. A 1072 basepair fragment, corresponding to the ligand-binding domain and beginning of the 3' untranslated region, was subcloned into a pGEM 3Z plasmid vector. Following linearization with Dra I and reverse transcription with T7 RNA polymerase, a 262 basepair GR riboprobe was generated. A rat MR cDNA pGEM 4Z construct corresponding to nucleotides 2809-3321 (Arriza *et al.*, 1987) was kindly provided by Dr. R Evans, Salk Institute. This construct generated a 196 basepair riboprobe complementary to the ligand-binding domain and beginning of the 3' untranslated region of the rat MR mRNA following linearization (Stu I) and reverse transcription with SP6 RNA polymerase. A 141 basepair long *in vitro* transcribed AR cRNA complementary to the 5' translated region (nucleotides 963-1104) of the rat AR mRNA (Tan *et al.*,

1988) was generated as previously described (Kerr *et al.*, 1995a). All cRNA probes had specific activities averaging 10^9 cpm/ug. Aliquots of all probes were analyzed on denaturing 5% acrylamide, 7.5 M urea gels to confirm their integrity. Only those probes >90% full length were used for *in situ* hybridization.

The *in situ* hybridization procedure used in the present study was based on the method described by Handa *et al.* (1993) with slight modifications. Briefly, coronal brain sections (16 μm) were made with a Leitz 1600 cyrostat, mounted onto superfrost plus slides (Fisher Scientific, Pittsburgh, PA), and stored at -70°C until use. The sections were brought to room temperature, pretreated in 4% buffered formaldehyde, acetylated in acetic anhydride (0.25% in triethylamine), dehydrated in ethanols, and delipidated in chloroform. Slides were air dried. For hybridization, the probe was heated to 65°C for 5 min and diluted in hybridization buffer containing 50% formamide, 20% dextran sulfate, 1.2 M NaCl, 20 mM Tris, 0.04% Denhart's, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.02% salmon sperm DNA, 0.1% yeast RNA, 0.01% yeast tRNA, 0.1% sodium thiosulfate, 100 mM dithiothreitol (DTT), 0.1% sodium dodecyl sulfate (SDS) to a final concentration of 20 x 10^6 cpm/ml. Approximately 85 μl of the hybridization buffer was applied to each slide and coverslipped. Hybridization was carried out in a 65°C humidified incubator for 16-20 h. Following hybridization, the coverslips were removed and the sections were repeatedly rinsed in 2 x SSC then subjected to RNase A treatment (20 μg/ml at 37°C for 30 min) to digest any nonhybridized RNA. The sections were washed to a final stringency of 0.1 x SSC at 65°C and dehydrated in increasing concentrations of

ethanol. Autoradiographs were obtained by exposing slides to x-ray film (Hyperfilm β-max, Amersham, Arlington Heights, IL) for 9 days (MR mRNA and GR mRNA) or 21 days (AR mRNA). After film exposure, slides were dipped in nuclear tract emulsion (Kodak NTB-3, Eastman Kodak, Rochester, NY) and exposed for 21-35 days before development and cresyl violet staining. Sections were examined under darkfield illumination using a Zeiss Axioplan microscope (Zeiss, New York, NY).

Image Analysis

Quantification of steady-state levels of mRNAs coding for GR, MR and AR was accomplished by digitizing autoradiographic images with the Macintosh-based software NIH IMAGE v.1.54. Optical densities were converted into dpm/mg protein by a third order polynomial equation based on ^{35}S standards co-expressed on each film. This method has been described in more detail by Brady *et al.* (1992).

Hybridization density in cell body regions of the dorsal hippocampus were obtained by individually tracing the upper blade of the DG granule cell layer, as well as the entire CA1, CA2 and CA3 pyramidal cell layers defined in accordance with the stereotaxic atlas of Paxinos and Watson (1982). Both the left and right hemispheres were measured. A background sample from the molecular layer of the hippocampus was subtracted from each measurement. Measurements from four sections from each animal were averaged to obtain a final density value for each hippocampal subfield. The large scale of these experiments necessitated the use of multiple film autoradiographs for the MR and GR probes. To minimize error between film

autoradiographs, hybridization density values were transformed to the percent of the mean obtained from the gonadally-intact, sham ADX rat sections included on each film. Percent data were then grouped and subjected to statistical analysis. For AR mRNA *in situ* hybridization, the sections were processed together using the same probe and a single film. Therefore, these data were expressed as dpm/mg protein.

In vitro Androgen Receptor Binding Assay

Cytosolic (ARc) and nuclear (ARn) AR were measured using modifications of previously described methods (Handa *et al.*, 1986). All procedures were carried out at 0-4°C. Hippocampi and pituitaries were placed into chilled Dounce tissue grinders (Wheaton Scientific, Millville, NJ) and homogenized in 500 μl (hippocampus) or 200 μl (pituitary) TEGMD buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, 25 mM molybdate, 1 mM DTT, pH 7.4). The homogenates were transferred with an additional 200 μl wash to ultracentrifuge tubes and centrifuged at 1500 x g for 15 min. The purified cytosols were prepared from the resultant supernatants by recentrifugation at 100,000 x g for 30 min. The high speed supernatant was saved to measure ARc levels and 10 μl was used to determine protein content by the method of Lowry *et al.* (1951).

The crude nuclear pellets obtained from the first low speed spin were further purified by resuspending the pellets in 400 μl of low sucrose buffer (Buffer A, 1 mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, 1 mM DTT, 10% glycerol) containing 0.25% triton x-100 and then were centrifuged at 1500 x g for 15 min to separate.

The supernatant was discarded and the pellet was washed with 400 μl Buffer A (without triton x-100) and centrifuged (1500 x g, 15 min). The supernatant was discarded and 400 μl of high sucrose buffer (Buffer B, 1 mM KH_2PO_4, 2.1 M sucrose, 3 mM $MgCl_2$, 1 mM DTT, 10% glycerol) was added. The tubes were vortexed and centrifuged at 50,000 x g for 30 min to obtain a purified nuclear pellet. ARn complexes were salt extracted from each nuclear pellet by adding 250 μl TEBD buffer (10 mM Tris, 1.5 mM EDTA, 0.5 mM bacitracin, 1 mM DTT, pH 7.4) and 5 min later adding an equal volume of TEBDK (TEBD containing 1.6 M KCl). Tubes were vortexed repeatedly for an additional 25 min and the suspension was again centrifuged (37,000 x g for 15 min) to separate the nuclear extract (supernatant) from DNA material (pellet). DNA content in each pellet was measured using a modified version of the method of Burton (1956). Single point receptor measurements were made using 5α-(1,2,4,5,6,7-N-^{3}H)androstan-17β-ol-3-one (^{3}H-DHT, 110-150 Ci/mmol; New England Nuclear Research Products, Boston, MA) as the specific AR ligand. The ^{3}H-DHT was stored in 100% ethanol and was purified by thin layer chromatography to assure low levels of non-specific binding.

Total binding was measured using 100 μl aliquots of the cytosolic and nuclear extracts that were incubated with 2 nM and 5 nM ^{3}H-DHT, respectively. To determine non-specific binding, 1 μM (200-500 fold excess) of radioinert R1881 (an AR specific agonist) was incubated in parallel tubes with ^{3}H-DHT and cytosols. Cytosolic and nuclear samples were incubated at 4°C for 24 h and 48 h, respectively. To separate bound from free ligand, samples were passed through miniature Sephadex

LH-20 columns using 600 μl of the appropriate buffer. Four ml of Ultima Gold scintillation fluid (Packard Inc., Downers Grove, IL) was added to each eluate and the radioactivity was counted for 5 min in a Packard 1900 LA liquid scintillation counter (Packard Instruments, Downers Grove, IL) at approximately 37% efficiency. Specific binding was determined by subtracting non-specific from total binding. Receptor data were expressed as femtomoles (fmol) per mg protein (ARc) or per mg DNA (ARn).

In vitro Competition Binding Assay

To determine whether DHT binds to MR or GR in the hippocampus, we examined the binding of [1,2,4,6,7-^{3}H]Dexamethasone (^{3}H-Dex, 92 Ci/mmol, Amersham, Arlington Heights, IL) to hippocampal cytosols in competition with increasing concentrations of radioinert 5α-DHT (0.1 - 10,000 nM, Steraloids, Inc., Wilton, NH). Specificity of ^{3}H-Dex binding was determined by competition of ^{3}H-Dex with increasing concentrations (0.1 - 10 nM) of radioinert CORT (Steraloids, Inc.), RU 28362 (Roussel-UCLAF, Romainville, France), and dexamethasone (Sigma, St. Louis, MO). Hippocampal cytosolic fractions from ADX male rats were prepared as described above and were pooled together. Purified cytosol (100 μl) was incubated with 2 nM ^{3}H-Dex with or without competitor at 4°C overnight. Bound and free ligand were separated by Sephadex LH-20 column chromatography and radioactivity counted as described for the ARc assay. Data were converted to percent of total ^{3}H-Dex binding.

Statistics

Data from in situ hybridization histochemistry were analyzed using a two-way analysis of variance (ANOVA) with androgen treatment (intact, GDX, GDX + DHTP) and corticosteroid treatment (ADX, sham ADX) as main factors. Subsequent analyses used a one-way ANOVA across treatment groups followed by Student Newman-Keuls' post-hoc tests. A P value less than 0.05 was considered significant.

Results

GR, MR and AR mRNA Regulation in the Hippocampus

As shown in **figure 16,** *in situ* hybridization analysis demonstrated unique patterns of MR, GR and AR mRNA expression in the hippocampus of control rats (gonad and adrenal intact). Consistent with several earlier studies (Van Eekelen *et al.*, 1988; Herman *et al.*, 1989; Seckl and Fink, 1991), high levels of GR mRNA were found in the CA1 and DG cell body regions of the hippocampus and expression was somewhat lower in the CA2 and CA3 pyramidal cell regions. MR mRNA levels were high in all regions with particularly dense hybridization in CA2 pyramidal cells. AR mRNA was also uniquely distributed across hippocampal subfields with high levels present in the CA1 area, lower levels in CA2/CA3 cells and little to no expression in DG granule cells.

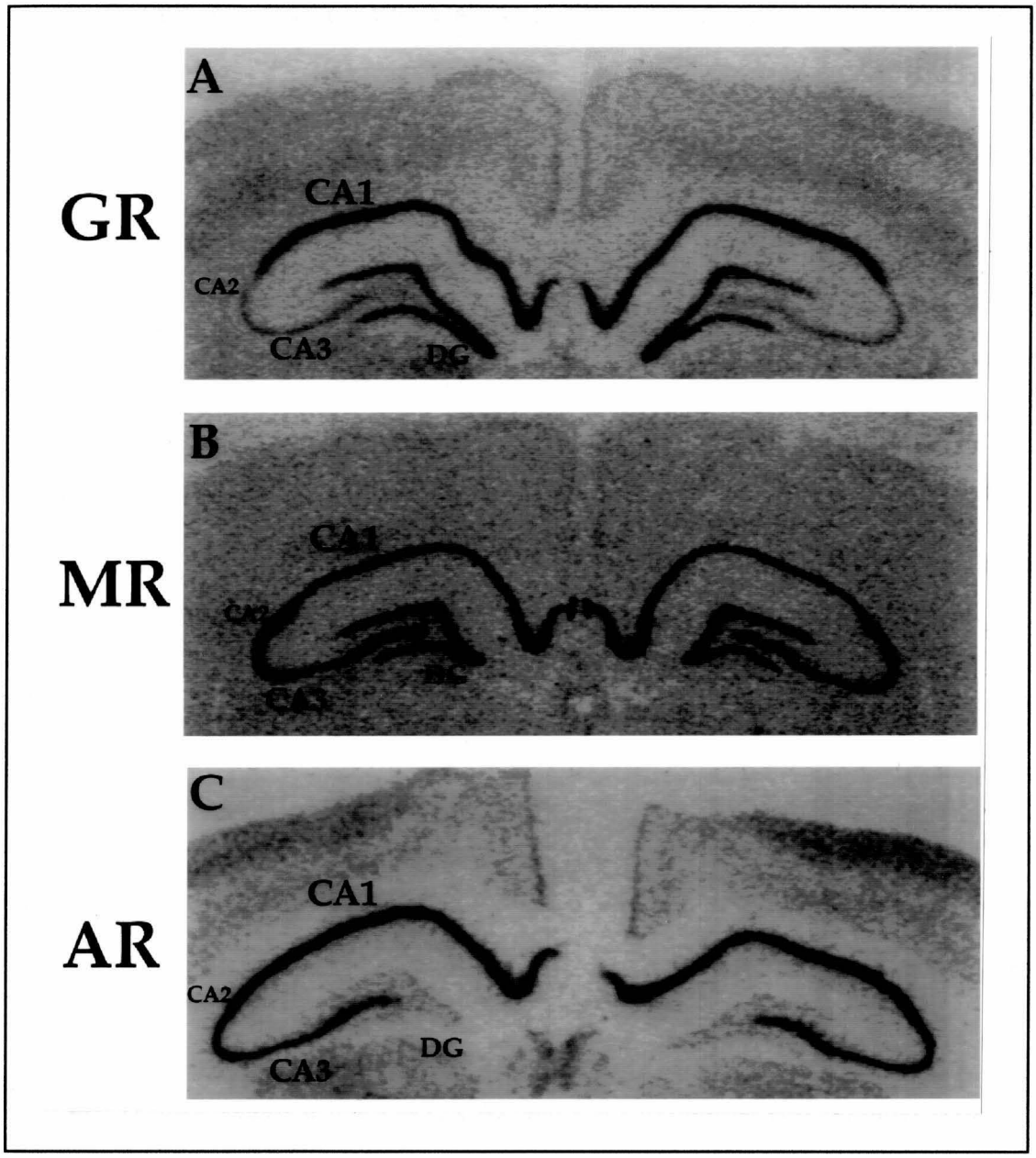

Figure 16. In situ hybridization autoradiographic films demonstrating the distribution of GR mRNA (A), MR mRNA (B) and AR mRNA (C) in the male rat hippocampus. Overlapping expression of AR, GR and MR mRNA is evident in the CA1 pyramidal cell region. CA1 = CA1 pyramidal cell region, CA3 = CA3 pyramidal cell region, DG = dentate gyrus granule cell region.

