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REGULATION OF ANDROGEN RECEPTOR-IMMUNOREACTIVE CELLS DURING PUBERTAL MATURATION WITHIN THE CENTRAL NERVOUS SYSTEM OF THE MALE EUROPEAN FERRET: EFFECTS OF GONADAL STEROIDS

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ABSTRACT

REGULATION OF ANDROGEN RECEPTOR-IMMUNOREACTIVE CELLS DURING PUBERTAL MATURATION WITHIN THE CENTRAL NERVOUS SYSTEM OF THE MALE EUROPEAN FERRET: EFFECTS OF GONADAL STEROIDS

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Puberty in males is characterized by onset of gonadal maturation brought about by an increase in the: a) frequency of episodic release of luteinizing hormone-releasing hormone from the hypothalamus, b) frequency of release of luteinizing hormone from the anterior pituitary, and c) testosterone from the testes. Two well known actions of testosterone on the central nervous system are suppression of gonadotropin secretion from the anterior pituitary, and activation of male reproductive behavior. During pubertal maturation, interaction of testosterone with target cells in the central nervous system is dynamic. First, there is a decline in the ability of testosterone to suppress gonadotropin secretion as the animal undergoes sexual maturation. Second, there is an increase in the ability of testosterone to activate male reproductive behavior. The biological actions of testosterone are mediated by the androgen receptor, or through the estrogen receptor following conversion of testosterone to estrogen. Experiments in this dissertation tested the hypothesis that the pubertal increase in behavioral responsiveness to testosterone is due to an increase in number of androgen receptor containing cells in regions of the brain which are involved in control of reproductive behavior. Immunocytochemical staining for androgen receptor was greatest in number of cells in

hypothalamic and limbic structures in ferrets undergoing pubertal maturation relative to prepubertal ferrets. This increase in androgen receptor-immunoreactive cells was linked to the increased concentrations of testosterone which accompany pubertal maturation. Since androgens regulate the level of their own receptors, it was proposed that prepubertal ferrets may be unable to respond to testosterone treatment with a sufficient upregulation of androgen receptors, and that this may be involved in mediating responsiveness to the behavioral effects of testosterone. However, both prepubertal and adult male ferrets displayed identical patterns of androgen receptor-immunoreactive cells following 10 days of treatment with testosterone, indicating that an upregulation of brain androgen receptors in response to testosterone treatment is not a limiting factor for the precocious display of male reproductive behaviors.

To my wife and best friend Kris,

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LIST OF ABBREVIATIONS

Anatomical Abbreviations

3V	third ventricle
AA	anterior amygdaloid area
ac	anterior commisure
Acb	nucleus accumbens
ACo	cortical amygdaloid nucleus, anterior
AHA	anterior hypothalamic area
AHi	amygdalo-hippocampal transition area
ALA	lateral amygdaloid area
APir	amygdalo-piriform transition area
BMA	basomedial amygdaloid nucleus
BNST	bed nucleus of the stria terminalis
Ca	caudate nucleus
CA	cornu ammonis
cc	corpus callosum
Ce	central amygdaloid nucleus
DG	dentate gyrus
DM	dorsomedial hypothalamic nucleus
f	fornix
FF	fields of Forel
HDB	nucleus of the diagonal band, horizontal limb
ic	internal capsule
LD	lateral dorsal nucleus of the thalamus
LHA	lateral hypothalamic area
LM	lateral mammillary nucleus
lo	lateral olfactory tract
LOT	nucleus of the lateral olfactory tract
LS	lateral septal nucleus
LV	lateral ventricle
ME	median eminence
MeA	medial amygdaloid nucleus, anterior
MeP	medial amygdaloid nucleus, posterior
MM	medial mammillary nucleus
MnPO	median preoptic nucleus
MPA	medial preoptic area
MS	medial septum
mt	mammilothalamic tract
MV	medioventral thalamic nucleus
mVMH	ventromedial hypothalamic nucleus, medial

opt	optic tract
OVLT	vascular organ of the lamina terminalis
ox	optic chiasm
PC	paracentral thalamic nucleus
PCo	cortical amygdaloid nucleus, posterior
PH	posterior hypothalamic area
Pir	piriform cortex
PMV	premammillary nucleus, ventral
POA	preoptic area
PT	paratenial thalamic nucleus
PV	paraventricular thalamic nucleus
PVH	paraventricular hypothalamic nucleus
RCh	retrochiasmatic area
Sch	suprachiasmatic nucleus
SFi	septofimbrial nucleus
SFO	subfornical organ
SHy	septohypothalamic nucleus
sm	stria medularis
SOa	nucleus, anterior
SOc	supraoptic nucleus, caudal
SuM	supramammillary nucleus
Tu	olfactory tubercle
VDB	nucleus of the diagonal band, vertical limb
VMH	ventromedial nucleus of the hypothalamus
VP	ventral pallidum
ZI	zona incerta

General Abbreviations

ANOVA	analysis of variance
AR	androgen receptor
AR-IR	androgen receptor-immunoreactive
ATD	androstendione .....
DAB	diaminobenzidine
DHT	dihydrotestosterone
E	estradiol
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
PBS	phosphate buffered saline
T	testosterone
TBS	tris buffered saline

INTRODUCTION

Puberty in males is characterized by the onset of gonadal maturation brought about by an increase in the frequency of episodic release of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus, a concomitant increase in the frequency of release of luteinizing hormone from the anterior pituitary, and testosterone from the testes. Two well known actions of testosterone on the central nervous system are the suppression of gonadotropin secretion from the anterior pituitary, and the activation of male reproductive behavior. The interaction of testosterone with target cells in the central nervous system is dynamic during pubertal maturation. First, there is a decline in the ability of testosterone to suppress gonadotropin secretion as the animal undergoes sexual maturation, (i.e., a change in the setpoint for steroid negative feedback regulation of gonadotropin secretion). In some species, this appears to be the mechanism by which frequent LHRH release is initiated at the onset of puberty. Second, there is an increase in the ability of testosterone to activate male reproductive behavior (i.e., the CNS becomes more responsive to behavioral actions of gonadal steroids). The timing of puberty is variable between species and within species depending upon environmental and nutritional factors, however regardless of when pubertal maturation occurs, these patterns of neuroendocrine and behavioral changes occur in virtually all mammalian species examined. Understanding the mechanisms underlying these changes in

responsiveness to testosterone during sexual maturation will increase our understanding of one of the fundamental developmental periods of an animal's reproductive life.

The working hypothesis of the Sisk laboratory is that target tissue responsiveness to steroid hormones is in part mediated by the number of steroid receptor containing cells and/or the number of steroid receptors per cell. One prediction of this hypothesis is that pubertal changes in the responsiveness to steroid hormones are positively correlated with changes in the number of brain cells which contain steroid receptors. Experiments in this dissertation address one aspect of this working hypothesis, namely that changes in the abundance of androgen receptor-containing cells during the process of pubertal maturation is one potential mechanism for pubertal shifts in responsiveness to testosterone. Using the European ferret as an animal model, the experiments in this dissertation investigate 1) the distribution of androgen receptor containing cells in forebrain structures, 2) pubertal changes in the density of androgen receptor-containing brain cells, and 3) changes in the density of androgen receptor-containing cells as a function of steroidal milieu. Particular attention will be focused on brain regions implicated in the control of both reproductive behaviors and negative feedback on gonadotropin secretion.

Background and Significance

The European ferret was used as an animal model for these experiments. In this section, some characteristics of this model will be described and the significance of this research will be placed in the context of understanding the process of pubertal maturation.

Photoperiodic Regulation of Pubertal Maturation

The European ferret (*Mustela putorius furo*) is a photoperiod-sensitive mammal that is reproductively active in the long days of spring and early summer (Neal, Murphy, Moger, & Oliphant, 1977). Sexual maturation of juvenile male ferrets, as measured by increased testicular size and serum testosterone concentrations, begins in December when ferrets are 20-25 wks old and, as daylength increases following the winter solstice. However, this increase in daylength is not required for sexual maturation, since ferrets maintained continuously in short day lengths (e.g. 8 hr light/day) under artificial light conditions also experience the onset of puberty at about 18-20 wks of age (Sisk, 1990). Nevertheless, early onset of sexual maturation can be induced in laboratory housed male ferrets by transferring them from short day to long day (e.g. 18 hr light/day) conditions at 12 wk of age (Sisk, 1990). Whether sexual maturation occurs spontaneously in short days, or following a photoperiod transition to long days, quantitative characteristics of luteinizing hormone (LH) pulse frequency and testosterone (T) levels are similar once sexual maturation is reached (Sisk, 1987; Sisk, 1990).

Activation of Hypothalamic-Pituitary-Gonadal Axis

The transition from an immature (low frequency) pattern of LH release to a reproductively mature (high frequency) pattern is the result of a shift in responsiveness of steroid sensitive neural circuits to the negative feedback effects of T. The immature ferret is fully capable of secreting adult levels of LH in the absence of gonadal steroids, and a dose of T which virtually abolishes LH secretion in the castrated prepubertal male ferret is less effective in lowering LH secretion in a castrated adult male ferret (Sisk,

1987). Furthermore, the pituitary gland of gonadally intact prepubertal male ferrets is capable of secreting LH in response to exogenous administration of LHRH (Berglund & Sisk, 1990). Therefore the pubertal increase in LH pulse frequency is the result of a reduction in the negative feedback effects of T on LHRH release.

Activation of Reproductive Behavior

Studies in the Sisk laboratory have demonstrated that pubertal maturation of sexual behavior patterns also involves a shift in responsiveness to gonadal steroids. In this component of puberty, steroid sensitive neural circuits in the brain become more responsive to the activational effects of T on male sexual behavior. Prepubertal male ferrets display almost none of the characteristic reproductive behaviors (i.e. neck gripping, mounting and thrusting) when tested with a receptive female (Sisk, Berglund, Tang, & Venier, 1992). Although some of these characteristic behavior patterns begin to emerge in castrated prepubertal males when injected with high doses of T, these behavior patterns can be activated in castrated adult males with a much lower dose of T. Interestingly, adults that undergo pubertal maturation following a photoperiod transition show an increased responsiveness to the behavioral actions of T on male sexual behavior compared to adults that undergo pubertal maturation in short days. When castrated and injected with T, adults raised in long days respond with an increase in sexual behaviors to lower doses of T than either adults raised in short days, or prepubertal males. Adults raised in short days in turn respond to lower doses of T than prepubertal males. These data indicate that steroid sensitive neural circuits are more responsive to T in adults compared with those in prepubertal males, and that the photoperiod conditions under

which pubertal maturation occur, and/or the photoperiod under which the animals were tested, influence the responsiveness to the activational effects of T in adulthood.

