ONTOGENY OF THE ANDROGEN RECEPTOR IN THE HIPPOCAMPUS OF THE SPRAGUE-DAWLEY RAT.

DORIS M. BABSTOCK


CENTRE FOR NFLD. STUDIES
MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

Ontogeny of the Androgen Receptor in the Hippocampus of the Sprague-Dawley Rat.

BY

Doris M. Babstock, B.Sc.

A thesis submitted to the
School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Department of Psychology
Memorial University of Newfoundland

December 1999

St. John's Newfoundland

Abstract

Immunocytochemistry (ICC) with a polyclonal antibody (PG-21) was used to investigate the ontogeny of the androgen receptor (AR) in the hippocampus of Sprague-Dawley rats. Brains of five male and female littermates were examined at each age covering the neonatal (PND 2, 5, 7), juvenile (PND 14), prepubertal (PND 25), and pubertal (PND 40) period with one male and female examined at PND 60. As well, two sets of PND 7 and PND 25 male and female littermates were injected 30 minutes before perfusion with dihydrotestosterone benzoate (DHTB) to look for a possible ligand-dependent increase in staining. The density of staining in the hippocampal CA1, CA3 and DG cell layers was determined in each section with the density of the DG subtracted from the density of the pyramidal cell layers as a control for background staining.

In the hippocampus of both sexes, AR staining density is described by an inverted U-shaped function between the ages of PND 2 and PND 25 with a peak at PND 7. AR staining density in the CA1 cell layer is at its highest density in both sexes during puberty (PND 40), with significantly greater density in the male. In contrast, at PND 40, AR staining density remains low in the CA3 cell layer. The only other age that exhibits a possible sex difference is PND 7 with a trend within the CA1 cell layer for greater AR staining density in the male.

In the neonatal and prepubertal animal, the AR staining is dispersed equally throughout the cell with nuclear enhanced density observed at PND 40.

The effect of DHTB treatment on AR density depends on age. Increased staining is observed in both sexes at PND 7 whereas, in the prepubertal animals (PND 25), DHTB appears to decrease AR density in females with no consistent effect in males.

The ontogeny of the AR within the hippocampus is compared to the ontogeny of the AR in other brain areas and to the ontogeny of the estrogen receptor (ER). It is concluded that neonatal and prepubertal hippocampal AR may be regulated by factors other than circulating androgen as AR density is not clearly related to serum testosterone ('T') levels and DHTB-treatment does not elicit a nuclear translocation as demonstrated in adult animals. Instead, the AR is equally dispersed throughout the cell, regardless of androgen treatment. Thus, the neonatal and adult AR may be differently regulated and may have different functions.

Key words: Androgen receptor (AR), immunocytochemistry (ICC), PG-21, ontogeny, hippocampus, CA1, CA3, dihydrotestosterone benzoate (DHTB).

Acknowledgements

I wish to express my gratitude to Drs. J. McLean and R. Adamec who served on my committee. I also acknowledge and appreciate the contribution of my supervisors, Drs. C. Harley and C. Malsbury for their moral and technical support throughout the many stages of this project. A special thanks to Dr. Malsbury for his encouragement and insightful comments as well as for providing the facilities that enabled this work to be completed.

I would also like to thank NSERC and the School of Graduate Studies for their financial support during the early stages of my post graduate studies. As well, I wish to express a very special thanks to Dr. Gail Prins, University of Illinois at Chicago. Dr Prins generous gift of AR antibody and control peptides ensured the survival and completion of this project.

Finally, very deep and heartfelt thanks go to my family, without whose support, friendship and love, I could never have considered striving for and possibly realizing the fulfillment of this dream. Thank you Cindie, Caryn & Krista and a very special thank-you to my husband and life companion, Reginald.

TABLE OF CONTENTS

List of Figures

List of Abbreviations

AR21	A peptide used to raise the antibody
AR462	A distant peptide
AR	androgen receptor
BSA	bovine serum albumin
cAMP	cyclic adenosine 3'.5'-phosphate
DG	dentate gyrus
DAB	diaminobenzidine tetrahydrochloride
DHT	dihydrotestosterone
DHTB	dihydrotestosterone benzoate
E	embryonic day
EB	estradiol benzoate
E_2	estrogen / estradiol
ER	estrogen receptor
EPSP	extracellular population spike potential
GR	glucocorticoid receptor
hER hAR	human ER or AR
ICC	immunocytochemical / immunocytochemistry
-ir	immunoreactive staining
ISO	International Standard Organization
LTP	long-term potentiation
mRNA	messenger RNA
ORX	orchidectomy
OVX	ovariectomized
PND	postnatal day
PR	progesterone receptor
SNB	spinal nucleus of the bulbocavernosus
s.	stratum (e.g., s. oriens)
sc	subcutaneously
R1881	synthetic non-aromatizable androgen, methyltrienolone
'T'	testosterone
TP	testosterone propionate

Ontogeny of the Androgen Receptor in the Hippocampus of the Sprague-Dawley Rat.

CHAPTER 1: INTRODUCTION

The brain regulates a variety of functions that are different between males and females. Some of these differences, termed 'organizational effects', are produced by perinatal exposure to the sex steroids, which permanently alters neural connections and functions within the brain. Other functional differences occur throughout life with temporary effects upon neural substrates or circuits, thus earning the nomenclature 'activational effects'. Both types result from the endogenous hormonal environment that differs between males and females. Although there may not be a true dichotomy between these two classes (see Arnold & Breedlove [6]), the terminology remains generally useful and will be used as defined above. The most obvious and best studied functional differences between males and females are those involved in reproductive physiology and behavior. Consequently, much is now known about the effect of the sex steroids on behavior, physiology and neuroanatomy that is linked to mating (for review see [18,26,43,51,55,88,94,109,114,119,133]). However, relatively recent discoveries of the interaction between progesterone and the GABA receptor [19,77,90,92,93,100,139,140,154] as well as the effect of estrogen (E_2) and the estrous cycle on dendritic processes within the hippocampus [54,106,178,179] suggest that the sex steroids may be involved in more than reproductive behavior [180].

As a means of introducing the classical conception of the androgen receptor (AR) within the central nervous system, the introduction will review the involvement of androgens and the AR in reproductive behavior as defined by defeminization and masculinization. This will encompass such topics as the synthesis of steroid hormones, the ontogeny of androgen secretion, the presence and function of α-fetoprotein, and the role of aromatization in brain development. As well, results from treatment with the non-aromatizable androgen, dihydrotestosterone (DHT), will be reviewed to help define the involvement of the AR in these processes. The introduction will then examine the composition and properties of the AR protein, as this protein is the means of exploration utilized by the current study. Because this thesis is concerned with the presence of the AR within the hippocampus of the neonate, the ontogeny of the hippocampal formation will then be introduced as well as what is currently known about the presence of this receptor within this brain area. Finally, the introduction will review hippocampal-related sex differences to provide a framework for functions that may be related to the presence of the AR within the hippocampal formation.

In general, before a receptor can initiate either a single or a cascade of events, it must first bind to its activating ligand. This is also true of the steroid receptors. Thus, the first topic to be covered is when and how the activating ligands of the steroid receptors are first synthesized.

1.1 Synthesis of Steroid Hormones

As reviewed by Brown [21], hormones are classified into two distinct groups, steroids and peptide/protein hormones. The latter are synthesized from amino acids within the ribosomes of the rough endoplasmic reticulum [21]. Their function and description will not be elaborated here as they do not relate to this project. The sex hormones belong to the steroid group, synthesized in the smooth endoplasmic reticulum of the cell body within the gonads and the adrenal cortex [21]. Following synthesis, they are transported to their respective sites of action within the brain via the endocrine system (blood stream). Steroids are synthesized from plasma cholesterol, which is initially converted into pregnenolone, and then into progesterone [21]. Both of these substances can be hydroxylated. Within the gonads of both sexes, hydroxyprogesterone acts as a prohormone to testosterone ('T') and DHT, which are secreted from the testes [21]. Within the ovaries, small amounts of 'T' are excreted without conversion but most is further converted to estradiol (E_2) [21]. In the adrenal gland, progesterone can be secreted as a steroid hormone or act as a prohormone (precursor) to the corticosteroids, corticosterone and aldosterone [21]. However, the hydroxylated form of progesterone acts as a prohormone to the corticosteroid, hydrocortisone or to the sex steroids, 'T' and E_2 [21]. Although the gonads and adrenal gland are proven sources of the sex steroids, recent work suggests that steroids may also be synthesized within the brain, earning the nomenclature neurosteroids [125]. However, although neurons metabolize gonadal steroids (e.g., aromatization),

there is no evidence that *de novo* synthesis of sex steroids within the CNS plays a role in its sexual differentiation.

1.2 Ontogeny of Androgen Secretion

The perinatal period is a critical period for the organizing effects of 'T' on brain and behavior. In order to understand the organizing process it is necessary to know a) when the brain is exposed to 'T' and b) when the particular brain regions begin to express steroid receptors. This section will explore what is known about the ontogeny of the ligand that activates the AR.

Using radioimmunoassay, 'T' is first detected in the testicles of the Sprague-Dawley [33] and Wistar [34] male rat by the 17th-18th day of gestation and in the ovaries of the female by postnatal day (PND) 10 [34]. In males of the Sherman strain, plasma sampled from rats born on day 21 of gestation show a 'T' surge between birth and 2-h *ex utero*, (2820 pg/ml) falling to pre-birth levels by 4-h *ex utero* (885 pg/ml) [32]. A similar surge in plasma 'T' is reported in Wistar male pups [34,50,153] with pre-birth levels of serum 'T' tripling at birth and then declining sharply within the first 6 hours. Levels return to pre-surge values by 12- [32,50,153] to 24 h [34] *post partum*. The sampling time relative to birth appears to be critical as the peak is not observed in blood from Sprague-Dawley males and females sampled between 1700-1800 h on the day of birth without consideration to the time of birth [41]. However, serum 'T' from Sprague-Dawley male and female pups sacrificed at 0.5-2 h *post partum* is 3 times greater than from pups sacrificed

within 2-24 h *post partum* [116]. This suggests the occurrence of a *post partum* surge in Sprague-Dawley neonates as well. The surge is not likely related to factors surrounding parturition as similar results were obtained in newborns taken by cesarean [32]. The early postnatal surge in plasma 'T' is suggested to be functional in the masculinization of the hypothalamus [32]. Although a direct equivalency is not made between the neonatal ages of monkey and rat, plasma androgen levels are elevated at birth in male monkeys as discussed above for the rat [8].

In the ovaries of the female rat, 'T' is either extremely low [153] or not detectable until PND 10 [34] and in the adrenals, significant levels are detected only after PND 17 in both sexes [34]. Although the female serum 'T' is significantly lower than in males from PND 1 up to PND 19 [41,116,153], the amount at PND 1 is twice the level reported for every day up to PND 5 [116] and for every day examined up to PND 49 [41,116]. The source of plasma 'T' found in the *post partum* female might be the maternal adrenal gland, which could contribute to the level observed at PND 1. However, as reported for the male, a similar but significantly smaller *post partum* 'T' surge is also observed in female Sprague-Dawley [116] and Wistar [153] pups. As well, the sex difference in neonatal plasma 'T' suggests that the level found in males cannot be accounted for by the maternal adrenal gland but must be dependent upon an additional source present in males only. Testosterone is present in the testes of the perinatal male [33,34,153] with the suggestion [153] that the rise in plasma 'T' immediately after birth is due to the lack

of metabolic clearance by the mother. The mother provides such metabolic clearance when the fetus is in the womb. The newborn then quickly establishes a new equilibrium [153].

Within the CNS, the level of 'T' is lower than in plasma [23] due to the low diffusion/transport rate from plasma to the cerebrospinal or interstitial fluid compartments [117]. In the PND 1-2 neonate, brain 'T' is 15-30% lower than plasma values in the male and 25-70% lower in females [23]. As expected, 12 to 48 h after birth, PND 1 and PND 2 males show significantly greater values compared to females for 'T' (>13x) and DHT (>4x) in both plasma and brain [23]. The amount of 'T' in both plasma and brain of the neonatal male is 100 times greater than the amount of E_2 [23]. Surprisingly, in the neonatal female rat, the amount of 'T' reported in the plasma and brain is also greater than the amount of E_2 by a factor of 5-10 [23]. Brain levels of E_2 are very low [23]. Although no sex difference is reported in plasma E_2 levels, the low level of E_2 found in the male brain is still greater than the reported value for the female brain [23]. Thus, the male brain contains more E_2 than the female brain, whereas both male and female brains contain more 'T' than E_2.

The continued pursuit of the ontogeny of steroid hormone binding to their respective receptors and the implications for sexual differentiation of mating behavior lead to the discovery of a neonatal binding protein for E_2. The binding protein is no longer present following three-four weeks of life.

1.3 α-FETOPROTEIN

The higher levels of E_2 in the male compared to the female brain supports the suggestion that E_2 is involved in male sexual differentiation (for review see Gorski [52], Goy and McEwen [55]). However, activation of the estrogen receptor (ER) in the perinatal brain is not dependent upon plasma levels of E_2. This is because E_2 is sequestered in both males and females by the serum estradiol binding protein, α-fetoprotein (for review see Lieberburg [82], Toran-Allerand [153]). Although various functions have been proposed for α-fetoprotein including that it 1) acts as a 'reservoir', releasing E_2 slowly to prolong the half-life of maternal estrogens and/or 2) provides intraneuronal transport of E_2 [40,163], the accepted function of α-fetoprotein is that it protects females from defeminization (see Dohler [40] and references therein) by limiting activity at the ER.

The properties of α-fetoprotein were defined using various methods including charcoal assay of *in vitro* cytosol binding, density gradients, and DNA binding via DNA cellulose chromatography [7,120,124]. α-Fetoprotein is differentiated from the ER by its sedimentation coefficient of approximately 4.5S compared to the coefficient of 8S identifying the ER [7,120,124]. In the perinatal mouse [7] and rat brain [120,124], α-fetoprotein is found at high concentrations in the extracellular fluid and in neuronal cytoplasm (see review by [40,163]). As well, α-fetoprotein shows high capacity and greater affinity than the ER for binding E_2, but does not bind 'T' nor non-estrogenic steroids (including DES) [7,120,124]. The high capacity and

greater binding affinity of α-fetoprotein for E_2 [120,124] prevents this steroid from entering the cell nucleus and activating the ER. α-Fetoprotein is present in both sexes of the 20-day old rat fetus [124] and in the neonate at PND 6 [7,120,124]. It decreases to half the concentration by PND 15, with very low presence by PND 22 [120] and is undetectable by PND 28-30 [124]. In the mouse, α-fetoprotein is undetectable by PND 21 [7].

Because α-fetoprotein is diffuse in cytoplasm of neonatal brain neurons [7,40,120,163] and in particular within the hypothalamic, preoptic, amygdala, and midbrain areas [120], it seems reasonable to assume that aromatized E_2 would also be sequestered in the neonate. However, it is not known if specific neurons containing α-fetoprotein within the CNS are actually involved in neonatal defeminization. On the other hand, α-fetoprotein could be actually providing a steady supply in these target neurons by slowly releasing earlier sequestered E_2 [40,163]. Alternatively perhaps, in the male neonate, large enough quantities of E_2 are synthesized in the cytoplasm such that significant amounts escape sequestering. Regardless, the unbound aromatized E_2 enters the nucleus where it attaches to the ER, thereby inducing a cascade of genomic events.

1.4 Aromatization

As indicated in the previous section, the high capacity α-fetoprotein sequesters E_2 within the cytoplasm [7,120,163] of the neonatal brain, thereby severely restricting action at the ER. However, E_2 is believed to mediate defeminization of

the male brain [30,40,52,62,94] (and others, see below) and play a role in masculine sexual behavior in the adult rat through activation of the ER [10,28,30,166]. The E_2 that activates the brain ERs in the neonate does not arrive via the endocrine system, but rather, is metabolized within the brain from 'T', in a process referred to as aromatization. Thus, although α-fetoprotein sequesters E_2 metabolized within the gonads, aromatization within specific areas of the brain provides a metabolic pathway that ensures local sources of E_2. Testosterone, which is not bound by α-fetoprotein, enters the cytoplasm of the cell and in the presence of the aromatase enzyme is catalyzed into E_2 [49,78,107,161,162]. Thus, aromatase must be present in target areas where 'T'-derived E_2 is believed to exert its effects (see Brown [20], Goy & McEwen [55], Hutchison & Steimer [62], McEwen et al. [94] for review).

Although aromatase activity is mediated via the aromatizing enzyme, such activity is enhanced by the presence of the AR with aromatase activity increasing with increased levels of 'T' [130]. Both aromatase activity and aromatase messenger mRNA (mRNA) is decreased in the hypothalamic-preoptic area of castrated male Sprague-Dawley rats and restored by concurrent treatment with Silastic implants containing either 'T' or DHT but not by E_2 [1]. The lack of a significant effect of E_2 treatment and the equivalent effect reported for both 'T' and DHT replacement in castrated males implies a regulatory role for the AR in aromatase activity and aromatase mRNA. The AR is further implicated by treatment with an anti-androgen [131]. Whereas implanting Silastic capsules containing 'T' at the time of castration restores aromatase activity in adult male

rats relative to controls, concomitant treatment with the AR antagonist, flutamide, decreases aromatase activity comparable to that observed in the untreated castrate [131]. Furthermore, as cited in a review by Roselli [132], androgen deficient *Tfm* male rats have significantly less aromatase activity compared to normal littermates; the decrease is suggested to be secondary to the deficit in AR function [132]. Thus, it appears pertinent to examine available information on aromatase activity within the hippocampus.

In adult mammals, aromatase activity has been reported to occur mainly in the limbic system, which includes the hippocampus (Callard [24,25]; cited in Brown [20] and in Hutchison & Steimer [62]). Although, aromatase activity is reported to be either negligible or very low in the hippocampus, cortex [1,128,129,131] and midbrain [128] of the adult rat, it is three to four-fold greater in the hippocampus than in the cortex or midbrain [1,128]. It has recently been reported that the intensity of aromatase immunoreactive staining (-ir) increases in the presence of the aromatase inhibitor, R76713, permitting increased sensitivity for detecting low level aromatase activity in specific brain areas [44]. As expected, Silastic capsules containing R76713 implanted in male mice 15 days before sacrifice did increase the density of aromatase-ir in various regions of the CNS. R76713 revealed aromatase-ir of medium intensity within the hippocampus and cortex of the adult male mouse [44]. Perusal of their photomicrographs suggests aromatase-ir within both Ammon's horn and the dentate gyrus (DG) [44]. The low but detectable presence of aromatase within the hippocampus of the adult rat is further

emphasized in an experiment by Abdelgadir et al. [1]. Autoradiograms were exposed for an extended period (72 vs. 6 h), to enhance the visibility of $P450_{AROM}$ within the hippocampus [1]. In the neonatal rat, data on aromatase activity in the hippocampus is very limited. Low levels of aromatase activity [86] and aromatase mRNA [78] occur in the hippocampal pyramidal cell layer and DG granule cell layer of the PND 5 male and female [86] and in the PND 6 and PND 15 male rat [78].

Thus, in the hippocampus of the adult rat, aromatase activity appears to be negligible to low [1,128,129,131], with a paucity of information available for the hippocampus of the neonate. Although the AR [1,131,132] may be functional in increasing aromatase activity, thereby optimizing activity at the ER in specific areas of the brain, such a function within the hippocampus of the neonatal rat is not yet tenable. An initial prerequisite is the presence of the AR within the hippocampus of the neonate at a time when aromatized derived E_2 is reported to exert its effect. This requirement will be addressed by the current experiment. However, the presence of the AR in the neonatal hippocampus is not sufficient. It will also be necessary to compare the level of aromatase activity in the presence of 'T' and DHT relative to the castrate as has been determined for other brain areas [1,131,132]. As hippocampal information has not been included in experiments manipulating aromatase activity in the adult rat [1,131,132], it is possible that the hippocampal aromatase levels are too low to assess such manipulations. Available data for the hippocampus of the neonate also indicate

low levels of aromatase activity and it is not known if treatment with the aromatase inhibitor, R76713, would be sufficient to permit evaluation of steroid manipulations.

1.5 5α-Reductase & DHT

As indicated, 'T' is a major source of aromatized E_2 in the neonatal brain. An alternate metabolic pathway, under the enzyme 5α-reductase, results in a second biologically active, but non-aromatizable androgen, DHT. Although both androgens activate the AR, they exhibit different affinities and binding kinetics as described later. In the rat, DHT, administered alone, does not promote either masculinization of male sexual behavior nor defeminization. Both of these appear to be under control of 'T' and E_2 (see Gorski [52], Goy & McEwen [55] pg. 139, Plapinger and McEwen [119], Toran-Allerand [163] for review). The function of DHT is not well understood but as reviewed by Brown [20], DHT activation of ARs within the hypothalamic-pituitary area is reported to inhibit gonadotropic hormone release. In a review paper, Plapinger [119] reports that DHT is potent in stimulating peripheral reproductive tissue in the adult male rat. As well, although DHT may not be involved in defeminization of the rat brain, there is evidence that it may interact with E_2 to invoke normal male sexual behavior (see [114] for review).

Early work from the 1970s investigated the presence of 5α-reductase activity in the rat using thin-layer chromatography of homogenized tissue from

different brain regions following 1-h [35,36,91,162] or 3-h [91] incubation of brain slices with ^{3}H-'T'. The adult (PND 70-90) male [35,36,91] and female [36,91] rat and ferret [162] show the formation of DHT in many areas of the brain and pituitary [35,36], including the hippocampus [35]. In general, the level of 5α-reductase observed in the adult is significantly lower than the level reported for the neonatal rat [36,91] and ferret [162]. In the midbrain [36], hypothalamus and cortex [36,91] of the rat brain, the highest levels of 5α-reductase activity occur within the first postnatal week in both males and females [36,91]. The adult value is observed by the end of the first month of life [91]. The pattern is slightly different in the ferret [162], as developmental levels appear to be region specific. In the ferret, 5α-reductase activity in the preoptic area peaks at the end of the second week of life whereas in the cortex, higher levels are observed between PND 30 and PND 51 [162].

The presence of 5α-reductase activity in specific brain regions suggests the expression of the AR in these same regions. Indeed, incubation of brain cytosols with ^{3}H-DHT to measure bound levels in specific brain areas reveals the interaction of DHT and the AR. In the hypothalamic-preoptic area of the untreated female mouse brain, specific binding assayed in cytosols incubated with ^{3}H-DHT jumps sharply between birth and PND 9-12 with very little change through the prepubertal period [7]. ^{3}H-DHT binding in the cortex of the untreated female mouse brain appears to be similar to the developmental profile of 5α-reductase observed in the ferret [162]. That is, low levels of DHT binding present in

cytosols during the first postnatal week increase during the second week and increase further during the third week of life [7]. This experiment [7], however, does not fully address the ontogeny of the AR, as the developmental profile of nuclear ARs was not determined. Thus, it is possible that part of the cytosolic increase is due to a decrease in nuclear AR.

1.6 Defeminization & Masculinization

Defeminization and masculinization will next be explored, not only to demonstrate the possible involvement of the AR in such processes but because, as will be seen, the data suggests an interdependence between the AR and the ER for maximal effect. Indeed, such interdependence may extend beyond sex differences and sexual behavior. Following aromatization of 'T' to E_2 out of reach of plasma (but not cytoplasmic) α-fetoprotein, E_2 is free to act at nuclear ERs. It is obvious, therefore, that higher levels of 'T' present in the perinatal male brain [23] result in greater ER activation. Defeminization, defined as the suppression of female-typical traits and behaviors such as the estrogen-dependent surge of luteinizing hormone and the lordosis reflex, occurs postnatally. The postnatal occurrence effectively protects the female *in utero* from possible 'organizing effects' due to exposure to 'T' produced by male littermates [94]. As stated by McEwen et al. [94] and reviewed by Olsen [114], the aromatization pathway appears to be critical to defeminization. However, not all data supports this generally accepted belief. Dohler [40], who cites many reports supporting the contention that

aromatized E_2 is involved in defeminization, also reports that neonatal exposure of the female rat to tamoxifen induces defeminization in a dose-dependent manner. Tamoxifen is an E_2 antagonist that binds to, thereby blocking, intracellular ERs. Compared to controls, the neonatally tamoxifen-treated females, primed as adults with E_2 and progesterone, do not ovulate and display decreased female sexual receptivity [40] as would be expected from agonistic activation of the ER in neonates. However, because tamoxifen is now known to elicit weak agonistic as well as antagonistic estrogenic effects in peripheral tissue such as the uterus, liver and bone [69], it is possible that agonistic estrogenic effects also occur centrally in tamoxifen-treated neonates. If tamoxifen elicits its effect on defeminization by agonistic action at the ER, the tamoxifen-treated females should display masculine sexual behavior when exposed to 'T' as adults. The opposite effect occurs. These neonatally tamoxifen-treated females, implanted as adults with a Silastic capsule containing 1 mg testosterone propionate (TP) and tested three weeks later, show a tamoxifen-related dose-dependent decrease in male sexual behavior [40]. The treated females show decreased mounting and intromission behavior relative to control males [40]. Thus, the possibility that tamoxifen was acting as a true agonist at brain ERs is not tenable at this time. However, interpretation of ER-related results is further complicated by the recent identification of two versions of the ER within the rat brain [80,147].

Recent experiments have explored the presence of two versions of ER mRNA [147] and ER protein [80] within the CNS of the adult rat. The ER-α version is designated as the classical or primary ER responsible for most of E_2's actions with the second being the newly discovered ER-β version. Whereas a number of regions of the brain and spinal cord contain either ER-α or ER-β mRNA exclusively, other regions, such as the amygdala, bed nucleus of the stria terminalis and the preoptic area contain both signals [147]. Within the hippocampus of the adult Sprague-Dawley female, the signal is dispersed throughout the dorsal to ventral extent for both ER-α and ER-β mRNA but more cells are labeled for ER-β than for ER-α mRNA [147]. However, the intensity of the hybridization signal for both types of the ER is reported to be weak [147]. Although the presence of ER-β mRNA does not ensure the presence of the receptor protein, ER immunocytochemistry (ICC) indicates ER-β-ir within the CA1, CA2 and DG subfields of the hippocampus, but not in CA3 [80]. In the ICC study, only data from the CA2 cell layer is presented, making it difficult to assess the intensity of the signal for ER-β in the CA1 cell layer [80]. Thus, it is possible that more cells label within CA2 than within CA1. Regardless, within CA1 and CA2, both interneurons and pyramidal cells contain ER-β-ir [80]. However, only the pyramidal neurons contain both nuclear and non-nuclear immunoreactivity with ER-β-ir also present in the proximal fibers. The interneurons show only nuclear ER-β-ir [80]. The distinctive partitioning of the ER-β depending upon cell type

(pyramidal or interneuron) suggests a multiple role for this receptor. As well, the presence of two forms of the ER within the CNS suggests at least two distinct roles for this protein. It will be of interest to discover if the antiestrogens, such as tamoxifen, elicit differential antagonistic or agonistic effects at the two versions of the ER and whether the defeminization process involves just one or both ERs.

In spite of the aromatization hypothesis, the AR may also be involved in the defeminization process. Olsen [114] found that the implantation of Silastic capsules containing 'T' for the first 10 days of life completely induced defeminization in females tested as adults. Injections of 100 µg of 'T' for 5 days from PND 1 to PND 5 were less effective [114]. The 'critical period' for defeminization, utilizing the lordotic response, was further defined by examining neonatal females and castrated males given a single subcutaneous (sc) injection of 500 µg of TP on one day from PND 3 through 9 [37]. Whereas the early neonatally TP treated rats (PND 3-7) display diminished lordosis, those treated at PND 8 or 9 are comparable to untreated females [37]. Thus, it appears that exposure to TP from PND 3 through PND 7 maximizes the defeminization process. However, implanting or injecting the synthetic non-aromatizable androgen, methyltrienolone (R1881), in neonatal females also partially inhibits the expression of female mating behaviors in the hormone-treated adult [114]. Thus, although DHT treatment does not affect lordosis or proceptive behavior [114], the inhibition by R1881 suggests that defeminization may not be induced exclusively by aromatization to E_2. Several possibilities for the effect of R1881

are presented, including the suggestion that defeminization in the neonate could be partially mediated by the AR [114]. The ineffectiveness of DHT does not weaken this suggestion for several reasons. DHT is more rapidly converted than R1881 into less potent androgens and DHT also shows less affinity than the synthetic androgen for the AR [114]. As well, there is autoradiographic data showing a lack of ^{3}H-DHT localization within the brain during the first days of life [144], suggesting the possibility that a difference exists between DHT and R1881 binding to neonatal ARs. Thus, the slower inactivation of R1881 and greater affinity for the AR may have demonstrated a direct role for the AR in defeminization.

The exceptions just noted do not, however, negate the many experiments that provide compelling evidence of the involvement of aromatized E_2 in defeminization as cited in reviews such as Olsen [114]. It simply suggests that while activation of the ER may be sufficient, activity at the AR also appears capable of inducing defeminization. As indicated earlier, the AR may also be indirectly involved in defeminization by enhancing the degree of aromatization that occurs in specific areas of the brain such as the preoptic area and hypothalamus. However, although androgenic stimulation of aromatase has been demonstrated in adult male [1,128,131] and female [128,131] rats, the effect of androgen on neonatal aromatase activity within the CNS is still to be determined.

Masculinization, the enhancement of male typical traits displayed in mating behavior, is also dependent upon the activity of sex steroids in the

neonatal brain, but unlike defeminization, masculinization begins before birth [114]. As reviewed by Olsen [114], neonatally castrated male rats given E_2 during development and androgen treatment as adults show mounting and intromission behavior, but ejaculatory behavior is significantly reduced. Similar results for specific behaviors occur in studies that replace neonatal E_2 treatment with either DHT or a synthetic non-aromatizable androgen, fluoxymesterone [114]. Thus, neither ER nor AR stimulation alone produces the pattern of masculine behavior observed in the intact male. Male rats, castrated at birth and given DHT (sc) ranging from 0.1 mg/day on PND 1, 3 and 5 [168] to 0.20 mg/day for 10 days beginning at PND 2 [60], display intromission behavior comparable to males given either 'T' or DHT + E_2 [60,157,168]. However, the comparison between neonatal treatment with E_2 and DHT on mounting behavior depends upon the parameter examined. The percentage of castrated rats showing at least one mounting response does not differ between DHT-, TP- or estradiol benzoate (EB)-treatment [60]. Likewise, hormone treatment does not effect the percentage of tests in which mounts occur with no difference reported between groups [60,157]. However, the number of mounts per minute in DHT-treated castrates is comparable to untreated castrates and significantly lower than the number observed in EB-treated castrates [157]. Although one experiment cites zero intromissions in male rats treated with DHT at 0.50 mg/day for the first five days of life [16], the data from that experiment are complicated by too many variables to be seriously considered. That is, neonatally castrated and DHT-treated males

were implanted with ovaries from female littermates at five weeks of age and given hormonal treatment to elicit female typical mating behavior. Following testing, the same animals were then subjected to 'T'-priming to elicit male-related sexual behavior [16].