Quantitative densitometric analysis of film autoradiographs revealed region and treatment specific regulation of hippocampal GR mRNA. The large scale of the MR and GR mRNA experiments required the use of multiple film autoradiograms and riboprobes which can generate variability between films and from study to study. Therefore, it was necessary to transform the mean dpm/mg protein values from each animal to percent of the intact + sham ADX mean from each film autoradiogram. As shown in **figure 17A**, ADX treatment upregulated GR mRNA levels in the CA1 region an average of 33% as compared to the sham operated control. In the CA1 region, DHTP treatment of castrates significantly decreased GR mRNA to 69 percent of levels found in gonadally intact rats ($P < 0.01$) and prevented the ADX-induced increases in GR mRNA observed in the gonadally intact and castrated animals ($P < 0.01$). In the CA2 and CA3 subfields where GR mRNA levels were considerably lower, ADX increased GR mRNA expression as compared to sham operated controls ($P < 0.01$), however androgen treatment had no effect (data not shown). In contrast, GR mRNA levels in the DG were unaltered by androgen status or ADX **(figure 17B)**.

MR mRNA levels in ADX animals were significantly increased above sham operated control values in the CA1, CA2 and CA3 pyramidal cell regions, however, androgen treatment or GDX failed to modulate MR mRNA expression **(figure 18A**, CA1 region data shown). Similar to GR mRNA, MR mRNA levels in the DG were unchanged by ADX or androgen treatments **(figure 18B)**.

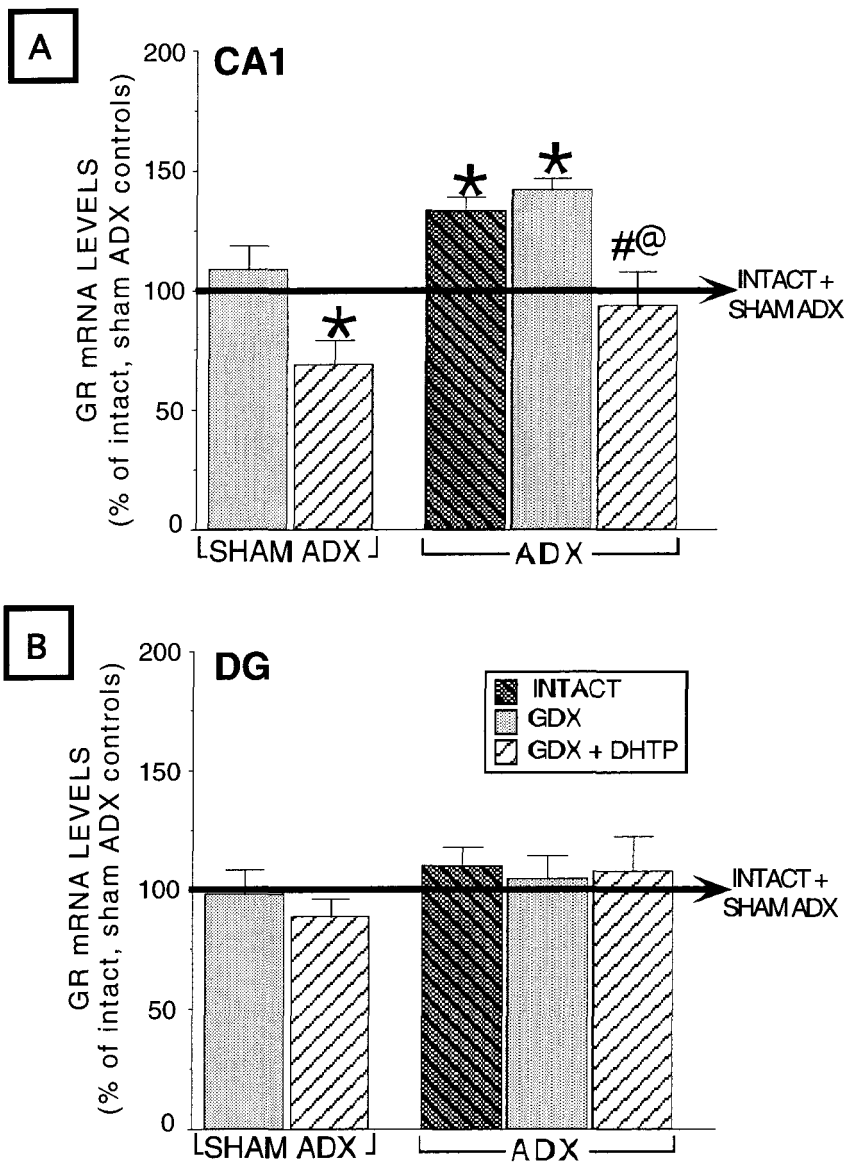

Figure 17. Effects of three week castration (GDX) or dihydrotestosterone propionate treatment of castrates (GDX + DHTP) on GR mRNA levels in the hippocampal CA1 and DG cell regions of sham operated (SHAM ADX) or adrenalectomized (ADX) male rats. *, Significantly different from intact + sham ADX value, @, significantly greater than DHT + sham ADX value, and #, significantly different from intact + ADX value as determined by Newman-Keuls' *post-hoc* analysis ($P < 0.01$). *In situ* hybridization densities are expressed as percent of intact + sham ADX mean from individual film autoradiograms (100%, black line). Each bar represents the mean $\pm$ SEM of 3-5 animals.

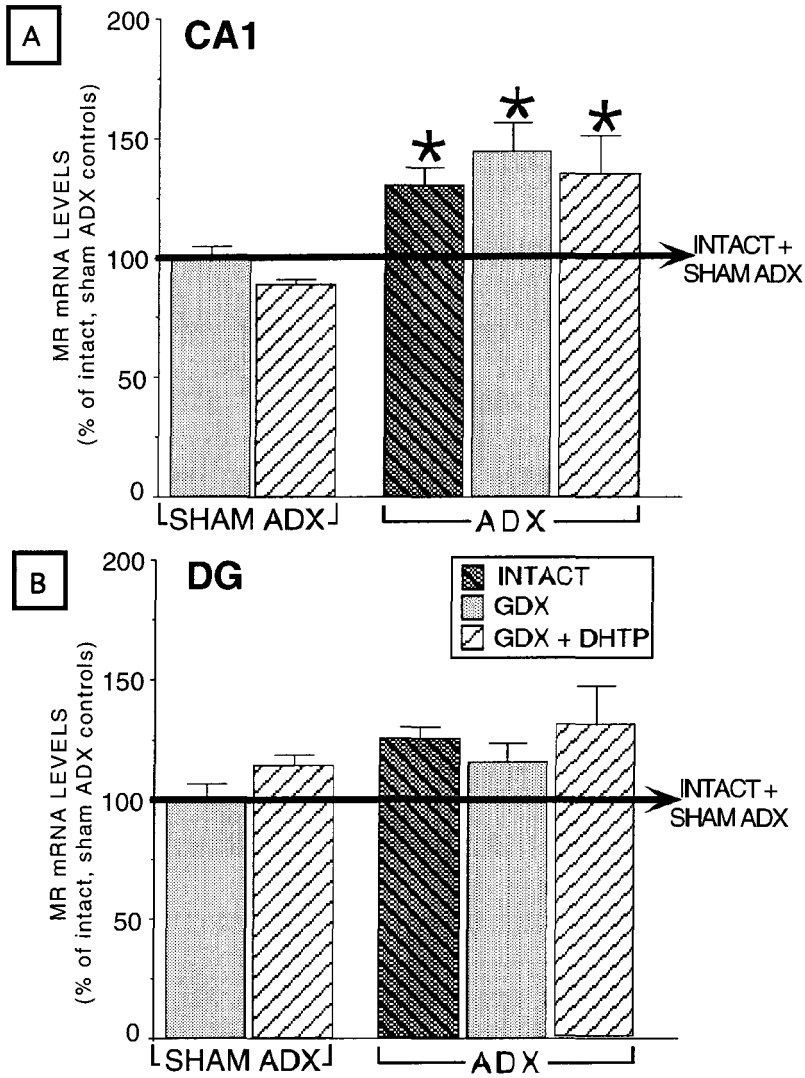

Figure 18. Effects of three week castration (GDX) and dihydrotestosterone propionate treatment of castrates (GDX + DHTP) on hippocampal MR mRNA levels in sham operated (SHAM ADX) and male rats adrenalectomized four days prior to sacrifice (ADX). (A) Hippocampal CA1 pyramidal cell region. **(B)** Dentate gyrus granule cell region (DG). Density values are expressed as percent of the intact + sham ADX control mean obtained from the corresponding *in situ* hybridization autoradiogram. Each bar represents the mean ± SEM from 3-5 rats. *, Denotes significantly different from corresponding sham ADX value ($P < 0.05$). Androgen treatment had no effect on MR mRNA levels.

AR mRNA levels were also examined in hippocampal sections from the same animals. These sections were all processed together using one AR ribopobe and were developed on a single film autoradiogram. Therefore, the dpm/mg protein hybridization density means from each hippocampal subfield in each animal could be directly compared and statistically analyzed. In the CA1 region which contains the highest concentration of AR mRNA of all the hippocampal subfields, steady state AR mRNA levels were not altered by 3 week androgen removal or replacement either alone or in combination with ADX 4 days prior to sacrifice (**Figure 19**). AR mRNA levels also remained constant in the CA2, CA3 and DG regions (data not shown).

Differential AR Occupancy by GDX and DHT Treatment

To confirm that the androgen treatments used in this study were sufficient to occupy AR in the hippocampus, we examined ARc (cytosolic, unbound form) and ARn (nuclear, bound form) concentrations in purified extracts from the hippocampus and of intact, GDX and GDX + DHTP treated rats (**figure 20**). Three weeks after GDX there were significantly higher ARc levels as compared to intact controls. Concomitant decreases in ARn following GDX did not reach statistical significance. In contrast, the administration of DHTP to castrates resulted in the dramatic accumulation of ARn ($P < 0.05$). The appearance of AR in the nuclear fraction of DHTP treated animals was accompanied by decreased AR in the cytosolic fraction ($P < 0.05$). As inter-assay controls, ARc and ARn concentrations were also measured in the anterior pituitary gland of selected rats. Mean ARc and ARn

concentrations in intact rat pituitary were 4- to 20-fold higher than found in the hippocampus (ARc: 6.3 ± 0.7 *vs.* 1.5 ± 0.2 fmol/mg protein and ARn: 193.6 ± 25.1 *vs.* 10.54 ± 4.5 fmol/mg DNA). Regardless of the differences in overall AR content in the pituitary and hippocampus, the relative changes in AR occupancy following androgen treatment or castration were similar in both tissues.

3H-Dexamethasone Competition Binding

To test the possibility that DHT might be promiscuously binding MR or GR in the hippocampus we incubated hippocampal cytosol with ^{3}H-Dex and several radioinert corticosteroids or DHT (**figure 21**). In the presence of 50-fold molar excess of DHT, ^{3}H-Dex binding was decreased only slightly. A 500-fold molar excess of DHT (1000 nM) was necessary to achieve any appreciable competition for ^{3}H-Dex binding (Approximate K_i=1500 nM). RU 28362, CORT and dexamethasone were all excellent competitors of ^{3}H-Dex for the corticosteroid receptor with approximate K_i values in the 2-8 nM range.

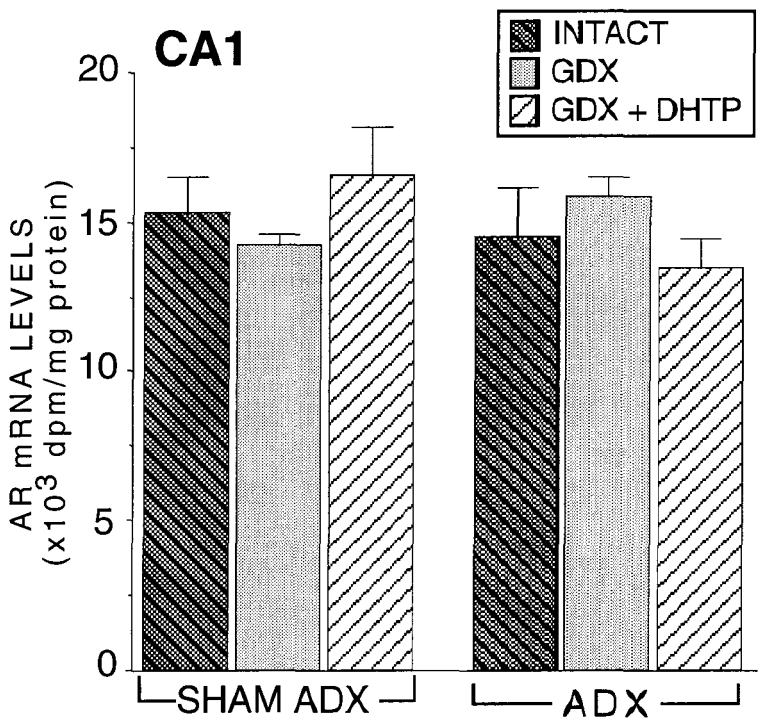

Figure 19. Effect of castration (GDX) and dihydrotestosterone propionate treatment of castrates (GDX + DHTP) on the magnitude of AR mRNA expression in the CA1 region of the hippocampus from adrenalectomized (ADX) and sham operated (SHAM ADX) male rats. Results from semi-quantitative densitometry of *in situ* hybridization histochemistry are shown. Each bar represents the mean ± SEM from 3-5 animals. No changes in AR mRNA were observed.