Contribution of Testosterone and its Metabolites

Negative Feedback: The central actions of T are often mediated following its conversion to either estrogen (E) following aromatization, or dihydrotestosterone (DHT) following 5α-reduction (Figure 1). These steroids then bind to their respective steroid receptors and alter transcriptional activity of genes which contain steroid response elements (Beato, 1989; Evans, 1988; O'Malley, Schrader, & Tsai, 1986; Truss & Beato, 1993; Yamamoto, 1985). It has been clearly demonstrated that estrogens can mediate negative feedback on gonadotropin secretion (Ellinwood, Hess, Roselli, Spies, & Resko, 1984; Kalra & Kalra, 1980; Schanbacher, 1984; Worgul, Santen, Samojlik, Irwin, & Falvo, 1981). Administration of the aromatase inhibitor ATD to male ferrets in breeding season elevates the number of LH pulses and the level of circulating LH (Carroll & Baum, 1989). Furthermore, mean LH levels, and LH pulse amplitudes were not significantly different in castrated males when compared to intact males given ATD (Carroll & Baum, 1989). However, androgens themselves induce negative feedback on gonadotropin secretion. Implantation of DHT directly into the mediobasal hypothalamus of intact rats decreased circulating levels of LH, without increasing the circulating level of DHT, indicating that hormone leakage into the periphery and a possible direct negative feedback action on the pituitary are unlikely to fully account for the decline in LH secretion (Kalra & Kalra, 1980). This is further supported by the reduced effectiveness of peripheral implantation of DHT or T filled silastic capsules to

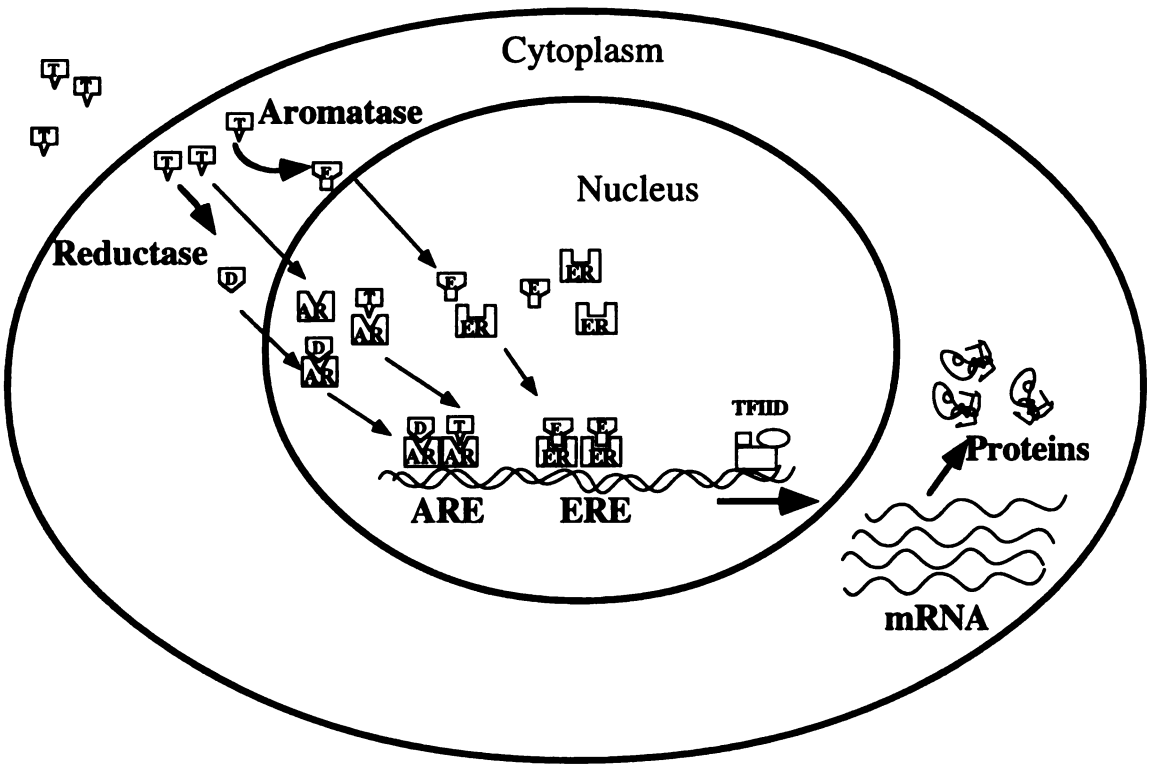


Figure 1. Schematic diagram of a cell indicating the cellular mechanism of steroid hormones. Testosterone (T) can alter cellular function by binding to the androgen receptor (AR) as itself, or as dihydrotestosterone (D) following 5α-reduction. T can also exert its actions by acting through the estrogen receptor (E) following aromatization. Steroid-receptor cfomplexes alter transcription of specific genes which contain androgen (ARE) or estrogen (ERE) response elements.

induce negative feedback following peripheral administration of the androgen receptor blocker flutamide (Kalra & Kalra, 1980). In the castrated adult male ferret, analysis of plasma LH levels following implantation of silastic capsules of DHT has shown that DHT is a potent inhibitor of LH secretion (Tang & Sisk, 1988). Although a direct negative feedback effect on the pituitary gland cannot be ruled out when DHT or flutamide are administered peripherally, the effectiveness of central implants of DHT in reducing LH secretion indicate that the binding of an androgen to the androgen receptor within the CNS may be directly involved in the negative feedback of gonadotropin secretion.

Reproductive Behavior: A great deal of research has been directed toward the investigation of whether T itself, or a metabolite of T such as E, is responsible for the activation of male reproductive behavior. This work led to the "aromatization hypothesis" stating that the metabolic conversion from T to E via aromatization is essential for the activation of male sexual behavior in a variety of species. This was based on evidence from many species, including the ferret, indicating that androgens subject to aromatization such as T, or androstenedione, are effective in maintaining copulation, while androgens not subject to aromatization, such as DHT, are ineffective (Meisel & Sachs, 1994). Furthermore, administration of E alone to castrated adult male ferrets can restore neck gripping, mounting and pelvic thrusting (Baum, 1976), while blocking the conversion of androgens to estrogens can inhibit male reproductive behaviors. In intact adult male ferrets, silastic capsules containing the aromatase inhibitor ATD significantly reduced the occurrence of neck gripping, mounting and intromissive behaviors compared to ferrets implanted with empty capsules (Carroll,

Weaver, & Baum, 1988).

In spite of good evidence that aromatization of T to E and, by implication, action of the estrogen receptor, is important for the behavioral effects of T, there are several lines of converging evidence indicating that androgens, acting on the androgen receptor, also contribute directly to the activation of male reproductive behaviors. For example, the combined treatment with E and DHT more closely approximates the behavior pattern seen when castrate male ferrets receive T replacement, than when E is administered alone (Baum & Vreeburg, 1983). In the castrated rat, DHT is effective in activating copulatory behavior when administered in conjunction with doses of E that are ineffective when administered alone (Baum & Vreeburg, 1973; Feder, Naftolin, & Ryan, 1974; Larrson, Perez-Palacios, Morali, & Beyer, 1975; Morali, Lemus, Oropeza, Garcia, & Perez-Palacios, 1990). Since DHT cannot be aromatized to E, these data suggest that androgen receptors themselves are mediating these effects. In this same context, administration of the androgen receptor blocker flutamide eliminates the restoration of both intromissions and ejaculations following T treatment (Gladue & Clemens, 1980). Flutamide treatment is most effective when the dose of T used activates a moderate (Gladue & Clemens, 1980) rather than a high (Gray, 1977) level of copulatory behavior, and is likely due to the low affinity of flutamide for the androgen receptor (Liao, Howell, & Chang, 1974). Further support for this hypothesis is data indicating that a metabolite of flutamide, Sch 16423, which has a higher affinity for the androgen receptor was able to eliminate mounting in 80% of rats treated with a dose of T which restored ejaculation in all control animals (McGinnis & Mirth, 1986).

Indirect Role of Androgen Receptors: The activity of the aromatase enzyme is regulated by androgens in several species (Connolly, Roselli, & Resko, 1990; Roselli, Ellinwood, & Resko, 1984; Steimer & Hutchison, 1981; Weaver & Baum, 1991), presumably through an androgen receptor mechanism (Roselli, Horton, & Resko, 1987; Roselli & Resko, 1984). The regulatory role of androgens on aromatase activity is brain region specific. In castrated adult male ferrets the magnitude of aromatase activity is similar in the bed nucleus of the stria terminalis, medial and lateral preoptic area, medial and lateral amygdala, and the ventromedial hypothalamic nucleus (Weaver & Baum, 1991). However, following treatment with DHT, aromatase activity was stimulated in the medial preoptic area, medial amygdala, and ventromedial hypothalamic nucleus, but this same treatment did not stimulate aromatase activity in other brain regions. Further evidence for androgen dependent regulation of aromatase activity is seen in the rat. Castration reduces aromatase activity in the preoptic area, but not the amygdala of male rats, while androgen administration restores aromatase activity in the preoptic area to levels greater than intact controls (Roselli, et al., 1984; Roselli, Horton, & Resko, 1985; Roselli & Resko, 1984). Furthermore, treatment of intact male rats with flutamide reduces aromatase activity in the hypothalamus-preoptic area (Roselli & Resko, 1984). Thus, even in cases where the effects of T on reproductive behaviors and gonadotropin secretion are ultimately mediated by E, the presence of androgen receptors may be required for this aromatization to occur. Further support for this hypothesis is the fact that in male hamsters housed in short days, a situation in which steroid responsiveness to the effects of T on behavioral and endocrine parameters is similar to that of a prepubertal male, there is a reduction in hypothalamic aromatase activity compared with those

hamsters housed in long days, and androgen administration to short day hamsters fails to increase hypothalamic aromatase activity compared with those housed in long days (Callard, Mak, & Solomon, 1986). Therefore, changes in the relative abundance of androgen receptors during the process of pubertal maturation may both directly and indirectly modulate responsiveness to the actions of testosterone on the nervous system.