As already stated, neonatal treatment with either DHT or E_2 elicits equivalent intromission behavior in males whereas ejaculatory behavior is severely inhibited [60] or delayed [157,168] relative to neonatal treatment with 'T' alone [60,157,168] or with DHT plus E_2 combined [157,168]. Thus, partial masculinization can be elicited by activity at either the AR or the ER whereas complete male sexual behavior appears to be dependent upon both AR and ER activity [114]. As well, as reviewed by Olsen [114], the use of antiestrogens and antiandrogens supports the assumption that both sex steroids are required; perinatal treatment with either alone fails to produce male sex behavior in the 'T'-treated adult that is equal to that in the intact male.

Activation of masculine traits, 'organized' in the intact neonate is also dependent upon the presence of sex steroids within the male brain; again, it remains unclear exactly which of the sex steroids are involved. As indicated above, DHT binding to the AR stimulates peripheral reproductive tissue in the adult rat [119] and inhibits gonadotropic hormone release within the hypothalamic-pituitary area [143]. As reviewed by Hutchison & Steimer [62], the AR is involved in copulatory behavior in the male rat as R1881, the synthetic non-aromatizable androgen, restores copulatory behavior in adult castrated males as effectively as

'T' [10,156]. This suggests that activity at the AR elicits such behavior but the finding that DHT, the non-aromatizable endogenous androgen, has no effect on 'activating' copulatory behavior [10,156] negates this suggestion. It would appear that either two different receptors, one for 'T' (that also binds R1881) and one for DHT (that may or may not bind R1881) or a specific conformational change in a single receptor, which is not elicited by DHT, is required to make this suggestion tenable. This possibility is discussed more fully in the following section ('Functional Domains and Neuronal Location of the AR') but has not yet been experimentally investigated. Other work supports the contention that only aromatizable androgens stimulate sexual behavior in the adult male rat. Copulatory behavior is decreased in the male rat by inhibitors of aromatase as demonstrated by intracerebral infusion of ATD [28] or Fadrozole [30,166] in the lateral ventral [166] or the medial preoptic area of either the intact [30] or 'T'-treated adult castrate [28,166]. The decrease in copulatory behavior by blocking aromatization suggests that this behavior is dependent upon the conversion of 'T' into E_2. Indeed, unlike masculinization of the neonatal brain, a low dose of E_2 (by a factor of 100 compared to 'T'-treatment) is effective in 'activating' copulatory behavior in the adult male [10]. However, as reviewed by Hutchison and Steimer [62] and demonstrated by Vagell and McGinnis [166], a caveat exists in applying the aromatization hypothesis. Inducing male sexual behavior in castrated rats by the application of E_2 is dependent upon the presence of either DHT [62] or 'T' [166]. Even the direct application of E_2 into the hypothalamus requires, at least, systemic

administration of DHT to induce male copulatory behavior [62]. This was originally believed to involve the DHT effect on peripheral sex organs such as the penis [62]. However, given the effect of R1881 indicated above, an alternate suggestion made by Sodersten [155] appears plausible; E_2 may act, not only at the ER but also by interfering with the metabolism of DHT into inactive metabolites.

It is possible that steroid binding to both the ER and the AR is required for maximal 'activation' of adult male sexual behavior. For example, recent work by Vagell and McGinnis [166] indicates that activity limited to either the ER (via E_2-filled Silastic implants) or the AR (via 'T'-filled Silastic implants plus brain infusion of the aromatase inhibitor, Fadrozole) does not restore ejaculatory behavior in male rats castrated as adults [166]. However, simultaneous implantation of 'T'-filled and E_2-filled Silastic capsules combined with the aromatase inhibitor (by brain infusion) or implanting 'T'-filled capsules without the aromatase block [166] completely restores male sexual behavior. However, the function of AR activity in male sexual behavior may be even greater than formerly believed.

In an effort to address the conflicting data regarding the involvement of the AR and/or ER in 'activation' of masculine copulating behavior, a recent experiment by Vagell and McGinnis [167] assessed ejaculatory behavior in the presence of either the ER antagonist, RU 58668, or the AR antagonist, hydroxyflutamide. RU 58668 is reported to be a 'pure' ER antagonist that does not exhibit any agonistic properties [167]. Thirteen days after implant surgery,

100% of the gonadectomized 'T'-treated male rats administered the ER antagonist ejaculated [167]. However, only 25% of the gonadectomized 'T'-treated males administered the AR antagonist did so [167] indicating that activity at the AR plays a major role in the 'activation' of male mating behavior. Although the difficulty in ejaculation experienced under hydroxyflutamide treatment may not originate within the CNS, but rather be peripherally-related by the blockage of penile ARs with a subsequent decrease in penile spines, there was also a reduction in mounting and intromitting behavior [167]. It is of interest that the behavioral data is supported by cell nuclear exchange assays of brain tissue. The ER antagonist reduced the level of nuclear ER present in the 'T'-treated male (from approximately 16 to 7 fmol/mg DNA) [167]. Likewise, the AR antagonist reduced the amount of AR present in nuclear exchange assays from 45 to less than 10 fmol/mg DNA [167]. Thus, the ER antagonist blocked brain ER binding but not male copulatory behavior whereas the AR antagonist not only blocked brain AR binding but also inhibited sexual behavior in the male.

Activity at the AR may also mediate many other 'organized' sexually dimorphic behaviors. Such an example is play-fighting, reported to involve the 'organization' of neuronal substrates. In the neonatal rat, castration or treatment with the androgen antagonist, flutamide, results in female typical levels of play-fighting [98] whereas females treated with 'T' or 5α-DHT within the first two days of life show the masculine pattern [97]. Treatment with E_2 has no effect [97]. Likewise,

Tfm mutations, who lack or have relatively few ARs, show the female pattern of play-fighting [98].

As indicated for 'organization', 'activation' of the AR in the adult is also implicated in behaviors indirectly linked to copulation. For example, intact, untreated male rats spend more time with a 'receptive' compared to a non-receptive female in behavior that is referred to as sociosexual recognition or partner preference [167]. While the mating advantages are implicit in the seeking and interacting with a receptive versus a non-receptive female, the neural substrates underlying such action are not necessarily the same neural pathways involved in copulation behavior *per se*. However, data suggests that the sociosexual recognition pathway involves activity at the AR. Treatment with the antiandrogen, hydroxyflutamide, eliminates any difference in the amount of time males spend with receptive compared to non-receptive females whereas males treated with the antiestrogen spend significantly more time with a receptive than with a non-receptive female [167]. In fact, antiestrogen-treated males do not differ from males treated with 'T' alone [167]. Thus, the AR is involved in 'organizing' and in 'activating' the brain with respect to masculine traits and behavior either in synergism with the ER or alone. While activity at brain ARs is functional in initiating sexual behavior, it is doubtful whether this involves hippocampal ARs. However, it is not known if partner preference is related to AR activity within the hippocampus.

Before focusing specifically upon the hippocampal formation and what is known about the AR within this area, it may be helpful to examine the structure of the AR protein as well as its neuronal location and other properties. Such an examination will add insight to the present study, which uses an antibody to the AR via ICC methods to define the ontogeny of this receptor within specific areas of the hippocampus.

1.7 Functional Domains and Neuronal Location of the Androgen Receptor

As reviewed by Zhou [186], the AR is a ligand-activated transcriptional regulatory protein composed of approximately 900 amino acids in the rat and human. In both species, over half of this sequence (from one to approximately 540) beginning at the NH_2-terminal, makes up the transcriptional activation region. This region is so named because it is required for full transcriptional activity within the DNA binding zone following steroid binding [151,165]. The middle segment of approximately 100 amino acids constitutes the DNA binding zone and, the hormone-binding domain, comprised of the final 250 amino acid sequence, is located at the carboxy-terminal [151,165,186]. Peptide sequences used in raising antibodies are generally located within the initial transcriptional activation domain [151,186] where antibodies will not interfere with steroid binding.

In whole cell competitive binding assay using monkey kidney COS-7 & CV1 cells, the hormone-binding domain of the AR protein displays high-affinity for the synthetic androgen, R1881 and for the two biologically active androgens,

'T' and DHT [72,186]. The ovarian hormones, E_2 and progesterone, are relatively ineffective at binding to the AR as 100-fold molar excess is required to limit binding of ^{3}H-R1881 to the AR by 60-80% [72]. Although the affinity of the AR for both 'T' and DHT is reported to be similar, the binding kinetics differ between the two endogenous androgens. Testosterone binds and dissociates faster than DHT as determined in testis and prostate tissue [175], monkey COS cells [185], and in brain cytosols from female mice at PND 23 [7]. Possible functional significance of this difference, as suggested by Zhou [186], is that higher levels of 'T' would be required to induce comparable biological effects to DHT. Alternatively, an altered conformation of the AR protein under these two steroids could regulate the transcription of different genes.

The location of the AR within neurons depends upon whether the receptor is bound to its ligand. Immunoreactive staining of monkey kidney COS cells [72,151] and CNS of Brazilian opossums, castrated four days earlier [63] reveals that the AR is predominantly cytoplasmic in the absence of androgen, with staining largely in the perinuclear region [151]. The presence of R1881 or the addition of androgen 2-h before fixation causes strong nuclear staining [63,72,151]. Findings extend to the brains of the male ferret [70], Syrian hamster [177] and Sprague-Dawley rat [75,99], where AR-ICC [70,99,177] and nuclear exchange assay using ^{3}H-R1881 [75] reveal that nuclear AR is decreased in long-term castrates (>14 days) and restored 4-8 h following systemic 'T', DHT or R1881 treatment [70,75,177]. In contrast to androgen treatment for 4-8 h, 30 minutes of treatment reduces nuclear AR-ir

density in five out of eight brain areas, relative to the castrated male ferret; however, the difference collapsed across brain areas is not significant [70]. Indeed, the lack of a rapid increase in nuclear AR-ir density, relative to the untreated castrate, in any of the eight brain areas examined 30 minutes following androgen treatment suggests that the AR does not simply translocate from the cytoplasm to the nucleus in long-term castrates given short-term androgen treatment [70]. However, AR occupation and translocation, measured by the nuclear exchange technique, is reported to increase in the long-term castrated Sprague-Dawley rat 30 minutes following treatment (sc) with androgen [75]. Several possibilities exist for these differences. Most obviously, the results could be species dependent. Alternatively, the nuclear exchange assay may label ARs that are not identified via immunocytochemistry. It is also possible that the AR down-regulates or undergoes a conformational change that is not reversed by the short-term presence of androgen. Such a conformational change may limit recognition of the protein by the AR antibody. As well, in specific brain areas of the male rat, androgen treatment for 10-14 days elicits significantly greater AR-ir staining compared to intact controls [70,71,99,177]. This gives additional support to the suggestion that, at least in those areas (including CA1 [99]), androgen-induced AR-ir within the nucleus involves more than translocation and may involve androgen-induced up-regulation of synthesis.

The recognized androgens ('T', DHT, and R1881) are not the only substances to produce translocation of the AR from the cytoplasm to the

nucleus. At high doses, both hydroxyflutamide [72] and cyproterone acetate [9,72], recognized as antiandrogens [82], also cause nuclear transport of the AR [72]. Cyproterone acetate displays further agonist activity by inducing transcription in kidney COS cells, whereas hydroxyflutamide does not elicit transcription, thus acting as a true antagonist [72]. Along with the androgens, high levels (100-fold molar excess) of progesterone and E_2 result in nuclear translocation of the AR and induce transcription [72].

As reviewed by Zhou [186] and discussed by Simental [151], the ER and progesterone receptor (PR) contrast with the AR as they are reported to be predominantly nuclear in the presence or absence of hormone whereas the glucocorticoid (GR) receptor is both nuclear and cytoplasmic in the absence of dexamethasone (see Zhou [186], Simental [151] and references therein). Thus, ligand binding does not appear necessary for nuclear location of ER, PR or GR whereas it appears to be a prerequisite for nuclear location of the AR.

Although a high level (100-1000 fold excess) of E_2 may induce AR agonist activity in some tissues, 100 µg (sc) does not reverse a castration-induced increase in AR mRNA observed in the ventral prostate, whereas 'T' effectively lowers AR mRNA to intact levels as measured 24 h later [123]. However, a comparison between the effect of the two steroids is difficult as the dosage of 'T' used was 20x greater (2 mg, im). 'T'-treatment elicits a similar decrease in a castration-induced increase in AR mRNA in brain tissue, but E_2 was not investigated [123]. Thus, although E_2 can mimic some of the effects of 'T' at the AR

in kidney cells *in vitro* [72], there is presently no report that such activity can induce functional changes in prostate or brain. However, the lack of effect could be dose-related.

As indicated above, the amount of AR present in the nucleus decreases in the absence, and increases in the presence of androgens. As well, without the presence of its androgenic ligand, the AR degrades relatively quickly and must then be replaced by protein synthesis. The up- and down-regulation of the AR protein has been explored by examining AR mRNA. While this signal may not be sufficient, it is necessary for production of the AR. Examination of the hormonal regulation of AR mRNA in neural tissues were conducted by Northern blot hybridization and ribonuclease (RNase) protection analysis [22,58]. Results are rather complex as regulation appears to be tissue specific and time related. As well, data involving regulation of the AR mRNA may be complicated by the complexity of the message itself, reportedly present in two forms [22].

Short-term (4 days) castration of the adult male rat increases AR mRNA in the hypothalamic-preoptic area [22], medial preoptic area [58], bed nucleus of the stria terminalis [58], and anterior pituitary gland [22] with no effect on the amygdala [22]. However, either short-term castration (4 days) or treatment of intact young adults with the AR antagonist, flutamide (15 mg/day) for four days decreases hippocampal AR mRNA [73]. Results following long-term (2 months) castration are similarly complex and contradictory. Northern blot analysis suggests that long-term castration of the adult male rat increases AR mRNA in the preoptic area,

medial basal hypothalamus, hippocampus, and the pituitary gland [22]. However, a later paper from the same group indicates that long-term castration of either the PND 25 or the adult male rat decreases, rather than increases, AR mRNA in the medial preoptic area and bed nucleus of the stria terminalis as measured by *in situ* hybridization two months later [58].

The short-term castration-induced increase in AR mRNA in the hypothalamic-preoptic area [22] and medial preoptic area [58] is reversed within one day of acute (2 mg, sc.) treatment with DHT. However, EB treatment for one [22,58] or five [22] days (10 µg/day) has no effect. Again, there is at least a four-fold difference in the dosage used for the two steroids.

The effect of hormone replacement on AR mRNA is similar in both the long-term and short-term castrate in several brain areas. For example, as in the short-term castrate, the long-term castration-induced increase is reversed in the medial basal hypothalamus [22] and bed nucleus of stria terminalis [58] by implantation of a Silastic capsule containing either 'T' (7-weeks) [22] or DHT (2-weeks) [58]. However, the effects of androgen replacement on preoptic area AR mRNA are less clear. As mentioned above, castration has been reported to have opposite effects on preoptic area AR mRNA in two studies from the same laboratory using different methods to measure AR mRNA. These studies also assessed the effects of androgen replacement. In the initial experiment, Northern blot analysis indicates that chronic treatment with 'T' decreases [22], whereas *in situ* hybridization analysis reveals that chronic treatment with DHT [58]

increases AR mRNA in the preoptic area relative to the gonadectomized male. Although the later study does not address the apparent discrepancy between these two findings, both methods report a restoration of AR mRNA to intact levels [22,58].

Results are further complicated by the Northern blot analysis revelation of two species of AR mRNA (a larger 11 kb and a smaller 9.3 kb version) found within all brain areas examined but not pituitary. The pituitary only contains the larger version [22]. It is not known if the smaller version is functional (i.e., will it bind to hormone and will it transcribe or translate?). Given the suggestion that 'T' and DHT may mediate transcription of different genes, albeit due to a variation in the conformation of the AR under 'T' and DHT [186], the possibility exists that the two forms of the AR mRNA regulate different proteins [22]. In turn, the different proteins may be associated with different functions.

1.8 Ontogeny of the Hippocampal Formation.

Because the present study is investigating the ontogeny of the AR within neurons of the hippocampal area, it appears necessary to review the data on the time course of the birth of hippocampal neurons. In this regard, it is possible that the AR is present in the hippocampus of the neonate and that its distribution differs from that observed in the adult. Consequently, both the time course of the appearance of the AR and its localization within Ammon's horn may suggest a role for this protein in differentiation, synaptogenesis and/or cell survival.

Within Ammon's horn, the earliest post-mitotic cells are large interneurons arising from embryonic day (E)15 to E17 within stratum (s.) oriens, s. radiatum and s. lacunosum-molecular [3,11,12]. Pyramidal cell neurogenesis occurs slightly later; the majority of CA3 cells form between E17-E18 and CA1 cells between E18-E19. Pyramidal cell neurogenesis is complete within four days, by E20 [2,3,11]. Because the pyramidal neurons sojourn from the site of their birth across the hippocampal intermediate zone, located between the future alveolar channel and s. pyramidale cell layer, the morphogenic profile of Ammon's horn takes slightly longer than neurogenesis but is completed by E22 [2,3] (defined using the first day of gestation as E1). Thus, all pyramidal neurons are born and in place prenatally.

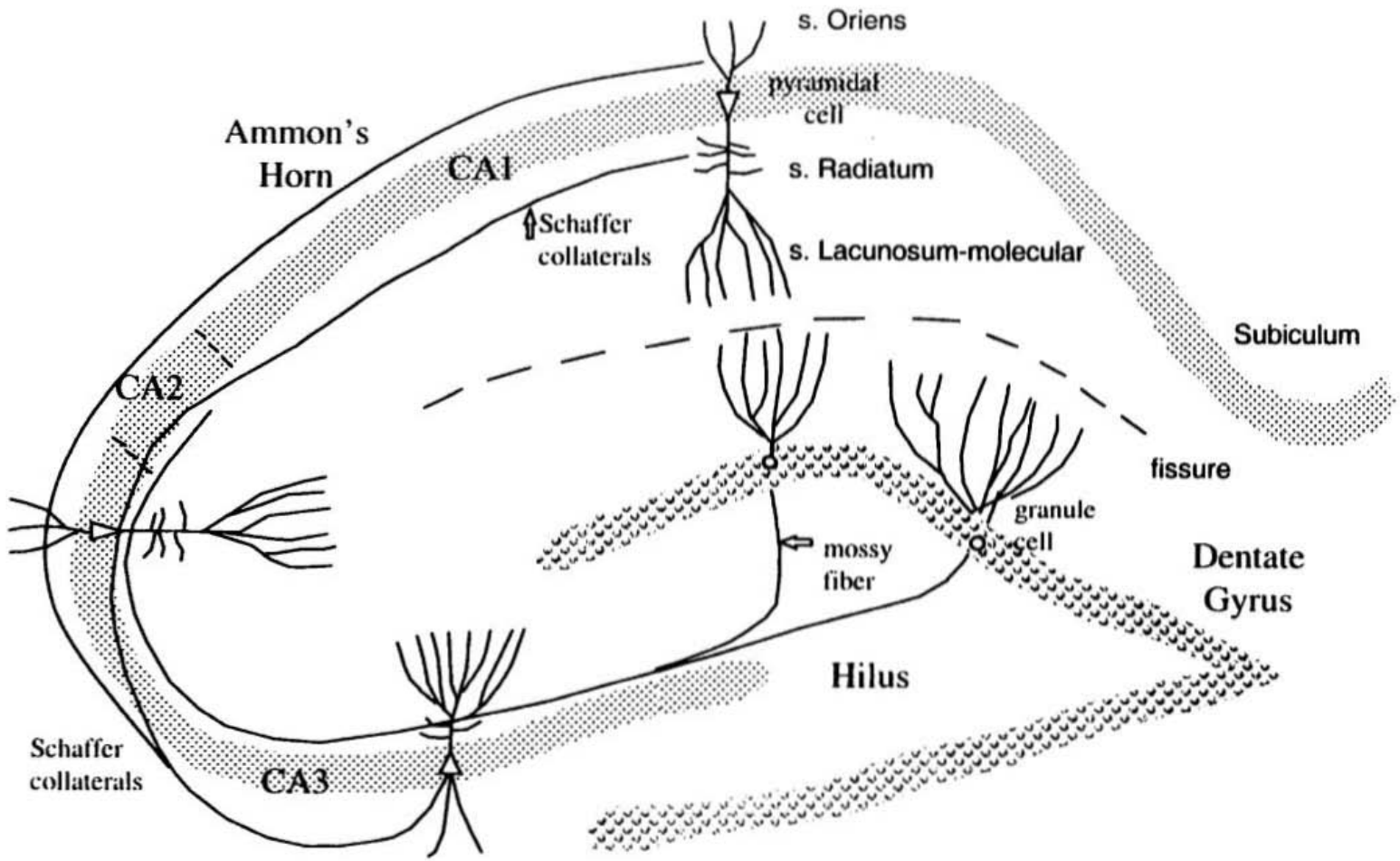
As reported with the interneurons of Ammon's horn, neurogenesis of hilar cells of the DG occurs mainly between E15-E17 and neurogenesis of molecular layer cells occur between E15-E19 with no postnatal genesis [11]. Dentate granule cells begin to originate on E17 [11]. By E21-E22, granule cells of the dorsal limb begin to display a mature profile with the ventral limb cells showing signs of differentiation [3,4]. However, 80-85% of granule cells form postnatally [11,12,138] with 40-50% forming by PND 5 and 5-10% still to differentiate later than PND 18 [4,11,12].

The completion of neurogenesis and the pyramidal cell layer alignment within Ammon's horn by birth [12] suggests that any organizational effects mediated by synaptogenesis could occur earlier in the pyramidal cell layer than

in the DG. However, a problem exists in that synaptogenesis occurs as part of the trisynaptic circuit. Thus, within the hippocampal area, DG granule cells extend their mossy fiber axons toward the CA3 pyramidal cells which, in turn extend their Schaffer collateral axons onto CA1 pyramidal neurons (see Figure 1.1). It is obvious, therefore, that the pyramidal cells are dependent upon the postnatal arrival of granule cell axons. Such organizational effects upon and perhaps even activation of these CA1 neurons would not be expected to occur until these connections are in place, possibly beginning within the first postnatal week. Indeed, Bayer and Altman [12] report that the pyramidal cell somatas are pushed further apart during the early postnatal period, possibly due to dendritic growth and that the expansion of the cell layer volume continues into adult life [12]. It appears relevant therefore, to begin exploration of the ontogeny of the AR within the hippocampus of the neonatal male and female from the earliest postnatal period possible. This is a period when synaptogenesis of hippocampal neurons and differentiation of granule cells can be expected to be at a very high level and when the activating ligands of the AR are known to be present as discussed earlier.

Having examined the available information on the AR and its message with respect to the brain in general, the introduction will now focus on relevant data concerning the AR within the hippocampal formation.

Figure 1.1 Drawing of the dorsal hippocampus of the rat showing both Ammon's horn and the dentate gyrus (DG). Ammon's horn consists of a continuous cell layer which can be divided into the three separate regions of CA1, CA2 and CA3. The DG consists of a dorsal and a ventral limb. The mossy fiber axons of the DG granule cells synapse onto the CA3 pyramidal cells, which in turn, project their axons (Schaffer collaterals) onto the CA1 neurons.


s. Oriens
pyramidal cell
Ammon's Horn
CA1
s. Radiatum
Schaffer collaterals
s. Lacunosum-molecular
Subiculum
CA2
fissure
granule cell
mossy fiber
Dentate Gyrus
Hilus
Schaffer collaterals
CA3

1.9 THE ANDROGEN RECEPTOR: WITHIN THE HIPPOCAMPUS

As indicated in a previous section, the message for AR is present in the hippocampus of the adult rat [22,73,152] and short-term (4 days) castration decreases [73] whereas long-term castration increases [22] the message. The level of AR mRNA is also decreased in the intact rat treated with the antiandrogen, flutamide, for four days (15 mg/day) [73] suggesting that agonistic activity at the AR is functional in maintaining AR mRNA levels. Along with the message [73], the AR protein is also present within CA1 of the adult male rat as measured by immunoreactivity [29,135] and autoradiography [137]. Orchidectomy of PND 26 and PND 60 male rats three days before injection (iv) of ^{3}H-DHT (10-20 µg/kg) [137] or ^{3}H-'T' (4 µg/kg) [136] 1-h prior to sacrifice reveals labeled neurons in CA1 and CA2 of the hippocampus [136,137]. Fewer cells are labeled for AR in CA3 and CA4 with no label found in the DG [136,137]. Injection of PND 26 males with unlabeled E_2, at either 20 or 200 µg/kg of body weight, five minutes prior to ^{3}H-DHT has no effect on the nuclear concentration of radioactivity in CA1 [137]. This indicates that E_2 does not interact with the AR in this area. Given that castration of the adult male rat decreases hippocampal AR-ir examined four weeks later [99], it is possible that a reduced number of neurons are actually labeled by autoradiography three days following orchidectomy in both the PND 26 and the PND 60 male [136,137].

While the above studies only investigated the male, the AR is not restricted to the hippocampus of the male rat. In the intact, untreated six-month old rat, AR-ir is present in the hippocampus of both sexes [29]. However, sex

differences were not investigated [29]. Sex differences were investigated with negative results in a study of hippocampal cytosolic ARs from adult Long-Evans male and female rats gonadectomized 2-3 weeks before sacrifice and incubated with ^{3}H-R1881 [95]. However, it must be realized that gonadectomy could obliterate any sex difference present in the intact animal. Thus, sex differences in hippocampal AR, even in the adult, are still to be determined.

A unique method employed to investigate the presence of the AR and the ER within the hippocampus bears examination. By using ^{3}H-'T' in the intact PND 2 Sprague-Dawley female [144] and the PND 24 Holtzman female (ovariectomized and adrenalectomized at PND 23) [143] rat, the ^{3}H-'T'$_{\alpha}$ marker is retained whether the steroid is metabolized or not. The ^{3}H-'T'$_{\beta}$ marker, on the other hand, is lost only under aromatization to unlabeled E_2, thereby labeling either the unmetabolized 'T' or presumably metabolized DHT. The difference observed between the two labels identifies the presence of the ER [143]. No hippocampal radioactivity occurred in the PND 2 female examined two hours following injection (sc) of either ^{3}H-'T'$_{\alpha}$ or ^{3}H-'T'$_{\beta}$ indicating neither ARs nor ERs were measurable at this time [144]. Ninety minutes following injection of ^{3}H-'T'$_{\alpha}$ in the PND 24 female, labeling was localized in many brain areas including the hippocampus whereas the ^{3}H-'T'$_{\beta}$ label was not found within the hippocampus [143]. The lack of label under ^{3}H-'T'$_{\beta}$ suggests that the AR is not present in the hippocampus of the PND 24 female rat [143]. Alternatively, perhaps at this age

aromatization in the hippocampus, at least in the female, is sufficient to metabolize all the injected ^{3}H-'T'$_{\beta}$ leading to the inaccurate assumption of a lack of ARs. In support of the aromatization proposition, autoradiography of the PND 26 male Holtzman rat, castrated three days before receiving an iv injection of ^{3}H-DHT and sacrificed one hour later, shows a high level of labeling in the hippocampus [137]. The radioactive label is comparable to that in the PND 60 male [137]. It is not currently known if results relate to a hippocampal sex difference, to differences in the sensitivity of the two methods or to other variables such as age.

The AR appears to be present in the hippocampus of the fetal, neonatal and adult monkey. However, a cautionary note is that such information generally comes from studies involving one or two animals at each age examined. In the adult male cynomolgus monkey (*Macaca fascicularis*), hippocampal neurons are not consistently labeled by AR-ir [29]. Likewise, although the AR-ir label is reported to be widely distributed throughout Ammon's horn and the DG in the rhesus monkey (*Macaca mulatta*), it is also widely dispersed within each area [27]. This contrasts to findings in the adult rat where the AR is localized in virtually every pyramidal neuron of CA1 and CA2 as identified by immunocytochemistry [29,135] and autoradiography using ^{3}H-DHT [137]. As well, compared to reports of the presence of the AR in the DG of the adult monkey [27], it is reported to be absent in the DG of the adult rat [135,137].

The AR is also reportedly present within the diencephalon of the fetal and neonatal monkey [27,31,57,101]. However, neonatal data is severely limited and an ontogenic profile is not yet available. The full gestational period for the monkey is approximately 166 days[146]. This permits a comparison of data between the monkey and the rat. Examination of various areas of the diencephalon of the rhesus monkey between gestation day 50 to 150 reveals both AR-ir [27] and the presence of AR within extracted cytosols (using ^{3}H-R1881) [57]. In the cynomolgus monkey, the AR is present in nuclear pellets (using ^{3}H-'T') [101] extracted from the hippocampus of the intact male and female at day 122 of gestation [101]. No sex difference in hippocampal AR occurs in the fetal rhesus [57] and cynomolgus [101] monkey. In the neonatal gonadectomized male and female cynomolgus monkeys, the hippocampal AR is present at PND 6-8 as labeled 'T' and DHT are extracted from nuclear pellets 60 min following injection [15]. As well, the 'allocortical' hippocampus from one 0-3 month-old female rhesus monkey exhibits high affinity and capacity for androgen binding relative to other sampled brain areas [31]. The affinity and capacity was determined by saturation binding analysis conducted on cytosol fractions using ^{3}H-R1881 [31].

Thus, although the AR appears to be present in the hippocampus of the adult rat and monkey, the distribution and quantity appear to differ. As well, in the hippocampus of the neonatal rat and primate, there is limited information about the presence of the AR with no data available on the ontogeny of this receptor within this brain area.

Although sex differences in hippocampal AR have not yet been reported, activation of ARs depends upon the presence of its activating ligand, either 'T' or DHT. Brain levels of both of these ligands are higher in the male, suggesting that AR activation would also be greater in the male. Thus, any function mediated by hippocampal ARs could potentially elicit one or more sex differences. It therefore appears relevant to explore information about sex differences with respect to the hippocampus and hippocampal-related functions.