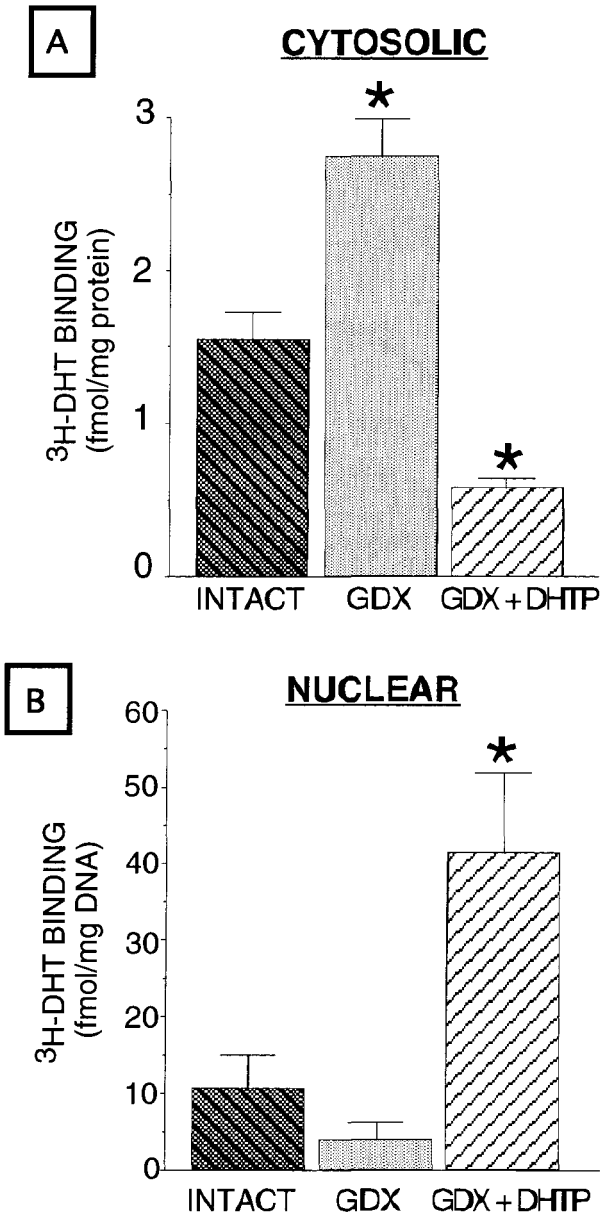

Figure 20. Quantification of AR in purified cytosolic (A) and nuclear (B) extracts from male rat hippocampus. Animals were left intact, gonadectomized (GDX) or GDX and implanted with two Silastic capsules of dihydrotestosterone propionate at the time of surgery (GDX + DHTP). All treatments lasted for three weeks. AR binding was determined using ^{3}H-DHT as the specific ligand. Each bar represents the mean $\pm$ SEM from 9 rats. *, Significantly different from intact value ($P < 0.05$).

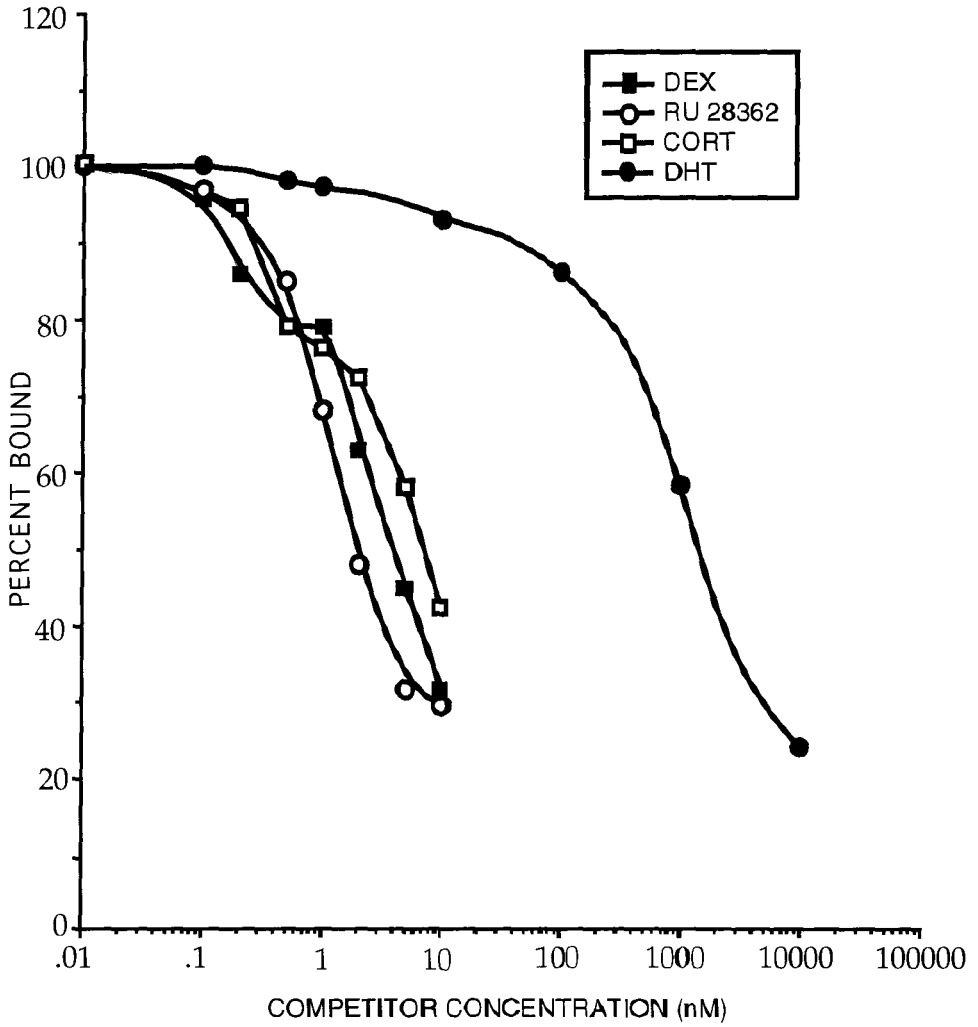

Figure 21. Competition of various radioinert steroids with the binding of ^{3}H-dexamethasone (^{3}H-Dex) in hippocampal cytosolic extracts from male rats adrenalectomized one day prior to sacrifice. ^{3}H-Dex was used at a concentration of 2 nM. Binding is expressed as percentage of that obtained in the presence and absence of cold competitor. Each point represents the mean of two replications. Approximate K_i values: 2 nM for RU 28362; 4 nM for dexamethasone (DEX); 8 nM for corticosterone (CORT); and 1500 nM for dihydrotestosterone (DHT).

Discussion

In the present study we have demonstrated a downregulation of GR mRNA by androgen treatment which occurs selectively in the CA1 pyramidal cell region of the hippocampus. In contrast, androgen treatment did not change MR or AR mRNA levels in any cell body region of hippocampus. Our results provide a plausible mechanism to explain recent studies by Bohn *et al.* (1994) showing lower GR mRNA content in the adult male hippocampus as compared to the female. These observations of message abundance are interesting when considered in conjunction with data from binding studies that show lower adrenocorticoid receptor binding capacity in the male rat hippocampus (Turner and Weaver, 1985). Whether our observed decline in CA1 pyramidal cell GR mRNA content following androgen treatment is translated into similar changes in GR protein has yet to be examined. However, the fact that data from *in vivo* receptor autoradiography using GR-selective ligands, and *in situ* hybridization with GR riboprobes have shown parallel distribution patterns of GR binding and GR mRNA in the hippocampus implies a correlation between GR mRNA expression and the level of expression of functional protein.

Our findings concerning downregulation of GR mRNA levels by androgen treatment are interesting in light of earlier work by Ahima and Harlan (1992) showing that the daily injection of high doses of anabolic-androgenic steroids increased the nuclear localization of GR immunoreactivity in the CA1 and DG regions of the male hippocampus. These authors suggested that circulating androgen present at levels

over and above that necessary to saturate hippocampal AR may bind non-specifically to GR thereby causing increased nuclear GR occupancy. These increases in GR occupancy could result in a downregulation of GR mRNA, as seen in our studies, however, our competition binding studies do not point to any appreciable binding of DHT to the hippocampal GR. The possibility of promiscuous binding of androgen to GR following extremely high levels of androgen cannot be ruled out. Additional studies examining the effects of various androgen concentrations on GR mRNA and protein levels are necessary to further elucidate the mechanism of this interaction.

Although we have not directly assessed the mechanisms governing androgen-mediated downregulation of GR mRNA observed in this study, we believe that DHT altered GR mRNA levels via AR binding and not through non-specific interactions with adrenocorticoid receptors. This is based on our results demonstrating that: 1) the majority of hippocampal AR was located in the nuclear fraction following DHTP treatment, 2) androgen treatment decreased GR mRNA levels selectively in the CA1 pyramidal cell region where AR mRNA expression predominates, and androgen treatment had no effect in area CA3 where GR mRNA is high, but AR mRNA is low and 3) there was little *in vitro* competition by DHT for hippocampal dexamethasone binding. Furthermore, the treatment of castrates with the non-aromatizable androgen, DHTP, eliminated the possibility of an estrogen receptor mediated effect that has been observed by others (Ferrini and DeNicola, 1991; Burgess and Handa, 1993b).

Since AR is a ligand-activated transcription factor, it is plausible that AR-mediated downregulation of GR expression is occurring at the level of gene

transcription. Although methological difficulties have prevented the direct colocalization of AR and GR expression in CA1 pyramidal cells, earlier work in our laboratory showing AR mRNA in most, if not all, CA1 neurons (See Chapter III and Kerr *et al.*, 1995a), and the current finding of even higher expression of GR mRNA in virtually every neuron in the CA1 region render colocalization of these two receptors in the majority of CA1 cells highly likely. It is known that AR, MR and GR regulate gene transcription by binding to an identical HRE (Beato, 1989; Chandler *et al.*, 1983). Since the GR gene contains this HRE sequence which likely mediates its autologous regulation (Burnstein and Cidowski, 1992), then activated AR complexes could act directly at this HRE to halt or repress transcription of the GR gene. Not surprisingly, activated MRs have been shown to regulate normally GR-responsive genes through a similar mechanism (O'Donnell and Meaney, 1994). If AR can act non-discriminately as an activated GR would at the same HRE, it is unclear why MR expression was not similarly affected. However, a consensus HRE has not been examined within or upstream of the rat MR gene.

Recently it has become apparent that MR mRNA autoregulation in the rodent hippocampus may be much more complex than originally thought. Similar to the finding of two distinct AR mRNA isoforms in the rat brain (McLachlan *et al.*, 1992), multiple MR mRNA forms that vary in their 5' untranslated regions have been found to exist in rat neural tissues (Kwak *et al.*, 1993). Interestingly, these three different sized MR mRNA isoforms were found to be unequally expressed in each subfield of the rodent hippocampus, and the expression of only one of these mRNA forms was

upregulated following ADX (Kwak *et al.*, 1993). As our MR riboprobe could not distinguish these three mRNA variants, limited regulation of just one form by GDX or DHTP treatment may not have been detected using our *in situ* hybridization methodology.

It appears that glucocorticoid regulation of brain adrenocorticoid receptors is complex. In these studies, ADX differentially affected hippocampal GR and MR mRNA expression in a subfield-specific manner. The moderate increases (30-45%) in GR and MR mRNA in each pyramidal cell field and no changes in MR mRNA levels in the DG region of the hippocampus following ADX were consistent with previous studies (Herman *et al.*, 1989; Herman, 1993). In contrast to our findings, earlier studies have demonstrated ADX-mediated increases in GR mRNA in the DG, however, the variability in the length of ADX appears to play a crucial role in the magnitude of the measured response. Taken together, the hippocampus shows diverse responses to glucocorticoid removal across its functionally heterogeneous subfields. These findings strengthen the hypothesis that multiple factors likely control adrenocorticoid receptor balance in this region.