Hypotheses and Predictions

The working hypothesis of this dissertation is that target tissue responsiveness to steroid hormones is in part mediated by the number of cells that express steroid receptor. While it is clear that both estrogen receptors and androgen receptors are involved in mediating the biological responses obtained following androgen administration, experiments in this dissertation only address the possible role of androgen receptors. Based on our working hypothesis, it was predicted that increases in androgen receptor containing cells would occur in brain regions which are known to be involved in the control of male sexual behavior. Second, it was predicted that a decrease in androgen receptor containing cells would occur in brain regions involved in the control of the negative feedback actions of T on gonadotropin secretion. Initial experiments in this dissertation demonstrate that there is indeed an increase in density of androgen receptor containing cells in some brain regions during pubertal maturation (Experiments I and II). Since the increase in circulating levels of T during pubertal maturation is one of the most dramatic endocrine changes during puberty, and several lines of evidence indicate that testosterone can regulate the expression of the androgen receptors, Experiments III and IV specifically address the hypothesis that the differential behavioral responsiveness to

the effects of T in pre- and postpubertal animals is related to differences in the regulation of the androgen receptor by androgens themselves. Thus if behavioral responsiveness to T is related to the ability of T to upregulate the level of AR in specific brain regions, then an identical dose of T to pre- and postpubertal ferrets would be predicted to result in a different pattern of AR immunostaining. Specifically, it was predicted that androgen treatment to prepubertal animals would result in a less robust increase in AR-IR cells than the same treatment to adults.

Experiment I. Distribution of androgen receptor containing cells in male ferrets before and after the onset of pubertal maturation.

As an initial investigation of developmental changes in androgen-sensitive brain regions during pubertal maturation, androgen receptor-immunoreactive (AR-IR) cells were identified in the brain of male ferrets before and after puberty onset.

Methods

Animals and Housing

All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals using protocols that were approved by the Michigan State University All-University Committee for Animal Use and Care. Thirty-six weanling (7 wk old) male ferrets were purchased from Marshall Farms (North Rose, NY) and housed under short days (8 hr light per day) in pairs in stainless steel cages (51 x 60 x 38 cm) in a temperature controlled (23° ± 1°C) colony room. Purina Ferret Chow (Ralston Purina, St. Louis, MO) and water were available ad libitum.

Procedure

At 12 wks of age, a group of ferrets (n=16) was transferred from short days to a long day photoperiod (16 hr light per day) to induce pubertal maturation, while the remaining ferrets (n=20) remained in short days to undergo spontaneous pubertal maturation. Under these two photoperiodic conditions, an increase in testicular size occurs within 2 wks following the transfer from short to long days (i.e., by 14 wk of age), while an increase in testicular size does not occur until approximately 18 wk of age in ferrets remaining in short days (Sisk, 1990). A group of four ferrets in short days was sacrificed at 12 wk of age, and additional groups of four ferrets in both short days and long days were sacrificed at 13.5, 15, 17.5, and 20 wks of age. Animals were anesthetized with methoxyflurane anesthesia (Metofane; Pittman-Moore; Washington Crossing, NJ), a blood sample (3 ml) was obtained via cardiac puncture, and the testes were removed via a mid-scrotal incision and weighed. Ferrets were then injected with Equithesin anesthetic (2.5 ml/kg ip) and perfused intracardially with 250 ml of 0.5 M Sorensen's phosphate buffer containing 0.8% NaCl, 0.8% sucrose, 0.4% ß-D-glucose and 3 IU/ml heparin, pH 7.4, followed by fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.5. Brains were removed from the skull, placed in fixative containing 15% sucrose, and stored at 4°C until sectioning. Brains were cut into 40 µm thick sections which were stored in a cryoprotectant solution at -20°C (Watson, Weigand, Clough, & Hoffman, 1986).

Testosterone Radioimmunoassay

Plasma concentrations of T in the terminal blood samples were measured with reagents in the Coat-a-Count Total Testosterone Kit (Diagnostic Products, Los Angeles,

CA). The limit of detectability of the assay was 0.26 ng/ml, and the intra-assay coefficient of variation was 9.8%.

Androgen Receptor Immunocytochemistry

Every sixth section from each brain was processed for immunocytochemistry, and tissue from both treatment groups was processed simultaneously. Sections were rinsed 10 times in 0.1 M PBS to remove the cryoprotectant solution, and then incubated sequentially in 0.1 M PBS containing 0.1 M glycine for 30 min, 0.5% H_2O_2 for 30 min, 4% normal goat serum for 30 min (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA), and rabbit anti-androgen receptor [PG-21 obtained from Dr. Gail Prins, Michael Reese Hospital, Chicago, IL, 0.5 µg/ml in 0.1 M PBS containing 0.2% Triton X-100 (PBS-TX); 36 h]. Sections were then incubated in secondary antibody (goat anti-rabbit immunoglobulins, Vectastain ABC Elite Kit, 1:200, 4 h), avidin-biotin-HRP complex (Vectastain ABC Elite Kit, 1:200, 2 h) and 1.0 % 3,3'-diaminobenzidine (DAB) containing 2% ß-D(+) glucose, 0.04% NH_4Cl, 0.038% imidazole, 50 µl/ml of 250mM $NiCl_2$, and 0.0075% H_2O_2 in 0.05 M Tris buffered saline (TBS; 10 min). Sections were rinsed 3 times in PBS-TX between each incubation with the exception of the rinse immediately preceding the DAB reaction which was in TBS. All incubations were at room temperature except primary antiserum, which was at 4°C. Following the DAB reaction, all sections were rinsed 5 times in PBS, mounted onto gelatin coated slides, dried, dehydrated and coverslipped.

Specificity of immunostaining was analyzed by competition and deletion studies. The primary antiserum is directed against a peptide fragment containing the first 21 amino acids of the human androgen receptor (Prins, Birch, & Greene, 1991).

Preincubation of the primary antiserum with 10 M excess of the peptide fragment against which it was raised virtually eliminated immunocytochemical staining (Figure 2B), while preincubation with a distant fragment of the human androgen receptor (amino acids 462-478) did not inhibit immunostaining (Figure 2C). Processing tissue in the absence of the primary antiserum resulted in no detectable immunostaining by the secondary antibody (Figure 2A). Specific immunostaining is shown in Figure 2D.

Tissue Analyzed in Experiment I

In this laboratory, brain tissue is typically processed into several sets of equally spaced brain sections allowing for the analysis of multiple anatomical dependent measures. For this initial study of pubertal regulation of androgen receptors, only tissue from 12 wk old prepubertal ferrets and 20 wk old ferrets housed in short days was processed for androgen receptor immunocytochemistry. This was the most efficient manner to begin answering the questions posed in the above sections, since it was important first to establish whether there are differences in AR expression before and after puberty onset.

Quantification of AR-IR Cells

A detailed description of the distribution of AR-IR cells in an adult T treated male is provided in Appendix A. As shown in Appendix A, literally thousands of cells that stain for AR can be found in a single section under the appropriate conditions. It was therefore not feasible to attempt to quantify the total number of AR-IR containing cells in each brain region. Estimates of AR-IR cell abundance were made by sampling the number of AR-IR cells within a circumscribed area at a high magnification. Thus the

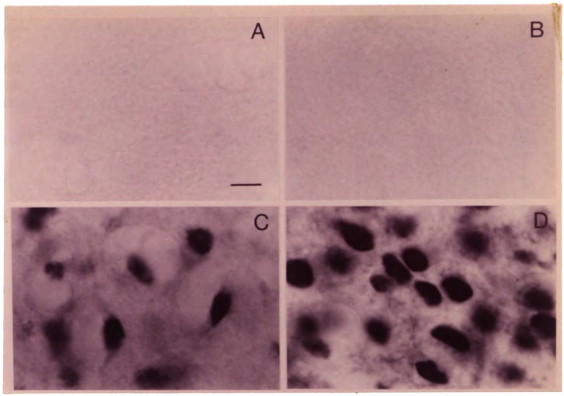


Figure 2. Specificity of androgen receptor immunoreactivity was confirmed by the lack of immunostaining when the tissue was processed in the absence of primary antiserum (A), or by preincubation of the primary antiserum with 10M excess of the peptide fragment against which it was raised (B). Preincubation of the primary antiserum with a distant peptide fragment of the human androgen receptor did not inhibit immunostaining (C). Androgen receptor-immunoreactivity using the standard immunocytochemical staining procedure is shown in D. Bar = 10 μm for all panels.

density of AR-IR cells was quantified in a given brain region by counting (at 1000x) the number of immunopositive cells present within an eyepiece grid (125μm by 125μm) positioned over the region. The grid was placed over this same relative position in two sections separated by 240 μm, and the two sections were anatomically matched across animals. Figure 3 schematically illustrates the position of the grid within each of the brain regions analyzed. Bilateral cell counts were made for each region, and the number of cells/grid/region was averaged across the 4 counts for each animal. Occasionally the tissue quality in a given region was not adequate for quantification, and in such cases, data from that region for that animal were omitted from the analysis.

The density of AR-IR cells was quantified in the lateral septum (LS), bed nucleus of the stria terminalis (BNST), medial preoptic area (MPA), periventricular preoptic area (pvPOA), retrochiasmatic area (RCh), anterior medial amygdala (MeA), arcuate nucleus (ARC), and the lateral portion of the ventromedial nucleus of the hypothalamus (lVMH). The LS, MPA, BNST and MeA are brain regions which have been identified as components of the steroid-sensitive neural circuit controlling male reproductive behaviors (Kondo, Shinoda, Yamanouchi, & Arai, 1990; Powers, Newman, & Bergondy, 1987). The ARC, RCh and VMH are structures known to contain androgen binding cells (Sar & W.E., 1975; Vito, Baum, Bloom, & Fox, 1985), but they have not been directly implicated in the control of male reproductive behavior. We have used the anatomical nomenclature adopted by Paxinos and Watson (1986) to refer to these areas, but cannot say whether responses observed in the precise areas examined in the present study would generalize to previously identified subdivisions in rodent brain.