1.10 Sex Differences and the Hippocampus

Sex differences in spatial behavior in the Meadow vole (*Microtus pennsylvanicus*) [47,48], rat [126,127], monkey [8] and human [13,61] and the correlations with the size of the hippocampus [65,66] and the DG granule cell layer [127] suggest that 'T' may be functional in behavior that is only indirectly related to copulation. For example, 'T' appears to be related to sex differences in learning reported for the monkey [8], rat [53,64,68,118,126,127] and human [13].

Sex differences in the development of learning abilities reported in the rhesus monkey are postulated to be related to the presence of gonadal steroids (for review see [8]). The argument is made that the presence of androgen promotes maturation of the orbital prefrontal cortex, as males perform an '*object discrimination reversal*' task, shown to be dependent upon this brain area, significantly better than females. This sex difference occurs only in the relatively young monkey (2-3 month old) and disappears with maturation of the female

cortical area [8]. Females treated with TP as neonates perform as well as intact males and better than intact females when tested at 75 days of age [8]. Analysis of a different visual discrimination task ('*concurrent object discrimination at a 24-h intertrial interval*'), shown to be related to the inferior temporal cortex in adults, also reveals a sex difference with females outperforming males at three months of age. Hormonal manipulation in the neonate by male orchidectomy (ORX) or by injecting ovariectomized (OVX) females with either TP or DHT three times/week from birth to three months of age reveals an androgen-related effect. ORX males perform better than intact males and as well as intact females, whereas DHT-treated OVX-females require significantly more trials to criterion than intact females. The sex difference disappears by six months of age and is not present in the mature (3-4 year old) monkey [8]. As discussed by Bachevalier and Hagger [8], androgen-derived estrogenic action on the orbital prefrontal cortex may affect object reversal learning, whereas the influence of androgens on the inferior temporal cortex (and associated pathways) may affect visual discrimination learning.

Studies in the rat reveal both positive and negative effects of 'T' on spatial learning. In the intact animal, three-month (Sprague-Dawley) [126] and six-month old (Zivic-Miller strain) [68] males make fewer errors and take fewer trials in various maze-learning problems (i.e., radial arm maze, and 'correct path' choices) compared to females [68]. Untreated control males also perform better than males treated neonatally with an androgen antagonist (cyproterone acetate,

5 mg/day at PND 1, 4, 7, and 10) [68]. Injection of intact females with 0.150 mg/day of TP at PND 3 and 5 [126] or with 5 mg/day of TP at PND 1, 4 [68] results in maze learning that is comparable to control males and better than cyproterone-treated males or control females [68]. Interestingly, intact males and females injected with cyproterone acetate perform better than either castrated males or normal females but, as already indicated, significantly worse than intact males or TP-treated females [68]. This result could relate to the finding that cyproterone acetate, at high doses, acts as an agonist, rather than an antagonist [9,72]. Similar sex differences occur for the Morris water maze. Males (Long-Evans, Sprague-Dawley) perform better than females in acquisition and retention of the spatial task [118,127]. As well, neonatally TP-injected (0.150 mg/day PND 3, 5) females perform better than control females [127].

In contrast to the positive effects of 'T' treatment in the female reported above, 'T' treatment of the intact male rat appears to have detrimental effects on spatial learning. In the intact adult male, implantation of 'T'-filled Silastic capsules one month before testing not only fails to reverse memory decline in aged (31 months) rats, measured in the hidden platform task of the Morris water maze, but it impairs retention in young (4.5 months) and middle-aged (20 months) animals (Brown-Norway) [53]. Results from TP and cyproterone acetate injections in the neonatal male rat indicate similar impairments of spatial learning when tested as adults. Both neonatally cyproterone-treated [68] and TP-treated [126,127] intact males make more errors and require significantly more trials to

criterion in a variety of mazes [68] including the radial arm maze [126] and Morris water maze [127] compared to control males, TP-treated females, or cyproterone-treated females. Again, the results from cyproterone acetate treatment suggest agonistic rather than antagonistic effects. Thus, it appears that neonatal exposure of the rat to 'T', either endogenous or exogenous, enhances spatial learning but an optimal level is already present in the neonatal male. An increase in 'T'-levels may reverse the positive effect, possibly by inducing a protective feedback mechanism to induce down-regulation of a 'T'-activated receptor (i.e., either the AR or ER), in both the neonate and the adult. Although it is realized that the ER may also be involved in organizational effects on maze learning, the involvement of the AR is supported by the finding that the AR antagonist, cyproterone acetate, is known to elicit agonistic effects at the AR (at least in kidney COS cells) [72], but not at the ER. The feedback regulating mechanism could also ensure maturation of the neonatal CNS at a controlled level as postulated earlier for the monkey [8].

While the above studies do not indicate the involvement of any specific area of the CNS in spatial learning, both lesion [104,105] and correlational studies [127,141,142] suggest the involvement of the hippocampus (for extensive review see [14]). Thus, if sex differences in spatial learning relate to maturation and or morphology of the hippocampal area (see Figure 1.1), then sex differences in anatomy would also be expected and such effects should correlate with behavioral data. With respect to androgen-induced maturation of hippocampal

tissue, Roof and Havens [127] reports that the hippocampal granule cell layer is wider in the male compared to the female. Furthermore, neonatal TP-treatment (0.15 mg/day on PND 3, 5) of the female increases the width relative to the control female [127]. As well, performance on the Morris water maze shows a strong correlation with the width of the granule cell layer [127].

Given the involvement of the DG in the tri-synaptic circuit from the entorhinal cortex, it is plausible that any modification of the DG granule cell layer should be reflected throughout this circuit which includes the CA3 and CA1 pyramidal cell layers. Indeed, in 3-4 month old males from a variety of mouse strains, high positive correlations are reported between the size of the infra- and intrapyramidal mossy fiber terminal field (projecting to CA3) and either water maze [142] or 8-arm radial maze [141] performance measuring spatial working memory (reentry to arm already visited). The relationship between the size of the mossy fiber terminal field and spatial memory is strengthened by the additional finding that there is no correlation with measures involving non-spatial reference memory [141]. Continuing further into the hippocampal tri-synaptic circuit, there is greater volume and neuron number in the hippocampal CA1 area of the male compared to the female [89]. This suggests that the Schaffer collaterals from CA3 interact with significantly more CA1 pyramidal cells in males than in females. Furthermore, the sex difference within area CA1 is present as early as PND 30 and still present at PND 180 [89]. Thus, the hippocampus proper (comprised of

Ammon's horn and the DG) displays morphological sex differences that are correlated with sex differences in spatial learning.

Sex differences in spatial performance discussed above suggest that androgen enhances spatial performance by an organizational, not an activational, effect. However, results do not indicate whether 'T' is directly involved or if the effect is induced by one of the metabolites, either DHT or E_2. None of the previously mentioned experiments addressed this issue except indirectly by the use of the antiandrogen, cyproterone acetate. However, work by Williams and her colleagues [173] suggest the involvement of E_2 in enhanced spatial behavior. Females given EB injections for five days from PND 1-10 perform as well as untreated males and better than either castrated males or untreated females, tested as young adults in a 12-arm radial maze spatial task [173]. The same group reports that ATD-treatment of neonatal (PND 1-10) males, to block aromatization of 'T' to E_2, impairs acquisition in maze performance compared to male controls tested as young adults [174]. Although it must be accepted that blocking aromatization in males inhibits spatial learning relative to male controls, the question remains whether 'T', either directly or via its metabolite DHT is also involved in spatial behavior. Support for such involvement is already indicated above as cyproterone acetate, an antagonist known to have agonistic properties at the AR at high doses (discussed above) enhances female performance but is not as effective as 'T'-treatment [68]. As well, in males, cyproterone acetate impedes performance [68] as reported for 'T'-

treatment [68,126,127]. Indeed, blocking aromatization in males by neonatal ADT-treatment may have induced its detrimental effect on spatial performance [174] by eliciting an increase in 'T'-activity at ARs, rather than by eliminating the E_2 metabolite. This is consistent with the idea of an 'optimal level' of 'T' required to enhance spatial performance.

Also, action by E_2 does not eliminate any possible contribution that may be made by either 'T' or DHT acting at the AR. Such a contribution could occur by an AR-mediated increase in aromatase activity as reported for many diencephalic nuclei [1,130,132]. In one such experiment, adult gonadectomized Sprague-Dawley male and female rats were either sham implanted or implanted with Silastic capsules filled with either crystalline 'T' or TP [130]. The capsules ranged from 2 to 40 mm in length in order to provide step increases in serum 'T' [130]. Aromatase activity was analyzed in the treatment groups with respect to circulating 'T'-levels measured at the time of sacrifice; data from sham implanted animals provide a baseline of comparison [130]. Aromatase activity is reported to increase with increasing levels of serum 'T' in both males and females in various brain areas including the bed nucleus of the stria terminalis, periventricular preoptic area, medial preoptic nucleus and ventromedial nucleus of the hypothalamus [130]. A sex difference accompanies the 'T'-induced increase in aromatase activity in all four areas with significantly greater activity in the male [130]. Indeed, the only area analyzed that exhibits increases in aromatase activity as a function of serum 'T' but does not exhibit a sex difference is the anterior

hypothalamus [130]. As indicated earlier under the topic of aromatization, the stimulatory effect of AR activity on aromatization within the hippocampus is not yet known.

The sex differences in spatial learning described above with males performing better than females in a variety of mazes may relate to gonadal hormone activity within the hippocampus. The influence of gonadal steroids upon the brain, either organizational or activational, depends not only on the presence of the relevant sex steroid but also upon the availability of its receptor within specific neural circuits. Thus, before 'T' or its metabolite DHT can elicit either genomic or possibly non-genomic effects, the steroid must interact with the AR whereas the metabolite, E_2 must act at the ER. The ontogeny of the ER and PR has been studied in the rodent in a number of brain areas [38,39,76,85,87,102,115,149,181] including the hippocampus [111-113,134,148,150,159,164]. The AR, however, has been studied mainly in areas of the brain known to be related to copulating behavior [45,67,91,96,135,137,144,145,169,182] with a paucity of information regarding the ontogeny of the AR within the hippocampus. Available data are from an experiment using autoradiography to show AR labeling within the hippocampal pyramidal cell layer of the PND 26 male [137] and PND 24 female [143] Holtzman rat. As well, AR mRNA is present in the fetal hippocampus. Both *in situ* hybridization [183] and cultured cells analyzed by Western blot analysis [176] indicate AR mRNA in the hippocampus at embryonic day 18 in the rat [176] and 15-16 [183] in the mouse. The lack of information on the AR could result from the absence of

this protein within the neonatal rodent hippocampus. However, both AR mRNA [22,73,152] and the AR protein are present in the hippocampus of the intact adult male [29,99,135-137] and female [29] rat as indicated by autoradiography [136,137] and (ICC) [29,99,135] as well as in hippocampal cytosols of the gonadectomized male and female rat analyzed with the micropunch technique [95]. Thus, the present experiment will address the question of the ontogeny of the AR within the hippocampus of the neonatal rat.

Given that 'T' is reported to exert organizational effects on spatial learning ability as indicated above, it appears pertinent to determine the ontogeny of the AR within this circuit that is implicated in spatial behavior. This thesis is therefore directed toward defining if the AR is present in the hippocampus of the neonatal male and female Sprague-Dawley rat, or if not, when it can first be detected. The newborn male and female are investigated at various stages of postnatal development. Using day of birth as PND 1, three days, PND 2, 5 and 7 cover the early and late neonatal critical period for the masculinization and defeminization for hypothalamic functions [37,157]. The juvenile rat is examined at PND 14, a dynamic period behaviorally, as ears open and pups begin to explore outside the nest with eyes opening two days later (at PND 16) [42]. PND 25 is the time period utilized to assess the prepubertal level of AR preceding the rise in 'T' observed between PND 25 and PND 35 in the Sprague-Dawley [41, 79] and the Holtzman [103] strain. The early pubertal stage is examined at PND 40.

CHAPTER 2: METHOD

2.1 Animals

All animals used in this study were treated under approved protocols and guidelines set by the Institutional Animal Care Committee of Memorial University of Newfoundland, which operates under the Canadian Council on Animal Care. Pairs of mature male and female Sprague-Dawley rats were housed in a large rectangular box over a three-day period to allow mating and initiation of pregnancy. Females were then removed and housed in pairs for 15 days in standard polycarbonate laboratory cages under a 12-h light-dark cycle with free access to food and water. During the last week of gestation, females were housed separately and checked at 9:00 a.m. and 6:00 p.m. every day. Pups born after 6:00 p.m. were considered born on the following day, with the day of birth designated as PND 1. Pups remained with the dam until PND 21 at which time littermates were housed in same sex triplets. At puberty, they were housed in same sex pairs. Male and female littermates were examined at PND 2, PND 5, PND 7 (newborn); PND 14 (juvenile); PND 25 (prepubertal), PND 40 (pubertal) and PND 60 (adult). Each age up to PND 25 contained five males and five females with four of each sex in the PND 40 group and one male-female set at PND 60. At least three different litters were sacrificed at each age (except PND 60) to ensure that reported differences in AR ontogeny would not be due to variability between litters. The ano-genital distance (mm) was used to identify

the sex of the newborn and juvenile pups. Pups were anesthetized by an injection (ip) of Somnotol (sodium pentobarbital, 65 mg/ml) diluted 1:10 in 0.9% saline and 1% heparin at a dosage ranging from 0.05 ml in PND 2 to 0.25 ml in PND 14. All other ages received undiluted Somnotol (ip) at a dosage of 0.09 ml/100 g. Twenty to 25 minutes later, the animals received a two minute pre-flush with 0.9% saline containing 1% heparin (20-35 ml) followed by 100-250 ml of 4% paraformaldehyde prepared in sodium phosphate buffer, pH 7.2. The fluids, kept ice cold by immersion in a pan of crushed ice, entered the left ventricle of the heart via a 23-gauge needle and drained from an incision made in the right auricle. The flow rate of the perfusate was adjusted from 9.2 ml/min for PND 2 up to 35 ml/min for PND 60 animals.

To investigate the possibility that the non-liganded AR may have less affinity for the antibody, possibly due to a conformational change in the AR as suggested earlier, two sets of male and female PND 7 and PND 25 pups were injected with dihydrotestosterone benzoate (DHTB) (20 mg/kg ip in ethyl oleate) 30 minutes before sacrifice. Following injection with DHTB, animals were either placed under a heat lamp (PND 7) or placed in a clean cage as defined above (PND 25). They were anesthetized 20 minutes following DHTB injection and perfused 10 minutes later. Thirty minutes is sufficient time for AR occupancy to occur in various brain areas and pituitary following 'T'-filled Silastic implants (sc) [75]. Hence, the 30 minute period was chosen because it is long enough for DHTB

to reach brain ARs, but not long enough to modify the amount of AR present via up- or down-regulation of transcription of the AR gene.

Following perfusion, brains were removed immediately and immersed in the perfusate for 24 hours, then cryoprotected by immersion in 20% sucrose in 4% paraformaldehyde for a further 24 hours or until sunk. Brains were then blocked by a vertical cut through the medulla at the caudal edge of the cerebellum, weighed, frozen by immersion for 2 minutes in isopentane (2-Methylbutane) that was cooled to $-70^{0}C$, wrapped and stored at $-70^{0}C$ until processed.

2.2 Androgen Receptor Antibody

The antibody and the peptide controls used in the present experiment were gifts from Dr. Gail Prins, University of Illinois at Chicago. PG-21 is an affinity purified, rabbit polyclonal antibody raised against the synthetic peptide corresponding to the first 21 amino acid sequence beginning at the NH_2-terminal end in the transactivation zone of the rat and human AR. The specific properties have been described in detail by the co-developers of this antibody [122] and it has been utilized in numerous experiments to explore properties of the AR. For competition experiments, tissue was incubated with the antibody PG-21 and a 10 molar excess of the peptide used to raise the antibody (AR21). A distant peptide (AR462) of amino acid sequence 462-478 of the AR was also synthesized (but not used to raise an antibody) and used as a noncompetitive control. As already

indicated, PG-21 binds to the first 21 amino acid sequence in the carboxy terminal zone and as such does not interfere with either the DNA binding sequence nor with hormonal binding to the receptor. Thus, it should react with either occupied, or unoccupied, receptors provided the unoccupied receptor does not exhibit a conformational change that prohibits antibody binding as discussed earlier.

2.3 ICC

Pre-testing was performed to determine the best method to investigate the presence of the AR in neonatal and adult tissue. Initially, brain tissue from the adult male was mounted onto gelatin-coated slides and processed for ICC using different dilutions. Although 1:5000 achieved adequate staining density, especially in hypothalamic areas, 1:2000 yielded better results in hippocampal tissue. However, neither dilution was effective when neonatal tissue was processed in this manner and the dilution was increased to 1:500. Again, the increase in antibody failed to reveal the presence of neonatal ARs in tissue mounted onto gelatin-coated slides before ICC processing. Neonatal tissue was eventually processed with alternate sections mounted on gelatin-coated slides or placed in plastic culture wells containing PBS using an antibody dilution of 1:500. Whereas staining density was not observed in the slide-mounted tissue, the AR was clearly present in the hippocampus of the free-floating sections. Although neonatal tissue was then tested with dilutions of 1:1000 and 1:2000 in

the free-floating procedure and compared with 1:500, the higher concentration of 1:500 was used to ensure maximizing the staining density of hippocampal ARs in the neonate.

Brains were sectioned in the coronal plane at 40 µm in a –20^{0}C cryostat. As they were cut, sections were placed into wells of plastic culture trays containing cold PBS. All ICC reactions were performed on free-floating sections within the wells of the trays. Alternate sections throughout the hippocampus, from the rostral to the caudal extremes, were either stained with cresyl violet (0.1%) or processed for AR-ICC. Using a dilution of 1:500, the rabbit antiserum to AR was stored in the wells of the plastic culture tray and reused to stain multiple sets of brains. Yoked sets of brains consisted of sections from both males and females age PND 2 through PND 40 such that sections from a total of 12 animals were processed for ICC at the same time. This ensured that any variability in procedure was evenly distributed across conditions. In one set, the male and female PND 40 was replaced by the adult PND 60.

In the AR-ICC, sections were blocked for nonspecific staining in a solution of 0.2 M PBS, 0.2% Triton X-100, 1.0% H_2O_2 and 4% normal goat serum (NGS, Gibco, ON) for 1 h at room temperature. Following three washes of 10 minutes each in PBS, sections were transferred to the primary antibody prepared in a solution of 0.2 M PBS containing 1.0% bovine serum albumin (BSA), 0.2% Triton X-100 and 0.02% sodium azide. To check specificity of the primary antibody, control sections from each set were immersed in the AR antibody PG-21

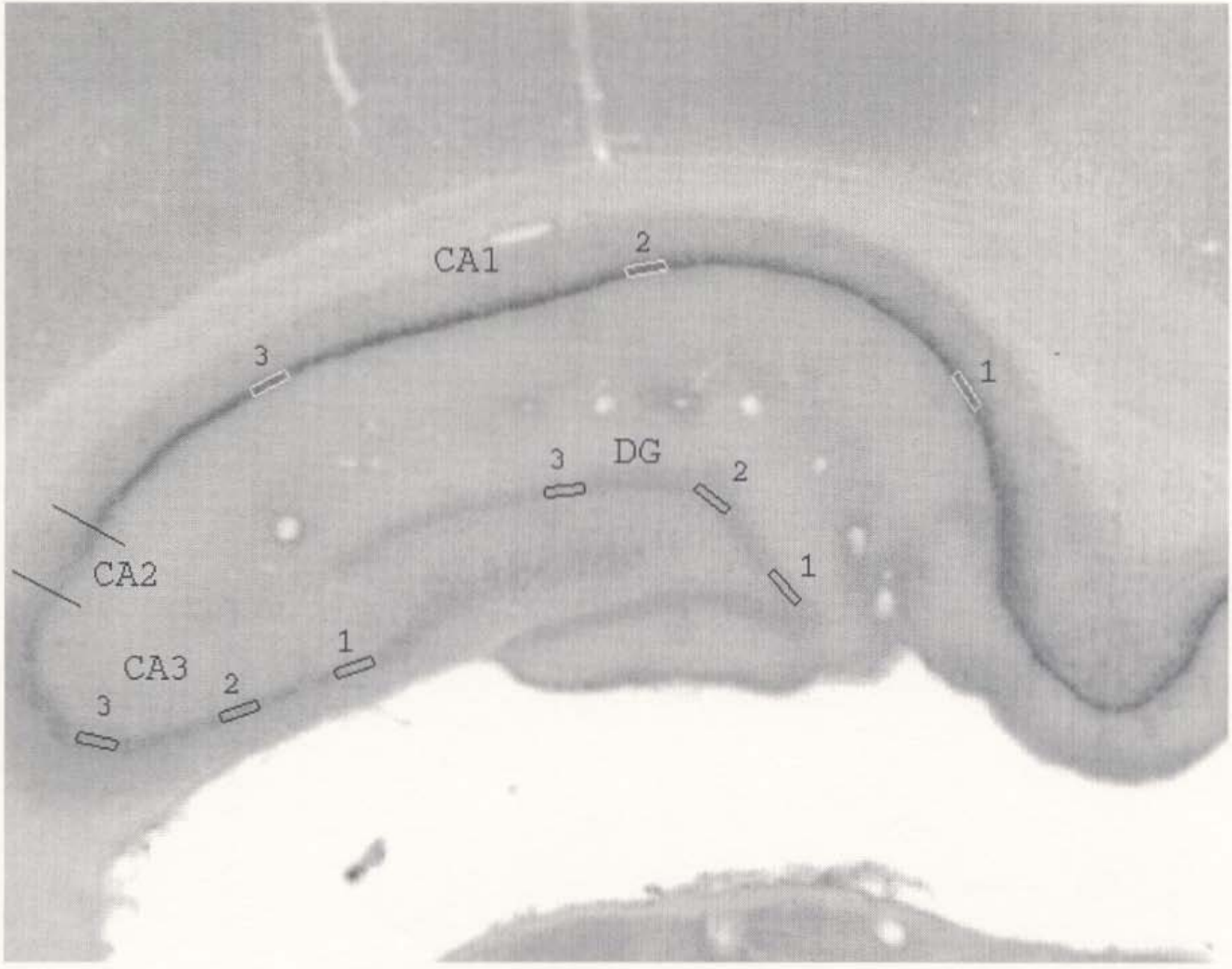
(1:1000) that also contained a 10 fold molar excess of, either AR21, the competitive peptide, or AR462, the unrelated peptide. Culture trays containing the sections were gently agitated for 1-2 hours and then stored, without agitation, at 4°C for the remainder of 48 hours.

All procedures following the primary antibody incubation were performed at room temperature. Sections were washed three times for 10 minutes each time in 0.2 M PBS before each of the following steps. Sections were immersed for 2 hours in a solution of normal goat serum (1:110) and anti-rabbit IgG biotinylated antibody (1:340) from the Vectastain *Elite* ABC kit, prepared in a diluent of 0.2 M PBS buffer with 0.2% Triton X-100 plus 0.1% BSA. Thirty minutes following the preparation of the Vectastain *Elite* avidin-biotin complex, prepared in the same diluent as the previous step, sections (washed in PBS) were immersed for an additional 2 hours. After the final washing, antibody binding was visualized by reacting with 0.05% diaminobenzidine tetrahydrochloride (DAB; Sigma) enhanced with 0.02% nickel ammonium sulphate and 0.025% cobalt chloride for 10 minutes. Sections were then immersed in the same solution with the addition of 0.01% hydrogen peroxide for 5 minutes. The reaction was terminated by incubating the tissue for 5 minutes in three changes of PBS. Sections were then mounted onto gelatin coated slides and air dried. Finally, sections were rinsed with distilled water, dehydrated in a series of alcohols, cleared by xylene and coverslipped with Microkit mounting media.

2.4 Analysis

A computer program, Micro Computer Imaging Device (MCID, M4 software, Imaging Research Inc., St. Catherines, ON) was used for densitometry. A video camera with a 123 mm lens extension captured images that were digitized by the MCID program. The system was initially calibrated to the International Standard Organization (ISO) density unit by a step scale (Eastman Kodak Co., Rochester, N.Y.) purchased from Imaging Research. The actual optical density read by the computer at each of 20 steps in the scale was compared to the pre-defined ISO density level at each step and adjusted accordingly. Following calibration, images of the hippocampus were captured, digitized and stored in the computer for later analysis. This procedure ensured that all images within a given set of animals were captured at the same time under equivalent conditions. The minimum number of sections that contributed to the data per animal was seven in the PND 2 male and female and the maximum number was 16 in the PND 60 male and female. Sections analyzed were evenly dispersed throughout the rostrocaudal extent of the dorsal hippocampus. A box measuring 20 by 4 pixels, corresponding to 0.14 by 0.04 mm, placed over the cell layer defined the area for a given density reading (see Figure 2.1). For each section, the density was determined by averaging three separate readings taken across the mediolateral extent of the hippocampus. For each animal, the density of a given anatomical area was determined by averaging across all the sections.

Figure 2.1 Computer digitized video image of AR-ir in a coronal section of the hippocampus from a PND 60 male illustrating the method used to gather data for analysis. The average density of three sampled areas of the DG was used to determine non-specific staining within a cell layer and subtracted from the average density of three sampled areas from either the CA1 or CA3 cell layer. The partitioning of Ammon's horn into CA1, CA2 and CA3 is also indicated and labeled as is the DG.


CA1
2
3
1
3
DG
2
1
CA2
CA3
1
2
3

The areas analyzed included CA1, CA3 and the DG. The density of the DG cell layer was used to generate a background density for each section for the following reasons, (1) little or no AR staining was seen in the DG at any age in the present study (2) the absence of staining in adult rat has also been reported by others using the PG-21 antibody [99] as well as the AR52 antibody [135] and (3) AR mRNA is not detectable in adult rat DG [15]. Thus, the density of the DG cell layer was subtracted from the density obtained within the CA1 and CA3 cell layers. Statistical analysis of results was performed by a two-factor (age and sex) ANOVA with five males and five females in each condition except PND 40, which had four of each sex. PND 60 was not included in the analysis, as only one male and female were processed. Post hoc analysis was subjected to the Fisher test for least significant difference based on the more stringent non-directional hypothesis (i.e., 2-tail test). The Fisher test was chosen because this test requires the condition of a significant omnibus F before implementation, thereby eliminating unnecessary comparisons, and because there is no previous data available on which to base specific planned comparisons. As well, results from the Bonferroni protection test are cited to bracket possible trends.

CHAPTER 3: RESULTS

There were no differences found between the medial to lateral measurements taken within each section ($F_{(2,162)} = 0.136$, $p>0.30$) nor were differences apparent between rostral to caudal sections. Consequently, analysis was as defined in the methods section above.

3.1 DG

As indicated in Figure 3.1, there is no apparent change in density in the DG at any of the ages examined. Furthermore, using the DG granule cell layer as a means of evaluating AR density of both the CA1 and CA3 hippocampal cell layers is validated by statistical analysis. There is no effect of age ($F_{(5,46)} =1.44$, $p>0.22$) or sex ($F_{(1,46)} =0.001$, $p>0.97$) in AR density staining within the DG granule cell layer. Although there appears to be an increase in AR density at PND 40 (see Figure 3.1), the variability at this age is such that the mean trend is not significant (data ranging from 0.17 to 0.44 in the male and from 0.16 to 0.46 in the female). Indeed, this spurious effect in DG-AR density validates using this cell layer as a background density control for each section as variability in ICC across experiments could have obviated the AR density results.

3.2 CA1

As illustrated in Figure 3.2 and the photomicrographs in Figure 3.3, the ontogeny of the AR staining in CA1 of the hippocampus shows an effect of age

Figure 3.1 Ontogenic profile of the average AR density ± SEM within the DG area of the dorsal hippocampus. The unit of measure is the integrated optical density (IOD) within a sampled area pre-calibrated with the International Standards Organization (ISO) step scale as defined in the text. Data obtained from the entire rostral-caudal extent. An 'n' of 5 contributed to the data for the earlier ages with the 'n' indicated above the bars for PND 40 and PND 60.

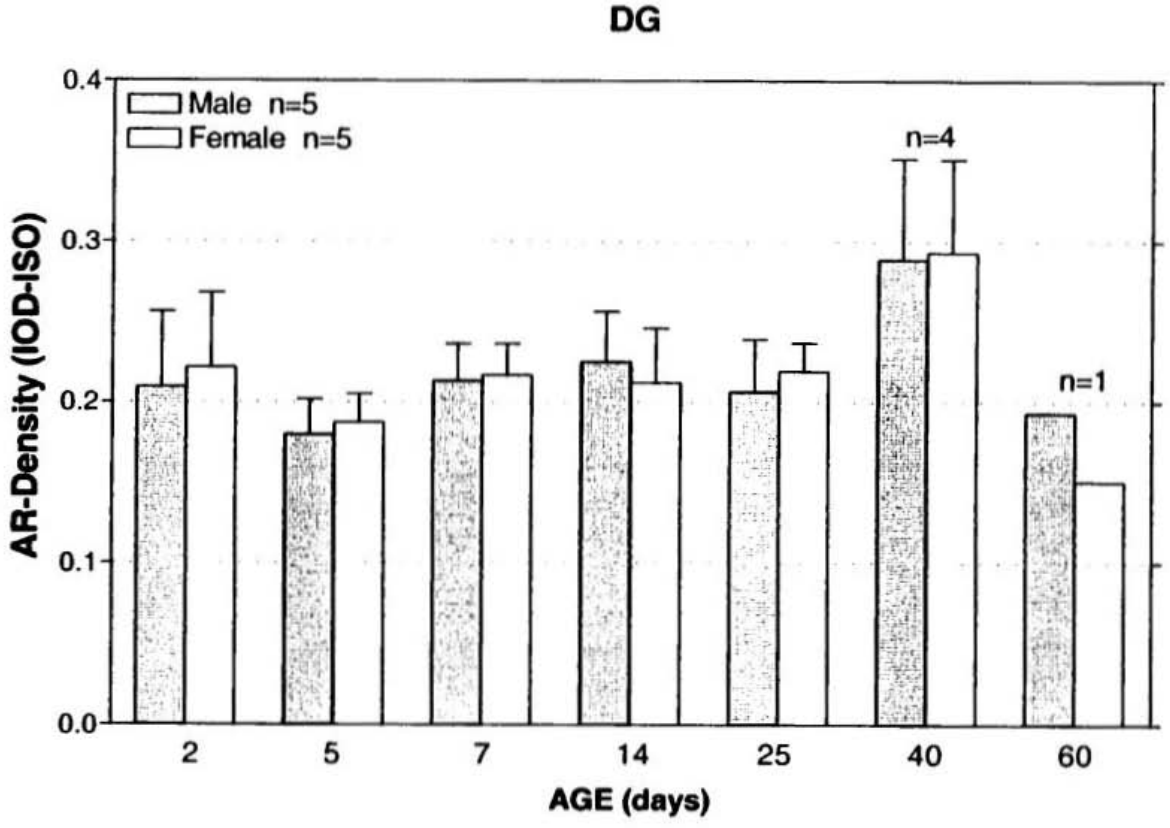

DG
Male n=5
Female n=5
n=4
n=1
AR-Density (IOD-ISO)
0.4
0.3
0.2
0.1
0.0
2
5
7
14
25
40
60
AGE (days)

Figure 3.2 Ontogenic profile of the average AR density ± SEM within the pyramidal cell layer of CA1 of the dorsal hippocampus. Data obtained as indicated in Figure 3.1. Heavy solid arrows indicate ages that AR density is significantly greater than ages indicated by linked smaller dashed arrows. The asterisks at PND 40 indicates significantly greater AR density relative to all other ages.