The lack of hippocampal AR mRNA regulation by castration, androgen treatment for three weeks, or short-term ADX was intriguing, yet not unexpected. Upregulation of AR expression following GDX and decreases in AR expression by androgen treatment have been found in peripheral male reproductive tissues such as the testes and ventral prostate (Blok *et al.*, 1992a; Abdelgadir *et al.*, 1993). However, studies examining autologous regulation of AR mRNA in brain regions are

more difficult to interpret. In particular, earlier studies have detected attenuated AR mRNA levels in the whole male rat hippocampus following four day castration, whereas in the aged rat, AR mRNA content was increased (See Chapter III and Kerr *et al.*, 1995a). In both cases, concomitant changes in AR binding levels were not found. As the present studies suggest, AR expression can be maintained in the hippocampus after three week androgen removal or treatment.

Previous studies have demonstrated many different effects of androgen on hippocampal physiology (Roof and Havens, 1992; Handa *et al.*, 1994a, Kerr *et al.*, 1995c, Pouliot *et al.*, 1995, Hampson and Kimura, 1992). Some of these androgenic effects are similar to reported glucocorticoid effects in the brain (Roof and Havens, 1992; Handa *et al.*, 1994a), whereas others are very different (Kerr *et al.*, 1995c, Pouliot *et al.*, 1995) from effects attributed to glucocorticoids (reviewed in De Kloet *et al.*, 1993b; Dubrovsky *et al.*, 1993; McEwen *et al.*, 1994). Based on my results, the effects of androgen in the hippocampus may be, in one respect, to mimic that of glucocorticoids, as evidenced by the reduction of GR mRNA in a fashion similar to that seen after glucocorticoid administration. An example of this is demonstrated by our recent studies showing that androgen treatment can inhibit stress-related corticosterone secretion, presumably by acting at the level of the hippocampus or hypothalamus (Handa *et al.*, 1994a). Conversely, androgens may act to antagonize glucocorticoid action by decreasing the synthesis of GR, and thus, sensitivity to circulating glucocorticoids. This possibility has been evidenced by studies demonstrating increased cell death in hippocampal pyramidal cells following chronic

stress of gonadectomized animals, but not intact or androgen treated animals (Mitzoguchi *et al.*, 1992).

In summary, it appears that AR, GR and MR are embedded in a complex network of transcriptional regulatory factors and our studies indicate some level of interaction of these networks in hippocampal CA1 pyramidal cells. The process of androgen-induced GR mRNA downregulation may prove to be an important influence on the ability of hippocampal CA1 pyramidal cells to adapt appropriately to hormonal stimuli, especially at times of heightened stress or during the aging process when hippocampal neurons are more susceptible to damage by glucocorticoids (McEwen, 1992). Further study of AR, GR and MR expression and regulation at the gene, mRNA and protein level following various hormonal challenges is necessary to determine the exact functional significance of the potential molecular interactions of AR, GR and MR in defined neuronal circuits.

CHAPTER V

ANDROGENS SELECTIVELY MODULATE *c-fos* mRNA INDUCTION IN THE RAT HIPPOCAMPUS FOLLOWING NOVELTY

Abstract

Earlier studies have shown that ARs are found in high concentrations in hippocampal CA1 pyramidal cells. To begin to explore the possible roles for AR in this area of the brain, the effects of endogenous and exogenous androgen on the behaviorally-induced expression of cIEG mRNAs were examined. Adult male Fischer 344 rats were either gonadectomized, gonadectomized and given two Silastic capsules of DHTP at the time of surgery, or left intact. Three weeks later, animals were placed into a novel open field for twenty minutes. This behavioral paradigm caused region- and gene-specific increases of *c-fos*, *jun-B*, *c-jun* and *zif268* mRNA in the hippocampus as determined by semi-quantitative *in situ* hybridization histochemistry. The removal of circulating androgen by GDX potentiated, whereas DHTP treatment of castrates attenuated, the behaviorally-induced expression of *c-fos* mRNA in the CA1 region of the hippocampus. No changes in *c-fos* mRNA expression were detected in the CA3 or DG regions where AR levels are low. Androgen status did not affect either the basal or stimulated expression of *jun-B*, *c-jun* or *zif268* mRNA in

any of the three cellular regions of the hippocampus examined.

These results implicate ARs in modulating the active response of hippocampal neurons to a behaviorally relevant stimulus. Since the products of cIEGs can function to alter an array of downstream genes, the modulation of these genes in the hippocampus by gonadal hormones may have important ramifications for hippocampal function.

Introduction

Androgens have a profound modulatory role in the mammalian CNS by not only directing the formation of neuronal pathways during fetal development (for reviews, see McEwen, 1983; Breedlove, 1992), but also through the maintenance and modulation of existing neural circuitry in adults (Arnold and Breedlove, 1985; Handa *et al.*, 1994b). Androgens initiate many of these effects by specifically binding to AR in the cytoplasm and nucleus of target cells (Barley *et al.*, 1975). These hormone-receptor complexes act as ligand-activated transcription factors at specific DNA sequences, termed HREs, upstream of target genes (Beato, 1989; Roche *et al.*, 1992).

Recent studies have found similar levels of AR mRNA and AR binding in the hypothalamus and hippocampus of the male rat (Burgess and Handa, 1993a; Kerr *et al.*, 1995a). In the hippocampus, AR expression was found to be particularly concentrated in the CA1 pyramidal cells (Kerr *et al.*, 1995a). These neurons form the major efferents of the hippocampal formation to various cortical and limbic areas

of the brain (Van Groen and Wyss, 1990). In the rat hypothalamus, androgen action has been well characterized and is known to mediate some aspects of reproductive behavior (Davidson, 1966) and hormonal feedback (Messi *et al.*, 1988; Zeitler *et al.*, 1990; Handa *et al.*, 1994b). Presently, the role of AR in the hippocampus is unclear, however, androgens have been shown to modulate some hippocampal-mediated behaviors including learning and memory (Flood *et al.*, 1992; Hampson and Kimura, 1992; Roof and Havens, 1992; Janowsky *et al.*, 1994) and emotionality (Hubert, 1990; Lumina *et al.*, 1994).

Despite accumulating molecular data on the interaction of steroid hormone-receptor complexes actions with HREs, the cellular machinery initiated by hormonal signals which leads to neuronal plasticity remains poorly defined. The identification of target genes in the brain whose expression is modulated by androgens would begin to clarify the role this hormone plays in selected brain areas, such as the hippocampus. Recent approaches to such questions have led to the observation that *in vivo* and *in vitro* stimulation of neurons causes the production of second messengers that rapidly activate the transcription of a family of genes termed cIEGs (for review, see Morgan and Curran, 1989). The protein products of these genes function as transcription factors that regulate the expression of additional genes over extended periods of time (for review, see Morgan and Curran, 1991). Both the pattern and magnitude of cIEG expression in the brain appears to be dependent on the stimulus employed (Bartel *et al.*, 1989; Wisden *et al.*, 1990) and the relative concentrations of cIEG protein products likely confers some level of specificity in the long-term cellular

response (Rausher *et al.*, 1988; Schütte *et al.*, 1989; Lin *et al.*, 1993b). In the rodent hippocampus, several cIEGs including the *fos* and *jun* family members, and *zif268* (also known as *NGFI-A*, *krox-24* or *egr-1*) are of particular interest as they are readily induced following stimulation paradigms relating to seizure (White and Gall, 1987; Wisden *et al.*, 1990; Gass *et al.*, 1992), memory formation (Tischmeyer *et al.*, 1990; Wisden *et al.*, 1990; Nikolaev *et al.*, 1992; Richardson *et al.*, 1992; Demmer *et al.*, 1993; Heurteaux *et al.*, 1993) and stress (Handa *et al.*, 1993; Imaki *et al.*, 1993; also see review, Robertson, 1992). Thus, the high levels of AR in neuronal populations that express cIEGs following various behavioral stimuli strongly suggests the presence of cross-talk between these two signal transduction pathways. Therefore, it was hypothesized that androgen status may alter cIEG induction in the hippocampus.

In the following study *in situ* hybridization was used to examine the pattern and magnitude of *c-fos*, *jun-B*, *c-jun*, and *zif268* mRNA induction in the male rat hippocampus following behavioral testing in the novel open field; a paradigm which has previously been shown to activate hippocampal neurons (Handa *et al.*, 1993). The novel open field has been used to monitor changes in fear, emotionality, anxiety and depression in rats (Denenberg, 1969). As a consequence of the exposure to a novel environment, rats show mild stress responses as measured by increases in ACTH and CORT secretion (Handa *et al.*, 1994a). In addition, the influence of the removal and subsequent addition of circulating androgens on the level of expression of these cIEGs was explored in discrete cellular regions of the hippocampus. Such modulation would implicate androgen in the alteration of hippocampal function and

would suggest that the hormonal status of the animal can affect the active response of hippocampal cells to incoming information.

Materials and Methods

Animals

Three month old Fischer 344 rats (Harlan Inc., Indianapolis, IN) were used in these studies. Animals were maintained in temperature (72°C) and humidity controlled rooms on a 12 h light/dark cycle (lights on at 0700 h) and were given free access to food and water. Bilateral GDX was performed under ether anesthesia. Some gonadectomized rats received hormone replacement by the subcutaneous implantation of two, 2.5 cm long Silastic capsules (0.07" i.d., 0.125" o.d.) containing the non-aromatizable androgen, DHTP (Steraloids Inc., Wilton, NH), immediately following GDX (GDX + DHTP group). These capsules have previously shown to provide a constant level of DHT 2-5 times that of circulating DHT found in intact male rats (Pouliot *et al.*, 1995). All androgen treatments lasted for three weeks. All rats were handled daily (2-5 min) for at least 10 days prior to sacrifice to reduce any stress responses associated with handling. Animals were killed by decapitation and their brains were removed immediately, frozen in isopentane (-30°C), and stored at -70°C.

Behavior testing was performed by placing animals in the center of the novel open field and allowing them to roam free for 20 min. The novel environment

apparatus consists of a wooden box measuring 100 cm x 100 cm x 40 cm high. The floor is painted white and divided into 25 squares with thin black lines. Four holes (3.5 cm diameter) are located in the four corner squares of the central nine squares. The open field was placed in a dark, quiet room next to the animal quarters and was illuminated by a 40W bulb positioned over the center of the chamber. Behaviors in the open field were monitored by a remote videocamera and videotaped for later analysis. Scores for a) the number of squares entered in the first 5 min, b) the total number of squares entered during the 20 min testing, c) the number of rears, and d) the number of nose pokes (rat enters snout into one of the holes) were tabulated for each animal.

Experiment 1. Time-course of cIEG mRNA Induction in the Hippocampus Following Novel Open Field.

With the exception of *c-fos* mRNA (Handa *et al.*, 1993), no previous studies have examined the time-course of cIEG expression in the hippocampus following exposure to a novel environment. Therefore, a preliminary experiment was performed to examine the levels of *c-fos*, *c-jun*, *jun-B*, and *zif268* mRNA induction in the hippocampus of intact male rats using *in situ* hybridization and to determine the time point where cIEG induction is maximal for later studies. Animals were sacrificed either directly from their home cage (HC), immediately following 20 min in the open field environment (20 min OF), or at 0.5 h (20 min OF + 0.5h), 2 h (20 min OF + 2h), or 8 h (20 min OF + 8h) following open field and return to the home

cage. Hybridization density from film autoradiograms of the CA1, CA3 and DG regions of the hippocampus were quantitated using an image analysis system. Each treatment group contained two animals.

Experiment 2. Effect of Castration and Androgen Replacement on novelty induced cIEG mRNA levels in the Hippocampus and Behaviors in the Open Field.

To determine if androgen status modulated the pattern or magnitude of cIEG mRNA induction in the hippocampus, intact, castrated, and castrated + DHTP treated rats (3 week treatment) were sacrificed either directly from their home cage or were exposed to the novel open field and sacrificed immediately upon removal from the apparatus (n = 6-13 rats per group). *In situ* hybridization to detect *c-jun*, *c-fos*, *jun-B*, and *zif268* mRNAs was performed on separate series of brain sections from each animal. The resulting film autoradiographs were analyzed using an image analysis system to quantitate hybridization density in the CA1, CA3 and DG cell regions of the hippocampus. To determine whether androgen status effects the behavioral response to novelty (which in turn could effect the magnitude of cIEG induction); intact, castrated or castrated + DHTP rats (3 week treatment, n = 6 per group) were scored in the novel open field environment as described above.

In situ Hybridization

For the *in situ* hybridization experiments, oligonucleotide probes were used to detect c-*jun* mRNA [48mer, probe sequence 5'-GGCGTTGAGGGCATCGTCGTAGA AGGTCGTTTCCATCTTTGCAGTCAT-3'; complementary to bases 353-400 of the rat *c-jun* mRNA (Sakai *et al.*, 1989)], *jun-B* mRNA [45mer, probe sequence 5'-GAAGGCGTGTCCC TTGACCCCTAGCAGCAACTGGCAGCCGTTGCT-3'; complementary to bases 1278-1322 of the rat *jun-B* mRNA (Ryder *et al.*, 1988)], and *zif268* mRNA (40mer, Oncogene Science). Each probe was 3' end-labelled with ^{35}S-dATP and terminal deoxynucleotidyltransferase (Promega, Madison, WI). A ^{35}S-labelled cRNA probe to detect *c-fos* mRNA was reverse transcribed as previously described by Handa *et al.* (1993). This probe was complementary to nucleotides 1838-2116 of the rat *c-fos* mRNA.