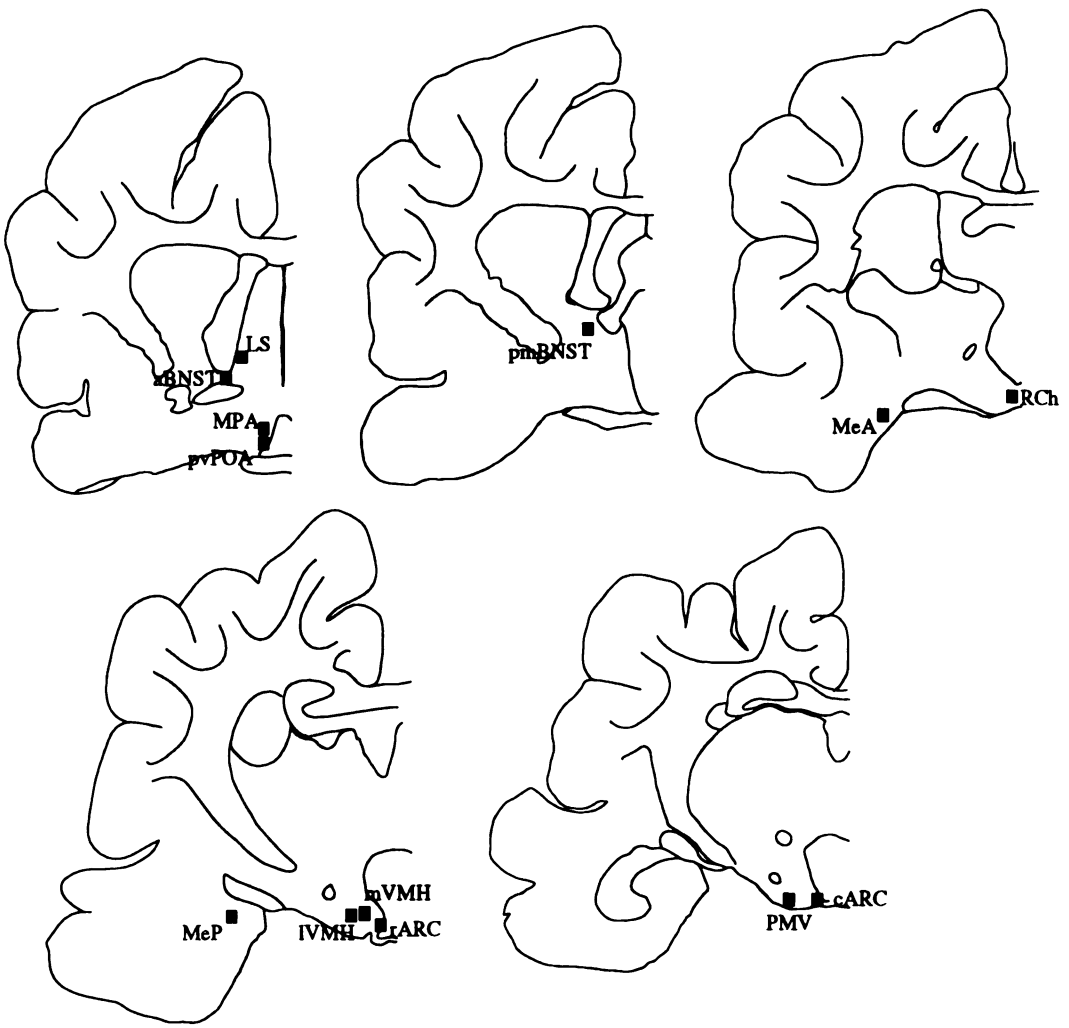


Figure 3. Line drawings of representative brain sections which were anatomically matched for quantification of the number of AR-IR cells. The position in which the reticle grid was placed in each region is marked by the square (■). Not all regions were examined in each experiment.

Data Analysis

Group differences in mean plasma T concentration, mean testis weight, and the density of AR-IR cells within each brain region were assessed by Student's t-tests (2 tailed).

Results

Mean paired testis weight and plasma T concentrations were significantly greater in 20 wk old ferrets compared to 12 wk old ferrets (Figure 4; $t(5) = 13.96$, $p < .01$, and $t(5) = 3.21$, $p < .05$, respectively). Of the four 12 wk old ferrets, only one had detectable levels of plasma T (1.8 ng/ml) at the time of sacrifice, whereas all of the 20 wk old ferrets had detectable levels of plasma T (range of 5.4-15.2 ng/ml). The mean density of AR-IR cells in the regions analyzed is shown in Figure 5. The density of AR-IR cells was significantly greater in 20 wk old ferrets compared to prepubertal ferrets in the ARC, $t(5) = 4.12$, $p < .01$, MPA, $t(5) = 3.29$, $p < .05$, pvPOA, $t(5) = 5.11$, $p < .01$, MeA, $t(5) = 4.07$, $p < .01$, RCh, $t(5) = 3.29$, $p < .05$, and lVMH, $t(5) = 2.98$, $p < .05$. The mean density of AR-IR cells in the LS or the BNST was not significantly different between 12 and 20 wk old ferrets, both $p > 0.05$.

Cellular immunoreactivity in brains of both prepubertal and 20 wk old ferrets was restricted to the nucleus (Figure 6, C&D). Nucleoli were unlabeled. The intensity of intracellular staining was heterogeneous within most of the cell groups analyzed, ranging from a very intense dark reaction product in some cells to a light reaction product in others. This was particularly true of the VMH, POA and MeA. Overall, immunostaining was noticeably more intense in 20 wk old ferrets than in prepubertal animals, however optical density was not quantified (cf Figure 6C & D).

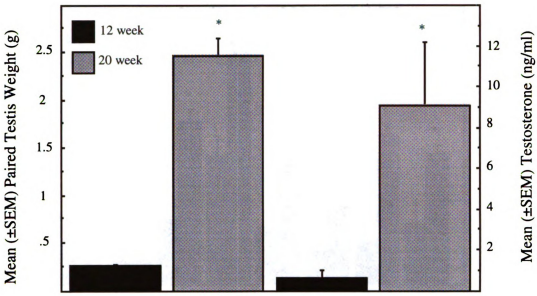


Figure 4. Mean (±SEM) paired testis weight (g) and plasma testosterone concentration (ng/ml) in 12 wk old and 20 wk old male ferrets. Asterisk designates statistically significant differences between the two groups ($p < .05$).

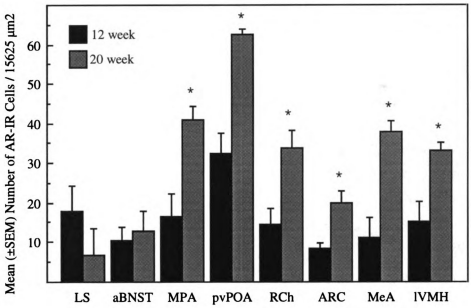


Figure 5. Mean (±SEM) number of androgen receptor-immunoreactive (AR-IR) cells / 15,625 μm^2 in specific brain regions of 12 wk old and 20 wk old male ferrets. Asterisk designates statistically significant differences between the two groups ($p < .05$).

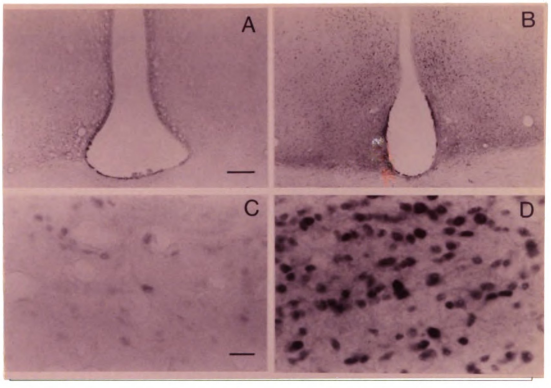


Figure 6. Low (A and B) and high (C and D) power photomicrographs of androgen receptor-immunoreactivity in the POA from representative 12 wk old (A and C) and 20 wk old (B and D) male ferrets. Bar = 200 μm (A and B) or 20 μm (C and D).

Discussion

This experiment is the first comparison of brain AR-immunoreactivity in males at different stages of pubertal maturation, and demonstrates that sexual maturation is characterized not only by gonadal growth and elevated circulating T concentrations, but also by an increase in the density of AR-IR cells in specific brain regions. The increase in the density of AR-IR cells in behaviorally relevant brain regions such as the MPA and MeA is consistent with the hypothesis that pubertal increases in behavioral responsiveness to the activational effects of T on reproductive behavior may be in part mediated by an increase in the number of cells responsive to androgens.

With respect to the neuroendocrine changes associated with pubertal maturation, this experiment found no evidence to support the hypothesis stated in the introduction that the pubertal decrease in responsiveness to the negative feedback effects of T may be due to a reduction in the density of AR-IR cells in brain regions implicated in the control of negative feedback. There is some evidence indicating that negative feedback on gonadotropin secretion can be achieved by direct implantation of gonadal steroids into the region of the mediobasal hypothalamus (Cheung & Davidson, 1977; Kalra & Kalra, 1980). However, this includes several nuclei and including the VMH, RCh, ARC, any or all of which may be involved in mediating negative feedback on gonadotropin secretion. Furthermore, these data do not rule out the possibility that other regions may also be involved in mediating negative feedback. Given that the precise anatomical location and/or locations which mediate the negative feedback effects of T on gonadotropin is unclear, it is possible that there is a brain region that shows a reduction in the density of AR-IR cells that was not quantified in the present study.

The results of this initial experiment leave several other issues unresolved that form the basis for the remainder of this dissertation. First, is the increase in the density of AR-IR cells observed in Experiment I linked to the process of pubertal maturation, or to some other unknown developmental event associated with age? Experiment II addresses this issue by using a photoperiod manipulation to alter the timing of the onset of pubertal maturation. Second, what is the mechanism responsible for the increase in the density of AR-IR cells during pubertal maturation? Experiment III addresses the hypothesis that the pubertal rise in circulating T is responsible for the increased density of AR-IR cells as animals undergo pubertal maturation. Experiment IVa addresses the hypothesis that the differential behavioral response of prepubertal and adult male ferrets to androgen administration is due to the differential regulation of androgen receptors by androgens themselves. Finally, Experiment IVb examines the short-term effects (30 min to 8 hrs) of T exposure on androgen receptor immunocytochemical staining in castrated adult ferrets. This experiment was designed to provide evidence against the possibility that changes in AR-IR staining following steroid treatment are solely due to translocation of receptor proteins in the presence of ligand, as well as to begin to assess the time-course of androgenic regulation of androgen receptors. The results of these experiments will lay the groundwork for experiments designed to test specifically whether the increase in AR-IR cell density is functionally linked to the changes in responsiveness to T which occur during pubertal maturation.