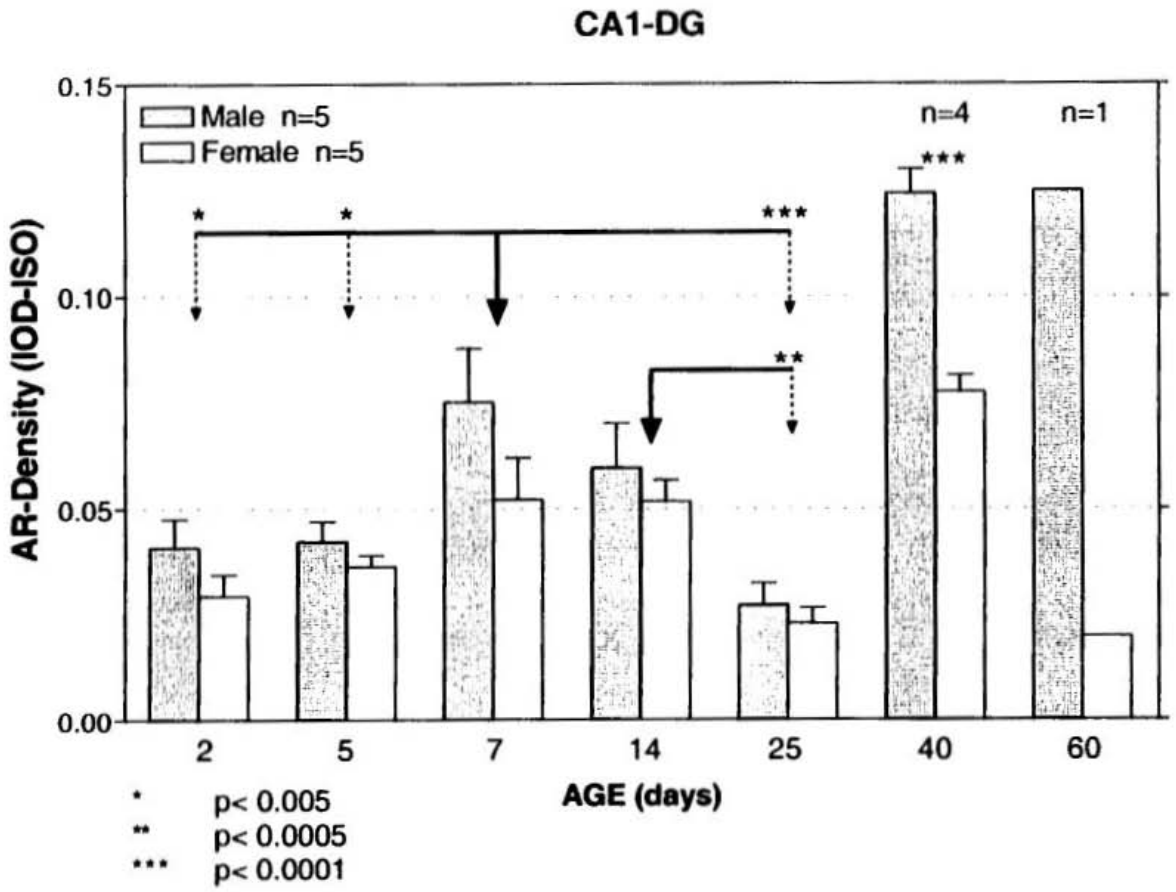
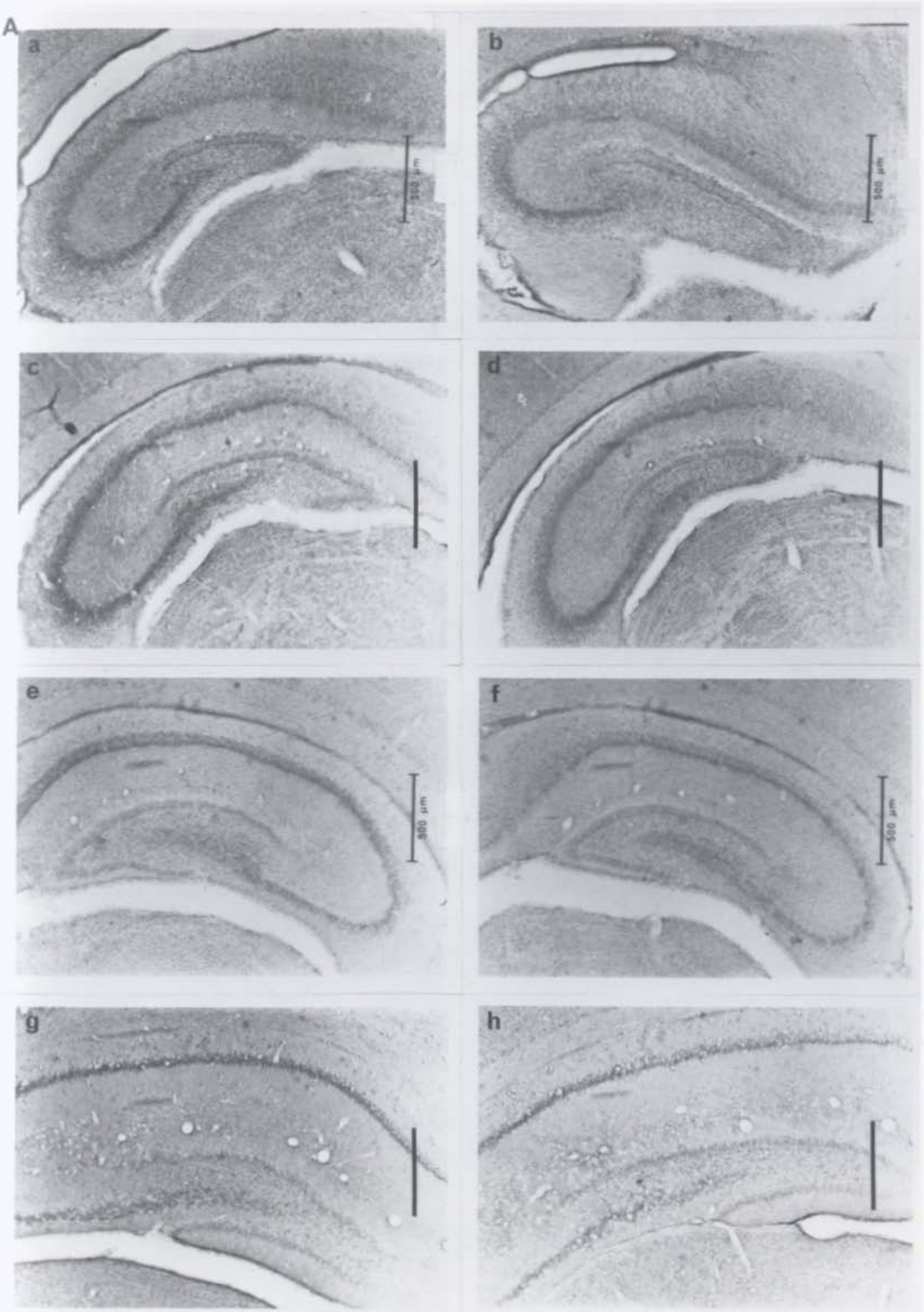
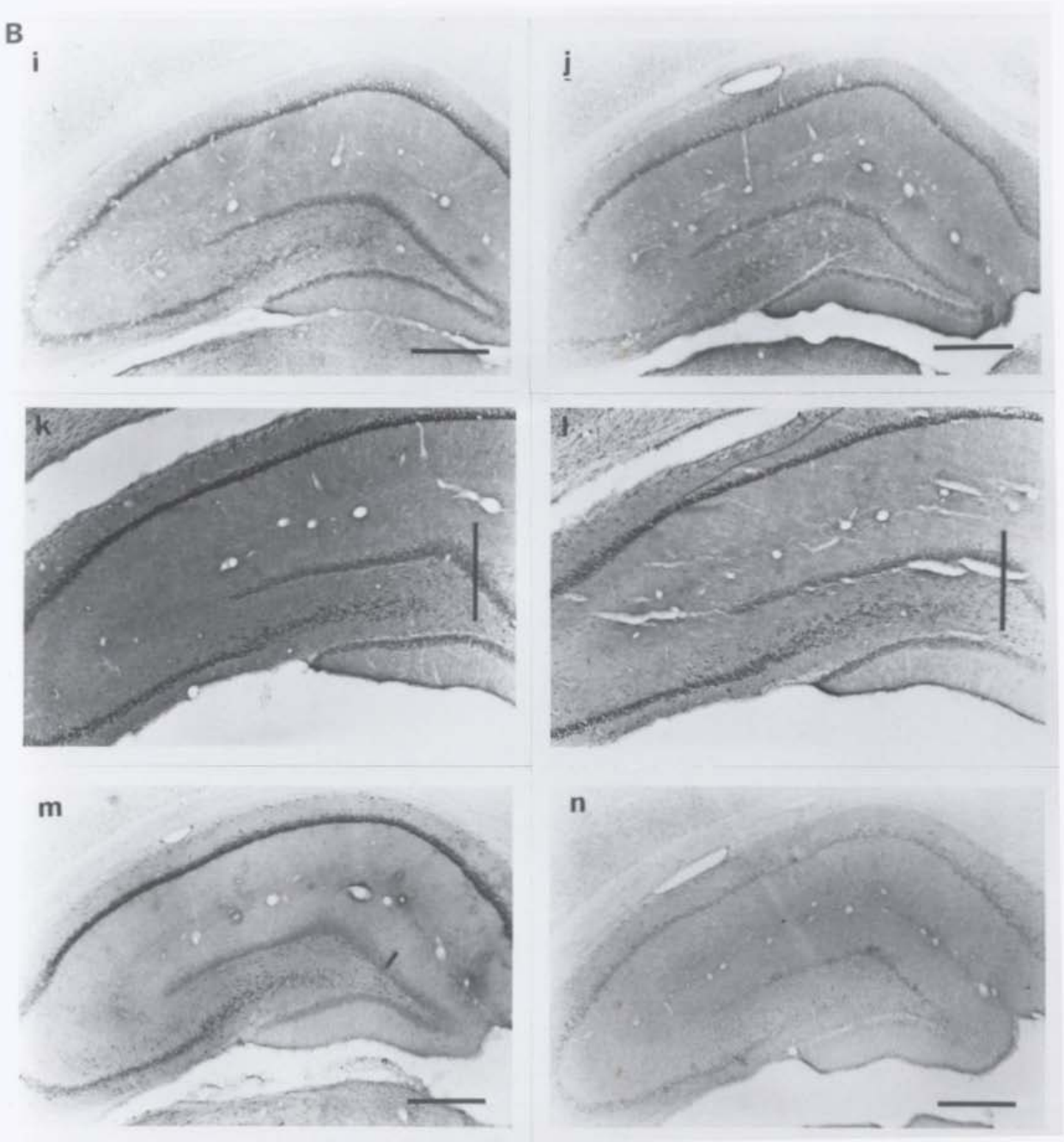

CA1-DG
Male n=5
Female n=5
n=4
n=1
***
*
*
***
**
AR-Density (IOD-ISO)
0.15
0.10
0.05
0.00
2
5
7
14
25
40
60
AGE (days)
* p< 0.005
** p< 0.0005
*** p< 0.0001

Figure 3.3 (A) and (B). Photomicrograph of representative sections showing the CA1, CA3 and DG from male and female rats at each age analyzed. The male is on the left and the female is on the right. Age increases with each descending picture such that PND 2 is at the top followed by PND 5, PND 7, PND 14, PND 25, PND 40 with PND 60 at the bottom. All scale bars are 500 μm with different magnifications used in microphotography represented by the orientation of the bar.


A
a
b
c
d
e
f
g
h
500 µm


B
i
j
k
l
m
n

($F_{(5,46)}$ = 26.187, p< 0.0001) and sex ($F_{(1,46)}$ = 14.141, p< 0.0005) with a trend toward a significant interaction ($F_{(5,46)}$ = 2.270, p< 0.065). Post hoc analysis reveals that the significant difference for age is mainly derived from the PND 40 and PND 7 age groups. At PND 7, the AR density is significantly greater than at the earlier ages, PND 2 or PND 5 (p< 0.005) or at PND 25 (p< 0.0001) but it does not differ from the PND 14 (p> 0.30) group. The inverted U shape indicates that AR density levels increase between PND 5 and PND 14, possibly peaking around PND 7, at which time they begin to decline to the lower density observed at birth. Indeed, the density of labeling in PND 25 animals is lower than the level at either of the two preceding ages, PND 7 (p<0.0001) and PND 14 (p< 0.0005), with staining comparable to the PND 2 (p> 0.15) age group. At puberty, AR levels again increase and surpass earlier levels as AR density is greater at PND 40 than at any other age examined (p < 0.0001).

As indicated above, AR density in the hippocampal CA1 cell layer averaged over ages PND 2 to PND 40, is greater in males (mean = 0.059 ± sem of 0.007) compared to females (mean = 0.044 ± sem of 0.004). Although statistical analysis of the interaction did not reach significance, the trend (p< 0.065) indicates that a cursory look is warranted. The interaction between gender and age, analyzed by the Fisher least significant difference, suggests that the sex difference is localized to the PND 7 (male: 0.0751, females: 0.0521; p <0.025) and PND 40 (males: 1.1240, females: 0.0776; p < 0.0005) ages. No significant differences between males and females occur in the other four age

groups. However, analysis by the more stringent Bonferroni method (requiring a p value < 0.008) indicates that the sex difference exists only at PND 40. Consequently, it may be more accurate to suggest that males show greater AR density staining than females at PND 40 with a trend at PND 7. Thus, the PND 40 and PND 7 age groups not only have greater AR density than other ages but, compared to females, there is greater AR staining density in the male at PND 40 with a similar trend at PND 7.

Given the stringent level of probability necessitated by Bonferroni protection, a general description of the interaction of the ontogeny of the AR in females compared to males will be by observation rather than by quantitative evaluation. As shown in Figure 3.2, AR ontogeny in both sexes follows the same pattern between PND 2 and PND 25, that of an inverted U. Thus, although the trend of a sex difference is suggested at PND 7, this effect does not seem to be due to a lack of increase in the female. The female appears to have greater AR density at PND 7 than at any earlier age and comparable to PND 14. As indicated above, by PND 40, a robust sex difference occurs with greater AR density in the male ($p< 0.0005$). However, again, the greater AR density seen in the male is not due to the lack of an increase in the female. Indeed, the AR density staining observed in the female at PND 40 appears to be greater than that observed at any earlier age. Although only one PND 60 male and female were processed in the present experiment, it is of interest that the level of AR staining in the male is comparable to the PND 40 male whereas the PND 60

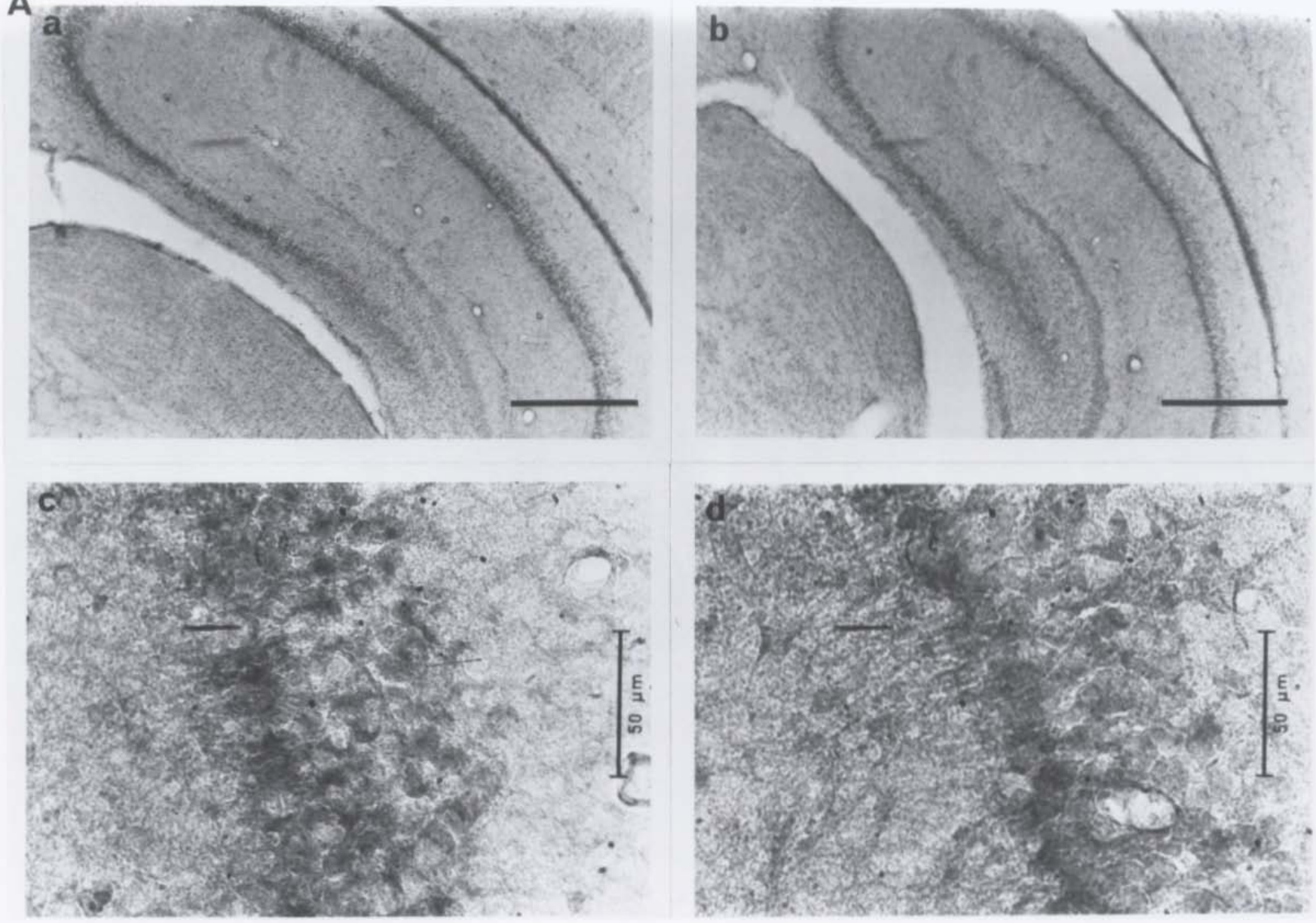
female contains extremely low levels (see Figure 3.1). This result remains to be verified by further experimentation, as it is possible that the stage of the estrous cycle is a factor at PND 60.

The analysis presented above does not present the complete picture of the presence of the AR within the hippocampus as there appears to be a qualitative difference over time as well (see Figure 3.4). Although the sections are too thick (40 µm) to do a quantitative evaluation of neuronal location of the AR there appears to be little or no enhanced nuclear staining in the neonatal hippocampus. The AR appears to be evenly dispersed throughout the cell. The earliest age that nuclear density is clearly visible is in the PND 40 male and female, at which time the density of nuclear AR is much stronger than the apparent density of the cytoplasmic AR.

3.3 CA3

A perusal of Figure 3.5 reveals that the pattern of the ontogeny of the AR in CA3 is different from that in CA1 (compare with Figure 3.2). In CA3, ANOVA reveals a significant effect of age ($F_{(5,46)} = 14.06$, $p < 0.0001$) but no gender difference ($F_{(1,46)} = 0.254$, $p > 0.60$). Density at PND 7 is greater than at all other ages examined ($p < 0.05$). Unlike the CA1 cell layer, however, by PND 14, the AR density has receded from the high level found at PND 7 ($p < 0.0001$) and is also significantly lower than the density in PND 2 ($p < 0.02$) and PND 5 ($p < 0.03$) neonates. The downward trend of the AR appears to culminate at PND 25 when

Figure 3.4 (A) and (B) Photomicrograph of representative sections showing low and high magnification of the CA1 cell layer in the PND 7 and PND 40 animal. The male is on the left and the female is on the right. The PND 7 animals are represented in (A), the first four photomicrographs (a-d) whereas the PND 40 animals are shown in (B) the last four (e-h). Notice the lack of consistent AR nuclear definition with low level density throughout the cell in the PND 7 male and female. At PND 40, nuclear definition may be visible in a few neurons in the female but is clearly defined in significantly more neurons in the male. The horizontal scale bar represents 500 µm and the vertical bar is 50 µm.


A
a
b
c
d
50 μm
50 μm

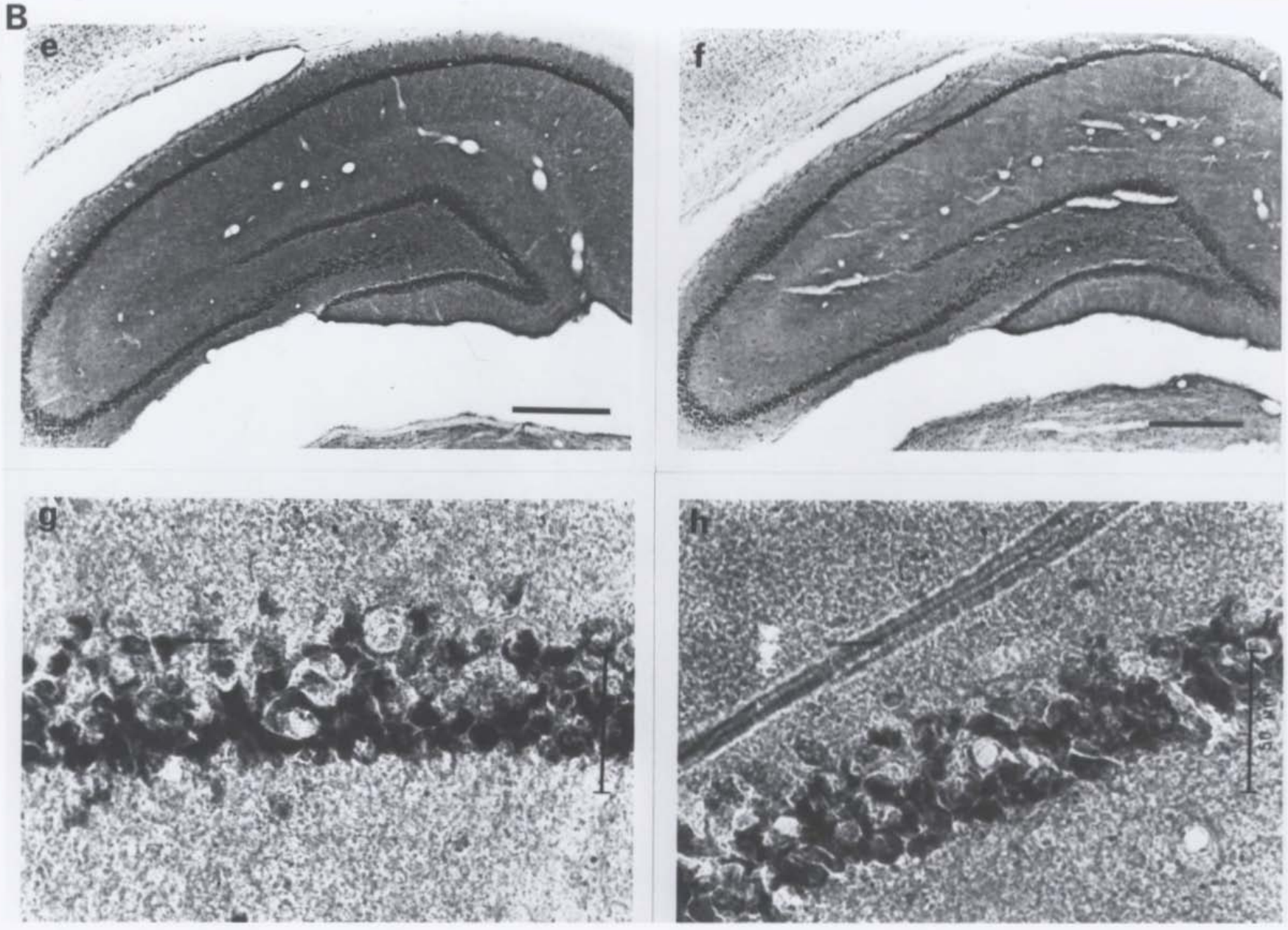
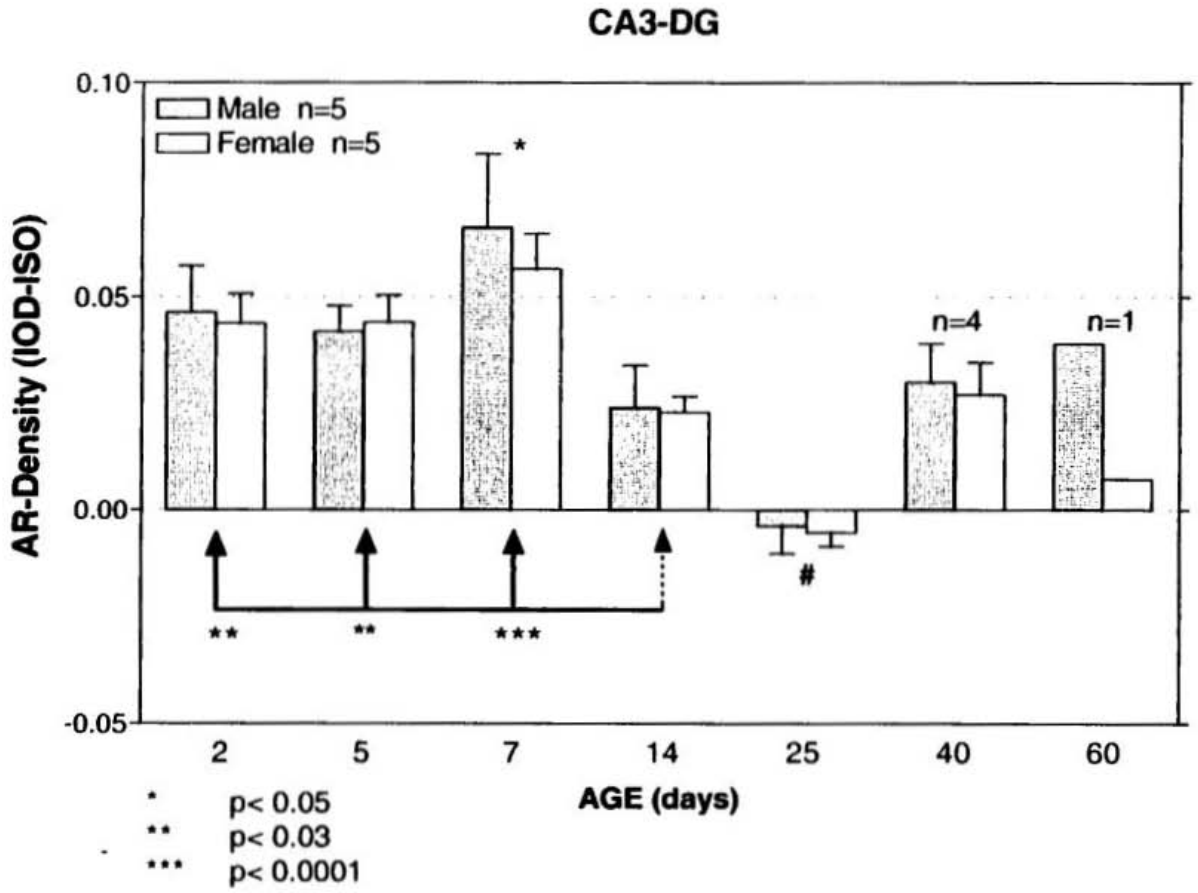

B
e
f
g
h

Figure 3.5 Ontogenic profile of the average AR density ± SEM within the CA3 area of the dorsal hippocampus. Data obtained as indicated in Figure 3.1. Heavy solid arrows indicate ages that AR density is significantly greater than ages indicated by the linked smaller dashed arrow. The asterisk at PND 7 indicates significantly greater, whereas the # at PND 40 indicates significantly less AR density relative to all other ages.


CA3-DG
Male n=5
Female n=5
AR-Density (IOD-ISO)
0.10
0.05
0.00
-0.05
n=4
n=1
*
**
**
***
#
2
5
7
14
25
40
60
AGE (days)
* p< 0.05
** p< 0.03
*** p< 0.0001

there is virtually an absence of AR staining as defined by subtracting the density of the DG granule cell layer. This complete absence of AR staining is reversed slightly at PND 40 at which time AR density does not differ from PND 14 levels ($p > 0.60$).

Several differences exist between CA3 and CA1. For example, in CA3, there is a rapid decrease in AR staining between PND 7 and PND 14 and a relative lack of AR staining at PND 25. Another difference is that following the pubertal burst, the AR does not return to CA3 at PND 40 to the same degree that it returns to CA1. A third striking difference is the lack of a sex difference in CA3 at any age examined up to and including PND 40. A sex difference may be present in CA3 by PND 60 but these data are preliminary and inconclusive.

3.4 Control Sections

Several sections from every animal were processed either without immersion in the PG-21 antibody or by including the competitive peptide (AR21) in the incubation with PG-21. Under both conditions, immunostaining indicating the presence of the AR was eliminated. However, sections incubated with the non-competitive peptide (AR462) showed comparable immunostaining to sections incubated with PG-21 alone. Although not all sections were analyzed, samples from three PND 7, one PND 14 and one PND 40 males were subjected to analysis with tissue from each animal incubated in both peptides for comparison. AR density was significantly greater in sections incubated with

AR462 compared to AR21 ($F_{(1,8)}$ = 10.462, p<0.01). Examples of sections exposed to these conditions are presented for the PND 7 and PND 40 male in Figure 3.6.