Coronal brain sections (16 μm) were made with a Leitz 1600 cryostat and mounted onto superfrost plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -70°C. *In situ* hybridization using the oligonucleotide and cRNA probes were performed as previously described by Hammer *et al.* (1993) and Handa *et al.* (1993), respectively. Briefly, tissue was postfixed in 4% buffered formaldehyde, acetylated with acetic anhydride (0.25% in TEA), dehydrated in ethanols and delipidated in chloroform. Approximately 85 μl of a 20 x 10^6 cpm/mL hybridization solution (50% formamide, 20% dextran sulfate, 1.2 M NaCl, 20 mM Tris, 0.04% Denhart's, 2 mM EDTA, 0.02% salmon sperm DNA, 0.1% yeast RNA, 0.01% yeast tRNA, 0.1% sodium thiosulfate, 100 mM DTT, 0.1% SDS) were placed on each slide,

coverslipped and incubated for 16 h at 65°C (for cRNA probe) or at 45°C (for oligonucleotide probes). cRNA-hybridized probes were rinsed in 2 x SSC, subjected to RNase A treatment (20 μg/ml at 37°C for 30 min) to digest any nonhybridized RNA, and washed to a final stringency of 0.1 x SSC at 65°C. Oligonucleotide probes did not undergo RNase A digestion and were washed to final stringency of 2 x SSC/50% formamide at 40°C. Autoradiographs were obtained by exposing slides to x-ray film (Hyperfilm βmax, Amersham, Arlington Heights, IL) for 9-15 days.

Image Analysis

NIH Image software was used to analyze film autoradiography. Hybridization density in the brain area of interest was expressed in terms of dpm/mg protein. To obtain a standard curve, a brain mash standard was made using increasing amounts of ^{35}S/mg protein. Co-exposure of this curve alongside a C^{14} plastic standard curve and subsequent exposure of the C^{14} standard in the cassette with hybridized tissue allowed for quantitation of density. This method has been described by Brady *et al.* (1992).

Brains were analyzed at the level of the dorsal hippocampus. Hybridization density within cell body regions of the hippocampus were obtained by separately tracing the entire upper blade of the DG granule cell layer, as well as the entire CA1 and CA3 pyramidal cell layers as defined by the atlas of Paxinos and Watson (1982). A background sample taken from the molecular layer of the hippocampus was subtracted from every measurement from each brain section. For each section, both the right and left hemispheres of the hippocampus were sampled. Values from four

brain sections were averaged to obtain a final density for each hippocampal field in every animal. To confirm our observations, experiment 2 was repeated three times for *c-fos* mRNA measurement. Thus, the use of multiple film autoradiographs for the analysis of *c-fos* mRNA expression necessitated the transformation of hybridization density values to the percent of the mean obtained from the gonadally intact rats on each film. Percent of intact data from all films were then grouped and subjected to statistical analysis.

Statistics

Statistical analysis was performed using a two-way analysis of variance (ANOVA) with treatment (intact, GDX, GDX + DHTP) and testing (HC vs. OF) as factors. Subsequent analyses used a one-way ANOVA across treatment groups and Student Newman-Keuls post-hoc tests. A P value less than 0.05 was considered significant for all tests.

Results

Experiment 1. cIEG mRNA Time-course.

As shown in **figure 22**, a preliminary time-course study indicated that open field behavior induced the rapid and transient expression of *c-fos, jun-B, c-jun* and *zif268* mRNAs in the CA1 region of the rat hippocampus. cIEG mRNA levels were

low to non-existent in the hippocampus of home cage (HC) rats except for *c-jun* and *zif268* mRNAs which had relatively high constitutive expression (**figure 22**). For all four of the cIEGs studied, mRNA induction reached between 85% and 100% of maximum immediately following the removal of the animal from the open field environment (20 min OF, **figure 22**, only CA1 region shown). Subsequently, in all later experiments, animals were sacrificed immediately following removal from the open field when it was now known that cIEG mRNA was at or near its peak expression in all areas of the hippocampus. All but *zif268* mRNA returned to HC levels within 8 h after open field exposure (20 min OF + 8h, **figure 22**).

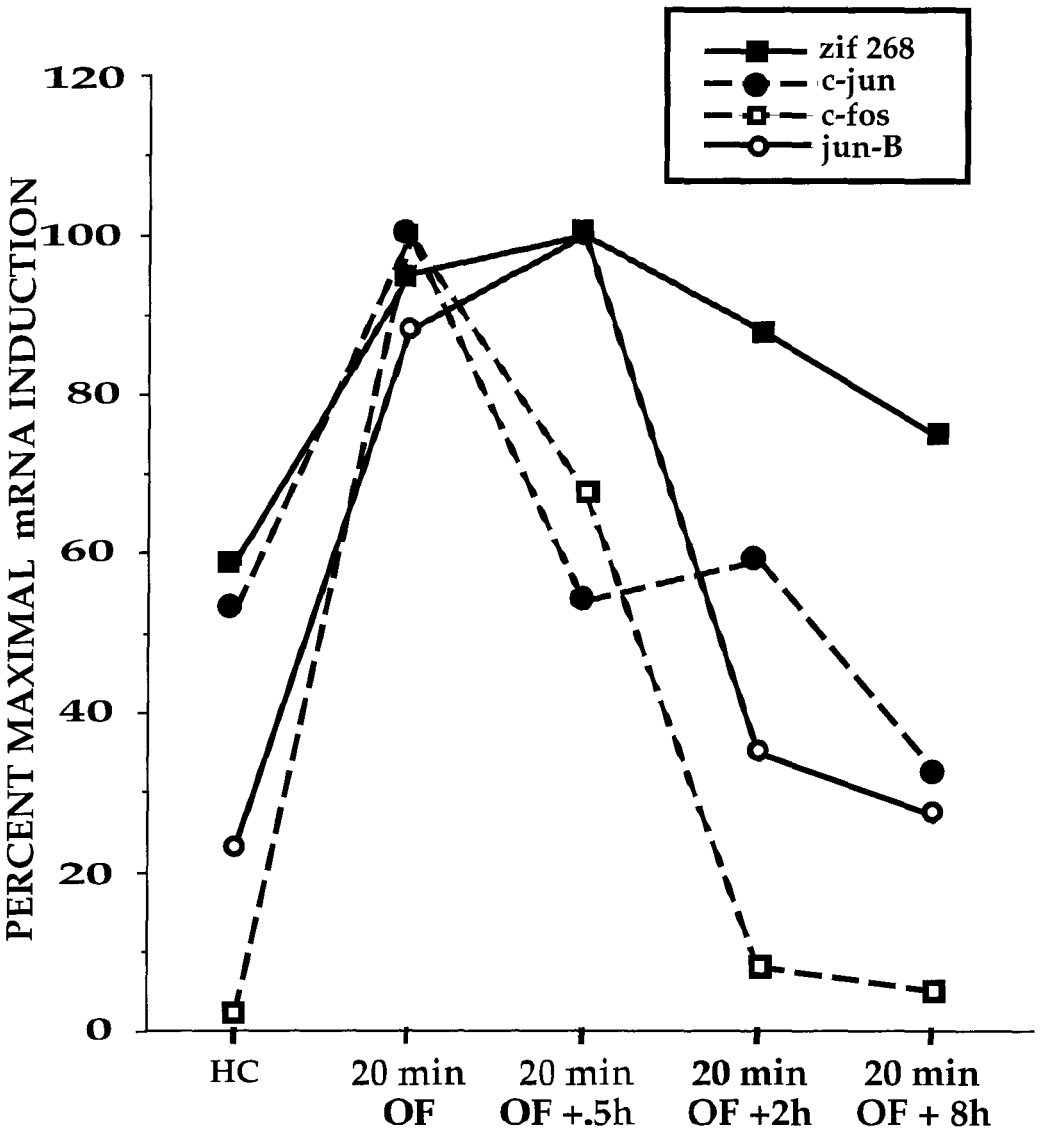

Figure 22. The time-course of cIEG mRNA induction in the CA1 pyramidal cell region of the hippocampus following introduction to a novel open field. Rats were sacrificed from their home cage (HC), after 20 min in the open field (20 min OF), or 0.5 h (20 min OF + .5h), 2 h (20 min OF + 2h), or 8 h (20 min OF + 8h) following open field and return to their home cage. Hybridization densities from film autoradiographs were obtained using a computerized image analysis system. Each point represents the mean of two animals. Due to enormous differences in basal levels between cIEGs, the time point at which the highest mean hybridization density value for each cIEG was obtained was considered 100% (maximal induction) and all other densities were transformed to percent of this maximal level for each cIEG.

Experiment 2. Effects of Castration and Androgen Treatment on cIEG mRNA Levels in the Hippocampus Following Novelty and Behavior in the Open Field.

As depicted in **figure 23**, *in situ* hybridization analysis revealed unique patterns of cIEG mRNA expression in response to novel open field testing in the intact male rat. *c-fos* mRNA was undetectable in the hippocampus of home cage animals, and was found in moderately high levels in the CA1 and DG regions of the hippocampus following novelty. The levels of both *jun-B* and *zif268* mRNA were low to moderate in the hippocampus of home cage animals and open field behavior resulted in increases in all areas. *zif268* mRNA levels were particularly high in the CA1 region. In contrast, *c-jun* mRNA was constitutively expressed in the CA3 and DG regions in home cage rats and no observable increases occurred as a result of behavioral testing. Neither castration nor androgen treatment altered the basal levels or distribution patterns of cIEG mRNA expression in the hippocampus.

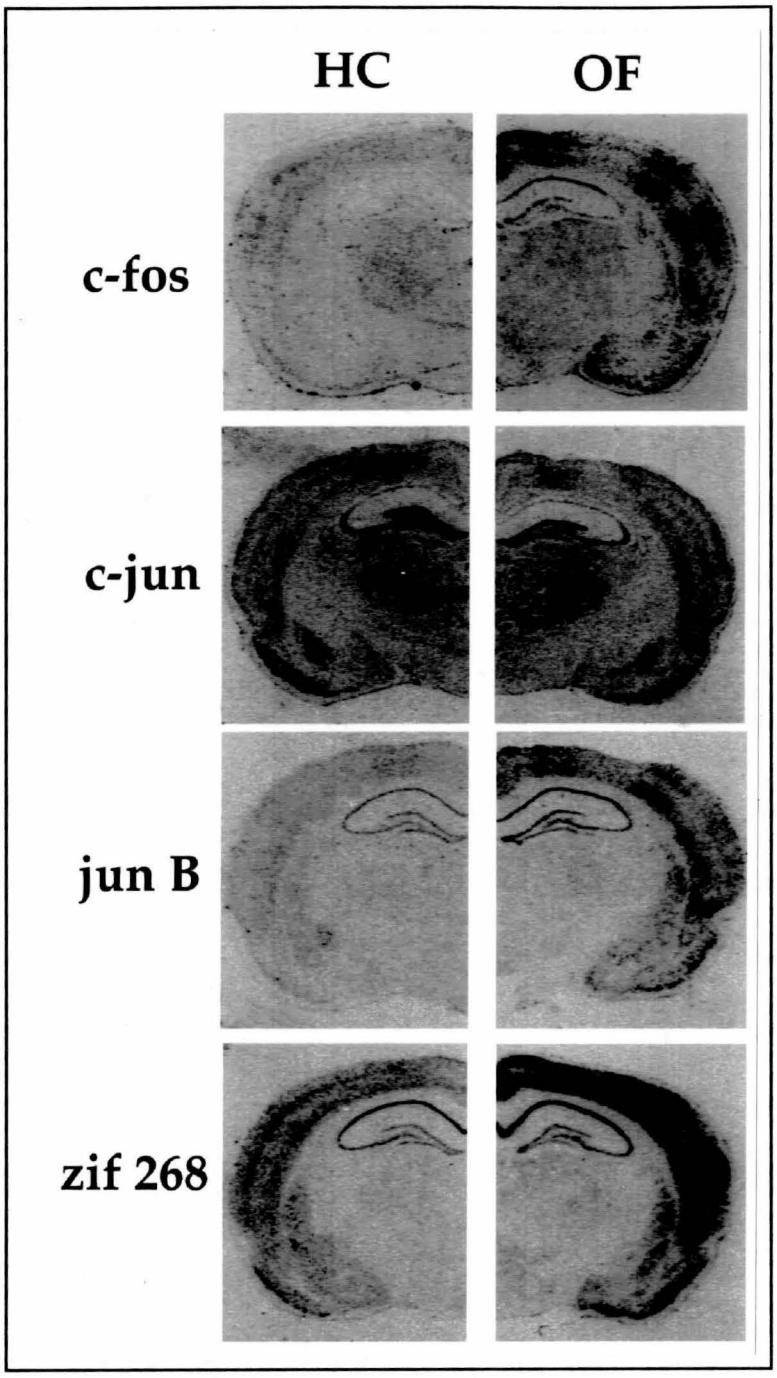

Figure 23. cIEG mRNA expression in home cage animals (HC, left panel) and in animals removed immediately following 20 min in the novel open field (OF, right panel). In the control hippocampus, *c-fos* mRNA was virtually absent. Novelty induced *c-fos, jun-B* and *zif268* mRNA in distinct regions of the hippocampus and cortex. *c-jun* mRNA is constitutively expressed at high levels in the CA3 and dentate gyrus cell regions of the hippocampus. Autoradiographs were digitized.