Experiment II. Time-course for the appearance of androgen receptor-immunoreactive cells in the brains of male ferrets undergoing pubertal maturation under different photoperiod conditions.

Rationale

Increases in gonadal size and in LH secretion have been directly linked to the process of pubertal maturation in male and female ferrets by demonstrations that the timing of the increase in these parameters is advanced during photostimulated pubertal maturation relative to that during spontaneous pubertal maturation (Ryan, 1985; Ryan & Robinson, 1985; Sisk, 1990). The present experiment tested the hypothesis that the increase in the density of AR-IR cells in specific regions of the male ferret brain observed in Experiment I is also directly linked with the process of pubertal maturation. If true, then advancing the age at onset of pubertal maturation by photostimulation should similarly advance the age at which the increase in the number of AR-IR cells in specific brain regions occurs, and the increase in AR-IR cell number should be temporally correlated with other indices of pubertal maturation.

Methods

Tissue utilized in Experiment II

Since Experiment I showed that there was an increase in AR-IR cell density after the onset of spontaneous puberty, sets of tissue from all animals described in the methods section of Experiment I were processed to determine if the increase in AR-IR cell density was linked directly to the process of pubertal maturation, or to a developmental process more closely related to the chronological age of the animal. Specific protocols are outlined in Experiment I.

Data analysis

A one-way analysis of variance (ANOVA) on the number of AR-IR cells/grid/region in 12 wk old prepubertal controls housed in short days, in 20 wk old ferrets undergoing photostimulated puberty in long days, and in 20 wk old ferrets undergoing spontaneous puberty in short days was performed in order to determine whether our previous experimental results showing a regional increase in the density of AR-IR cells was replicated across 2 separate immunocytochemical runs, and to determine if, at 20 wks of age, there was a difference in AR-IR cell density between ferrets in long days and ferrets in short days. Significant differences were probed with the Fisher's Protected Least Significant Difference (PLSD) test , and were considered significant at $p<0.05$. Because the group of 12 wk old ferrets in short days served as a baseline control group against which the other groups of ferrets of different ages and in different photoperiods were compared, Dunnett's tests (one tailed, $\alpha = 0.05$) were used to determine at what age mean testis weight, circulating T levels, and density of AR-IR cells within each region in ferrets undergoing photostimulated or spontaneous puberty first differed from these measures in 12 wk old prepubertal controls (Steel & Torrie, 1980). This analysis was used to determine if transfer of ferrets from short days to long days at 12 wks of age advanced the onset of gonadal growth and circulating T levels relative to ferrets remaining in short days, and if advancing the onset of gonadal growth similarly advanced the increase in the density of AR-IR cells in specific brain regions. Finally, to determine whether chronological age, testis weight, or concentrations of circulating T, was the best predictor of the density of AR-IR cells, a multiple regression analysis, forcing all three variables into the model, was performed on the combined data

from those regions in which an increase in the density of AR-IR cells during pubertal maturation was observed. Each independent variable in the model (i.e., age, testis weight, and circulating T) was assessed for statistical significance using a t-test (Steel & Torrie, 1980).

Results

Gonadal indices

Based on Dunnett's tests, the photoperiod transition from short to long days induced precocious gonadal maturation as evidenced by significant increases in testis weight and plasma T concentrations at younger ages in ferrets transferred to long days compared with ferrets that remained in short days (Figure 7). Relative to 12 wk prepubertal ferrets, testis weight significantly increased by 15 wks of age in ferrets transferred to long days at 12 wk of age, but did not significantly increase until 17.5 wks of age in ferrets that remained in short days. Mean plasma T concentration in ferrets in long days was significantly greater than that of 12 wk old prepubertal ferrets by 17.5 wks of age ($p<0.01$), while plasma T levels in ferrets remaining in short days did not significantly increase above levels seen in 12 wk old controls until 20 wks of age.

Androgen receptor immunoreactivity

Cellular AR immunoreactivity in brains of all ferrets was restricted to the nucleus with a lack of reaction product in the nucleoli. The intensity of intracellular staining was heterogeneous within most of the cell groups analyzed, ranging from a very intense dark

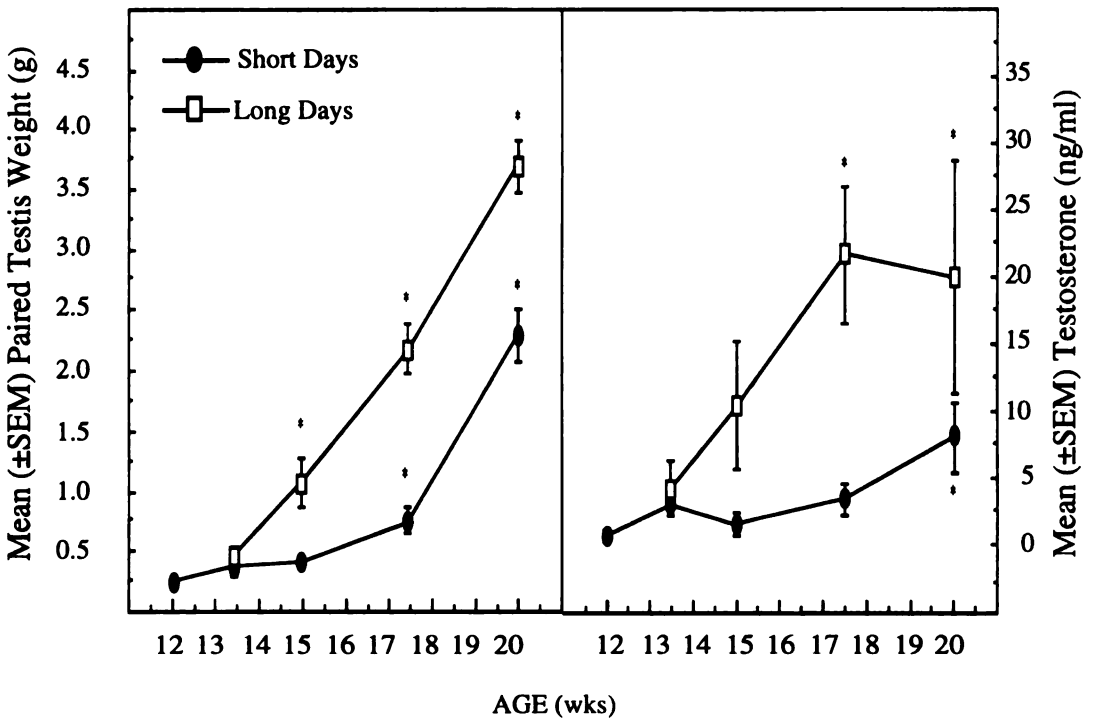


Figure 7. Mean (±SEM) paired testis weight (g) and plasma testosterone concentration (ng/ml) in male ferrets undergoing spontaneous pubertal maturation in short days or photoinduced pubertal maturation in long days. Asterisk designates significantly different than 12 wk old prepubertal controls ($p<.05$).

reaction product in some cells to a light reaction product in others. Overall, immunostaining increased in intensity as ferrets began undergoing pubertal maturation, however optical density was not quantified.

One-way ANOVA between 12 wk old prepubertal ferrets housed in short days, and 20 wk old ferrets undergoing photostimulated puberty in long days or spontaneous puberty in short days indicated that both groups of 20 wk old ferrets had a significantly greater density of AR-IR cells than 12 wk old prepubertal animals in the MPA [$F_{2,8}$=13.22] MeA [$F_{2,8}$=21.58], lVMH [$F_{2,7}$=13.25], and ARC [$F_{2,7}$=8.62] (Figure 8; all p's<0.05). No developmental increase in AR-IR cell density was observed in the BNST or LS (p's >0.05). Furthermore, 20 wk old ferrets undergoing photoinduced pubertal maturation had a significantly greater density of AR-IR cells in the MeA and MPA, than did 20 wk old ferrets undergoing spontaneous pubertal maturation in short days (p's<0.05). There were no differences between photoperiod conditions at 20 wks of age in the density of AR-IR cells in the ARC or lVMH (p's>0.05).

For those regions in which an increase in the density of AR-IR cells occurred, Dunnett's tests indicated that the photoperiod transition from short to long days resulted in an increased density of AR-IR cells at a younger age (Figure 8). The mean density of AR-IR cells in the MPA, MeA, lVMH and ARC was significantly greater than that of 12 wk old controls by 17.5 wks of age in ferrets transferred to long days, but did not significantly increase in ferrets that remained in short days until 20 wks of age.

Multiple regression analysis indicated that a significant proportion of the variance in the dependent variable (AR-IR cell density) could be accounted for by the three independent variables (age, testis weight, and T levels) in those brain regions in which