3.5 DHTB AT PND 7

The possibility that the non-liganded AR may have less affinity for the antibody was investigated in two sets of male and female PND 7 rat pups by pretreatment with DHTB as indicated in the methods section.

Figures 3.7, 3.8 and 3.9 demonstrate the results of treatment with DHTB 30 minutes before sacrifice in PND 7 animals. Both the individual results and the average (showing the sem) are presented in Figure 3.7. Within CA1, analysis reveals an effect of sex ($F_{(1,4)}$ = 500.1, p<0.0001) and of DHTB treatment ($F_{(1,4)}$ = 44.391, p<0.003) as well as a trend for an interaction ($F_{(1,4)}$ = 7.148, p<0.06). Post hoc analysis revealed that DHTB increases the AR density of the CA1 cell layer in both males (control: 0.053, DHTB: 0.061; p <.005) and females (control: 0.0369, DHTB: 0.040; p < .030). However, the effect may be stronger in males resulting in the observed trend for an interaction. The strong sex effect from a 'n of 2' adds support to the sex effect at PND 7 indicated by a trend in the main ontogeny experiment. Unlike the CA1 cell layer, no sex effect ($F_{(1,4)}$ = 0.602, p>0.48) or interaction ($F_{(1,4)}$ = 0.026, p>0.88) was found in the CA3 cell layer of DHTB-treated animals compared to untreated controls (see Figure 3.10). Although DHTB was not effective in increasing AR density within the CA3 cell

Figure 3.6 Photomicrograph of sections processed in the control studies from males at PND 7 (a), (b) and at PND 40 (c), (d). Sections (a) and (c) were incubated in AR462, the distant peptide and sections (b) and (d) were processed in AR21, a competitive peptide. All scale bars equal 500 µm.

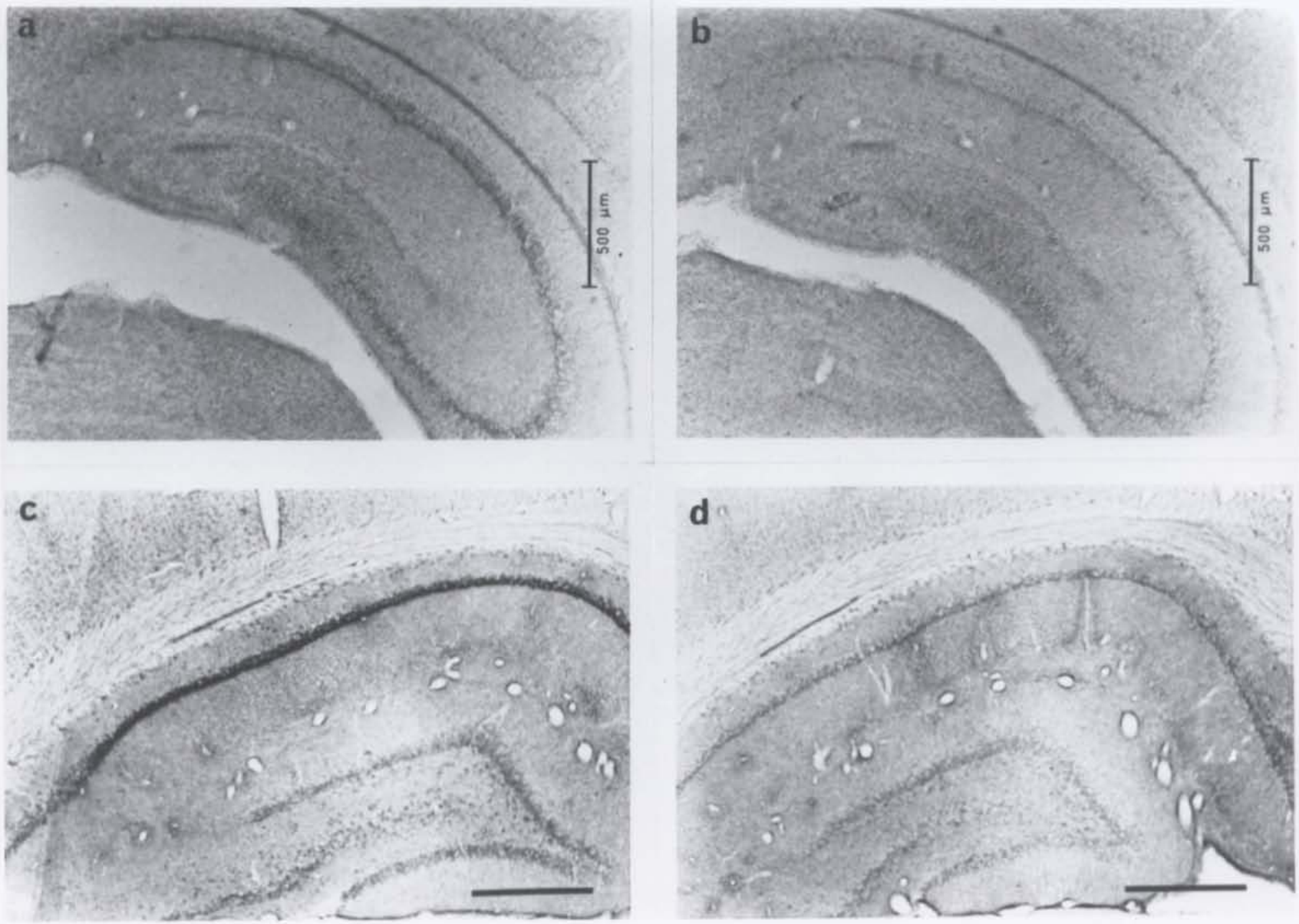

a
500 µm
b
500 µm
c
d

Figure 3.7 Results obtained within CA1 following treatment with DHTB (20 mg/kg, ip) 30 minutes before perfusion in PND 7 animals. In group '1', the control M and F were littermates as were the DHTB-treated animals but the control and DHTB-treated sets represent different litters. In group '2', all four animals were littermates. The heavy solid arrow indicates that AR density is significantly greater in males compared to the females (indicated with smaller dashed arrow). The asterisks indicate significantly greater AR density in the DHTB animals compared to their same sex controls.

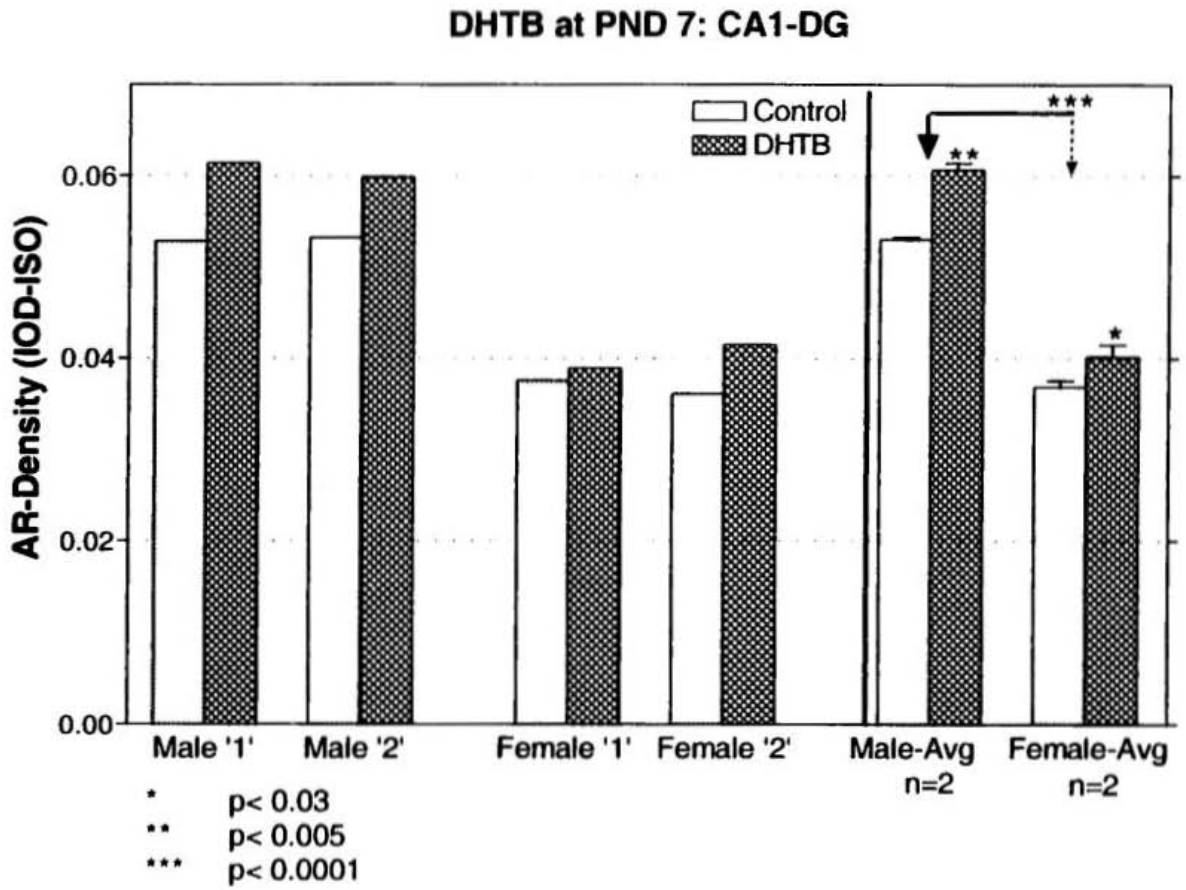

DHTB at PND 7: CA1-DG
Control
DHTB
AR-Density (IOD-ISO)
0.06
0.04
0.02
0.00
Male '1'
Male '2'
Female '1'
Female '2'
Male-Avg
n=2
Female-Avg
n=2
* p< 0.03
** p< 0.005
*** p< 0.0001

Figure 3.8 Photomicrograph of representative sections from PND 7 control and DHTB-treated males and females. (a) Control male, (b) control female, (c) DHTB-treated male, and (d) DHTB-treated female. The scale bar in (a) is 500 µm and apply to all.

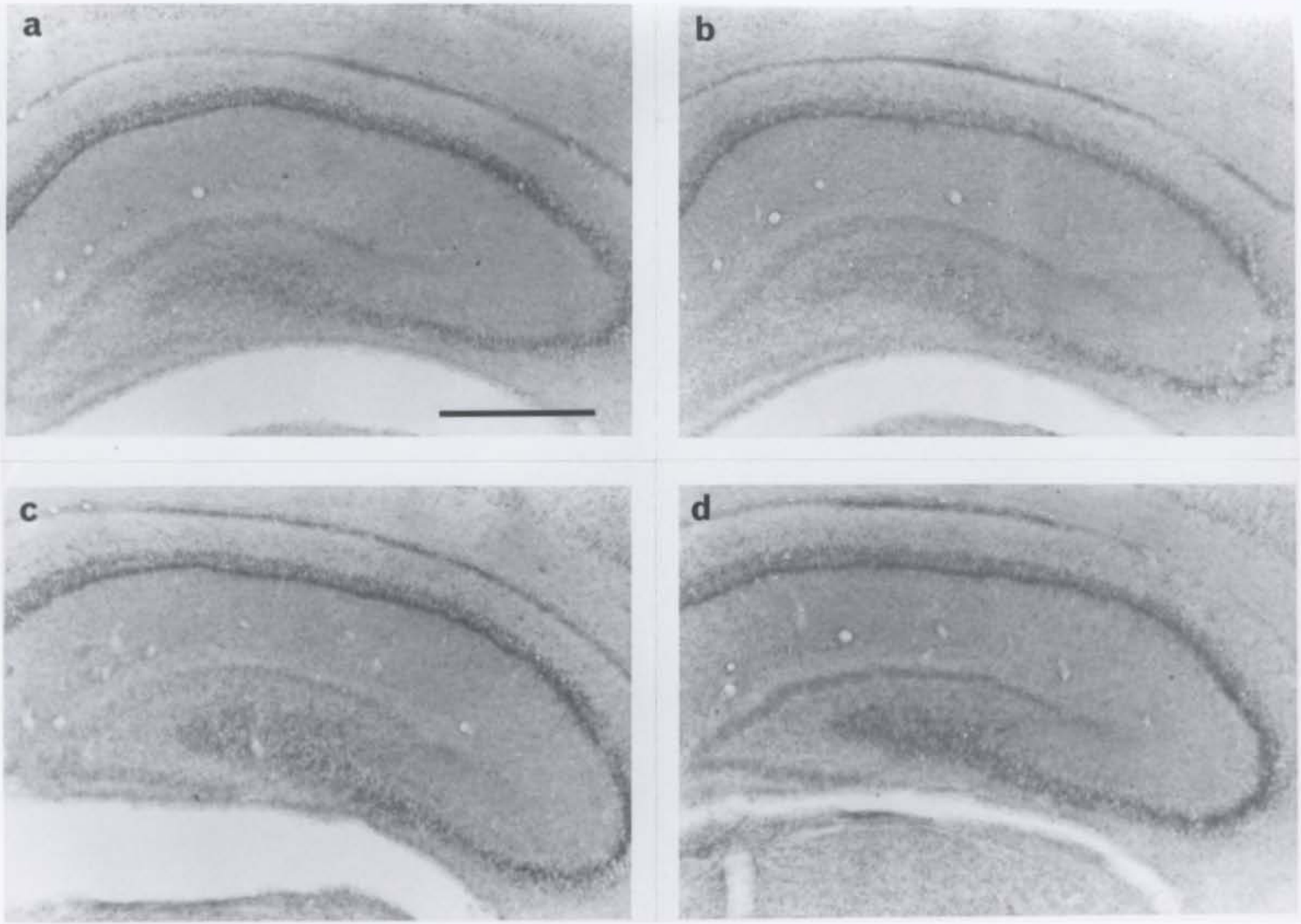

a
b
c
d

Figure 3.9 Photomicrograph of representative sections from PND 7 control and DHTB-treated males and females at high magnification showing the hippocampal CA1 cell layer. (a) Control male, (b) control female, (c) DHTB-treated male, and (d) DHTB-treated female. The scale bar in (a) is 50 μm and apply to all.

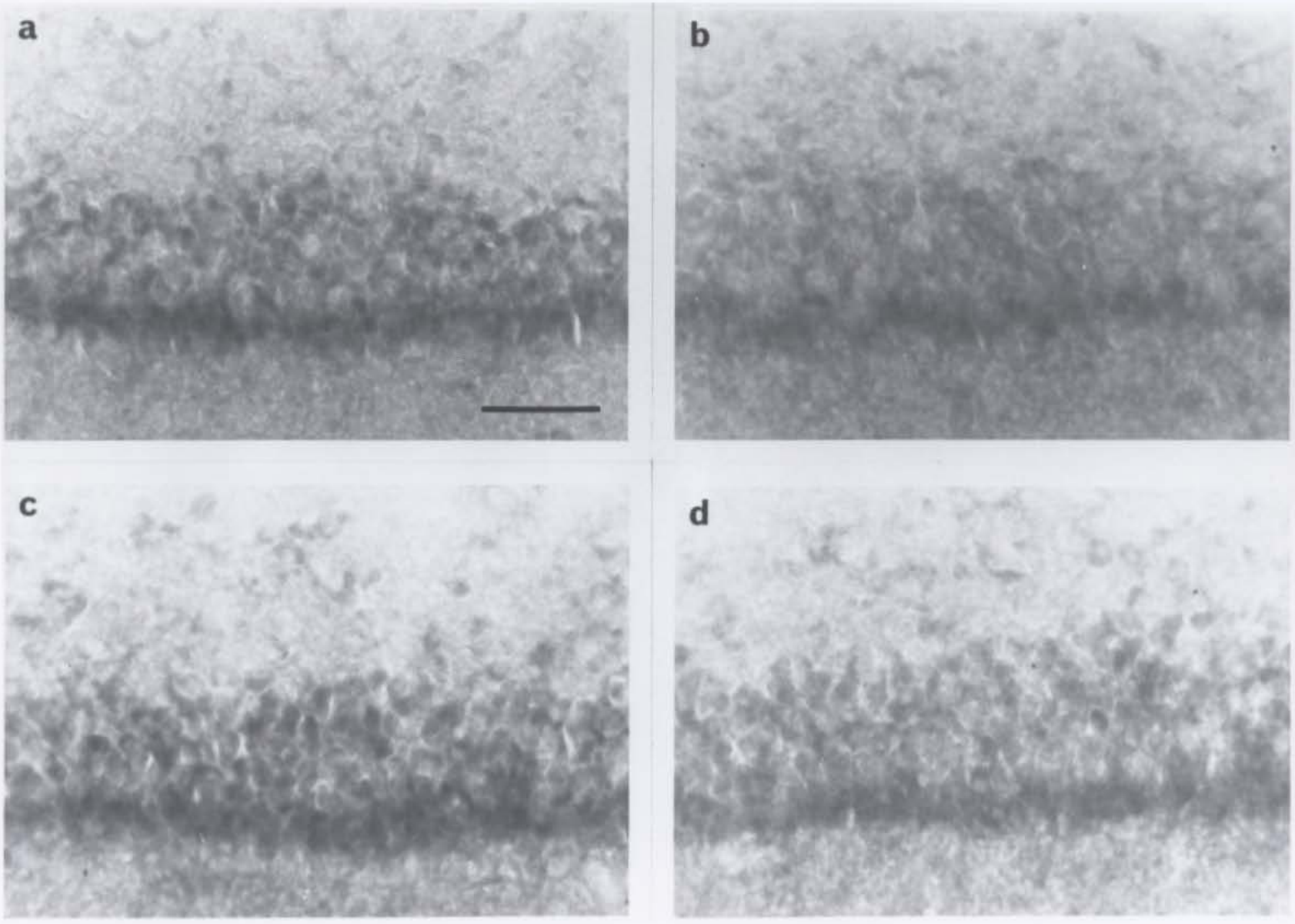
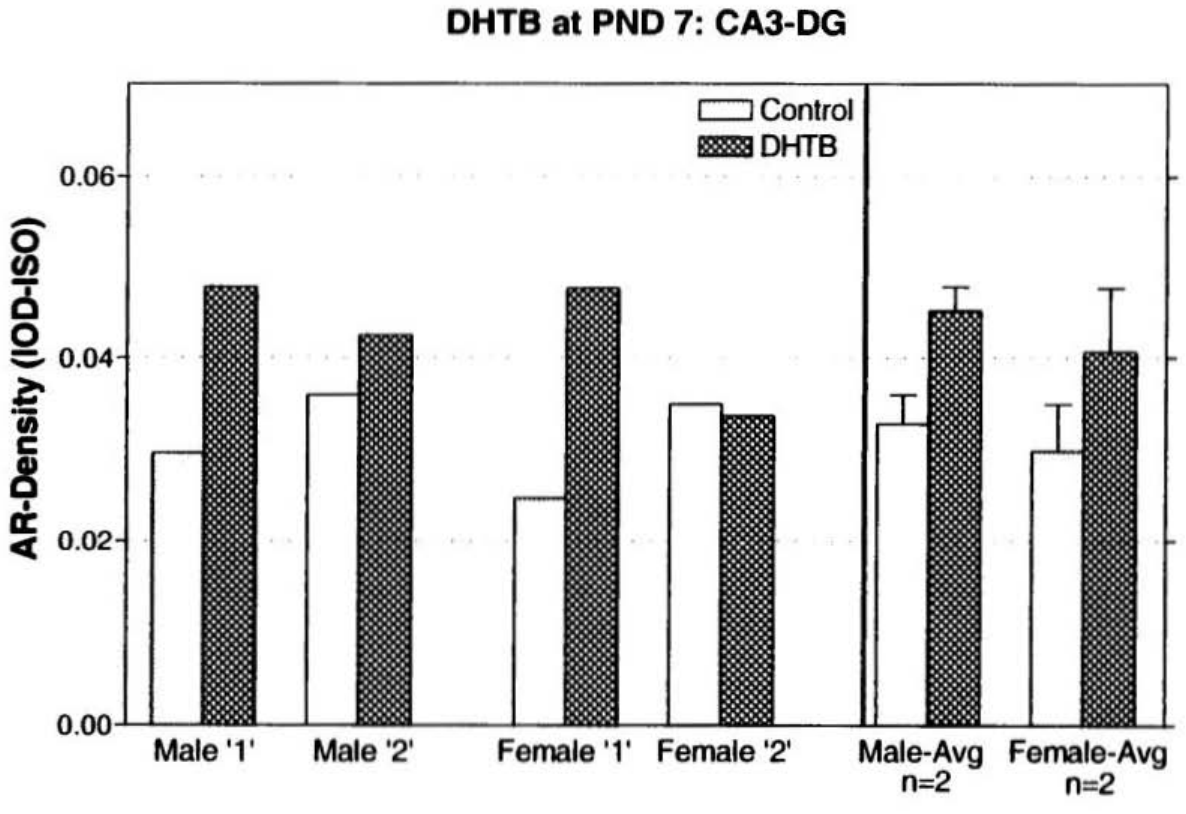

a
b
c
d

Figure 3.10 Results obtained within CA3 following treatment with DHTB 30 minutes before sacrifice in PND 7 animals.


DHTB at PND 7: CA3-DG
Control
DHTB
AR-Density (IOD-ISO)
0.06
0.04
0.02
0.00
Male '1'
Male '2'
Female '1'
Female '2'
Male-Avg n=2
Female-Avg n=2

layer at PND 7, a trend was apparent ($F_{(1,4)} = 5.84$ $p < 0.075$). The trend emerging from a 'n of 2' cannot be ignored and requires further investigation.

For comparison, the data obtained from the DG is presented in Figure 3.11. The density of this cell layer remains relatively stable and is unaffected by DHTB treatment in either males or females.

As far as could be ascertained, the cellular location of ARs within neurons of Ammon's horn in both DHTB-treated and untreated animals is both cytoplasmic and nuclear as indicated in the ontogeny experiment (see Figure 3.9). Thus, although AR density increases at PND 7 with DHTB treatment, the result is not related to AR translocation to the nucleus as there is no obvious increase in nuclear compared to cytoplasmic staining.

3.6 DHTB AT PND 25

Figures 3.12, 3.13, and 3.14 illustrate the effect of DHTB treatment at PND 25 in two males and two females from two different litters for a total of eight animals. Both the individual results and the average (showing the sem) are presented in Figure 3.12. The variability appears to be much greater at this age. Thus, with an 'n of 2' and no consistent trend from treatment, stringent analysis may be neither informative nor useful. However, it may be useful to describe results for each litter.

Within CA1, DHTB-treatment had opposite effects in the two males examined with increased staining in one male and either no difference or

Figure 3.11 Results obtained within DG following treatment with DHTB 30 minutes before sacrifice in PND 7 animals.

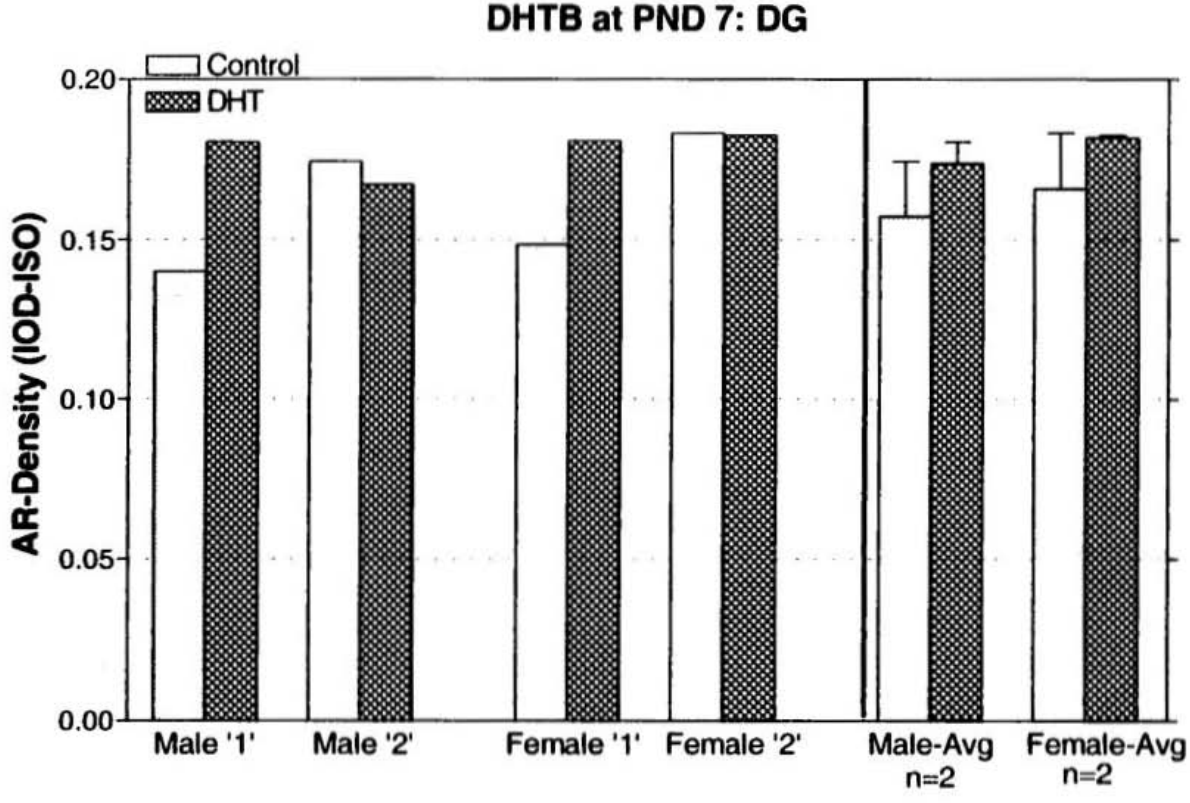

DHTB at PND 7: DG
Control
DHT
AR-Density (IOD-ISO)
0.20
0.15
0.10
0.05
0.00
Male '1'
Male '2'
Female '1'
Female '2'
Male-Avg
n=2
Female-Avg
n=2

Figure 3.12 Results obtained within CA1 following treatment with DHTB 30 minutes before sacrifice in PND 25 animals. Two different litters are represented and all four animals within a group are littermates.

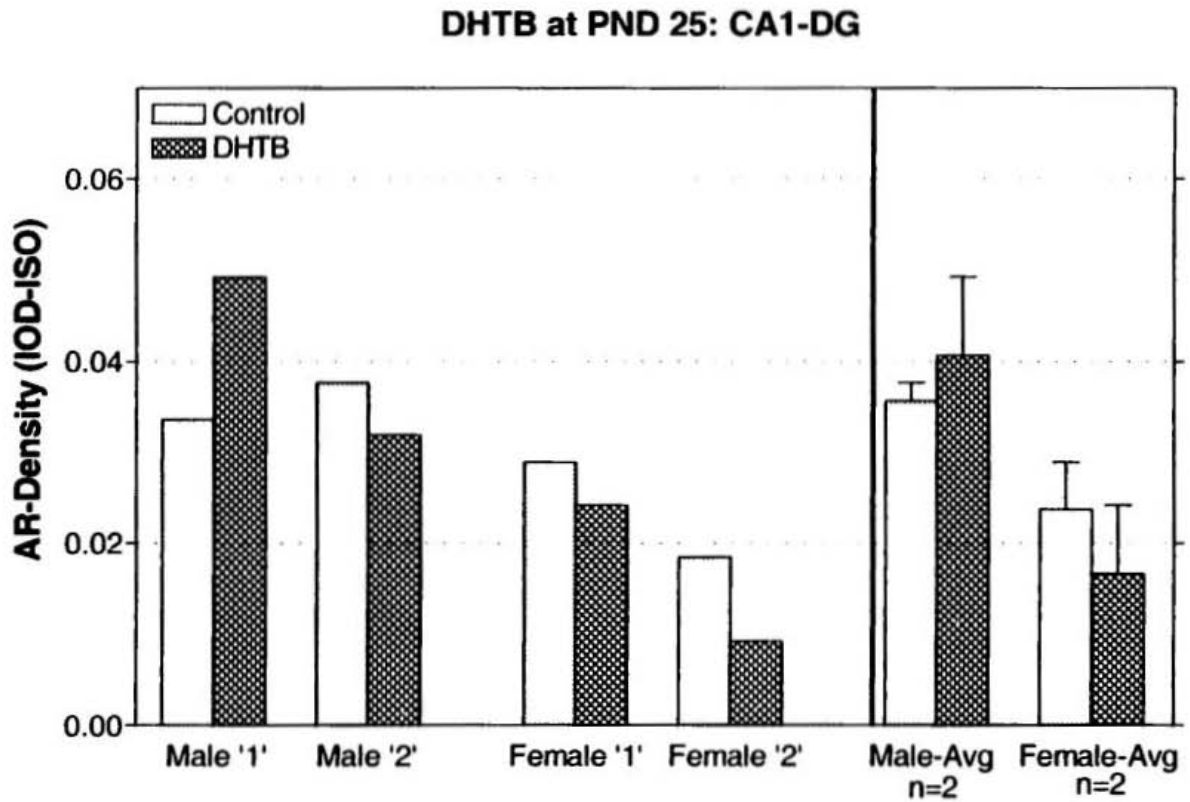

DHTB at PND 25: CA1-DG
Control
DHTB
AR-Density (IOD-ISO)
0.06
0.04
0.02
0.00
Male '1'
Male '2'
Female '1'
Female '2'
Male-Avg n=2
Female-Avg n=2

Figure 3.13 Photomicrograph of representative sections from PND 25 control and DHTB-treated males at high magnification showing the hippocampal CA1 cell layer. (a), (b) are from control males with the DHTB-treated littermate represented in (c) and (d) respectively. Data suggests an increase with DHTB-treatment in (c) relative to (a) and either a decrease or no difference with DHTB-treatment in (d) compared to (b). A Nissl-stained section from a PND 25 control male is shown in (e). The scale bar in (a) is 50 μm and apply to all.