Quantitative densitometric analysis of *jun-B* and *zif268* mRNA from film autoradiographs demonstrated that the open field stimulus induced both *jun-B* (**figure 24A**) and *zif268* (**figure 24B**) mRNA above home cage levels regardless of androgen treatment ($P < 0.05$). However, there were no effects of castration or DHTP treatment in any region of the hippocampus in either home cage or open field rats (CA1 and DG shown). In contrast, *c-jun* mRNA levels were unchanged by open field or androgen treatment in the CA3 and DG cell regions, where constitutive *c-jun* mRNA expression was high (**figure 24C**). The very low levels of *c-jun* mRNA in the CA1 region of both home cage and open field rats made quantitation of hybridization density in this area difficult. Since none of the density values obtained fell on the linear part of the film standard curve, statistical analysis of these data was not performed. In a single study of 6 rats per group, *c-fos* mRNA induction after novel open field was dramatically increased above home cage levels in the CA1 and DG regions (**figure 24D**). Essentially, *c-fos* mRNA hybridization was not above background levels in the hippocampus of home cage animals. In addition, *c-fos* mRNA levels in the CA1 region were attenuated in castrates treated with DHTP as compared to the castrate controls (**figure 24D**, $P < 0.05$). There were no effects of androgen treatment on any cIEG mRNA expression level in the CA3 or DG regions.

The finding that *c-fos* mRNA induction was attenuated by DHTP treatment in the CA1 region were consistent in three separately run groups of animals, therefore, combining the groups was warranted. However, due to variations in film autoradiogram intensities and the use of a newly transcribed *c-fos* cRNA probe for

each *in situ* hybridization run, it was not possible to compare dpm/mg protein hybridization densities between films without introducing an enormous amount of variability. To circumvent this problem, the results were expressed as percent of the density of gonadally intact mean for each film autoradiograph then these data were combined and statistically analyzed to generate the graph depicted in **figure 25** (only open field *c-fos* mRNA levels in the CA1 and DG regions are shown). When the studies were merged, thereby raising the number of animals per group to 11-13, androgen treatment significantly affected *c-fos* mRNA induction in the CA1 region (ANOVA: $F(2,33) = 12.32$, $P = 0.0002$). GDX increased inducible *c-fos* mRNA levels in the CA1 region of the hippocampus by 32% as compared to intact controls ($P < 0.05$) and DHTP treatment of castrated males prevented the effect of GDX and lowered *c-fos* mRNA expression to 69% of intact values (**figure 25**, $P < 0.05$). No effect of androgen treatment were found in the DG (ANOVA: $F(2,33) = 1.552$, $P = 0.23$).

TABLE 1.

Effect of androgen treatment on open field activity measures in the male F344 rat.

Treatment	*n*	Total Squares Entered		Rears	Nose Pokes
		First 5 min	20 min		
INTACT	6	21±9.1	102±27.9	22±4.4	11±4.2
GDX $^{\Psi}$	6	44±8.8*	133±27.4	28±7.5	14±4.3
GDX + DHTP $^{\varphi}$	6	9±3.3	60±21.1	20±4.0	4±1.6

Data are presented as group mean± SEM.
* Significantly different ($p < 0.05$) from intact group (ANOVA followed by Newman-Keuls' test).
Ψ Gonadectomized 3 weeks prior to testing.
φ Gonadectomized and given two 2.5cm Silastic capsules of dihydrotestosterone propionate (DHTP) at time of surgery.

Castrated males showed significant increases in exploratory behavior during the first 5 minutes of testing as compared to intact or hormone-replaced male rats (**Table 1**). This effect of hormone treatment was not present when data were examined over the entire 20 minute period. Androgen treatment did not significantly affect any other measures of open field behavior.

Correlation analysis of total squares entered within the first 5 min of open field exposure, as well as total squares entered within the entire 20 min, with the corresponding CA1 *c-fos* mRNA density in individual rats (n=18) revealed R^2 values of only 0.19 and 0.52, respectively (nonsignificant, data not shown).

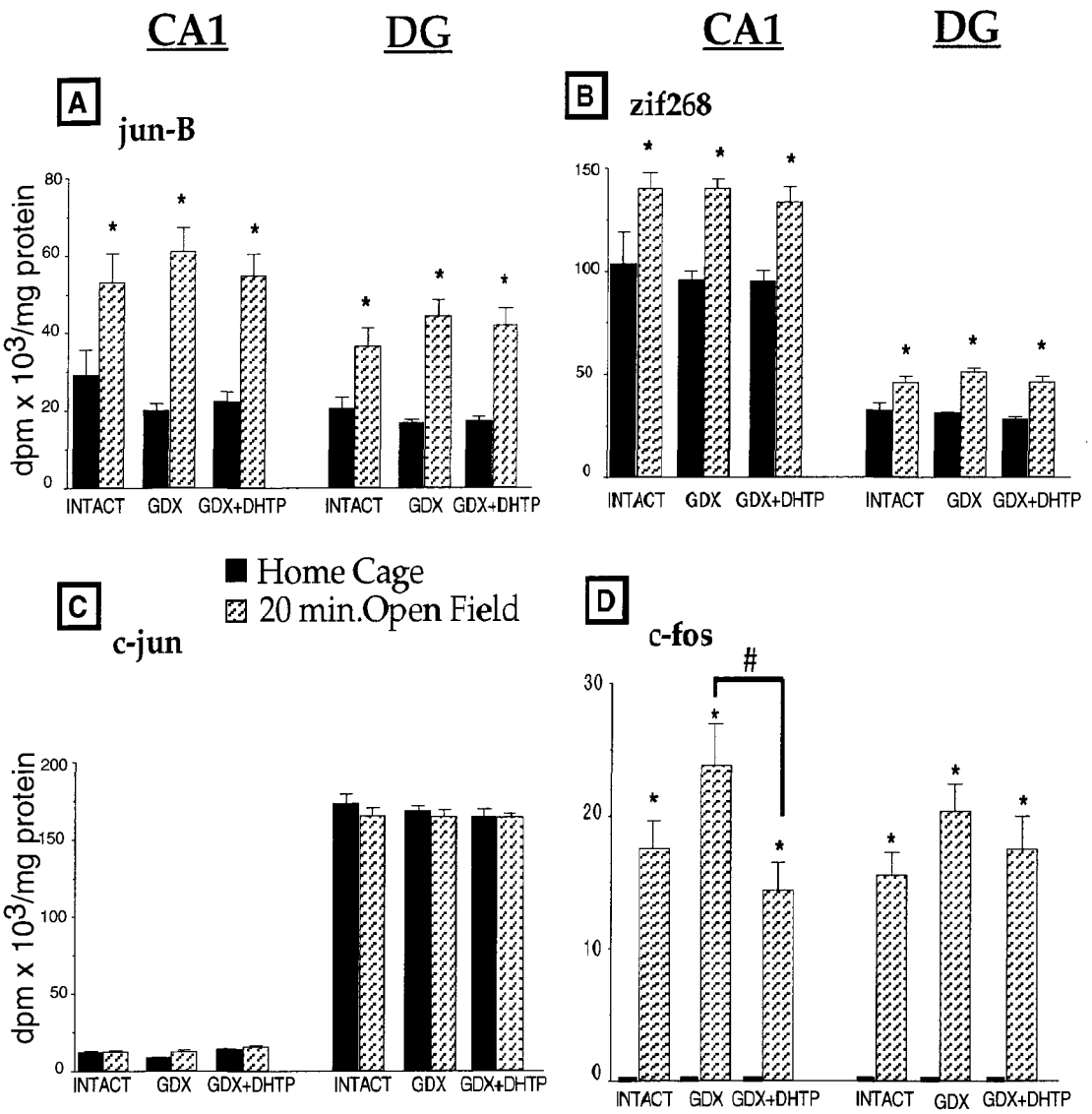

Figure 24. Quantitation of cIEG mRNA expression in the rat hippocampus. Effect of long-term castration (GDX) and DHTP treatment of castrates (GDX + DHTP) on the magnitude of **(A)** *jun-B*, **(B)** *zif268*, **(C)** *c-jun*, and **(D)** *c-fos* mRNA induction in the hippocampus of rats removed from their home cage (solid bars) or immediately following 20 min in the open field (hatched bars). Results from quantitative densitometry of *in situ* hybridization histochemistry in the CA1 region (left) and dentate gyrus (DG, right) are shown. Each bar represents the mean ± SEM from 6 animals. *, Significantly greater than home cage value ($P < 0.05$) and #, significantly different from each other ($P < 0.05$).

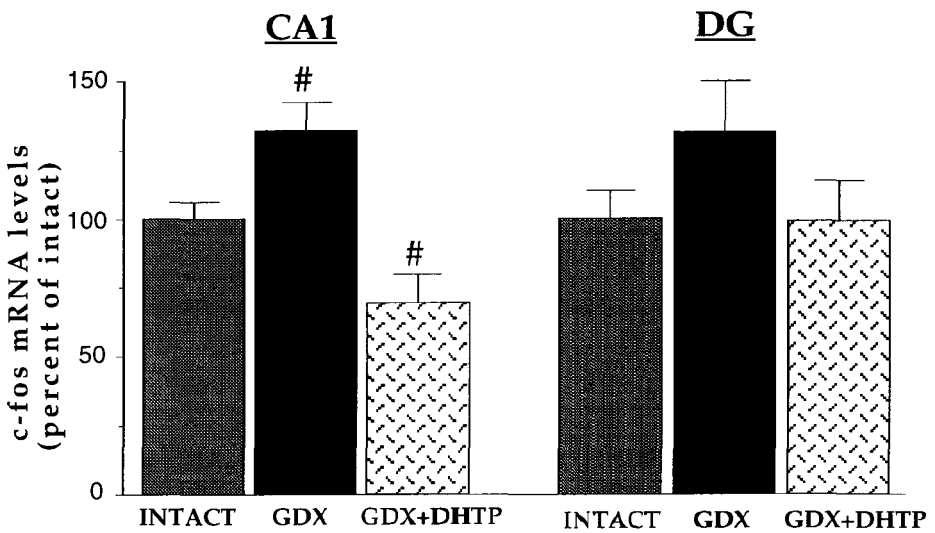

Figure 25. Effect of castration (GDX) and DHTP treatment of castrates (GDX + DHTP) on the magnitude of hippocampal *c-fos* mRNA induction following 20 min in the novel open field. CA1 = hippocampal CA1 pyramidal cell region; DG = dentate gyrus granule cell region. *c-fos* mRNA hybridization data were combined from three separately run studies. Due to inter-assay variability between the film autoradiograms, densitometry values from each animal are expressed as percent of the intact mean obtained from each film autoradiogram. Each bar represents the mean ± SEM from 11-13 rats. #, Significantly different from intact value ($P < 0.05$).

Discussion

The purpose of this study was to determine if androgens modulate the *in vivo* expression of cIEGs in the rat hippocampus following novelty. The hippocampus is a likely target for androgens based on earlier studies showing that AR and AR mRNA were expressed in this region with the greatest levels being found in the CA1 pyramidal cell region, lower levels in the CA3 region, and no expression in the DG granule cells.[27] Quantitative densitometry of *in situ* hybridization histochemical labelling detected by film autoradiography provided a means of assessing *c-fos, c-jun, jun-B*, and *zif268* mRNA levels in the densely packed cell body layers of the hippocampus. Since open field exploratory behavior had previously shown to stimulate *c-fos* mRNA (Handa *et al.*, 1993), as well as enhance the binding of hippocampal transcription factors to their DNA recognition elements (Kinney and Routtenberg, 1993), it was suspected that this behavior would be a simple, non-intrusive method of inducing cIEG expression in the hippocampus. In addition, scores for general activity in the open field apparatus could be tabulated and later related to gene induction.

Initially, novel open field exposure caused rapid increases of *c-fos, jun-B, c-jun* and *zif268* mRNA levels. However, there was also a region and gene specific pattern of expression which would argue against the possibility that this behavioral stimulus activates all hippocampal neurons leading to global, non-specific increases in mRNA transcription. In general, hippocampal *c-fos* and *jun-B* mRNA levels

increased more after novelty than did *zif268* or *c-jun* mRNA levels. The lower stimulation of *c-jun* and *zif268* mRNA levels appeared to be due to their relatively high basal expression; a finding that has been noted by others (Worley *et al.*, 1990; Gass *et al.*, 1992; Hughes *et al.*, 1992). *zif268*, the cIEG best correlated with the induction and maintenance of the hippocampal memory stimulus paradigm, LTP (Worley *et al.*, 1990; Richardson *et al.*, 1992), showed the longest time-course of expression of all the cIEGs studied. Hippocampal *zif268* mRNA levels were still higher than home cage levels 8 h after open field behavior and this protracted expression may play a role in memory formation. Preliminary studies demonstrated that novelty elicits specific cIEG signals in each hippocampal region. Since many of the cIEG protein products work in concert with each other to control transcription (Chiu *et al.*, 1988), this transcriptional network likely leads to the fine tuning of transcriptional activation of target genes.