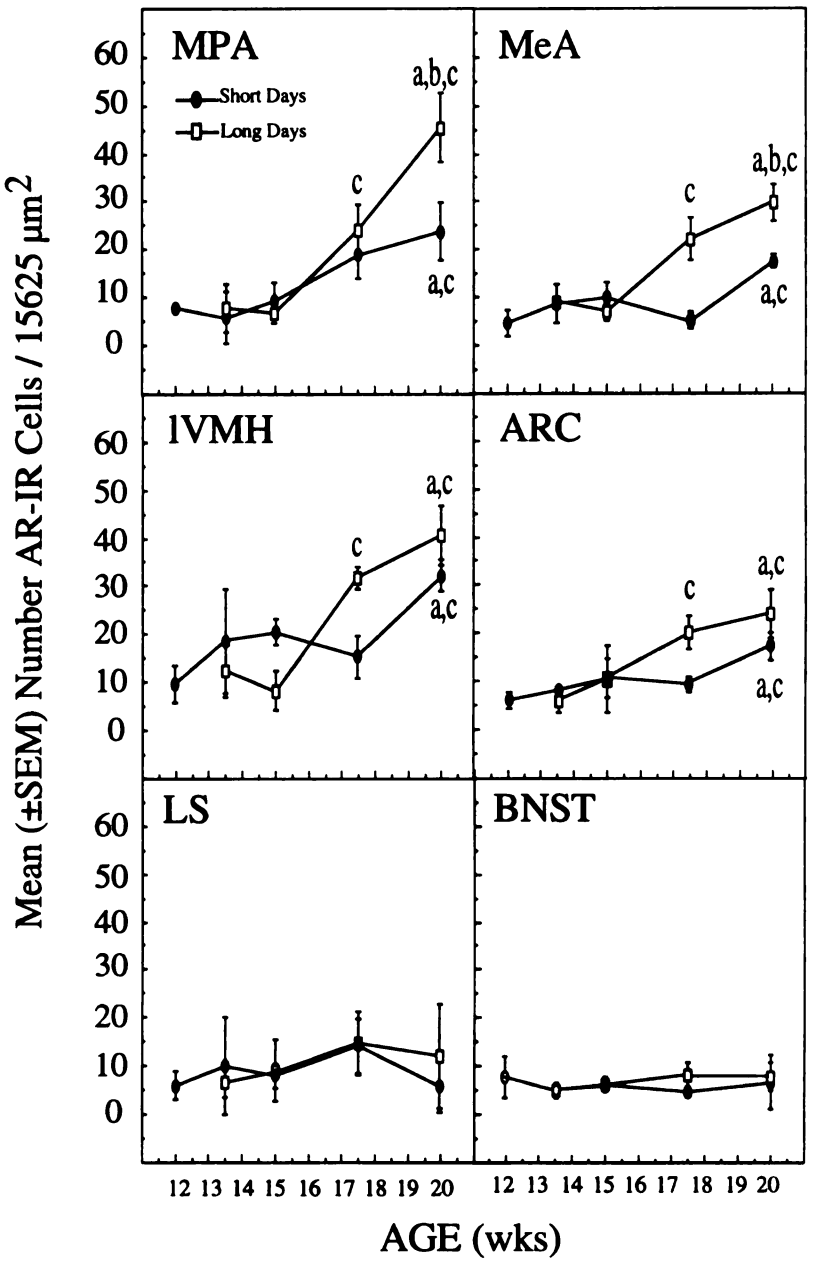


Figure 8. Mean (±SEM) number of androgen receptor-immunoreactive (AR-IR) cells / 15,625 µm2 in specific brain regions of male ferrets undergoing spontaneous pubertal maturation in short days or photoinduced pubertal maturation in long days. Symbols: (a) significantly different from 12 wk old prepubertal controls (ANOVA, $p<.05$), (b) significantly different from 20 wk old ferrets in short days (ANOVA, $p<.05$), (c) significantly different from 12 wk old prepubertal controls (Dunnett's tests, $p<.05$).

there was a pubertal increase in the density of AR-IR cells [$F_{3,121}$=37.60; $p<0.0001$, R^2=.482]. T-tests performed on each independent variable indicated that only testis weight was a significant predictor of the density of AR-IR cells [t=3.39, $p<0.01$].

Discussion

This experiment has confirmed our previous demonstration of an increase in the density of AR-IR cells in the POA, MeA, VMH and ARC of male ferrets undergoing spontaneous pubertal maturation (Kashon & Sisk, 1994), and extends this observation to male ferrets undergoing photoinduced pubertal maturation. We have further demonstrated that a photoperiod transition from short days to long days at 12 wks of age advances the age at which significant gonadal growth and increases in circulating T levels occur. This early onset of gonadal maturation stimulated by photoperiod is also reflected in the brain by an advance in the timing of the increase in the density of AR-IR cells in POA, MeA, VMH and ARC. In addition, testicular size, but not chronological age, is a significant predictor of AR-IR cell density in these regions. Increases in LH pulse frequency and circulating levels of gonadal steroids are well-known endocrine correlates of pubertal maturation, and in seasonal breeders, the timing of increases in these measures is influenced by environmental photoperiod (Ebling & Foster, 1989). This experiment provides evidence that an increase in the density of AR-IR cells in specific brain regions is a neuroanatomical correlate of pubertal maturation that is also influenced by environmental daylength.

The mechanism responsible for the pubertal increase in the density of AR-IR cells in POA, MeA, VMH, and ARC is unknown. There is ample evidence that androgens can

increase androgen receptor immunoreactivity, nuclear androgen receptor binding, and androgen receptor half-life (discussed in detail in Experiment IVb) (Bittman & Krey, 1988; Krey & McGinnis, 1990; Prins & Birch, 1993; Sar, Lubahn, French, & Wilson, 1990; Wood & Newman, 1993a). Thus, the increase in circulating levels of T which occurs during pubertal maturation, operating through one or more mechanisms could mediate the pubertal increase in the density of AR-IR cells in certain brain regions. In this experiment, multiple regression analysis indicated that T concentration was not a significant predictor of the density of AR-IR cells in any brain region. However, the single sample of blood collected in this experiment may not be an accurate estimate of circulating T due to the pulsatile nature of T secretion. This analysis did indicate that testis weight, which provides a more stable correlate of ongoing gonadal steroid secretion, is a significant predictor of the density of AR-IR cells in those regions of the brain in which a pubertal increase in the density of AR-IR cells occurred (i.e., POA, MeA, ARC, and VMH).

The density of AR-IR cells did not increase as a function of pubertal maturation in all brain regions examined. While clear increases in the density of AR-IR cells were seen in the POA, MeA, ARC, and VMH, there were no increases in the density of AR-IR cells in the BNST or the LS associated with pubertal maturation in either photoperiod condition. The lack of a pubertal increase in AR-IR cell density in the LS and BNST indicates that pubertal regulation of androgen receptor is different across brain regions. The factors that result in a greater density of AR-IR cells in the POA, VMH, ARC, and MeA of more reproductively mature ferrets apparently do not operate in the same fashion in LS or BNST. Other instances of brain region differences in the regulation of androgen

receptor immunoreactivity have been previously documented (Menard & Harlan, 1993). Regulation of AR is also different in separate lobes of the prostate (Prins & Birch, 1993), and regulation of estrogen receptor-immunoreactivity is also brain region specific (Koch, 1990; Sisk & DonCarlos, 1995). Differences in afferent input and in intracellular chemical milieu are likely to be involved in brain region-specific regulation of steroid hormone receptors.

Pubertal maturation is associated with increased responsiveness to the activational effects of T on steroid-dependent male reproductive behaviors (Sisk, et al., 1992). Prepubertal males display little reproductive behavior in the presence of a receptive female, and relatively high doses of T are required to induce reproductive behavior in prepubertal males (Beach, 1942; Sisk, et al., 1992; Sodersten, Damassa, & Smith, 1977). Furthermore, the lowest dose of T able to activate male reproductive behaviors decreases as animals undergo pubertal maturation, indicating a pubertal change in sensitivity to the behavioral actions of steroid hormones (Sisk, et al., 1992). Similarly, the photoperiod in which male ferrets undergo pubertal maturation, and/or in which they are tested as adults, modulates responsiveness to the behavioral actions of T in adulthood (Sisk, et al., 1992). As adults, ferrets that undergo spontaneous pubertal maturation in short days require a higher dose of T to activate male reproductive behavior patterns, and are thus less responsive to the activational effects of T on male reproductive behaviors, than animals that undergo photostimulated pubertal maturation following a photoperiod transition from short days to long days (Sisk, et al., 1992). This is true despite the fact that measures of gonadal function and LH secretion between spontaneous and photoinduced animals do not differ in adulthood (Sisk, 1990). The cellular basis for pubertal and

photoperiod modulation of behavioral responsiveness to T is unknown, but this experiment provides evidence consistent with the hypothesis that a pubertal increase in androgen receptor expression in brain regions involved in the control of male sexual behavior (e.g. MPA, MeA) may mediate the increased responsiveness to the behavioral effects of T. In addition, at 20 wk of age, AR-IR cell density in these brain regions is greater in animals undergoing photostimulated puberty compared with animals undergoing spontaneous puberty. It is unknown if this photoperiod difference in the density of AR-IR cells in POA and MeA will persist once pubertal maturation is complete (by approximately 28 wks (Sisk, 1990). If it does, then photoperiod modulation of responsiveness to behavioral actions of T may also be mediated by photoperiod-induced changes in AR-IR cell density in brain regions important for male reproductive behavior.

Experiment III. Regulation of androgen receptor-immunoreactive cells in the brains of prepubertal male ferrets by gonadal steroids.

Rationale

The temporal correlation between the pubertal rise in T secretion and the increased density of AR-IR cells in certain brain regions suggests that T may be responsible for the pubertal increase in AR-IR cell number. There is evidence from both immunocytochemical studies and androgen receptor binding studies that both the cellular location of androgen receptors (nuclear vs cytoplasmic) and the intracellular concentration of androgen receptors in brain can be altered by androgens (Bittman & Krey, 1988; Krey & McGinnis, 1990; Menard & Harlan, 1993; Wood & Newman,

1993a). Studies in other tissues or model systems also indicate, either directly or indirectly, that androgens may stimulate AR synthesis and/or increase AR protein stability (Blondeau, Corpéchot, Le Goascogne, Baulieu, & Robel, 1975; Grino, Griffin, & Wilson, 1990; Handa, Stadelman, & Resko, 1987b; Kemppainen, Lane, Sar, & Wislon, 1992; Krongrad, Wilson, Wilson, Allman, & McPhaul, 1991; Prins & Birch, 1993; Robel, Eychenne, Blondeau, Jung-Testas, Groyer, Mercier-Bodard, et al., 1983; Syms, Norris, Panko, & Smith, 1985; Verhoeven & Cailleau, 1988). Thus, the higher circulating levels of T, present after the onset of puberty, and operating through one or more mechanisms, could induce the pubertal increase in the number of AR-IR cells in brain.

This experiment was designed to examine the regulation of brain AR-IR cells by gonadal steroids in prepubertal male ferrets, and to test the hypothesis that the pubertal increase in AR-IR cell density is the result of the pubertal increase in circulating androgens. It was predicted that castration would reduce nuclear AR immunoreactivity, and that androgen treatment would reverse this effect of castration. Furthermore, if the previously observed pubertal increases in the number of AR-IR cells in POA, ARC, MeA, and VMH are induced by the pubertal increase in circulating T concentrations, then treatment with a dose of T that yields adult plasma levels of hormone in castrated prepubertal ferrets should result in a significant increase in the density of AR-IR cells in these brain regions compared with those in intact prepubertal controls.