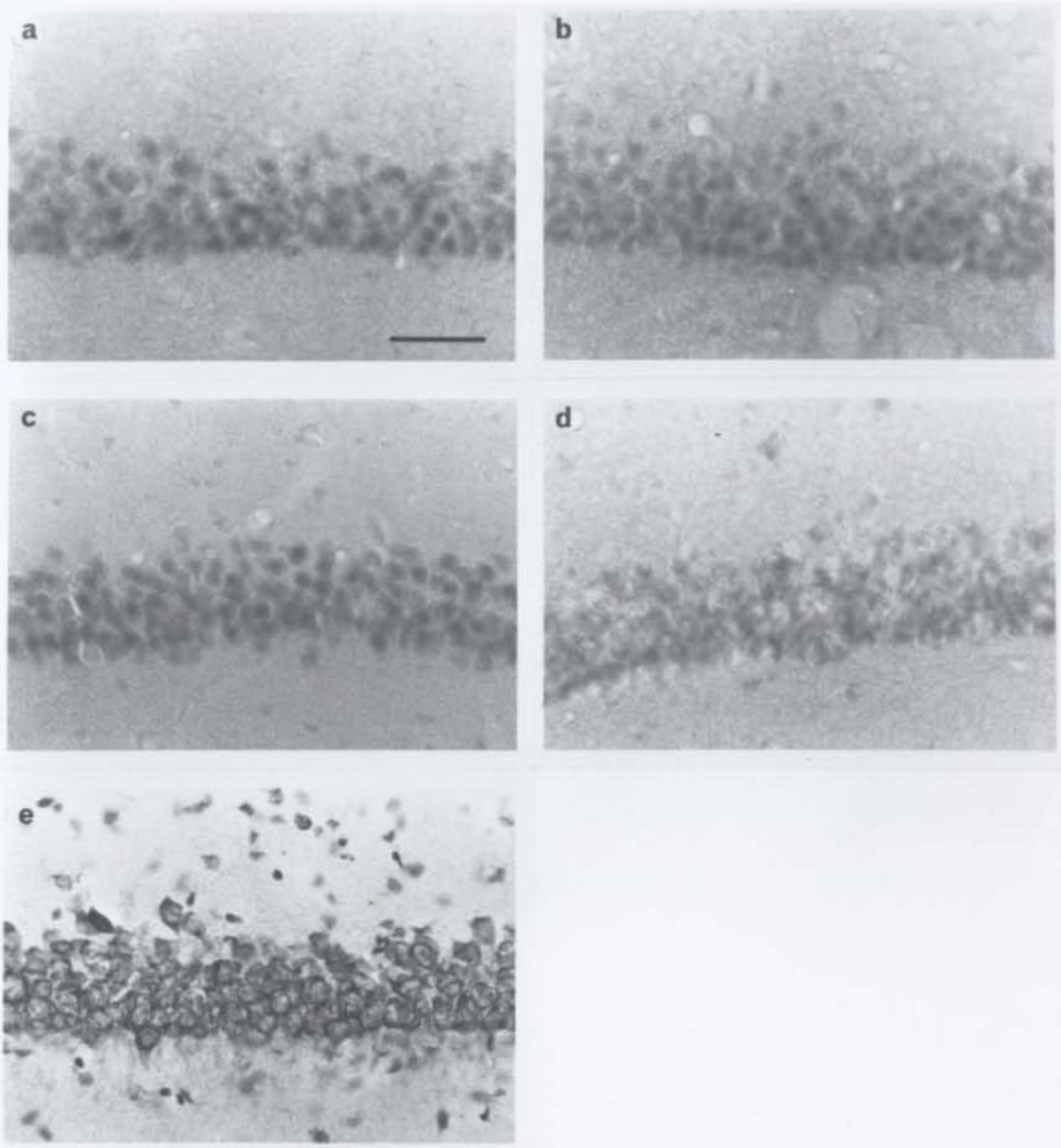
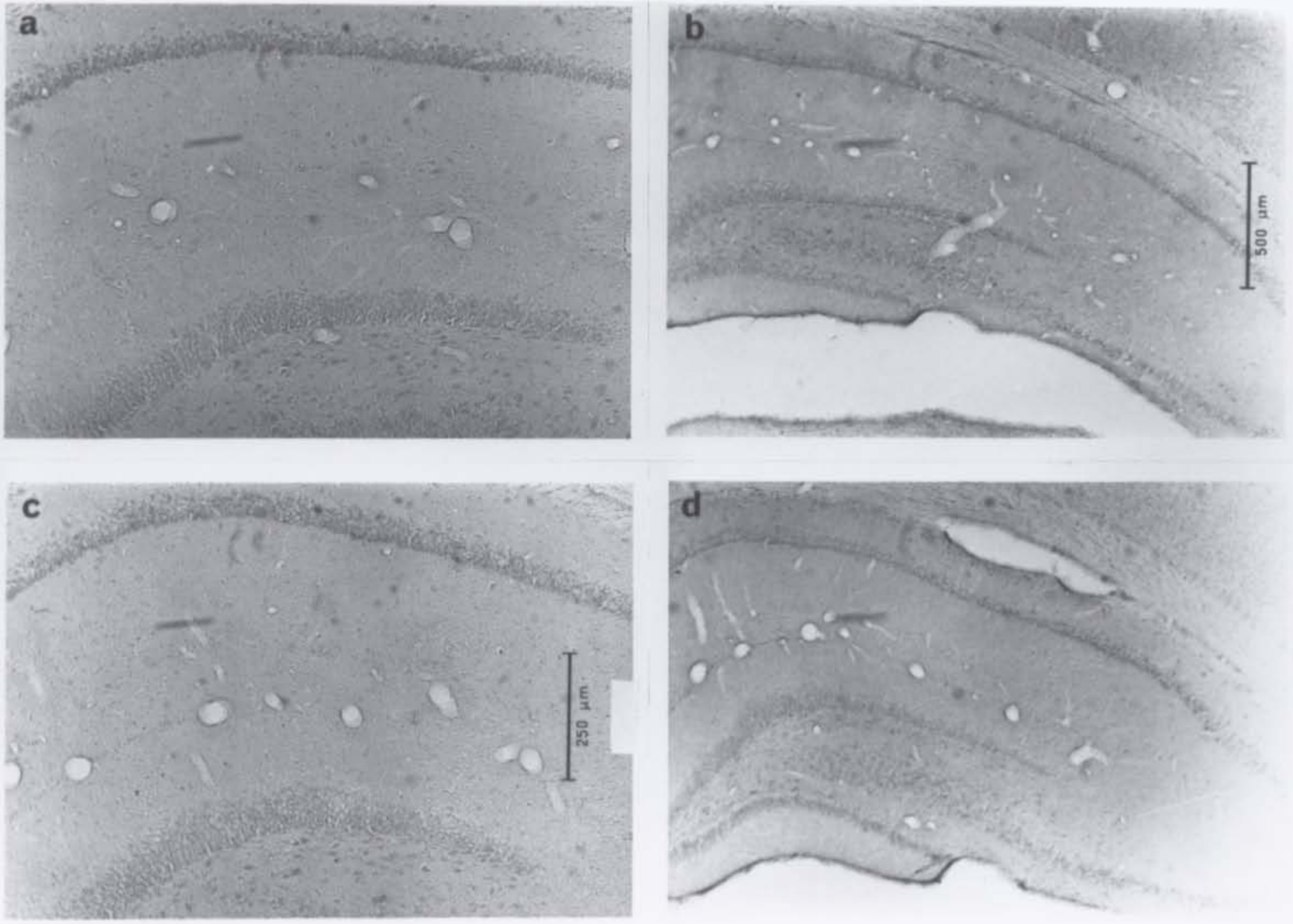

a
b
c
d
e

Figure 3.14 Photomicrograph of representative sections from PND 25 control and DHTB-treated females at two different magnifications. (a), (b) are from the control female, with (c), (d) from the DHTB-treated female. The scale bar in (c) is 250 μm and applies to (a) whereas it is 500 μm in (b) and applies to (d).


a
b
500 µm
c
250 µm
d

decreased staining in the other relative to same litter controls (see Figure 3.13). In the female, DHTB appears to decrease AR staining density in both litters relative to their untreated controls (Figure 3.14)

Within the CA3 cell layer, both litters show a similar effect from DHTB treatment with statistical analysis indicating a trend effect. Although AR density appears greater in DHTB-treated males and females compared to untreated littermates, this difference is not significant ($F_{(1,4)} = 5.04$, $p<0.10$). It remains to be determined whether a larger sample size would reveal a significant effect of DHTB treatment in this area. The data for CA3 are presented in Figure 3.15.

Figure 3.16 includes the data obtained from the DG cell layer. At PND 25, as at PND 7, the DG was not affected by DHTB treatment in either animal again supporting the use of this area as a stable cell layer background against which to compare AR densities in areas CA1 and CA3.

As indicated in the results for the ontogeny experiment and for the PND 7-DHTB experiment, there is no obvious difference in nuclear compared to cytoplasmic staining of either untreated or DHTB-treated animals.

Figure 3.15 Results obtained within CA3 following treatment with DHTB 30 minutes before sacrifice in PND 25 animals.

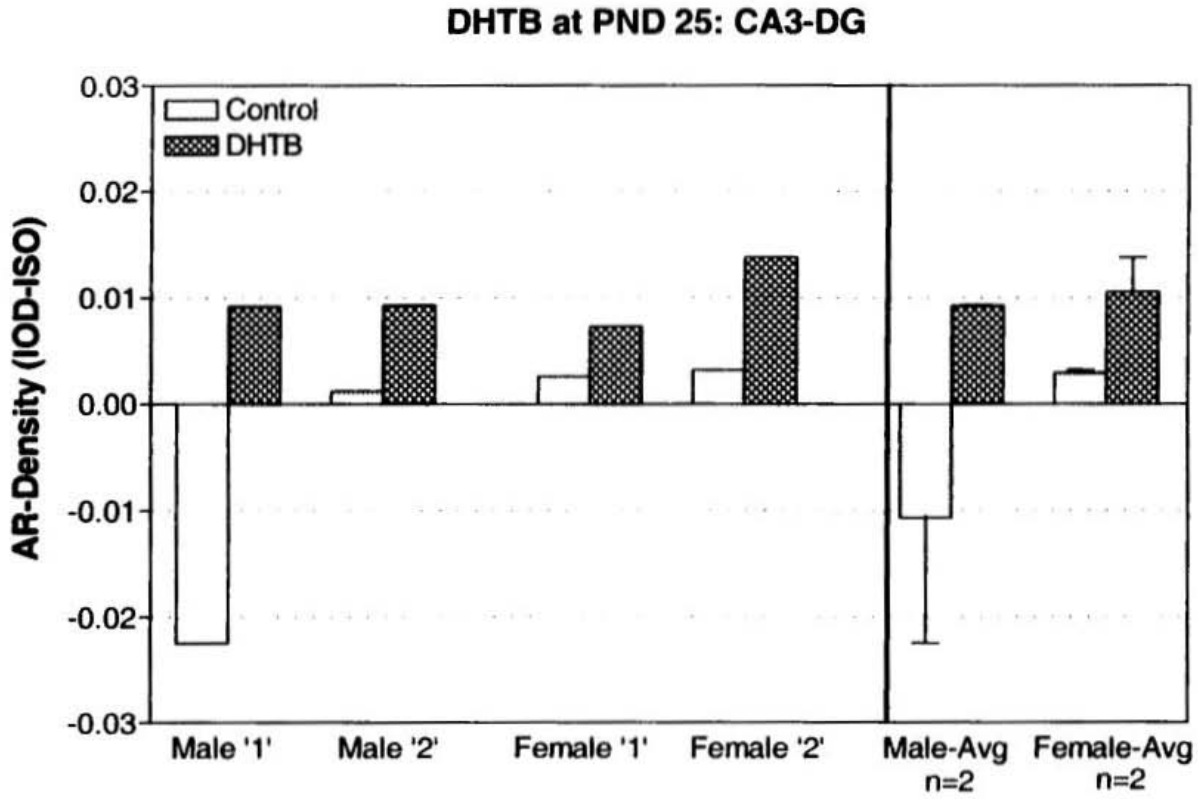
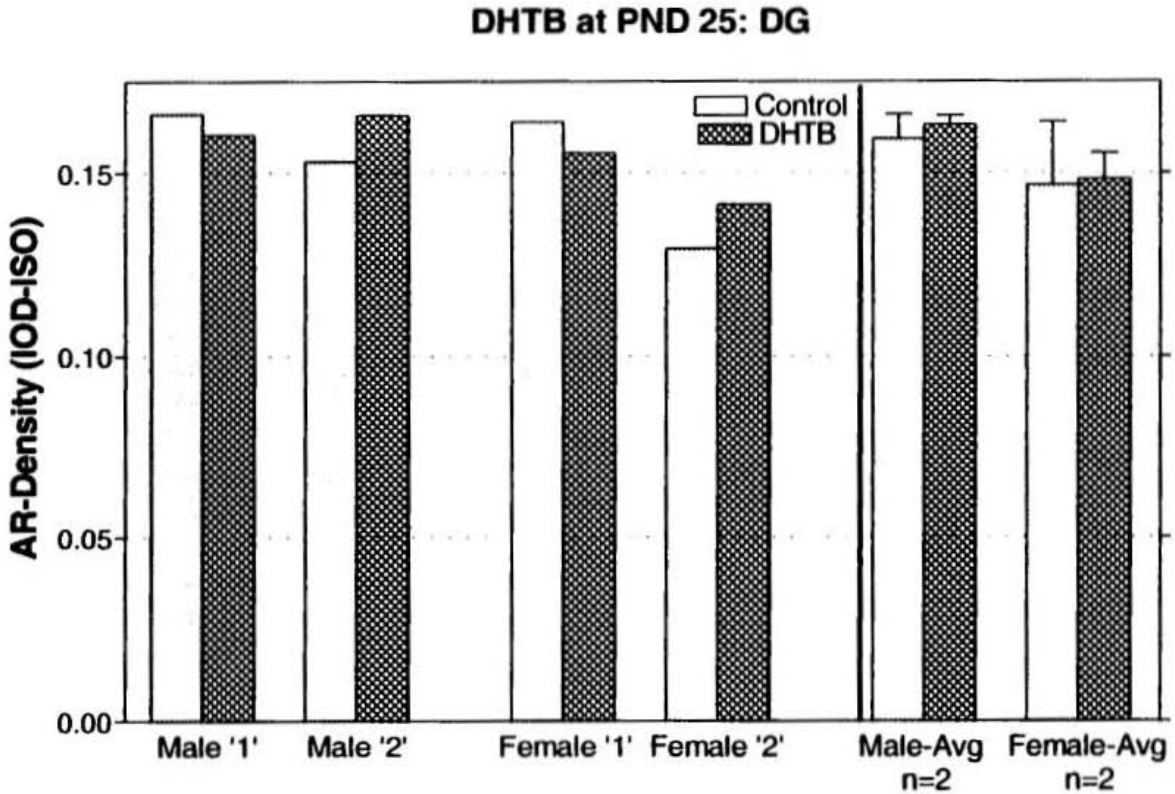

DHTB at PND 25: CA3-DG
AR-Density (IOD-ISO)
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
Control
DHTB
Male '1'
Male '2'
Female '1'
Female '2'
Male-Avg n=2
Female-Avg n=2

Figure 3.16 Results obtained within DG following treatment with DHTB 30 minutes before sacrifice in PND 25 animals.


DHTB at PND 25: DG
Control
DHTB
AR-Density (IOD-ISO)
0.15
0.10
0.05
0.00
Male '1'
Male '2'
Female '1'
Female '2'
Male-Avg n=2
Female-Avg n=2

CHAPTER 4: DISCUSSION

The present results provide the first description of the ontogeny of the AR in the hippocampus of the rat. Within CA1, AR staining is present in both sexes as early as PND 2, which is the earliest period examined. The density of AR staining then rises to peak at PND 7, a time when there may be a sex difference with greater density in the male. This peak is followed by a decline that reaches low values at PND 25. By PND 40, the AR density has increased again in both sexes but is approximately 1.6 times greater in the male compared to the female. The profile within CA3 is similar to that in CA1 at both PND 2 and PND 5. However, beginning at PND 7, several differences exist in AR staining between these two cell layers. Between PND 7 and PND 14, there is a steep decline in AR density within CA3 in both sexes. In contrast, within CA1 there is no difference between PND 7 and PND 14 AR density in either males or females. The rapid decrease in AR density observed in CA3 between PND 7 and PND 14 and the lack of staining at PND 25 suggests that the function of the AR in the neonatal hippocampus is completed earlier in CA3 then in CA1

A second notable difference occurs at PND 40. Within CA3, although AR density increases from PND 25 to PND 40, the increase only reaches levels observed at PND 14. However, within CA1, AR density reaches the highest level observed in the present study, with the male showing approximately 1.65 times the value found at PND 7. A further difference in AR density is the development-

related sex difference found in one cell layer of Ammon's horn but not in the other. Although no sex difference occurs at any age within CA3 in untreated animals, the male has greater AR staining within CA1 at PND 40 and possibly at PND 7.

4.1 Comparison of the Ontogeny of Hippocampal AR with Other Areas within the CNS

The ontogenic profile for the hippocampal AR contrasts with that demonstrated in other brain areas using a nuclear exchange assay [96]. A nuclear exchange assay only measures occupied receptors (translocated from the cytoplasm to the nucleus). Given the results of the present experiment indicating that DHTB treatment increases the AR density in PND 7 animals, suggesting that the number of occupied receptors is related to density level, a comparison of these two experiments appears reasonable. That is, the level of AR, determined by nuclear exchange assay, and the AR density, determined in the present experiment, might both relate to AR occupancy. In the Long-Evans untreated rat, the AR has been examined from PND 2 to PND 10 in nuclear exchange assays using ^{3}H-R1881 with results showing an area specific profile [96]. In the hypothalamus, amygdala, and septal areas, the nuclear AR binding peaks at PND 4 whereas in the preoptic area, this early peak is seen at PND 6 [96]. However, all areas reveal a sharp decline by PND 10 such as that observed in the present study at PND 25 for both CA1 and CA3.

Sex differences, with greater AR in the male compared to the female, are age as well as area dependent [96]. Although a sex difference is reported for the septum at PND 4 only, the male has more AR binding in the hypothalamic area at three ages, PND 2, 4, and 6 and the amygdala shows a sex difference at every age examined up to PND 10 [96]. No sex differences are reported for the preoptic area [96]. The sex difference reported here for CA1 at PND 7 in both untreated and DHTB-treated animals is at a time co-incidental with that observed in the hypothalamus and the amygdala. However, as indicated in the untreated animals, the neonatal sex difference is restricted to a shorter time period as observed for the septum. Indeed, unlike the hypothalamic-preoptic area, AR density in the hippocampal CA1 area appears very similar between males and females at every neonatal age except PND 7, separating again during puberty (at PND 40).

Thus, the comparison of the AR-ir density from the current study with the nuclear presence of AR in all brain areas examined reveals a similar ontogenic profile with a peak around the end of the first week [96]. However, such a similarity is not found in a comparison with cytoplasmic ARs concentrated in hypothalamic-preoptic cytosolic extracts. In such a preparation, AR concentration appears to increase from PND 2 up to PND 21, the latest age examined, with no decrease observed at any age [169].

The sex differences in the AR cited above for either the present study or the nuclear exchange assay study may reflect sex differences in either blood

androgen level, AR level, or a combination of both. As indicated in the introduction, a sex difference in brain levels of 'T' is present at PND 1-2 with significantly higher levels in the male [23]. Thus, if the level of serum 'T' is the sole determinant of the level of AR in the neonate, the sex difference should be present from the earliest ages examined. However, a sex difference in AR-ir staining does not occur at either PND 2 or PND 5 but appears to be specific for PND 7. In the current study, the question of whether or not there is a sex difference in AR level, not just a difference in AR occupation, was addressed by injecting PND 7 males and females with DHTB 30 minutes before sacrifice at a dose sufficient to ensure saturation of all ARs. As the data show, the sex difference still exists at PND 7 in the DHTB treated animals, suggesting that the AR staining density in CA1 is sexually dimorphic. However, regulation of AR-ir staining within the neonatal hippocampus could still be dependent upon the presence of serum 'T'. This question may be addressed in future experiments by long-term treatment with DHTB, an anti-androgen such as hydroxyflutamide, or by neonatal castration.

4.2 Comparison of the Ontogeny of Hippocampal AR with ER

In comparison to the ontogeny of the AR, the profile for the ontogeny of the ER within the hippocampus differs in several aspects. *In vitro* cytosolic binding of ^{3}H-estradiol in tissue from Sprague-Dawley rats assessed the ontogeny of the hippocampal ER [111]. Although the ER is now believed to be

nuclear bound, even in the absence of ligand [151,186], the *in vitro* study [111] used gonadectomized animals as a means of recycling the ER back to the cytoplasm. The report of ^3H-E_2 binding in hippocampal cytosols [111] therefore suggests that ^3H-E_2 is either binding to something other than the ER or that the ER is recycled back to the cytoplasm, at least in the hippocampus of the neonate. In a different study, the developmental profile of hippocampal ER mRNA was determined by RNase protection assay with 2-5 animals of the same sex pooled for each RNA sample [112]. The lack of a sex difference in the RNase protection assay resulted in the pooling of data for analysis [112]. While the ER [111] and ER mRNA [112] are present in the hippocampus as early as PND 2, the peak occurs at PND 5 [111,112], at least two days earlier than hippocampal AR and decreases again by PND 11 [111]. Whereas the hippocampal AR (present study) and ER [111] exhibit low levels before the onset of puberty, the AR increases dramatically in the early pubertal and adult CA1 region of the male whereas the ER is reported to remain at low levels in the adult [111].

The finding of a low level of the hippocampal ER may only apply to the classical ER-α, as the newly discovered ER-β receptor is reported to have a strong presence within hippocampal subfields in the adult [80]. However, although more cells within Ammon's horn contain ER-β mRNA than ER-α mRNA, the intensity of both signals is reportedly low [147] suggesting a limited presence of both versions of the protein. As well, the ontogeny of the newly discovered ER-β version has yet to be explored.

Unlike the hippocampal AR, the ER or ER mRNA within the hippocampus does not differ between males and females at any age examined [111,112]. A further difference between the AR and the ER relates to location. In the adult, the ER is located both within the CA1 cell layer, possibly within pyramidal neurons [80] and polymorphic interneurons [83], and in the interneurons of s. oriens, lucidum and radiatum [83]. However, the current results show that AR-ir staining is mainly in the CA1 and CA3 cell layers where density of staining is similar during the first postnatal week. At later ages, staining is much more prominent in CA1 than in CA3.

In summary, several differences exist between the AR and the ER in the hippocampus. In the neonate, the peak in AR density occurs two days later than the peak observed for the ER. In the adult, AR density appears to be significantly greater than that reported for the ER. As well, whereas the AR is localized within the pyramidal cell layers of Ammon's horn, the ER is present in both the pyramidal cell layer and in interneurons.

4.3 AR Regulation by Serum T?

As discussed in the introduction, in the adult the AR appears to be regulated by the presence of circulating 'T', with low levels decreasing and high levels increasing and/or stabilizing this protein. However, current data suggests that something other than the presence of serum 'T' is regulating the neonatal

AR. A review of serum 'T' levels in the neonatal rat, up to the third postnatal week may be helpful in the following discussion.

In the neonatal Sprague-Dawley and Wistar rat, serum 'T' is relatively stable from PND 2 up to PND 10 [34,81,116] and also up to PND 19 [41,50,79]. While serum 'T' is also stable in the Holtzman rat from PND 5 to PND 10, in contrast, it doubles from PND 10 up to PND 19, at which time it reverses and plummets to extremely low values by PND 25 [103].

The question may now be asked; is the level of circulating androgen related to the early presence of the AR within the CNS as determined by other studies and in particular by the present experiment? The relative stability of serum 'T' from PND 2 up to PND 10 (or PND 19) would predict no effect of age on AR density during this time and that any hippocampal area containing ARs would show a similar profile. The current data do not support such conclusions. Indeed, the ontogenic profile of CA1 indicates a significant increase in AR density from PND 2 to PND 7 with no difference between PND 7 and PND 14. However, within CA3, a different profile indicates a significant decrease from PND 7 to PND 14. Thus, not only does the hippocampal AR-ir staining vary with age whereas serum 'T' is constant during the first two postnatal weeks, but hippocampal AR density also varies with cell layer. Furthermore, although neonatal serum 'T' is stable in both males and females, all of the studies that examined both sexes indicate much lower levels of plasma 'T' and DHT in females compared to males [41,81,116,153]. Consequently, if circulating androgens

alone define AR levels within the neonate, then the male should contain more AR, not just at PND 7, but at every age examined. Current data again negate this supposition with no sex differences at PND 2, 5, 14 or 25. The cytosolic presence of the AR in non-hippocampal areas as defined by other studies also bears examination. Using untreated neonatal animals, cytosolic receptor assays from the hypothalamic-preoptic area were evaluated with either ^{3}H-R1881 in Long-Evans [96] or ^{3}H-DHT in the Sprague-Dawley [169] rat and mouse [45]. Results reveal an increasing presence of the AR from PND 2 to PND 6 and PND 10. Although such a profile would follow from decreasing levels of serum 'T', resulting in a shift of the AR from the nucleus to the cytoplasm, the level of serum 'T' is constant throughout this period. Thus, neither the increase in AR density from PND 2 to PND 7 observed in the present study nor the increase in cytoplasmic AR reported by others [45,96,169] for the hypothalamus can be accounted for by a change in serum 'T'.

The above discussion does not address whether serum 'T' plays any contributing role in AR density up to PND 14, but strongly implies that it is not the only contributor. Indeed, the present experiment provides support for the involvement of circulating androgens in AR density as indicated by DHTB treatment at PND 7. DHTB administered 30 minutes before sacrifice increases AR density in both males and females compared to controls, but elicits a stronger effect in males. However, the increased density in ARs detected under ICC may result from a DHTB-elicited conformational change in the receptor,

rather than by auto-regulation. Thus, androgens as well as a currently unknown factor may be involved in the ICC derived density of the AR for the first two to three weeks of life.

Assessment of the possible involvement of plasma 'T' with AR regulation at PND 25 also requires a review of serum 'T' levels at this age. The observation of extremely low values at PND 25 observed in the Holtzman rat extends to every other strain examined. Sprague-Dawley males are reported to have consistent levels of plasma 'T' up to approximately 3 weeks after birth, when values decline to the lowest levels observed at any time tested, increasing again 1-2 weeks later [41,79]. Although the fall is significant in males ($p = < 0.005$), a similar drop in serum 'T' observed in females is not [41], possibly due to the already low levels present in females. The decrease observed in serum 'T' within the third week of life in the Sprague-Dawley is of short duration, increasing within five days from a low of 0.37 (PND 21-25) to 0.95 (PND 26-30) ng/ml serum [79]. Maximum adult values occur from seven [41] to nine [79] weeks after birth. In the Wistar male, serum 'T' appears to show a 7-day cycle between PND 12 and PND 25 with low values at PND 17 and PND 23 [34]. The Ivanovac and Holtzman rat strains also show low levels of serum 'T' at PND 25 [56,103]. Although data is not available before PND 15 for the Ivanovac rat, low but stable levels of plasma 'T' and DHT are reported between PND 15 and PND 25 [56]. Plasma androgens begin to rise at PND 25 with DHT doubling and 'T' increasing by a factor of 5 to attain adult values within 3 days [56]. As reported for the Ivanovac

strain, plasma 'T' in the Holtzman rat increases significantly from PND 25 to double within 4 days [103].

The involvement of circulating androgens with AR regulation during the fourth postnatal week may be suggested by the co-incidence of decreasing values of serum 'T' and decreasing values of AR density observed at PND 25 in the present study. AR-ir density in both CA1 and CA3 is at its lowest level at this time. Within CA3, the density at PND 25 is significantly lower than at any other age examined whereas within CA1, it is comparable to that obtained at PND 2. While DHTB treatment at PND 7 indicates that circulating androgen increases hippocampal AR-ir density, the results of DHTB treatment at PND 25 suggests that the interaction of circulating androgens and the AR may involve a complex process. Thus, the influence of circulating androgens on hippocampal AR density in the untreated PND 25 animal remains to be resolved.

Regardless of whether or not serum 'T' exerts a primary influence on the presence of AR-ir in the neonatal hippocampus, circulating androgens do affect AR-ir density as indicated in DHTB-treated animals. As well, as reviewed in the introduction, serum 'T' is reported to affect AR density in the adult, perhaps by stimulating synthesis and/or reducing degradation of the AR. In both the neonate and the adult, circulating androgens may be necessary to activate the AR, whether nuclear or cytoplasmic. However, circulating androgens in the neonate may be equally important for stabilizing receptors located within the cytoplasm where degradation of unoccupied receptors by enzymes would be enhanced.

Although androgens increase the AR-ir density in the neonate, possibly by changing the conformation of the protein, there is little or no difference in nuclear compared to cytoplasmic staining observed in the neonatal ages. In both sexes, there is virtually no increased nuclear presence of the AR up to and including PND 25. While a faint (nuclear type) outline may be observed in a few scattered cells at PND 7 and 14, the thickness of the sections makes it virtually impossible to define whether the outline is related to increased nuclear stain or to the layering of cells. Thus, the AR in the neonate appears dispersed throughout the cell. Indeed, there is no obvious difference in AR cellular location whether the neonatal animal is DHTB-treated or not, suggesting that the presence of androgen does not elicit a nuclear shift. By PND 40, nuclear stain is visible and is much stronger relative to cytoplasmic stain in both sexes, suggesting a shift in AR. The nuclear presence of the AR in the PND 40 female may be short-lived as it is no longer present in the single PND 60 female examined in the current study. However, the male of the same age shows strong nuclear AR-ir density. In the neonate, the dispersed presence of the hippocampal AR may indicate an involvement in both non-genomic events, such as membrane activity, and nuclear-related genomic events. In the pubertal (and adult) male, the AR is predominantly nuclear and may involve only genomic-related events.

As previously noted, the AR undergoes a conformational change upon binding with its activating ligand. The change is thought to result in a

translocation from the cytoplasm to the cell nucleus. However, this does not imply that all binding-induced changes in the AR protein are limited to, and necessarily result in, translocation. The lack of greater 'nuclear' AR-ir density in the androgen-treated neonate may relate to a conformational change in the receptor that does not result in a nuclear shift. However, such androgen-induced activation of the AR may relate to non-genomic functions within the cytoplasm. Indeed, in the present study, DHTB treatment at PND 7 does not appear to evoke a conformational change in the AR protein resulting in translocation to the cell nucleus. It cannot be argued that DHTB was ineffective as it increased AR density in both males and females at PND 7. Thus, in the neonate, DHTB binds to hippocampal ARs, thereby enhancing density as measured by the PG-21 antibody, but may not elicit the conformational change required for nuclear translocation. Although it is possible that 30 minutes is not sufficient time for the nuclear shift to occur in neonates, nuclear immunostaining is reported in adult castrated rats within 15 minutes of DHT or 'T'-treatment [184]. It is therefore possible that some other factor is interfering with the AR conformational change that is associated with a nuclear shift. Thus, it would be of interest to determine what possible functions could be mediated by the cytoplasmic AR in the neonate.