To investigate the modulatory role of androgen on cIEG expression, GDX was used to eliminate endogenous androgen and hormone replacement of castrates with the non-aromatizable androgen, DHTP, was used to stimulate hippocampal ARs and isolate AR-mediated effects. The intact rat, which has high circulating levels of the aromatizable androgen, T, served as a physiological control. Castration of adult male rats for three weeks potentiated the behaviorally-induced *c-fos* mRNA levels in the CA1 region of the hippocampus as compared to intact rats. Furthermore, DHTP-treatment attenuated *c-fos* mRNA induction to 70% of the level found in intact rats, and to only 52% of that found in castrated animals. Since no significant changes in *c-*

fos mRNA occurred in the DG, where AR are not found (see Chapter III and Kerr *et al.*, 1995a), this finding strongly suggests that androgen acts through an AR-mediated process to initiate these effects. The intermediate expression of *c-fos* mRNA in intact rats may reflect the actions of the less potent androgen, T, on hippocampal AR activation or the possible counteractive effects of estrogen through estrogen receptors by the localized aromatization of T to estrogen in the hippocampus (Abdelgadir *et al.*, 1994). Unfortunately, RNase treatment of the tissue and the extreme density of cells in the CA1 cell body layer of the hippocampus makes examination of *c-fos* expression at the single-cell level difficult. In order to elucidate possible mechanisms of androgen action, it would be informative to know whether the decreases in behaviorally-induced *c-fos* mRNA levels that we have observed were the result of lower expression per cell, or if fewer CA1 cells expressed *c-fos* mRNA.

The findings concerning *c-fos* mRNA in these studies were perhaps in contrast to earlier work showing that seven days after castration or treatment with DHT, mating-induced Fos immunoreactive cell numbers were not altered in several areas of the rat brain (Baum and Wersinger, 1993). However, these researchers used a shorter androgen treatment duration which may not have allowed for the necessary AR-mediated changes in the cells to occur. Also, Fos was examined in hypothalamic brain areas, not in the hippocampus, and Fos immunireactivity was measured following a different stimulus (mating versus novelty). Finally, the androgenic effects on *c-fos* mRNA concentration that were observed in this study may not directly correlate with numbers of Fos-immunoreactive cells. To better understand this

cascade of cellular events and make assumptions on the role of Fos in hippocampal neuronal plasticity, as opposed to using *c-fos* mRNA induction strictly as a marker for neuronal activation, as was done in this study, it would be necessary to investigate whether changes in *c-fos* mRNA led to subsequent changes in Fos protein levels. In this regard, studies by Shultz *et al.* (1994) demonstrated that the induction of Fos immunoreactivity closely followed the induction of *c-fos* mRNA in the rat brain following novelty. This observation suggests that Fos protein levels would likely follow the same pattern of expression that was observed for *c-fos* mRNA.

The observation that castrated animals had increased activity in the novel open field during the first 5 min was intriguing. These increases in activity paralleled *c-fos* mRNA induction patterns in the CA1 region of the hippocampus and raised the possibility that main effects of *c-fos* were solely due to changes in activity. However, analysis of activity and the magnitude of CA1 *c-fos* mRNA levels on an individual animal basis revealed no significant correlations. Additionally, if treatment group differences in activity were the sole determinants of *c-fos* expression, then one would of expected to see significant changes in the CA3 and DG regions as well. The fact that the levels of *jun-B* mRNA, which was highly inducible by this behavioral stimulus, did not correlate with activity in individual animals, and did not change in response to androgen removal or treatment, also argues against activity level being the only factor regulating cIEG expression.

Earlier studies have revealed AR mRNA expression in virtually every hippocampal CA1 neuron (see chapter III and Kerr *et al.*, 1995a). This finding

enhances the probability that AR is present in the same CA1 neurons expressing *c-fos, jun-B* and *zif268* mRNA following novelty. Co-localization of mating-induced Fos and AR immunoreactivity has been described in the male hamster brain (Wood and Newman, 1993) and provides further evidence that these two transcriptional pathways are intertwined in several areas of the central nervous system. It is difficult to assess from these data why androgen status only affected *c-fos* mRNA levels, and not *c-jun, jun-B*, or *zif268* mRNA levels. Clearly, since *c-fos* was the most highly inducible mRNA following novelty, its expression had the greatest room for modulation by androgens. Since *c-jun* mRNA was not induced in the CA1 region, where AR expression is highest, it was not suprising that androgen had no effect on the expression of this cIEG. It can only be speculated that the cellular events triggering *zif268* and *jun-B* expression in CA1 neurons differ from that of *c-fos* and are not similarly altered by AR activation.

The consequences of altered *c-fos* expression in CA1 neurons are likely diverse. Earlier work has shown that Fos proteins must dimerize with Jun family member proteins to initiate its transcriptional regulation (Chiu *et al.*, 1988), and shifts in the relative concentrations of Fos and Jun can communicate very different messages in the cell nucleus (Diamond *et al.*, 1990). For example, differences in the amount of Fos expressed in cells *in vitro* relative to Jun expression allows for discrimination of transcriptional activation from transcriptional repression by GR acting at a composite HRE (Pearce, 1994). These studies suggest that changes in *c-fos* expression, without corresponding changes in *c-jun*, could alter Fos/Jun ratios, and thereby add another

level of transcriptional control within neurons.

Although the mechanisms accounting for the repression of *c-fos* mRNA levels by androgens were not explored in the present experiments, it appears likely that the long-term activation of AR in Fos-expressing CA1 cells was involved. Unlike what has been found for estrogen receptors (Weisz and Rosales, 1990), there is no evidence for a direct effect of androgen on the *c-fos* gene through the binding to an upstream HRE. Therefore, AR activation may lead to cellular changes which alter the ability of CA1 neurons to respond to *in vivo* stimuli and accounts for the observed changes in *c-fos* mRNA induction. Recently, it has been shown that DHT treatment attenuates the binding of MK-801, an NMDA receptor antagonist, in the CA1 region of the rat hippocampus (Kus *et al.*, 1995), and may subsequently inhibit the electrophysiological responses of CA1 pyramidal cells to NMDA. This decrease in membrane-bound excitatory receptor concentration is one possible mechanism by which androgens could alter synaptically mediated CA1 neuronal depolarization and/or lower the production of second messengers, thereby decreasing cIEG induction. The present findings concerning *c-fos* mRNA complement a recent study showing that removal of glucocorticoid hormones by ADX potentiated kainate-induced cIEG mRNAs in the hippocampus (Li *et al.*, 1992). Thus, androgen modulation of glucocorticoid receptor mediated events in the hippocampus are a possibility and are currently being investigated. If these mechanisms are occurring in CA1 hippocampal neurons, it is not yet clear why *c-fos* expression was preferentially affected.

In summary, these data have demonstrated that androgen modulates the

inducibility of certain cIEGs following a behaviorally relevant stimulus, most probably by acting through the androgen receptor. This may have been the result of changes in the excitability of existing neural circuits. Androgen modulation of behaviorally-induced cIEG levels within hippocampal neurons may result in large variations in transcription factor networks and may serve to fine tune androgen-mediated processes at the molecular level. In the hippocampus, these functions may include memory formation, cell maintenance, as well as cell survival.

CHAPTER VI

DISCUSSION

Growth, differentiation and plasticity of neurons involve the coordinated expression of many genes in a precise temporal sequence. In these studies, the expression of the receptor for androgens was characterized in the adult male rat hippocampus and this area of the brain was found to be sensitive to this potent class of steroids. This was emphasized by the fact that hormonal manipulations, in particular, selective, high level stimulation of AR for relatively prolonged periods, altered the expression of certain target genes within CA1 pyramidal cells.

Briefly, to summarize the results of this dissertation, it was shown that the male rat hippocampus contains a single, saturable, high-affinity binding site for androgen, and that this receptor has the same size and affinity characteristics as the AR found in other areas of the brain, and in peripheral tissues. AR and AR mRNA was expressed in the hippocampus in amounts comparable to that found in the hypothalamus -- an area where androgens act to control aspects of reproductive function and hormonal feedback. *In situ* hybridization revealed that AR mRNA expression is not uniformly distributed within the hippocampus. AR mRNA was concentrated in CA1 pyramidal neurons, and very little expression was found in the DG. Short term GDX and AR antagonism downregulated AR mRNA in the whole

hippocampus; however, AR levels (as determined by *in vitro* [^{3}H]DHT binding) were slightly elevated following similar treatment. These data suggest a unique AR autoregulatory process in hippocampal neurons. Additionally, steady state AR mRNA levels, but not AR binding levels, were higher in the hippocampus of old rats as compared to their young counterparts suggesting, at least, a maintenance of androgen sensitivity in this tissue throughout life. Sub-chronic treatment of young rats with the AR-selective androgen, DHTP, significantly decreased steady state GR mRNA expression, and prevented ADX-induced GR mRNA upregulation, selectively in the CA1 region of the hippocampus. Neither MR or AR mRNA levels were altered by the same androgen treatments. Finally, inducible gene expression was characterized in the hippocampus following exposure to novelty. Of the four cIEGs studied, *c-fos* mRNA was the most highly induced in the hippocampus by this stimulus, and DHTP treatment attenuated *c-fos* mRNA induction selectively in CA1 pyramidal cells.

As with most scientific endeavors, many questions have arisen from these studies. Certainly, two fundamental questions remain. 1) Through what cellular mechanisms does the ligand-activated AR regulate the expression of GR, *c-fos* and, possibly, other genes in hippocampal CA1 pyramidal neurons? 2) How might androgen-mediated regulation of GR and *c-fos* expression lead to physiologically relevant changes in hippocampal plasticity and, ultimately, affect hippocampal regulated behaviors? Unfortunately, at the current level of understanding neither of these questions can be answered definitively. Much of the following discussion is a theoretical scheme of potential molecular mechanisms and ramifications of androgen

action in the hippocampus. To support these theories, evidence from recent studies examining interactions among the several classes of transcription factors and cIEG protein products in neurons, cell culture and other molecular systems is discussed.

Mechanisms of Androgen Receptor Action

These dissertation studies have demonstrated that AR activation for sub-chronic periods attenuates steady state levels of constitutively expressed GR mRNA and behaviorally-induced *c-fos* mRNA selectively in hippocampal CA1 pyramidal cells. As neither *c-jun, junB, zif268*, MR or AR mRNA levels were similarly altered by this treatment, it is doubtful that generalized decreases in transcriptional efficiency would account for these results. More likely, other mechanisms account for the effect of androgens on the transcription of selective target genes in CA1 neurons. Potential mechanisms to explain AR-mediated decreases in GR mRNA levels include: direct or indirect androgen-induced alterations in the ability of GR to mediate its own transcriptional regulation, changes in GR mRNA processing or stability, and/or by direct AR inhibition of GR gene transcription through a simple HRE. Androgenic effects on inducible *c-fos* mRNA expression following a behavioral stimulus may be occurring through androgen modulation of membrane receptor levels, changes in other second messenger systems that have known effects on cIEG transcription, multi-synaptic changes in neuronal excitability, and/or direct modulation of cIEG

transcription or mRNA stability. A more detailed discussion of some of these theoretical mechanisms, and any available evidence for them, follows below.

Cellular Interactions Between Androgen and Glucocorticoid Receptors

AR and GR may be interacting in CA1 pyramidal cells at several levels of their transcriptional pathways to regulate GR gene expression. Three plausible mechanisms to account for androgen modulation of GR mRNA levels are depicted in **figure 26**. Activated AR may act non-discriminately at a simple HRE within or upstream of the GR gene and block its transcription **(figure 26A)**. In this scenario, AR mimics the normal GR effect and, at high enough levels, AR may displace GR dimers at this site. Both AR and GR have been shown to activate transcription *in vitro* from the simple HRE contained in the mouse mammary tumor virus promoter (Shemshedini et al., 1991) which lends some support to this theory. However, most AR-regulated genes thus far (including probasin and mouse sex-limited protein) contain complex response elements that were specific for AR as a result of selective protein-protein interactions and response element spacing within the promoter region (Adler et al., 1993). Further characterization of the HRE controlling steroid regulation of GR transcription would help to determine whether this mechanism could also occur in CA1 neurons.

Alternatively, as depicted in **figure 26B**, high levels of activated AR may use transcription factors and/or accessory proteins also necessary for normal GR gene transcription. One such protein, designated receptor accessory factor (RAF; later

found to have complete amino acid identity with insulin degrading enzyme), has been shown to directly interact with and enhance DNA binding of both AR and GR peptide fragments (Kupfer et al., 1993). This finding suggests that RAF may play a role in the transcriptional activity of these receptors. Along these lines, overexpression of ER significantly inhibited AR transcriptional activity in cell culture (Kumar et al., 1994) prompting the authors to suggest that these two receptors must compete for some unknown factor necessary for their transcriptional activity. Several studies have demonstrated that GR interacts with many other transcriptional activators *in vitro*, including Fos, Jun, and octamer transcription factor I (Yang-Yen et al., 1990; Jonat et al., 1990; Schüle et al., 1990; Kutoh et al., 1992). Although AR protein-protein interactions have yet to be studied in depth, the overlapping use of transcription factors by AR and GR may serve an important regulatory function in hippocampal pyramidal cells.