Methods

Animals and Experimental Design

Twenty five 7-wk-old weanling male ferrets were purchased from Marshall Farms (North Rose, NY) and housed in pairs in stainless steel cages (51 x 60 x 38 cm) in a temperature- (23° ± 1°C) and light- (8 hr light : 16 hr dark) controlled colony room. Under these conditions, testicular growth begins at about 18 wk of age, and therefore ferrets in this study were still juvenile at the time of sacrifice (mean body weight ± SEM = 816.3 ± 55.9 g). At 9 wk of age, 4 groups of 5 ferrets each were castrated under methoxyflurane anesthesia (Metofane; Pittman-Moore, Washington Crossing, NJ), and one group of 5 ferrets underwent sham castration and remained intact. Following a 1 wk recovery period animals were injected (sc) once daily with either 5 mg/kg testosterone propionate, 5 mg/kg dihydrotestosterone propionate, 10μg/kg estradiol benzoate, or oil vehicle (castrated and intact animals) for 10 days. The doses and injection regimen for T and E were chosen based on their ability to activate reproductive behavior in adult ferrets (Baum, Carroll, Cherry, & Tobet, 1990; Sisk, et al., 1992).

Animals were perfused 4 hr following the final steroid injection, animals were sacrificed, brains were prepared for AR immunocytochemistry , and plasma was prepared for radioimmunoassay for T as described in Experiment I. However, several modifications of the AR immunocytochemistry procedure were included in this experiment. Due to the report that visualization of brain AR immunostaining in castrated hamsters required a lengthy incubation of the tissue in the chromagen solution (Wood & Newman, 1993a), half of the sections from each animal were incubated in the DAB solution for 6 min, while the other half were incubated for 60 min. To further enhance

androgen receptor immunocytochemical staining, the incubation in secondary antibody was increased to 24 hours, and the concentration of $NiCl_2$ in the DAB was doubled to 50 µl/ml of 500mM $NiCl_2$. In addition to T levels measured in intact, castrate and T treated animals, a radioimmunoassay for E was performed using the terminal blood samples from intact, castrated, and E treated animals with reagents from the Coat-A-Count Estradiol Kit. All samples were run in duplicate and the minimum detectable levels of steroid were 0.10 ng/ml for T and 10.0 pg/ml for E.

Data Analysis

For each brain region, the mean density of AR-IR cells was analyzed using a two-way analysis of variance (steroid treatment x incubation time). Significant differences were probed with the Fisher's PLSD test (Steel & Torrie, 1980). All differences were considered significant at $p \leq 0.05$.

Results

Distribution and patterns of AR immunocytochemical staining

The general distribution of AR-IR cells throughout the forebrain and hypothalamus of the male ferret was similar to that seen in Experiments I and II, and previously reported in males of other species (Choate & Resko, 1992; Clancy, Bonsall, & Michael, 1992; Menard & Harlan, 1993; Sar, et al., 1990; Wood & Newman, 1993a). However, due to the modifications in the immunocytochemical staining procedure, AR-IR cells were much darker in this experiment than previously seen. There were noticeable differences in intracellular immunocytochemical staining intensity across the regions in which AR-IR cells were quantified in this experiment. In intact animals, AR-

IR cells were darkest in the LS and pvPOA, appeared to be slightly less dark in the MPA, VMH, and ARC, and were lightest in the MeA and BNST. Photomicrographs of AR-IR cells in the pvPOA and the lVMH from representative animals within each treatment group are shown in Figure 9. In intact and androgen-treated animals immunostaining was restricted to the nucleus (Fig. 9A, 9B, 9C, 9F, 9G, 9h). Subjectively, androgen treatment resulted in darker nuclear immunostaining within any given brain region when compared with that of any of the other treatment groups. However, even in the presence of androgen, AR-IR cells in the MeA and BNST remained more lightly stained compared with AR-IR cells in other regions. Castration reduced the intensity of nuclear immunocytochemical staining in all brain regions, and resulted in the appearance of visible immunocytochemical reaction product distributed throughout the cytoplasm of some, but not all, cells (Figure 9E and 9J, arrows). The qualitative appearance of AR-immunostaining in estrogen-treated castrates resembled that of oil-treated castrates, except that cytoplasmic staining, while observed in estrogen-treated ferrets, did not seem as prevalent as it was in oil-treated castrates (Fig. 9D and 9I).

Effect of incubation time in DAB on AR-immunoreactivity

Incubation of tissue in DAB for 60 min resulted in darker immunocytochemical staining intensity when compared with that in tissue incubated in DAB for only 6 minutes. However, ANOVA revealed that, of the eight brain regions examined, only in ARC [$F_{1,38}$=6.22, p=0.017] and in MeA [$F_{1,39}$=12.731, p=0.001] was there a significant main effect of incubation time in DAB on the density of AR-IR cells. In these two regions, tissue which was incubated for 60 min in chromagen had more AR-IR cells than

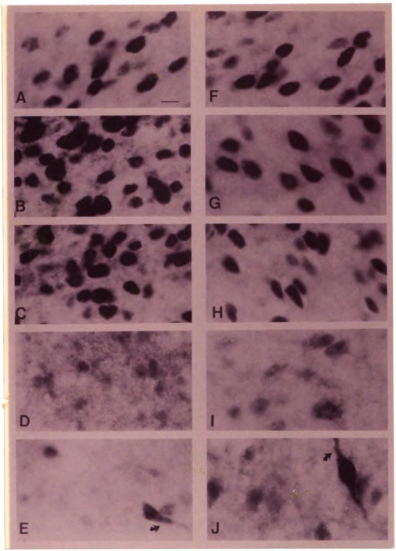


Figure 9. Photomicrographs of AR-IR cells taken from the periventricular preoptic area (pvPOA; A-E), and the lateral portion of the ventromedial hypothalamus (lVMH; F-J) from oil-injected intact animals (A,F), and those castrated and injected once daily for 10 days with either 5 mg/kg testosterone propionate (B,G), 5 mg/kg dihydrotestosterone propionate (C,H), 10μg/kg estradiol benzoate (D,I), or oil vehicle (E,J). The pvPOA is an example of a region in which androgen treatment (T or DHT) resulted in a significantly greater number of AR-IR cells compared with intact males (cf A with B and C). The lVMH is an example of a region where androgen treatment did not increase the number of AR-IR cells above the number seen in intact animals (cf F with G and H). Arrows in E and J point to AR-immunoreactivity in the cytoplasm, which was seen primarily only in tissue from castrated animals. All micrographs are of tissue incubated in DAB for 60 minutes. Magnification Bar= 10μm.

did tissue incubated for only 6 min. The mean density of AR-IR cells in the short and long incubation times, respectively, and collapsed across steroid treatment, were 27.5 $\pm$ 2.85 and 34.2 $\pm$ 3.2 for the ARC, and 17.0 $\pm$ 3.0 and 26.2 $\pm$ 3.1 for the MeA. However, no significant interactions existed between steroid treatment and incubation time on AR-IR cell density in any brain region examined, indicating that the long incubation in the chromagen did not preferentially increase the density of AR-IR cells in a given steroid treatment group. Therefore, comparisons between steroid treatments in each region were analyzed using data collapsed across incubation time (Keppel, 1982).

Effect of steroid treatment on the density of AR-IR cells

Two-way ANOVA revealed a significant main effect of steroid treatment on the density of AR-IR cells/grid in all brain regions (all p's < 0.01). Figure 10 depicts the mean density of AR-IR cells in intact and castrated ferrets treated with oil or steroids in each brain region collapsed across incubation time (Keppel, 1982). Several effects of steroid treatment were consistent across brain regions. First, castration resulted in a significant decrease in the density of AR-IR cells in all regions examined. Second, T or DHT administration to castrates resulted in a significantly greater density of AR-IR cells compared to treatment with oil or E in all brain regions. Finally, for all brain regions, there were no significant differences in density of AR-IR cells between T- and DHT-treated castrates, or between oil- and E-treated castrates.

Importantly, there were brain region differences with respect to the magnitude of the increase in AR-IR cell density following administration of T or DHT. The statistical analysis indicated that specific brain region responses to androgen treatment of castrates

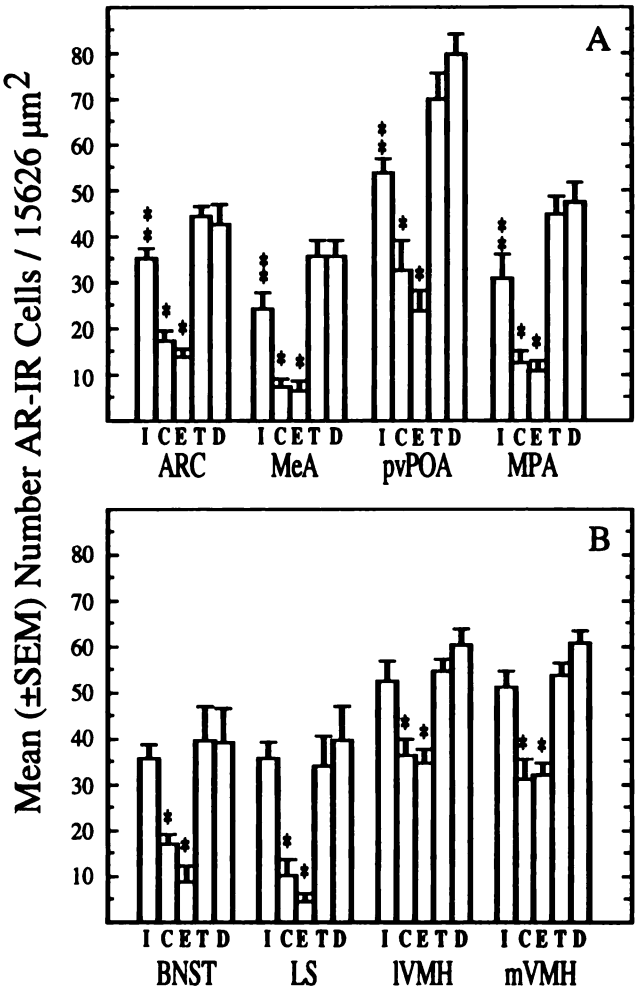


Figure 10. Mean (±SEM) number of androgen receptor-immunoreactive (AR-IR) cells/15,625 µm2 in specific brain regions of intact and castrated prepubertal male ferrets injected (sc) once daily with either 5 mg/kg testosterone propionate (T), 5 mg/kg dihydrotestosterone propionate (D), 10 µg/kg estradiol benzoate (E), or oil vehicle (castrate (C) and intact (I) animals) for 10 days. Animals were sacrificed 4 hr following the last injection. Regions in which androgen treatment significantly increased the number of AR-IR cells compared with intact animals are shown in A. Regions in which androgen treatment restored the number of AR-IR cells to that seen in intact animals are shown in B. Asterisk (*) designates significant difference from intact, T and DHT treatment. Double asterisk designates significant difference from T and DHT treated animals.

could be classified as one of two types: either a restoration of the density of AR-IR cells similar to that seen in intact animals, or a significant increase in the density of AR-IR cells compared with that of intact animals. These two types of response to androgen treatment are illustrated by the photomicrographs of AR-IR cells in pvPOA and lVMH in Figure 9, and in Figure 10, the 8 regions are separated in panels A and B on the basis of which category of response to androgen was observed in that region. In the BNST, LS, mVMH, and lVMH, androgen administration to castrates restored the density of AR-IR cells to that observed in these regions in intact animals (cf 9F with 9G and 9H; Figure 10B). In contrast, in the MPA, pvPOA, MeA, and ARC, the same androgen treatment not only reversed the effects of castration, but resulted in a significant increase in AR-IR cell density compared with that seen in these regions in intact animals (cf 9A with 9B and 9C; Figure 10A). Figure 11 depicts these data as the percent of increase or decrease in the density of AR-IR cells in steroid treatment groups relative to intact prepubertal males, and arranges brain regions in order of increasing magnitude of response to androgen treatment. Viewed in this way, brain region specific effects of androgen on AR immunoreactivity may be characterized as a continuum, in contrast to the dichotomy presented in Figure 10. Regardless of how the brain region responses to androgen are categorized, it is clear that there are brain region differences in response to androgen under the present experimental conditions.