4.4 Conformational Change and DHTB

Although it is not the intent of this discussion to pursue the molecular consequence of conformational changes in the AR, it must be noted that such modifications may not only interfere with antibody binding. Indeed, current data on the AR indicate that this receptor may be activated without the presence of its activating ligand [108,171]. As reported by Weigel and Zhang [171], steroid receptors, including the ER, PR and AR, all exhibit ligand-independent activation under appropriate conditions such as cell type, promoter and activator. Although the mechanisms involved in the induction of ligand-independent activation are still being explored, several such mechanisms have been identified. Data from both human prostate PC-3 cells and monkey kidney CV1 cells, indicate that the AR can be activated by a protein kinase A activator, forskolin, in the absence of androgen [108]. In a review paper, Tsai & O'Malley [165] indicate that phosphorylation not only enhances ligand-dependent activation of steroid receptors in a synergistic manner but induced phosphorylation can also activate receptors in the absence of ligand [165]. As well, both dopamine and growth factors, acting via membrane receptors, activate steroid receptors as demonstrated for the human ER and chicken PR [165]. Furthermore, steroid (glucocorticoid) receptors interact with the immediate early genes, Fos or Jun, within the nucleus to initiate down-regulation of the receptor [165]. It is therefore possible that at PND 25, the effect of DHTB will vary depending on the current conformation of the AR at the time of DHTB application. Perhaps phosphorylation via other signals (e.g., neurotrophic

factors such as brain-derived neurotrophic factor (BDNF) or neurotransmitters that increase cyclic adenosine 3'.5'-phosphate (cAMP)) changes the conformation of the AR so that it responds differently to DHTB.

It is of interest that DHTB treatment at PND 25 resulted in mixed effects in the CA1 area with a possible increase in one male and either no effect or a possible decrease in AR staining density in the other male and both females. DHTB treatment was administered to elicit an increase in AR-ir staining by countering the possible influence of a conformational change on PG-21 antibody staining and as such, either an increase or no effect was expected. The trend toward an increase in CA3 of the DHTB-treated males and females is consistent with the conformational change of the unoccupied AR reducing antibody binding that is reversed by the presence of the AR ligand. Likewise, the increase in AR-CA1 density in one of the DHTB-treated males (called the first male for clarity) at PND 25 agrees with the change in AR conformation. However, the lack of an effect or possible decrease in AR-CA1 density observed in the other (second) male and both females following DHTB-treatment suggests that the presence of the AR ligand evokes a conformational change that may limit (rather than enhance) antibody binding. The first male may already have been experiencing a pubertal-related increase in serum 'T' levels. If the pubertal-induced increase in serum 'T' initiates up-regulation of hippocampal ARs, then the presence of exogenous DHTB could ensure occupation of all AR proteins, especially if the amount of endogenous 'T' is not sufficient to bind all ARs. Alternatively, the AR

may be up-regulated at this time in the first male, by a signal independent of serum 'T' in advance of the pubertal surge, resulting in a DHTB-treated increase in enhanced density.

The data from CA3 appear to conflict with the suggestion of a difference between the males with respect to the presence or absence of an endogenous signal regulating the level of ARs within CA1. Both PND 25 males show increases under DHTB treatment. However, these two cell layers could be responding differently as indicated by AR-ir density observed at PND 40. At PND 40, the CA3-AR-ir density values remain low compared to the early neonatal ages whereas, within CA1, AR density reaches maximum values. Within CA3, the pubertal burst of 'T' does not elicit the increase observed within CA1 and again, the AR-ir density does not differ between males and females at any age examined. The CA1 cell layer may be more sensitive to AR activity and more susceptible to AR regulation at the PND 25 prepubertal stage. A further indication of the significance of this stage as being a sensitive period for CA1-AR activity comes from the data obtained from the DHTB-treated PND 7 animals.

DHTB treatment at PND 7 results in an AR-density increase in the CA1 region of both sexes with a trend in CA3. Thus, the data from PND 7 supports the suggestion that the decreased density in the CA1 cell layer produced by DHTB in the PND 25 male and both females is age-related and unique to this period of development. Again, the strong effect of DHTB treatment within the

CA1 area at PND 7 indicates that this area is sensitive to the presence of the AR ligand and responds to changes in 'T' levels.

Studies cited earlier may now be addressed in light of the current data. The presence of weakly labeled neurons in CA1 and CA2 in the PND 24 Holtzman female 90 minutes following an injection (sc) of ^{3}H-'T' [143] is in agreement with our data reporting low AR levels at PND 25. As well, autoradiograms from PND 26 Holtzman males, examined one hour following an injection (iv) of ^{3}H-DHT reveals the presence of nuclear AR in hippocampal CA1 and CA2 pyramidal neurons four days after combined castration and adrenalectomy [137]. Although the androgen label is reportedly weaker in the hippocampus than in other brain areas, it is stronger in CA1 and CA2 relative to CA3 [137] as found with AR-ir in the present study. Further interpretation of the autoradiographic data is limited by the lack of untreated controls for comparison [137]. However, in the adult, castration induces AR down-regulation/degradation and AR mRNA levels decrease in the hippocampus of the short-term (four days) male castrate [73]. Indeed, the autoradiographic data suggests that castration was effective in reducing the androgen label in the young adult as the distribution of the ^{3}H-DHT was reported to be similar between the PND 26 and PND 60 castrates [137]. This contrasts with data in the current study using intact animals, wherein the greatest difference observed in AR-ir occurs between PND 25 and PND 60, with the lowest density at PND 25. If regulation of the neonatal and adult ARs is similar, then castration at PND 22 should significantly reduce the

nuclear label by PND 26. Given the low density of AR-ir present at PND 25 in the intact animal, it is surprising that any detectable nuclear ^{3}H-DHT was observed in the short-term castrate of a similar age [137]. The lack of information about the AR and AR mRNA at neonatal and prepubertal ages makes it difficult to hypothesize about possible differences in the regulation of neonatal and adult AR. However, it is tempting to suggest that other factors control the regulation of the neonatal AR. One such factor could relate to the completed maturation of the CA1 hippocampal area.

Until the hippocampal AR is further explored, it is not known whether the neonatal and the adult presence of this receptor protein provide similar or different functions. It can be postulated that they provide different functions, with the apparent down-regulation observed at PND 25 signaling the end of the early period in both males and females. It is also not known at this time if the down-regulation of the AR occurring between PND 14 and PND 25 is due to autoregulation or other factors as suggested above. The function of the AR in the adult appears to be more restricted to the male as the AR may not return to the female hippocampus as observed by ICC in the single female examined at PND 60. However, as mentioned earlier, AR-ir, defined with a different antibody (PA1-110), is present in the hippocampus of the intact, untreated male and female rat at six months of age [29]. Sex differences were not reported [29]. As well, Simerly et al. [152] have demonstrated the presence of AR mRNA in CA1 of adult females.

One factor that must be considered in determining the AR-ir density within the adult female in the present study is that the antibody used in the present experiment may be more sensitive to the occupied, rather than the unoccupied, receptor. Such an effect has been demonstrated in the brain of the adult hamster [177], opossum [63] and rat [46], using the same antibody. The enhancement appears to be related to nuclear staining density [46,63,177], which is restored by 'T'-treatment of castrates. However, PG-21 is also capable of detecting cytoplasmic stain in the absence of androgen in the castrated adult [46,177]. Thus, this antibody can detect the unoccupied AR. Cytoplasmic staining of the AR using PG-21 appears to be dependent upon the length of time tissue is exposed to nickel-cobalt enhanced DAB [71,177], with 60 minutes giving maximum effect. However, the present study only used a total of 15 minutes in order to control background staining. Thus, although the current study clearly identifies AR density in the neonate, the level of AR present in the cytoplasm may be underestimated at each age and as such, may be especially applicable to the adult female. This issue relates to the PND 7 DHTB-treated animals, which show increased density when injected 30 minutes before perfusion. Alternatively, the difference in sensitivity of the PG-21 antibody to the occupied, versus the unoccupied, receptor in the adult could be due to a difference between the neonatal and adult AR. Thus, one could speculate about different 'isoforms' of the AR produced in neonatal versus adult brains, with the antibody, PG-21, binding

fairly well, but not maximally, to the unoccupied AR receptor in neonates, but not in adults.

4.5 Possible Functions of the AR within the Hippocampus

Within the hippocampus, possible functions related to the AR of the adult male include a role in the formation of neurotransmitter-stimulated cAMP. Incubation of hippocampal slices with histamine to induce cAMP formation reveals significant lower levels in adult castrated males compared to sham operated controls [59]. This is a sex-related effect as the opposite result occurs in females. OVX females show significantly higher levels of histamine-stimulated cAMP in hippocampal slices compared to OVX females treated with E_2 plus progesterone either 48 h or 24 h before sacrifice [59]. Thus, activity at the ER in the E_2 plus progesterone treated female or a lack of activity at the AR of the castrated male both achieve a similar effect; a decrease in histamine-stimulated cAMP, at least in hippocampal slices [59].

Activity at the AR in the hippocampus of the adult male modulates c-fos mRNA levels [74]. c-Fos mRNA induction in the hippocampus is related to exposure to a novel environment, as c-fos mRNA is not observed in animals sacrificed without, or at greater than two hour intervals following, such exposure [74]. Although not all 'immediate early genes' are affected by AR activity, gonadectomy of Fisher 344 male rats results in increased hippocampal levels of c-fos mRNA compared to intact controls following 20 minutes of open field

activity [74]. The effect is offset by the implantation of a Silastic capsule of DHT immediately following gonadectomy [74]. It is possible that c-fos mRNA increases in the gonadectomized compared to the intact animal, in response to inactivity at the AR. Thus, in the adult male, hippocampal AR activity is related to processes that may be linked to learning by limiting, thereby controlling the amount of c-fos mRNA induction following novel experience.

c-Fos was also explored by ICC in the prepubertal Sprague-Dawley male rat. Exposure of PND 16 or PND 23 males to a novel environment for 1 h before sacrifice has no effect on the level of CA1 hippocampal c-fos-ir [170]. However, exposure of PND 30 males to a novel environment increases c-fos-ir in the CA1 compared to controls [170]. Furthermore, the effect is not seen in the DG or in CA3 [170]. Whereas the involvement of other aspects of hippocampal maturation cannot be ruled out in the effect of exposure to a novel environment on c-fos-ir, the earlier discussion (see last section) indicating the involvement of c-fos in steroid receptor down-regulation [165] suggests a possible juvenile role for c-fos that is not related to learning and therefore not modified by exposure to a novel environment.

As well as the effect of age on novel exposure, Waters et al. [170] cites various references reporting similar age-related results for tasks involving spontaneous alteration, spatial learning, conditioning to contextual cues and working memory in which behavior impairment is observed until the 4th week of life. Electrophysiological data also suggests a difference in hippocampal

response between the age of PND 16 and 30 [170]. Long-term potentiation (LTP), the phenomenon reported to underlie the hippocampal involvement in memory formation, was induced by high-frequency stimulation (two 1 sec bursts of 100 Hz; separated by 10 sec) in hippocampal slices from both Sprague-Dawley control males and males exposed to a novel environment [170]. At both PND 16 and PND 23, there is no difference between the treated and control males in the LTP-induced slope of the extracellular population spike potential (EPSP). However, the EPSP slope is decreased in the PND 30 males exposed to the novel environment compared to controls. The effect is not related to the ability to induce hippocampal CA1-LTP, as adult-like LTP is present in the control animals at the earliest age tested (PND 16) [170]. Thus, the plasticity is functional but does not respond to the environment before PND 30 [170]. The decreased slope of the EPSP to LTP-stimulation at PND 30 is suggested to result from the prior activation of CA1 by the novel environment [170]. The lack of any effect on the EPSP slope earlier than PND 30 suggests that such novel exposure does not elicit a similar response in the juvenile CA1. The hippocampus of the juvenile rat is 'not ready' to process such information, possibly because 'other events' still demand priority. For example, perhaps it would be detrimental for a neonatal or juvenile animal to 'learn' that its 'food' is associated with a given site rather than with its mother, who will undoubtedly change location over time. Whatever mechanism is associated with such a functional effect in the juvenile must be similar in both males and females. This criterion is met by the neonatal presence

of the AR in both sexes, albeit there appears to be a slight dominance in favor of the male. Of course, there is presently no evidence to support the involvement of the AR in this function and it is realized that any other aspect of the juvenile hippocampus that is not sexually dimorphic may be involved, either exclusive of, or in synergy with, the AR. Consequently, whether the AR is associated with the 'inhibition' of these learning paradigms remains to be determined. However, it is of interest that serum 'T' levels in the Sprague-Dawley rise from 0.37 ng/ml recorded for the period between PND 21-25 up to 0.97 ng/ml observed by PND 26-30 [79], an increase of approximately 250%. Thus, increased AR production and activation in males at puberty could be a factor in producing the dramatic functional changes that occur in the hippocampus at this time.

A similar argument may be made for the monkey. As cited in the introduction, AR activation appears to be involved in visual discrimination learning in the anterior inferior temporal cortex with the immature female outperforming the immature male monkey [8]. The sex difference disappears with maturation. Thus, in the monkey, activity at the juvenile AR appears to be related to delaying a particular form of 'learning'. The interpretation of the sex difference in the monkey is suggested to involve an androgenic effect on maturation of the inferior temporal cortex, possibly by influencing neural connectivity. It is also possible that the AR is responsible for 'repressing' learning until maturation is complete, thereby promoting critical connections before any learning-induced weighting occurs. It is of particular interest that the temporal lobe also contains

the entorhinal cortex, the major input, via the subiculum, into the hippocampal tri-synaptic circuit involving the DG and Ammon's horn [121]. As well, hippocampal CA1 outputs return back to the entorhinal cortex [160]. Although it is not known whether a sex difference exists in the ontogeny of the AR in the hippocampus of the monkey, such a difference is inferred in this discussion and could be verified by experimentation.

The AR may also be functional in the prevention of hippocampal cell death given that 'T' has been shown to prevent cell death in the sexually dimorphic spinal nucleus of the bulbocavernosus (SNB) [110]. The motoneurons of the SNB and their target muscles, the bulbocavernosus and levator ani that attach to the penis, play a role in male copulatory behavior. Perinatal females treated with 'T' from embryonic day 16 through PND 5, examined from embryonic day 18 through PND 10, do not differ from control males in the number of SNB motorneurons [110]. However, the 'T'-treated females have significantly more neurons than control females [110]. As indicated by Freeman et al. [46], the presence of the *Tfm* mutation limits binding of 'T' to the AR by 85-90% and results in feminization of the SNB system. Surprisingly, as reviewed by Breedlove [17], the prevention of cell death in SNB motoneurons is secondary to the androgen-related prevention of atrophy of the bulbocavernosus and levator ani muscles. However, the effect on the SNB is related, either directly or indirectly, to the AR (rather than the ER) as the male pattern occurs in 'T'-treated neonatal females

and the female pattern occurs when androgen binding is pharmacologically blocked [17].

As well as a possible function in the prevention of cell death, the AR could play an active role in connectivity with target sites. The effect of activity at the AR was investigated *in vitro* using rat pheochromocytoma cells that were transfected with cDNA's for either the human ER (hER) or AR (hAR) [84]. In the presence of NGF, both hER and hAR cells exhibit induced neurite outgrowth. The addition of E_2 to hER cells in the presence of NGF increases the length of the neurite, increases neuritic spine formation and interneuritic connections [84]. Although the addition of DHT to hAR cells with NGF does not increase the length of neurite growth, it does promote increases in neurite outgrowth, primarily by increasing branching and arborization [84]. Thus, activation of the neonatal AR could be instrumental in providing a 'larger target area' to 'capture' incoming connections in general, with the ER functioning to increase specific synaptic connections by stimulating spine formation. It is of interest that the ER is reportedly located in hippocampal interneurons in adults [80,83,172] and in the hippocampus of the neonate [110]. The demonstrated presence of the AR in the neonatal hippocampus may indicate a function in maturation by providing an 'enhanced' target area for potential afferents.

The presence of the hippocampal AR in the neonate from birth to a time greater than PND 14 suggests that any related function occurs over an extended period. The AR is present during the extended birth of the DG granule cells,

which show 50% formation by PND 5 with a further 5-10% still to differentiate as late as PND 18 [4,11,12]. The DG mossy fiber axons presumably extend toward the CA3 pyramidal cells shortly after neuronal birth, with the peak of axonal projection possibly coinciding with the AR density peak observed in both CA3 and CA1 at PND 7. In fact, DG mossy fiber density is reported to increase 5-fold from PND 3 (1.1 μm) to PND 9 (5.7 μm) [5], with only a 2-fold increase observed between PND 9 and PND 77 [5]. The 5-fold increase in DG mossy fiber density occurs during the period of maximum AR density in CA3 (at PND 7).

Following the DG-CA3 connection, the CA3 Schaffer collaterals project toward CA1 pyramidal cells to form synapses onto the basal and apical dendrites. As reported above for the AR and the DG mossy fibers, a similar correlation exists between the AR and the CA1 synaptic area. Within CA1, the length of the apical dendritic shaft increases 50% and the total length of the lateral branches increases 3-fold from PND 5 to PND 10 (cited in Zimmer and Haug [187]). Furthermore, Timm silver sulphide stain indicates that the CA1 s. radiatum fibers increase gradually up to PND 8, then rapidly between PND 8-12 [187]. This is a period when AR density is decreasing in CA3 but remains at a high level in CA1.

Although it would be advantageous for the AR 'enhanced' target area to be induced prior to the arrival of incoming afferents, it would not be necessary to maintain the AR protein nor the stimulation of neurite outgrowth once synaptogenesis has occurred. Thus, if the AR is involved in organizing the

hippocampal tri-synaptic circuit between the DG, CA3 and CA1, it would be expected that the AR involvement would near completion in CA3 prior to completion within CA1. Using the previous logic, it would also follow that AR density would decrease earlier in the CA3 area relative to CA1. This corresponds to the finding reported here wherein AR density begins a significant decline in the CA3 area between PND 7 and PND 14, a period when the AR density within CA1 does not differ from the peak observed at PND 7.

The AR may also play an indirect role in promoting hippocampal development and synaptogenesis by increasing aromatization (discussed earlier). Although aromatase activity [86] and aromatase mRNA [78] appear to be relatively low in the hippocampus of the neonate, its presence has been documented. Indeed, the presence of the ER itself within Ammon's horn is reportedly low [83,158,172]. Whether this conclusion will change appreciably with the discovery of a new version of the ER is still to be determined. Although both the α- and newly discovered β- version of the ER mRNA is dispersed throughout the dorsal to ventral extent of the hippocampus, the intensity of the signal is reportedly low [147] and data on the level of the ER-β within most of the hippocampus is still lacking. However, activity at the neonatal AR may be functional in ensuring that ER activity is maximized by increasing the aromatization of 'T' to E_2, thereby providing a steady supply of ER ligand.

Whereas the AR may be related to sexual behavior by 'organizing' systems later 'activated' by gonadal hormones, this view of the function of the

AR is too narrow. Indeed, as indicated above, there are many possible functions for the neonatal AR within the hippocampus such as a modifying role in second messenger (cAMP) [59] and immediate early gene (c-fos) [74] responses. Such a role could provide the mechanism whereby AR activity induces an increase in neurite outgrowth [84] and prevents hippocampal cell death as demonstrated by the influence of target areas on the motoneurons of the SNB [17,110]. In addition, the hippocampal AR may be involved in 'organizing' (as indicated in the monkey [8]) and 'activating' circuitry that is involved in certain types of learning. As well, the AR could be functional in delaying the onset of hippocampal-related learning until maturation is complete as demonstrated by the lack of a learning-induced effect on the slope of the hippocampal EPSP in young rats [170]. Thus, electrophysiological data from the rat [170] and impairment on learning-related tasks in the rat (cited by Waters et al. [170]) and monkey [8] give credence to an early function for the AR that has higher priority in the survival of the animal than hippocampal-related types of learning. Such a function for the AR, as already postulated, could involve maturation and synaptogenesis.

Experiments involving possible functions of the neonatal AR in the hippocampus have not yet been fully explored except as noted above. It is possible that sex differences in the adult relate to the early presence of the hippocampal AR. However, it is also possible, given the presence of the neonatal AR in both sexes, that the function is not solely related to sex differences in the adult, but to the survival and synaptogenesis of information

processing systems in both sexes. The pursuit of possible functions of the AR in non-sexual behavior has just begun.

Ignoring the neonatal presence of this gonadal hormone receptor within the hippocampus is to refuse to acknowledge the presence of a phenomenon that has apparently been selected for and that may ultimately provide further insight into the magic of the central nervous system. We cannot simply allocate this receptor protein to a single, albeit, major purpose, that of copulatory behavior. Nature is far too frugal!

References

1 Abdelgadir, S.E., Resko, J.A., Ojeda, S.R., Lephart, E.D., McPhaul, M.J. and Roselli, C.E. Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain, *Endocrinol.* 135 (1994) 395-401.

2 Altman, J. and Bayer, S.A. Prolonged sojourn of developing pyramidal cells in the intermediate zone of the hippocampus and their settling in the stratum pyramidale, *J. Comp. Neurol.* 301 (1990) 343-364.

3 Altman, J. and Bayer, S.A. Mosaic organization of the hippocampal neuroepithelium and the multiple germinal sources of dentate granule cells, *J. Comp. Neurol.* 301 (1990) 325-342.

4 Altman, J. and Bayer, S.A. Migration and distribution of two populations of hippocampal granule cell precursors during the perinatal and postnatal periods, *J. Comp. Neurol.* 301 (1990) 365-381.

5 Amaral, D.G. and Dent, J.A. Development of the mossy fibers of the dentate gyrus: 1. A light and electron microscopic study of the mossy fibers and their expansions, *J. Comp. Neurol.* 195 (1981) 51-86.

6 Arnold, A.P. and Breedlove, S.M. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis, *Horm. Behav.* 19 (1985) 469-498.

7 Attardi, B. and Ohno, S. Androgen and estrogen receptors in the developing mouse brain, *Endocrinol.* 99 (1976) 1279-1290.

8 Bachevalier, J. and Hagger, C. Sex differences in the development of learning abilities in primates, *Psychoneuroendocrinol.* 16 (1991) 177-188.

9 Bardin, C.W., Brown, T., Isomaa, V.V. and Jänne, O.A. Progestins can mimic, inhibit and potentiate the actions of androgens, *Pharmacol. Ther.* 23 (1984) 443-459.

10 Baum, M.J. A comparison of the effects of methyltrienolone (R1881) and 5α-dihydrotestosterone on sexual behavior of castrated male rats, *Horm. Behav.* 13 (1979) 165-174.

11 Bayer, S.A. Development of the hippocampal region in the rat. I. Neurogenesis examined with ^{3}H-thymidine autoradiography, *J. Comp. Neurol.* 190 (1980) 87-114.

12 Bayer, S.A. and Altman, J. Hippocampal development in the rat: cytogenesis and morphogenesis examined with autoradiography and low-level x-irradiation, *J. Comp. Neurol.* 158 (1974) 55-80.

13 Berenbaum, S.A., Korman, K. and Leveroni, C. Early hormones and sex differences in cognitive abilities, *Learning and Individual Differences*, 7 (1995) 303-321.

14 Berger, T.W. and Bassett, J.L. System properties of the hippocampus. In I. Gormezano and E.A. Wasserman (Eds.) *Learning and Memory: The Behavioral and Biological Substrates*, Lawrence Erlbaum, Hillsdale, N.J. 1992, pp. 275-320.

15 Bonsall, R.W. and Michael, R.P. Developmental changes in the uptake of testosterone by the primate brain, *Neuroendocrinology*, 55 (1992) 84-91.

16 Booth, J.E. Sexual behaviour of neonatally castrated rats injected during infancy with oestrogen and dihydrotestosterone, *J. Endocrinol.* 72 (1977) 135-141.

17 Breedlove, S.M. Sexual dimorphism in the vertebrate nervous system, *J. Neurosci.* 12 (1992) 4133-4142.

18 Breedlove, S.M. Sexual differentiation of the human nervous system, *Annu. Rev. Psychol.* 45 (1994) 389-418.

19 Brinton, R.D. The neurosteroid 3α-hydroxy-5α-pregnan-20-one induces cytoarchitectural regression in cultured fetal hippocampal neurons, *J. Neurosci.* 14 (1994) 2763-2774.

20 Brown, R.E. Steroid and thyroid hormone receptors. In R.E. Brown (Ed.) *An Introduction to Neuroendocrinology*, Cambridge University, Cambridge, 1994, pp. 147

21 Brown, R.E. Regulation of hormone synthesis, storage, release, transport and deactivation. In R.E. Brown (Ed.) *An Introduction to Neuroendocrinology*, Cambridge, Cambridge, 1994, pp. 113-126.

22 Burgess, L.H. and Handa, R.J. Hormonal regulation of androgen receptor mRNA in the brain and anterior pituitary gland of the male rat, *Molecular Brain Research*, 19 (1993) 31-38.

23 Butte, J.C., Moore, J.A. and Kakihana, R. Brain and plasma levels of testosterone, dihydrotestosterone and estradiol in the one-day-old rat, *Life Sci.* 24 (1979) 2343-2350.

24 Callard, G.V. Androgen and estrogen actions in the vertebrate brain, *American Zoologist*, 23 (1983) 607-620.

25 Callard, G.V., Petro, Z. and Ryan, K.J. Conversion of androgen to estrogen and other steroids in the vertebrate brain, *American Zoologist*, 18 (1978) 511-523.

26 Carter, C.S. Neuroendocrinology of sexual behavior in the female. In J.B. Becker, S.M. Breedlove and D. Crews (Eds.) *Behavioral Endocrinology*, MIT Press, London, 1991, pp. 73-87.

27 Choate, J.V.A., Slayden, O.D. and Resko, J.A. Immunocytochemical localization of androgen receptors in brains of developing and adult male rhesus monkeys, *Endocrine*, 8 (1998) 51-60.

28 Christensen, L.W. and Clemens, L.G. Blockade of testosterone-induced mounting behavior in the male rat with intracranial application of the aromatization inhibitor androst-1,4,6-triene-3,17-dione, *Endocrinol.* 97 (1975) 1545-1551.

29 Clancy, A.N., Bonsall, R.W. and Michael, R.P. Immunohistochemical labeling of androgen receptors in the brain of rat and monkey, *Life Sci.* 50 (1992) 409-417.

30 Clancy, A.N., Zumpe, D. and Michael, R.P. Intracerebral infusion of an aromatase inhibitor, sexual behavior and brain estrogen receptor-like immunoreactivity in intact male rats, *Neuroendocrinol.* 61 (1995) 98-111.

31 Clark, A.S., MacLusky, N.J. and Goldman-Rakic, P.S. Androgen binding and metabolism in the cerebral cortex of the developing rhesus monkey, *Endocrinol.* 123 (1988) 932-940.

32 Corbier, P., Kerdelhue, B., Picon, R. and Roffi, J. Changes in testicular weight and serum gonadotropin and testosterone levels before, during, and after birth in the perinatal rat, *Endocrinol.* 103 (1978) 1985-1991.

33 Corpéchot, C., Baulieu, E-E. and Robel, P. Testosterone, dihydrotestosterone and androstanediols in plasma, testes and prostates of rats during development, *Acta Endocrinol.* 96 (1981) 127-135.

34 Csernus, V. Production of sexual steroids in rats during pre- and early postnatal life, *Exp. Clin. Endocrinol.* 88 (1986) 1-5.

35 Denef, C., Magnus, C. and McEwen, B.S. Sex differences and hormonal control of testosterone metabolism in rat pituitary and brain, *J. Endocrinol.* 59 (1973) 605-621.

36 Denef, C., Magnus, C. and McEwen, B.S. Sex-dependent changes in pituitary 5α-dihydrotestosterone and 3α-androstenediol formation during post-natal development and puberty in the rat, *Endocrinol.* 94 (1974) 1265-1274.

37 Diaz, D.R., Fleming, D.E. and Rhees, R.W. The hormone-sensitive early postnatal period for sexual differentiation of feminine behavior and luteinizing hormone secretion in male and female rats, *Dev. Brain Res.* 86 (1995) 227-232.

38 DonCarlos, L.L. and Handa, R.J. Developmental profile of estrogen receptor mRNA in the preoptic area of male and female neonatal rats, *Dev. Brain Res.* 79 (1994) 283-289.

39 DonCarlos, L.L., McAbee, M., Ramer-Quinn, D.S. and Stancik, D.M. Estrogen receptor mRNA in the preoptic area of neonatal rats are responsive to hormone manipulation, *Dev. Brain Res.* 84 (1995) 253-260.

40 Döhler, K.D., Hancke, J.L., Srivastava, S.S., Hofmann, C., Shryne, J.E. and Gorski, R.A. Participation of estrogens in female sexual differentiation of the brain; neuroanatomical, neuroendocrine and behavioral evidence. In G.J. De Vries, J.P.C. De Bruin, H.B.M. Uylings and M.A. Corner (Eds.) *Progress in Brain Research (Sex Differences in the Brain)*, Elsevier, Amsterdam, 1984, pp. 99-117.

41 Döhler, K.D. and Wuttke, W. Changes with age in levels of serum gonadotrophins, prolactin, and gonadal steroids in prepubertal male and female rats, *Endocrinol.* 97 (1975) 898-907.

42 Fahrbach, S.E. and Pfaff, D.W. *The Physiological Mechanisms of Motivation*, Springer-Verlag, New York, 1982, pp. 258

43 Feder, H.H. Specificity of steroid hormone activation of sexual behaviour in rodents. In J.B. Hutchison (Ed.) *Biological Determinants of Sexual Behaviour*, John Wiley & Sons, Toronto, 1978, pp. 395-424.

44 Foidart, A., Harada, N. and Balthazart, J. Aromatase-immunoreactive cells are present in mouse brain areas that are known to express high levels of aromatase activity, *Cell Tissue Research*, 280 (1995) 561-574.

45 Fox, T.O., Vito, C.C. and Wieland, S.J. Estrogen and androgen receptor proteins in embryonic and neonatal brain: hypotheses for roles in sexual differentiation and behavior, *Amer. Zool.* 18 (1978) 525-537.

46 Freeman, L.M., Padgett, B.A., Prins, G.S. and Breedlove, S.M. Distribution of androgen receptor immunoreactivity in the spinal cord of wild-type, androgen-sensitive and gonadectomized male rats, *J. Neurobiol.* 27 (1995) 51-59.

47 Gaulin, S.J.C. and Fitzgerald, R.W. Sex differences in spatial ability: an evolutionary hypothesis and test, *Am. Nat.* 127 (1986) 74-88.