Due to the long-term nature of the androgen treatments used in these dissertation studies, it is also possible that AR activation could have altered GR expression through more indirect means than discussed above. As mentioned earlier, there is mounting evidence that the transcriptional activity of GR is modulated by its interaction with other transcription factors traditionally thought to be stimulated by cell surface receptor signal transduction (Diamond et al., 1990; Hoeck et al., 1990; Jonat et al., 1990; Lucibello et al., 1990; Yang-Yen et al., 1990; Schüle et al., 1990; Shemshedini et al., 1991; Shüle and Evans, 1991; Unlap and Jope, 1994). In particular, the protein-protein interaction of GR with the AP1 transcription factor may

repress or activate the transcriptional activity of GR depending on the relative concentrations of cIEG family members, Fos and Jun, in the complex (Diamond et al., 1990). Since the studies present in this dissertation have revealed decreased levels of behaviorally-induced *c-fos* mRNA in the CA1 region of the hippocampus of DHT-treated castrates, this potential modulation of the Fos:Jun ratio within CA1 pyramidal cells following three week androgen treatment may in turn alter how GR acts at its HRE within or upstream of its own or other target genes (**figure 26C**). Along these lines, expression of *Ha-ras* and *v-mos* oncogenes in GR-expressing NIH 3T3 cells enhanced ligand-induced down-regulation of GR (Hoeck et al., 1990). Taken together, androgen modulation of such intermolecular interactions between GR and other transcription factors may be another mechanism mediating GR transcriptional activity, conferring steroid hormone specificity, or fine-tuning gene expression at the HRE resulting in our observed decreases in GR mRNA levels. It is also possible that AR-mediated downregulation of GR expression enhances androgen sensitivity within cells that express both GR and AR, as this mechanism would enhance the probability of AR action at HRE sites used by both AR and GR. Many additional studies examining the cross-talk between these signal transduction pathways are necessary to ascertain which, if any, of the previously mentioned mechanisms are occurring in CA1 neurons.

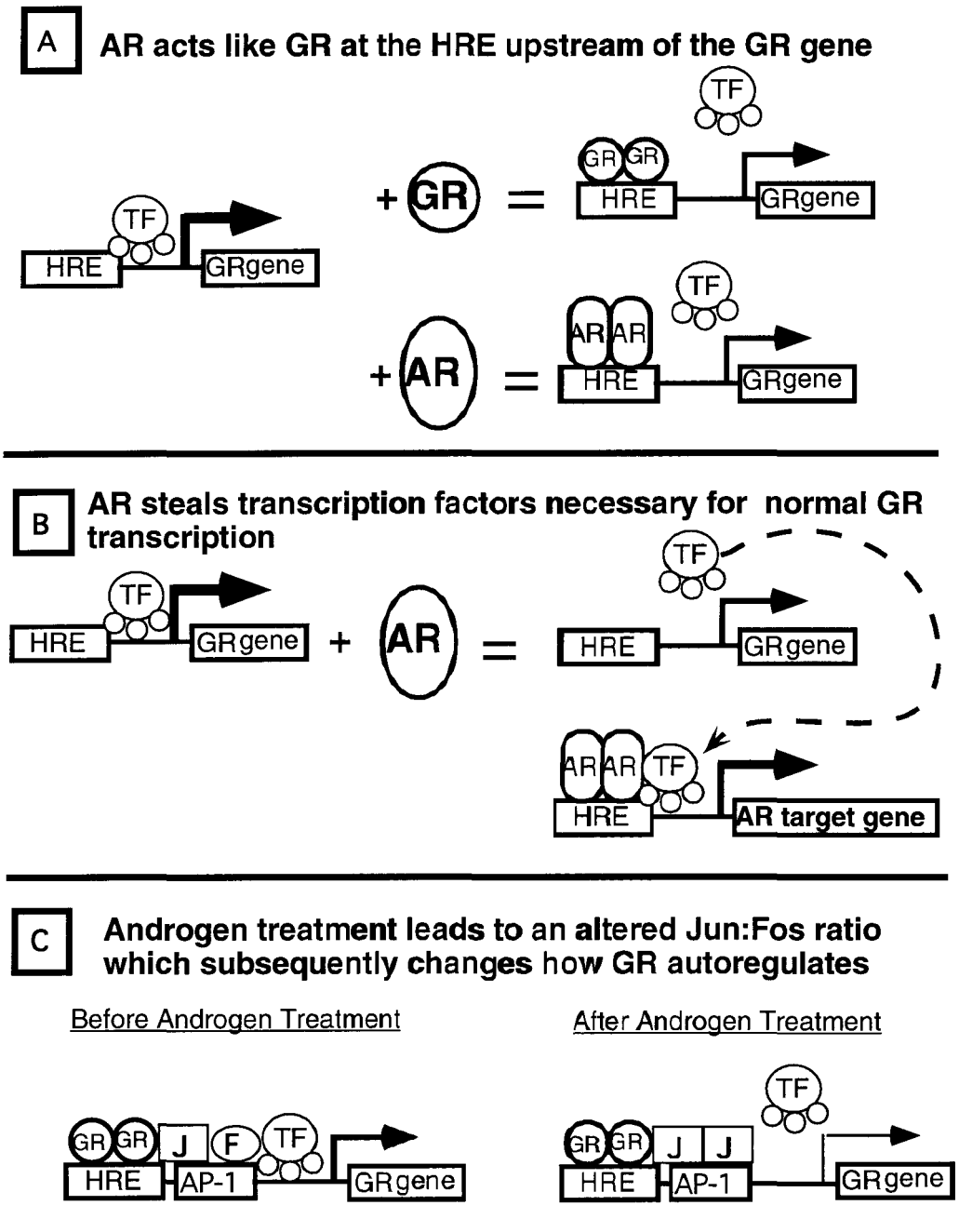

Figure 26. Schematic representation of three possible mechanisms by which activated androgen receptors (AR) could interact or interfere with glucocorticoid receptor(GR)-mediated autoregulation. Bent arrow thickness indicates strength of gene transcription.

Mechanisms of Androgen Modulation of Cellular Immediate Early Genes

Few studies have examined specific AR interactions with cIEG protein products, however, as mentioned above, there is increasing evidence that nuclear hormone receptor pathways do cross-talk with the cIEG pathways, thereby modulating each other's activity (see review by Hyder et al., 1994).

At our current level of understanding, the most plausible mechanism to explain androgen mediated attenuation of *c-fos* mRNA induction in the CA1 region is through the modulation of the function of a stimulating receptor in these neurons. In the CA1 region, the best example of a stimulatory receptor is the NMDA receptor. The expression of these receptors is highly concentrated in CA1 neurons (Mackler and Eberwine, 1993; Kus et al., 1995) and glutamate is thought to be the principle excitatory neurotransmitter in the hippocampal formation (Jahr and Stevens, 1987). In addition, rapid *c-fos* expression has been observed in the hippocampus following NMDA receptor activation (Sonnenberg et al., 1989). Similarly, studies have demonstrated that the administration of the NMDA receptor antagonist, MK801, strongly attenuates the rise in *c-fos* mRNA and protein in the DG following a kindling stimulus, but has a lesser effect on *jun-B* and *c-jun* mRNA and protein and does not markedly attenuate *zif268* mRNA and protein levels (see review by Hughes and Dragunow, 1995). This selectivity of the NMDA receptor for *c-fos* expression strongly suggests that NMDA sensitivity may play a key role in androgen modulation of *c-fos* expression. Studies are currently underway to investigate androgen regulation of NMDA receptor expression and action in the hippocampus. In support of this

hypothesis, initial studies by Kus et al. (1995) have found that androgen administration decreases MK801 binding in CA1 pyramidal cells. In accordance with these findings, Pouliot et al. (1995) have demonstrated that androgen treatment attenuates NMDA's excitotoxic electrophysiologic responses in CA1 neurons. In contrast, estrogen has been shown to increase NMDA agonist sites in the CA1 pyramidal cell region (Weiland, 1992). Such polarized effects of androgen and estrogen may underlie sex differences in hippocampus-mediated behaviors.

It can not be ruled out that other pyramidal cell membrane receptors could also be regulated by androgens thereby affecting neuronal excitability and cIEG induction. Interestingly, the induction of *c-fos* by administration of the nonselective muscarinic agonist, pilocarpine, was localized to the CA1 and CA2 cell body regions of the hippocampus (Hughes and Dragunow, 1993, 1994). Unfortunately, no studies have yet explored androgen regulation of muscarinic receptors to determine if such mechanisms could account for our results.

Alternatively, androgens may regulate the levels of second messenger molecules or transcription factors known to activate or control the rapid induction of cIEGs. Such possibilities include the protein kinase C-dependent serum response factor (SRF) and the Ca^{2+}/cAMP-activated CREB protein; both of which bind to upstream response elements in the Fos gene and stimulate its expression (Treisman, 1985; Sheng et al., 1990). Although androgen withdrawal has been shown to decrease CREB transcript in the adult rat testis (West et al., 1994), no such studies have been performed in brain tissue. Thus, it is still too early to predict if androgen

acts through this mechanism in CA1 pyramidal neurons.

Androgen may also be acting at a site distant from the hippocampus, but through multi-synaptic connections alters CA1 cell excitability and, in turn, modulates *c-fos* expression following a behavioral stimulus. Certainly when one considers the widespread connectivity to and from the hippocampus, such a complex process can not be ruled out. Future studies using more localized administration of androgen into the hippocampus, cultured pyramidal cells, or the hippocampal slice preparation will help to elucidate if androgen's actions are multi-synaptic.

Functional Implications of Androgen Sensitivity in the Hippocampus

Due to the fact that only subtle changes in gene expression following relative extreme alterations in circulating androgen levels were observed, it appears that androgens act in the adult hippocampus to fine-tune selective transcriptional responses. Interestingly, the presence of functional AR in the body is not necessary for life or normal intelligence. This information has been attained from genetic XY individuals who are born with mutations in the AR gene, and thus, are insensitive to androgen's developmental and activational effects despite having high levels of circulating T. In most cases, these individuals have severely malformed sexual organs and are typically raised as females, but, otherwise have normal IQs (Imperato-McGinley et al., 1991) and life spans (McPhaul et al., 1991). These findings

suggest that androgen's actions in the brain are delicate, or, it is possible that other mechanisms may compensate for a lack of direct androgen action in the brain. In light of these data, androgen insensitive individuals have been found to perform worse on hippocampally-mediated visuospatial tests as compared to both normal males and their own unafflicted sisters (Imperato-McGinley et al., 1991); and curiously, T supplementation to female-to-male transsexuals was associated with an increase in their spatial ability, and had a deteriorating effect on their verbal fluency (Van Goozen et al., 1994). These findings further support the studies in this dissertation suggesting subtle activational effects of androgens in the hippocampus.

Functional Implications of Androgen Regulation of GR and *c-fos* Expression

Although the studies in this dissertation did not explore the functional or behavioral significance of androgen-mediated changes in GR and *c-fos* mRNA expression in hippocampal pyramidal cells, it is still possible to speculate how changes in the expression of these genes may affect hippocampal function using evidence from studies that have investigated GR- and Fos-mediated functions within the hippocampal formation.

Activation of GRs in the hippocampus has been associated with decreased excitability within CA1 neurons (Joël and De Kloet, 1992), and in the process of information storage (Oitzl and De Kloet, 1992). In addition, the activation of

hippocampal GRs at high levels of circulating CORT contributes to the HPA axis hormonal feedback inhibition process, resulting in the termination of the stress response (Ratka et al., 1989). Potentially, all of these physiologic outcomes could be indirectly modulated by fluctuations in androgen levels. It has also been well documented that prolonged exposure to high levels of glucocorticoids, especially in older rats, is neurotoxic; with preferential injury to the hippocampus (Landfield et al., 1978; Sapolsky et al., 1985; Meaney et al., 1988; Woolley et al., 1990). In addition, exposure to physiological levels of glucocorticoids can "endanger" the hippocampus, making its neurons less likely to survive coincident challenges such as hypoxia-ischemia (Sapolsky and Pulsinelli, 1985; Morse and Davis, 1990), seizures (Sapolsky, 1985), and NMDA receptor-mediated excitotoxicity (Supko and Johnston, 1994). If androgen treatment proves to be effective in decreasing GR protein levels in CA1 pyramidal cells, such a mechanism may, in turn, be protective to these neurons.

The use of androgens to control the magnitude of *c-fos* induction in the hippocampus following a stimulus or stressor may also prove to be a useful tool to prevent cell loss or injury. The debate continues as to whether the induction of *c-fos* after stress, seizure or neurotoxin exposure is involved with the neuroprotective regeneration process, or if it sets into motion the genetic program for cell death. When this process is better understood, androgen sensitivity may play out to be an important modulator of this process.

Conclusion

These studies have demonstrated relatively high levels of functional AR in hippocampal CA1 pyramidal cells of the adult male rat. In addition, it was found that these receptors are sensitive to changes in circulating androgen levels by altering AR occupancy and the modification of selective transcriptional responses within these neurons. Although, it is still difficult at this time to pinpoint the functional significance of AR expression in the hippocampus, the preceding observations unveil a solid foundation for further investigation of the activational roles of androgen in hippocampal pyramidal cells and the cellular interactions between steroid hormone receptors and other transcription factor responses within neurons. Undoubtedly, AR action is complex and involves multiple signal transduction pathways. Future studies clarifying the molecular cascade of events following AR activation, as well as the precise behavioral outcomes of androgen manipulation, will provide crucial information in the aim of understanding androgen action in the brain.

APPENDIX 1.

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October 2, 1995

Shlomo Melmed, M.D.
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Dear Dr. Melmed,

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> **Kerr JE, Allore RJ, Beck SG, Handa RJ** 1995 Distribution and hormonal regulation of androgen receptor (AR) and AR messenger ribonucleic acid in the rat hippocampus. Endocrinology 136(8):3213-3221

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VITA

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The final copies have been examined by the co-directors of the dissertation and the signatures which appear below verify the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the committee with reference to content and form.

The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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