48 Gaulin, S.J.C. and Fitzgerald, R.W. Sexual selection for spatial-learning ability, *Anim. Behav.* 37 (1989) 322-331.

49 George, F.W. and Ojeda, S.R. Changes in aromatase activity in the rat brain during embryonic, neonatal, and infantile development, *Endocrinol.* 111 (1982) 522-529.

50 Gogan, F., Slama, A., Bizzini-Koutznetzova, B., Dray, F. and Kordon, C. Importance of perinatal testosterone in sexual differentiation in the male rat, *J. Endocrinol.* 91 (1981) 75-79.

51 Gorski, R.A. Critical role for the medial preoptic area in the sexual differentiation of the brain. In G.J. De Vries, J.P.C. De Bruin, H.B.M. Uylings and M.A. Corner (Eds.) *Progress in Brain Research*, Elsevier, Amsterdam, 1984, pp. 129-146.

52 Gorski, R.A. Sexual Differentiation of the Brain: Mechanisms and Implications for Neuroscience. In S.S. Easter, K.F. Barald and B.M. Carlson (Eds.) *From Message to Mind*, Sinauer Associates Inc, Sunderland, Mass, 1988, pp. 256-271.

53 Goudsmit, E., Van de Poll, N.E. and Swaab, D.F. Testosterone fails to reverse spatial memory decline in aged rats and impairs retention in young and middle-aged animals, *Behav. Neural. Biol.* 53 (1990) 6-20.

54 Gould, E., Woolley, C.S., Frankfurt, M. and McEwen, B.S. Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood, *J. Neurosci.* 10 (1990) 1286-1291.

55 Goy, R.W. and McEwen, B.S. *Sexual Differentiation of the Brain*, MIT Press, Cambridge, MA, 1980.

56 Gupta, D., Zarzychi, J. and Rager, K. Plasma testosterone and dihydrotestosterone in male rats during sexual maturation and following orchidectomy and experimental bilateral cryptopchidism, *Steroids*, 25 (1975) 33-42.

57 Handa, R.J., Connolly, P.B. and Resko, J.A. Ontogeny of cytosolic androgen receptors in the brain of the fetal rhesus monkey, *Endocrinol.* 122 (1988) 1890-1896.

58 Handa, R.J., Kerr, J.E., DonCarlos, L.L., McGivern, R.F. and Hejna, G. Hormonal regulation of androgen receptor messenger RNA in the medial preoptic area of the male rat, *Molecular Brain Research*, 39 (1996) 57-67.

59 Harrelson, A. and McEwen, B. Gonadal steroid modulation of neurotransmitter-stimulated cAMP accumulation in the hippocampus of the rat, *Brain Res.* 404 (1987) 89-94.

60 Hart, B.L. Neonatal dihydrotestosterone and estrogen stimulation: effects on sexual behavior of male rats, *Horm. Behav.* 8 (1977) 193-200.

61 Hines, M. and Sandberg, E.C. Sexual differentiation of cognitive abilities in women exposed to diethylstilbestrol (DES) prenatally, *Horm. Behav.* 30 (1996) 354-363.

62 Hutchison, J.B. and Steimer, T. Androgen metabolism in the brain: behavioural correlates. In G.J. De Vries, J.P.C. De Bruin, H.B.M. Uylings and M.A. Corner (Eds.) *Sex Differences in the Brain (Progress in Brain Research)*, Elsevier, New York, 1984, pp. 23-51.

63 Iqbal, J., Swanson, J.J., Prins, G.S. and Jacobson, C.D. Androgen receptor-like immunoreactivity in the Brazilian opossum brain and pituitary: distribution and effects of castration and testosterone replacement in the adult brain, *Brain Res.* 703 (1995) 1-18.

64 Isgor, C. and Sengelaub, D.R. Prenatal gonadal steroids affect adult spatial behavior, CA1 and CA3 pyramidal cell morphology in rats, *Horm. Behav.* 34 (1998) 183-198.

65 Jacobs, L.F., Gaulin, S.J.C., Sherry, D.F. and Hoffman, G.E. Evolution of spatial cognition: sex-specific patterns of spatial behavior predict hippocampal size, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 6349-6352.

66 Jacobs, L.F. and Spencer, W. Patterns of natural spatial behavior predict hippocampal size in kangaroo rats, *Soc. Neurosci. Abst.* 21 (1991) 134

67 Jordan, C.L., Padgett, B., Hershey, J., Prins, G. and Arnold, A. Ontogeny of androgen receptor immunoreactivity in lumbar motorneurons and in the sexually dimorphic levator ani muscle of male rats, *J. Comp. Neurol.* 379 (1997) 88-98.

68 Joseph, R., Hess, S. and Birecree, E. Effects of hormone manipulations and exploration on sex differences in maze learning, *Behav. Biol.* 24 (1978) 364-377.

69 Kangas, L. Agonistic and antagonistic effects of antiestrogens in different target organs, *Acta Oncologica*, 31 (1992) 143-146.

70 Kashon, M.L., Arbogast, J.A. and Sisk, C.L. Distribution and hormonal regulation of androgen receptor immunoreactivity in the forebrain of the male European ferret, *J. Comp. Neurol.* 376 (1996) 567-586.

71 Kashon, M.L., Hayes, M.J., Shek, P.P. and Sisk, C.L. Regulation of brain androgen receptor immunoreactivity by androgen in prepubertal male ferrets, *Biol. Reprod.* 52 (1995) 1198-1205.

72 Kemppainen, J.A., Lane, M.V., Sar, M. and Wilson, E.M. Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation, *J. Biol. Chem.* 267 (1992) 968-974.

73 Kerr, J.E., Allore, R.J., Beck, S.G. and Handa, R.J. Distribution and hormonal regulation of androgen receptor (AR) and AR messenger ribonucleic acid in the rat hippocampus, *Endocrinol.* 136 (1995) 3213-3221.

74 Kerr, J.E., Beck, S.G. and Handa, R.J. Androgens selectively modulate *c-fos* messenger RNA induction in the rat hippocampus following novelty, *Neurosci.* 74 (1996) 757-766.

75 Krey, L.C. and McGinnis, M.Y. Time-courses of the appearance/disappearance of nuclear androgen + receptor complexes in the brain and adenohypophysis following testosterone administration/withdrawal to castrated male rats: relationships with gonadotropin secretion, *J. Steroid Biochem.* 35 (1990) 403-408.

76 Kühnemann, S., Brown, T.J., Hochberg, R.B. and MacLusky, N.J. Sex differences in the development of estrogen receptors in the rat brain, *Horm. Behav.* 28 (1994) 483-491.

77 Landgren, S.O. Pregnanolone (3α-hydroxy-5α-pregnane-20-one), a progesterone metabolite, facilitates inhibition of synaptic transmission in the Schaffer collateral pathway of the guinea pig hippocampus in vitro. *Epilepsy Res.* 10(2-3) (1991) 156-165.

78 Lauber, M.E. and Lichtensteiger, W. Pre- and postnatal ontogeny of aromatase cytochrome P450 messenger ribonucleic acid expression in the male rat brain studied by *in situ* hybridization, *Endocrinol.* 135 (1994) 1661-1668.

79 Lee, V.W.K., De Kretser, D.M., Hudson, B. and Wang, C. Variations in serum FSH, LH and testosterone levels in male rats from birth to sexual maturity, *J. Reprod. Fert.* 42 (1975) 121-126.

80 Li, X., Schwartz, P.E. and Rissman, E.F. Distribution of estrogen receptor-β-like immunoreactivity in rat forebrain, *Neuroendocrinology*, 66 (1997) 63-67.

81 Lieberburg, I., Krey, L.C. and McEwen, B.S. Sex differences in serum testosterone and in exchangeable brain cell nuclear estradiol during the neonatal period in rats, *Brain Res.* 178 (1979) 207-212.

82 Lieberburg, I., MacLusky, N.J., Roy, E.J. and McEwen, B.S. Sex steroid receptors in the perinatal rat brain, *Amer. Zool.* 18 (1978) 539-544.

83 Loy, R., Gerlach, J.L. and McEwen, B.S. Autoradiographic localization of estradiol-binding neurons in the rat hippocampal formation and entorhinal cortex, *Dev. Brain Res.* 39 (1988) 245-251.

84 Lustig, R.H. Sex hormone modulation of neural development *in vitro*, *Horm. Behav.* 28 (1994) 383-395.

85 MacLusky, N.J., Lieberburg, I. and McEwen, B.S. The development of estrogen receptor systems in the rat brain: perinatal development, *Brain Res.* 178 (1979) 129-142.

86 MacLusky, N.J., Clark, A.S., Naftolin, F. and Goldman-Rakic, P.S. Estrogen formation in the mammalian brain: possible role of aromatase in sexual differentiation of the hippocampus and neocortex, *Steroids*, 50 (1987) 459-474.

87 MacLusky, N.J., Chaptal, C. and McEwen, B.S. The development of estrogen receptor systems in the rat brain and pituitary: postnatal development, *Brain Res.* 178 (1979) 143-160.

88 Madeira, M.D. and Lieberman, A.R. Sexual dimorphism in the mammalian limbic system, *Progress in Neurobiology*, 45 (1995) 275-333.

89 Madeira, M.D., Sousa, N., Lima-Andrade, M.T., Calheiros, F., Cadete-Leite, A. and Paula-Barbosa, M.M. Selective vulnerability of the hippocampal pyramidal neurons to hypothyroidism in male and female rats, *J. Comp. Neurol.* 322 (1992) 501-518.

90 Majewska, M.D., Harrison, N.L., Schwartz, R.D., Barker, J.L. and Paul, S.M. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor, *Science*, 232 (1986) 1004-1007.

91 Massa, R., Justo, S. and Martini, L. Conversion of testosterone to 5α-reduced metabolites in the anterior pituitary and in the brain of maturing rats, *J. Steroid Biochem.* 6 (1975) 567-571.

92 McCarthy, M.M., Coirini, H., Schumacher, M., Johnson, A.E., Pfaff, D.W., Schwartz-Giblin, S. and McEwen, B.S. Steroid regulation and sex differences in [^{3}H]muscimol binding in hippocampus, hypothalamus and midbrain in rats, *J. Neuroendocrinol.* 4 (1992) 393-399.

93 McEwen, B.S. Non-genomic and genomic effects of steroids on neural activity, *Trends Pharmacol. Sci.* 12 (1991) 141-147.

94 McEwen, B.S., Luine, V.N. and Fischette, C.T. Developmental actions of hormones: from receptors to function. In S.S. Easter, K.F. Barald and B.M. Carlson (Eds.) *From Message to Mind*, Sinauer Assoc Inc. Sunderland Mass, 1988, pp. 272-287.

95 McGinnis, M.Y. and Katz, S.E. Sex differences in cytosolic androgen receptors in gonadectomized male and female rats, *J. Neuroendocrinol.* 8 (1996) 193-197.

96 Meaney, M.J., Aitken, D.H., Jensen, L.K., McGinnis, M.Y. and McEwen, B.S. Nuclear and cytosolic androgen receptor levels in the limbic brain of neonatal male and female rats, *Dev. Brain Res.* 23 (1985) 179-185.

97 Meaney, M.J. and Stewart, J. Neonatal androgens influence the social play of prepubescent rats, *Horm. Behav.* 15 (1981) 197-213.

98 Meaney, M.J., Stewart, J., Poulin, P. and McEwen, B.S. Sexual differentiation of social play in rat pups is mediated by the neonatal androgen-receptor system, *Neuroendocrinology*, 37 (1983) 85-90.

99 Menard, C.S. and Harlan, R.E. Up-regulation of androgen receptor immunoreactivity in the rat brain by androgenic-anabolic steroids, *Brain Res.* 622 (1993) 226-236.

100 Meyer, J.H. and Gruol, D.L. Dehydroepiandrosterone sulfate alters synaptic potentials in area CA1 of the hippocampal slice, *Brain Res.* 633 (1994) 253-261.

101 Michael, R.P., Zumpe, D. and Bonsall, R.W. The interaction of testosterone with the brain of the orchidectomized primate fetus, *Brain Res.* 570 (1992) 68-74.

102 Miranda, R.C. and Toran-Allerand, C.D. Developmental expression of estrogen receptor mRNA in the rat cerebral cortex: a nonisotopic *in situ* hypbidization histochemistry study, *Cerebral Cortex*, 2 (1992) 1-15.

103 Miyachi, Y., Nieschlag, E. and Lipsett, M.B. The secretion of gonadotropins and testosterone by the neonatal male rat, *Endocrinol.* 92 (1973) 1-5.

104 Morris, R.G.M., Garrud, P., Rawlins, J.N.P. and O'Keefe, J. Place navigation impaired in rats with hippocampal lesions, *Nature*, 297 (1982) 681-683.

105 Moser, E., Moser, M.-B. and Anderson, P. Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions, *J. Neurosci.* 13 (1993) 3916-3925.

106 Murphy, D.D. and Segal, M. Regulation of dendritic spine density in cultured rat hippocampal neurons by steroid hormones, *J. Neurosci.* 16 (1996) 4059-4068.

107 Naftolin, F., Ryan, K.J. and Petro, Z. Aromatization of androstenedione by the diencephalon, *J. Clin. Endocrinol. Metab.* 33 (1971) 368-370.

108 Nazareth, L.V. and Weigel, N.L. Activation of the human androgen receptor through a protein kinase A signaling pathway, *J. Biol. Chem.* 271 (1996) 19900-19907.

109 Nock, B. and Feder, H.H. Neurotransmitter modulation of steroid action in target cells that mediate reproduction and reproductive behavior, *Neurosci. Biobehav. Rev.* 5 (1981) 437-447.

110 Nordeen, E.J., Nordeen, K.W., Sengelaub, D.R. and Arnold, A.P. Androgens prevent normally occurring cell death in a sexually dimorphic spinal nucleus, *Science*, 229 (1985) 671-673.

111 O'Keefe, J.A. and Handa, R.J. Transient elevation of estrogen receptors in the neonatal rat hippocampus, *Dev. Brain Res.* 57 (1990) 119-127.

112 O'Keefe, J.A., Li, Y., Burgess, L.H. and Handa, R.J. Estrogen receptor mRNA alterations in the developing rat hippocampus, *Mol. Brain Res.* 30 (1995) 115-124.

113 O'Keefe, J.A., Pedersen, E.B., Castro, A.J. and Handa, R.J. The ontogeny of estrogen receptors in heterochronic hippocampal and neocortical transplants demonstrates an intrinsic developmental program, *Dev. Brain Res.* 75 (1993) 105-112.

114 Olsen, K.L. Genetic determinants of sexual differentiation. In J. Balthazart, E. Pröve and R. Gilles (Eds.) *Hormones and Behaviour in Higher Vertebrates*, Springer-Verlag, Berlin, 1983, pp. 138-158.

115 Orikasa, C., Okamura, H. and Hayashi, S. Estrogen receptor found in facial nucleus of the newborn rat is suppressed by exogenous estrogen: immuno- and in situ hybridization histochemical studies, *Dev. Brain Res.* 82 (1994) 9-17.

116 Pang, S.F., Caggiula, A.R., Gay, V.L., Goodman, R.L. and Pang, C.S.F. Serum concentrations of testosterone, oestrogens, luteinizing hormone and follicle-stimulating hormone in male and female rats during the critical period of neural sexual differentiation, *J. Endocrinol.* 80 (1979) 103-110.

117 Pardridge, W.M. Steroid hormone transport through blood-brain barrier: methods and concepts. In P.M. Conn, E.R. De Kloet and W. Sutanto (Eds.) *Methods in Neurosciences*, Academic Press, New York, 1994, pp. 3-22.

118 Perrot-Sinal, T.S., Kostenuik, M.A., Ossenkopp, K.-P. and Kavaliers, M. Sex differences in performance in the Morris water maze and the effects of initial nonstationary hidden platform training, *Behav. Neurosci.* 110 (1996) 1-12.

119 Plapinger, L. and McEwen, B.S. Gonadal steroid - brain interactions in sexual differentiation. In J.B. Hutchison (Ed.) *Biological Determinants of Sexual Behavior*, John Wiley, Chichester, NY, 1978, pp. 153-218.

120 Plapinger, L., McEwen, B.S. and Clemens, L.E. Ontogeny of estradiol-binding sites in rat brain. II. Characteristics of a neonatal binding macromolecule, *Endocrinol.* 93 (1973) 1129-1139.

121 Primus, R.J. and Kellogg, C.K. Gonadal hormones during puberty organize environment-related social interaction in the male rat, *Horm. Behav.* 24 (1990) 311-323.

122 Prins, G.S., Birch, L. and Greene, G.L. Androgen receptor localization in different cell types of the adult rat prostate, *Endocrinol.* 129(6) (1991) 3187-3199.

123 Quarmby, V.E., Yarbrough, W.G., Lubahn, D.B., French, F.S. and Wilson, E.M. Autologous down-regulation of androgen receptor messenger ribonucleic acid, *Mol. Endocrinol.* 4 (1990) 22-28.

124 Raynaud, J.P., Mercier-Bodard, C. and Baulieu, E.-E. Rat estradiol binding plasma protein (EBP), *Steroids*, 18 (1971) 767-788.

125 Robel, P. and Baulieu, E.-E. Neurosteroids: biosynthesis and functions. In E.R. De Kloet and W. Sutanto (Eds.) *Methods in Neurosciences*, Academic Press, New York, 1994, pp. 36-50.

126 Roof, R.L. Neonatal exogenous testosterone modifies sex difference in radial arm and Morris water maze performance in prepubescent and adult rats, *Behav. Brain Res.* 53 (1993) 1-10.

127 Roof, R.L. and Havens, M.D. Testosterone improves maze performance and induces development of a male hippocampus in females, *Brain Res.* 572 (1992) 310-313.

128 Roselli, C.E., Ellinwood, W.E. and Resko, J.A. Regulation of brain aromatase activity in rats, *Endocrinol.* 114 (1984) 192-200.

129 Roselli, C.E., Horton, L.E. and Resko, J.A. Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system, *Endocrinol.* 117 (1985) 2471-2477.

130 Roselli, C.E., Klosterman, S.A. and Fasasi, T.A. Sex differences in androgen responsiveness in the rat brain: regional differences in the induction of aromatase activity, *Neuroendocrinology*, 64 (1996) 139-145.

131 Roselli, C.E. and Resko, J.A. The distribution and regulation of aromatase activity in the central nervous system, *Steroids*, 50 (1987) 495-508.

132 Roselli, C.E. and Resko, J.A. Aromatase activity in the rat brain: hormonal regulation and sex differences, *J. Steroid Biochem. Molec. Biol.* 44 (1993) 499-508.

133 Sachs, B.D. and Meisel, R.L. The physiology of male sexual behavior. In E. Knobil and J. Neill (Eds.) *The Physiology of Reproduction*, Raven Press, New York, 1988, pp. 1393-1485.

134 Sar, M. Distribution of progestin-concentrating cells in rat brain: colocalization of [^{3}H]ORG.2058, a synthetic progestin, and antibodies to tyrosine hydroxylase in hypothalamus by combined autoradiography and immunocytochemistry, *Endocrinol.* 123 (1988) 1110-1118.

135 Sar, M., Lubahn, D.B., French, F.S. and Wilson, E.M. Immunohistochemical localization of the androgen receptor in rat and human tissues, *Endocrinol.* 127 (1990) 3180-3186.

136 Sar, M. and Stumpf, W.E. Distribution of androgen-concentrating neurons in rat brain. In W.E. Stumpf and L.D. Grant (Eds.) *Anatomical Neuroendocrinology*, S.Karger, New York, 1975, pp. 120-133.

137 Sar, M. and Stumpf, W.E. Distribution of androgen target cells in rat forebrain and pituitary after [^{3}H]-dihydrotestosterone administration, *J. Steroid Biochem.* 8 (1977) 1131-1135.

138 Schlessinger, A.R., Cowan, W.M. and Gottlieb, D.I. An autoradiographic study of the time of origin and the pattern of granule cell migration in the dentate gyrus of the rat, *J. Comp. Neurol.* 159 (1975) 149-176.

139 Schumacher, M. Rapid membrane effects of steroid hormones: an emerging concept in neuroendocrinology, *Trends Neurosci.* 13 (1990) 359-362.

140 Schumacher, M., Coirini, H. and McEwen, B.S. Regulation of high-affinity $GABA_A$ receptors in the dorsal hippocampus by estradiol and progesterone, *Brain Res.* 487 (1989) 178-183.

141 Schwegler, H., Crusio, W.E. and Brust, I. Hippocampal mossy fibers and radial-maze learning in the mouse: a correlation with spatial working memory but not with non-spatial reference memory, *Neurosci.* 34 (1990) 293-298.

142 Schwegler, H., Crusio, W.E., Lipp, H.-P. and Heimrich, B. Water-maze learning in the mouse correlates with variation in hippocampal morphology, *Behav. Genet.* 18 (1988) 153-165.

143 Sheridan, P.J. The nucleus interstitialis striae terminalis and the nucleus amygdaloideus medialis: prime targets for androgen in the rat forebrain. *Endocrinol.* 104 (1979) 130-136.

144 Sheridan, P.J. Unaromatized androgen is taken up by the neonatal rat brain: two receptor systems for androgen, *Dev. Neurosci.* 4 (1981) 46-54.

145 Sheridan, P.J., Sar, M. and Stumpf, W.E. Estrogen and androgen distribution in the brain of neonatal rats. In W.E. Stumpf and L.D. Grant (Eds.) *Anatomical Neuroendocrinology*, S.Karger, Basel, 1975, pp. 134-141.

146 Sholl, S.A., Goy, R.W. and Kim, K.L., 5α-Reductase, aromatase, and androgen receptor levels in the monkey brain during fetal development, *Endocrinol.* 124(2) (1989) 627-634.

147 Shughrue, P.J., Lane, M.V. and Merchenthaler, I. Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system, *J. Comp. Neurol.* 388 (1997) 507-525.

148 Shughrue, P.J., Sar, M. and Stumpf, W.E. Progestin target cell distribution in forebrain and midbrain regions of the 8-day postnatal mouse brain, *Endocrinol.* 130 (1992) 3650-3659.

149 Shughrue, P.J., Stumpf, W.E., MacLusky, N.J., Zielinski, J.E. and Hochberg, R.B. Developmental changes in estrogen receptors in mouse cerebral cortex between birth and postweaning: studied by autoradiography with 11β-methoxy-16α-[^{125}I]iodoestradiol, *Endocrinol.* 126 (1990) 1112-1124.

150 Sibug, R.M., Stumpf, W.E., Shughrue, P.J., Hochberg, R.B. and Drews, U. Distribution of estrogen target sites in the 2-day-old mouse forebrain and pituitary gland during the 'critical period' of sexual differentiation, *Dev. Brain Res.* 61 (1991) 11-22.

151 Simental, J.A., Sar, M., Lane, M.V., French, F.S. and Wilson, E.M. Transcriptional activation and nuclear targeting signals of the human androgen receptor, *J. Biol. Chem.* 266 (1991) 510-518.

152 Simerly, R.B., Chang, C., Muramatsu, M. and Swanson, L.W. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study, *J. Comp. Neurol.* 294 (1990) 76-95.

153 Slob, A.K., Ooms, M.P. and Vreeburg, J.T.M. Prenatal and early postnatal sex differences in plasma and gonadal testosterone and plasma luteinizing hormone in female and male rats, *J. Endocrinol.* 87 (1980) 81-87.

154 Smith, S.S. Female sex steroid hormones: from receptors to networks to performance - actions on the sensorimotor system, *Prog. Neurobiol.* 44 (1994) 55-86.

155 Södersten, P. A way in which estradiol might play a role in the sexual behavior of male rats, *Horm. Behav.* 14 (1980) 271-274.

156 Södersten, P. and Gustafsson, J.-Å. Activation of sexual behaviour in castrated rats with the synthetic androgen 17β-hydroxy-17α-methyl-estra-4,9,11-triene-3-one (R 1881), *J. Endocrinol.* 87 (1980) 279-283.

157 Södersten, P. and Hansen, S. Effects of castration and testosterone, dihydrotestosterone or oestradiol replacement treatment in neonatal rats on mounting behaviour in the adult, *J. Endocrinol.* 76 (1978) 251-260.

158 Stumpf, W.E. and Sar, M. Hormone-architecture of the mouse brain with ^{3}H-estradiol. In W.E. Stumpf and L.D. Grant (Eds.) *Anatomical Neuroendocrinology*, S.Karger, Basel, 1975, pp. 82-103.

159 Stumpf, W.E., Sar, M. and Keefer, D.A. Atlas of estrogen target cells in rat brain. In W.E. Stumpf and L.D. Grant (Eds.) *Anatomical Neuroendocrinology*, S.Karger, New York, 1975, pp. 104-119.

160 Swanson, L.W., Wyss, J.M. and Cowan, W.M. An autoradiographic study of the organization of intrahippocampal association pathways in the rat, *J. Comp. Neurol.* 181 (1978) 681-716.

161 Tobet, S.A., Baum, M.J., Tang, H.B., Shim, J.H. and Canick, J.A. Aromatase activity in the perinatal rat forebrain: effects of age, sex and intrauterine position, *Dev. Brain Res.* 23 (1985) 171-178.

162 Tobet, S.A., Shim, J.H., Osiecki, S.T., Baum, M.J. and Canick, J.A. Androgen aromatization and 5α-reduction in ferret brain during perinatal development: effects of sex and testosterone manipulation, *Endocrinol.* 116 (1985) 1869-1877.

163 Toran-Allerand, C.D. On the genesis of sexual differentiation of the central nervous system: morphogenetic consequences of steroidal exposure and possible role of α-fetoprotein. In G.J. De Vries, J.P.C. De Bruin, H.B.M. Uylings and M.A. Corner (Eds.) *Progress in Brain Research: Sex Differences in the Brain*, Elsevier, Amsterdam, 1984, pp. 63-98.

164 Toran-Allerand, C.D., Miranda, R.C., Hochberg, R.B. and MacLusky, N.J. Cellular variations in estrogen receptor mRNA translation in the developing brain: evidence from combined [^{125}I]estrogen autoradiography and non-isotopic in situ hybridization histochemistry, *Brain Res.* 576 (1992) 25-41.

165 Tsai, M.-J. and O'Malley, B.W. Molecular mechanisms of action of steroid/thyroid receptor superfamily members, *Annu. Rev. Biochem.* 63 (1994) 451-486.

166 Vagell, M.E. and McGinnis, M.Y. The role of aromatization in the restoration of male rat reproductive behavior, *J. Neuroendocrinol.* 9 (1997) 415-421.

167 Vagell, M.E. and McGinnis, M.Y. The role of gonadal steroid receptor activation in the restoration of sociosexual behavior in adult male rats, *Horm. Behav.* 33 (1998) 163-179.

168 Van der Schoot, P. Effects of dihydrotestosterone and oestradiol on sexual differentiation in male rats, *J. Endocrinol.* 84 (1980) 397-407.

169 Vito, C.C. and Fox, T.O. Androgen and estrogen receptors in embryonic and neonatal rat brain, *Dev. Brain Res.* 2 (1982) 97-110.

170 Waters, N.S., Klintsova, A.Y. and Foster, T.C. Insensitivity of the hippocampus to environmental stimulation during postnatal development, *J. Neurosci.* 17 (1997) 7967-7973.

171 Weigel, N.L. and Zhang, Y.X. Ligand-independent activation of steroid hormone receptors, *J. Mol. Med.* 76 (1998) 469-479.

172 Weiland, N.G., Orikasa, C., Hayashi, S. and McEwen, B.S. Distribution and hormone regulation of estrogen receptor immunoreactive cells in the hippocampus of male and female rats, *J. Comp. Neurol.* 388 (1997) 603-612.

173 Williams, C.L., Barnett, A.M. and Meck, W.H. Organizational effects of early gonadal secretions on sexual differentiation in spatial memory, *Behav. Neurosci.* 104 (1990) 84-97.

174 Williams, C.L. and Meck, W.H. The organizational effects of gonadal steroids on sexually dimorphic spatial ability, *Psychoneuroendocrinol.* 16 (1991) 155-176.

175 Wilson, E.M. and French, F.S. Binding properties of androgen receptors, *J. Biol. Chem.* 251 (1976) 5620-5629.

176 Wilson, M.E., O'Keefe, J.A., Price, R.H. and Handa, R.J. Androgen receptor (AR) and AR mRNA in primary cultures of rat hippocampus, *Soc. Neurosci. Abst.* 21 (1995)

177 Wood, R.I. and Newman, S.W. Intracellular partitioning of androgen receptor immunoreactivity in the brain of the male Syrian hamster: effects of castration and steroid replacement, *J. Neurobiol.* 24 (1993) 925-938.

178 Woolley, C.S., Gould, E., Frankfurt, M. and McEwen, B.S. Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons, *J. Neurosci.* 10 (1990) 4035-4039.

179 Woolley, C.S. and McEwen, B.S. Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat, *J. Neurosci.* 12 (1992) 2549-2554.

180 Woolley, C.S., Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus, *Horm. Behav.* 34 (1998) 140-148.

181 Yokosuka, M., Okamura, H. and Hayashi, S. Transient expression of estrogen receptor-immunoreactivity (ER-IR) in the layer V of the developing rat cerebral cortex, *Dev. Brain Res.* 84 (1995) 99-108.

182 Yokosuka, M., Prins, G.S. and Hayashi, S. Co-localization of androgen receptor and nitric oxide synthase in the ventral premammillary nucleus of the newborn rat: an immunohistochemical study, *Dev. Brain Res.* 99 (1997) 226-233.

183 Young, W.J. and Chang, C. Ontogeny and autoregulation of androgen receptor mRNA expression in the nervous system, *Endocrine*, 9 (1998) 79-88.

184 Zhou, L., Blaustein, J.D. and De Vries, G.J. Distribution of androgen receptor immunoreactivity in vasopressin- and oxytocin-immunoreactive neurons in the male rat brain. *Endocrinol.* 134 (1994) 2622-2627.

185 Zhou, Z., Lane, M.V., Kemppainen, J.A., French, F.S. and Wilson, E.M. Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability, *Mol. Endocrinol.* 9 (1995) 208-218.

186 Zhou, Z., Wong, C., Sar, M. and Wilson, E.M. The androgen receptor: an overview, *Recent Progress In Hormone Research*, 49 (1994) 249-274.

187 Zimmer, J. and Haug, F-M.S. Laminar differentiation of the hippocampus, fascia dentata and subiculum in developing rats, observed with the Timm sulphide silver method, *J. Comp. Neurol.*, 179 (1978) 581-618.