The factors that contribute to regional differences in androgen receptor immunocytochemistry are unknown. It is conceivable that regional differences in AR expression are due to regional differences in cell and/or nuclear size, which result from steroid treatment, and thus a bigger cell may be more likely to be detected by

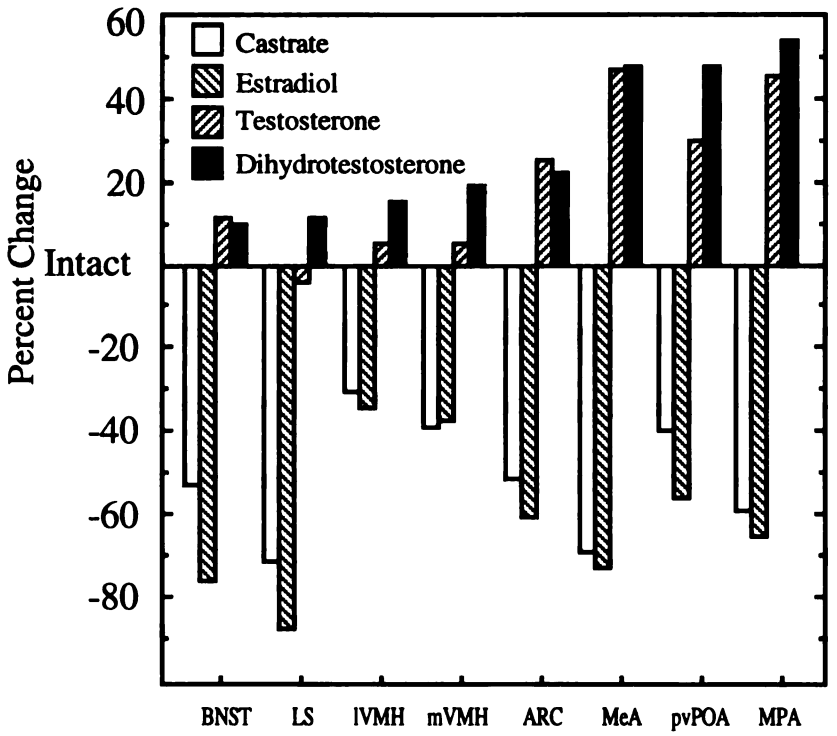


Figure 11. Percent change relative to intact prepubertal male ferrets in the number of AR-IR cells/15,625 μm2 in specific brain regions of castrated prepubertal male ferrets treated with either 5.0 mg/kg testosterone propionate, 5.0 mg/kg dihydrotestosterone propionate, 10 μg/kg estradiol benzoate, or oil vehicle (sc) once daily for 10 days.

immunocytochemistry. Androgen treatment increases dendritic sprouting (Cherry, Tobet, DeVoogd, & Baum, 1992) and increase cellular size (Tobet, Zahniser, & Baum, 1986b) in some regions of the ferret brain such as the male nucleus of the POA-AH. Thus increases in cellular size within a given region may lead to what appears to be an increase in cell number within that region. However, whether the regional differences in AR found in the present experiment are positively correlated with an increase in cell and/or nuclear size following androgen treatment remains to be determined.

Since there was an effect of incubation time on the density of AR-IR cell in the ARC, and since the effect of androgens on the density of AR-IR cells in this region fell approximately in the middle of the continuum depicted in Figure 11, a one way ANOVA was performed on cell counts for the short and long incubation times separately for this region, even though two way ANOVA did not reveal a significant interaction between steroid treatment and incubation time. Androgen treated groups were not significantly different from intact groups when short incubation time data were analyzed alone, and when long incubation time data were analyzed alone, the number of cells/grid in the ARC of intact animals was significantly different from that of T treated ferrets, but not from that of DHT treated ferrets. Therefore, under the hormone treatment paradigm used in this study, the effect of androgen on the number of AR-IR cells/grid in ARC is somewhat ambiguous.

Steroid Hormone Levels

Plasma T concentrations (mean ng/ml ± SEM) were 0.644 ± 0.315, 0.116 ± 0.014, and 23.328 ± 2.393 for intact, castrated, and T-treated ferrets, respectively. The

levels of T in T-treated ferrets are high, but within the normal physiologic range seen in adult male ferrets (Sisk & Desjardins, 1986). Estradiol levels in all castrated ferrets, and in 3 of 5 intact ferrets, were below the assay limit of detectability. Mean (±SEM) plasma E concentration was 43.1 ± 11.5 pg/ml in E-treated castrates.

Discussion

This experiment demonstrated that castration of prepubertal male ferrets results in a decrease in immunocytochemical staining intensity and in the density of AR-IR cells in specific brain regions, and that AR immunostaining can be restored by treatment of castrates with androgens, but not estrogen. The dramatic decrease in AR immunostaining following castration indicates that brain AR immunoreactivity is influenced even by the very low levels of circulating T characteristic of prepubertal males. The castration-induced decrease and androgen-induced restoration in AR immunoreactivity in prepubertal male ferrets are in general agreement with two other reports of the effects of castration and steroid replacement on brain AR immunoreactivity (Menard & Harlan, 1993; Wood & Newman, 1993a). In a detailed study of intracellular partitioning of AR immunoreactivity in adult male hamsters under different steroid conditions, Wood and Newman (Wood & Newman, 1993a) found that nuclear staining intensity decreased, while cytoplasmic staining increased, after castration. Furthermore, androgen, but not estrogen, replacement reversed these effects, and the low levels of circulating T in males in the nonbreeding condition were sufficient to restrict immunoreactivity to the nucleus. Using a quantitative analysis similar to that used in this

experiment, Menard and Harlan (Menard & Harlan, 1993) reported that a cocktail of anabolic-androgenic steroids, either administered to castrated rats, or superimposed over endogenous steroids in intact males, increased the number of AR-IR cells/unit area in several brain regions. Thus, the ability of androgens to enhance brain AR-immunoreactivity appears to be consistent across a variety of androgen treatment paradigms and species (but see (Choate & Resko, 1992; Clancy, Whitman, Michael, & Albers, 1994) for possible exceptions).

When a quantitative analysis is used to assess the effect of androgen on AR immunoreactivity, as it was in this experiment and in the Menard and Harlan (1993) study, it becomes apparent that the magnitude of the androgen-induced increase in AR-IR cells/unit area varies across brain regions. In the present study, 10 days of androgen treatment, designed to approximate adult circulating concentrations of steroid in castrated prepubertal ferrets, restored the density of AR-IR cells in the BNST, LS, mVMH, and lVMH to that seen in intact prepubertal ferrets. In contrast, the same androgen treatment resulted in a significantly greater density of AR-IR cells in the pvPOA, MPA, and MeA (and possibly in ARC) compared with corresponding densities in intact animals. Differences in the magnitude of brain region responses to androgen have also been documented in male rats. Administration of an androgenic-anabolic steroid cocktail to intact adult male rats increased the number of AR-IR cells/area in VMH above that seen in untreated intact animals, but not in the MPA or MeA (Menard & Harlan, 1993). The data from the present study and those from the Menard and Harlan study (Menard & Harlan, 1993) are not directly comparable because of differences in experimental protocols (steroid treatment of castrated vs intact animals), the particular steroids and

doses administered, species, and reproductive status of the animals (prepubertal vs adult males). The conclusion from both sets of data, however, is that the regulation of AR immunoreactivity by androgens is different across brain regions. Clearly, under experimental conditions, the specific response evoked by androgen in cells in a given brain region will depend upon many factors, including afferent input and neurochemical phenotype of the cells at the time of androgen action. The challenge is to understand what accounts for cell specificity in the regulation of AR by androgen under normal physiological and developmental conditions.

Even under physiological conditions, regulation of AR varies across brain regions. The first two experiments of this dissertation showed that during normal pubertal maturation, the number of AR-IR cells/area increases in the POA, MeA, ARC, and VMH, but not in the BNST and LS. The fact that the regional increases in the density of AR-IR cells were correlated with the increased levels of T that accompany pubertal maturation prompted the present attempt to test the hypothesis that, under experimental conditions, adult levels of T could increase AR-IR cell density in these regions in prepubertal males. Under the replacement paradigm used in the present study, androgens increased the density of AR-IR cells above those seen in intact prepubertal males in POA, MeA, and possibly in ARC, but not in the VMH. Thus, this experiment suggests that the pubertal increase in AR-IR cell density in at least POA and MeA is stimulated by the pubertal increase in T. It is possible that a longer exposure to androgens, a different pattern of hormone administration, or exposure to androgens at an older age, would have simulated the pubertal increase in AR-IR cell density in VMH and ARC as well. Because AR-IR cell density in BNST and LS have not been shown to

increase as a function of puberty, the lack of a significant androgen-induced increase in the density of AR-IR cells in these regions relative to prepubertal controls was expected.

We found no effect of E treatment on the density of AR-IR cells in any brain region. An earlier study reported that E treatment to castrated male rats resulted in a small increase in the amount of radiolabeled androgen binding in the cytosolic fraction of micropunches from the MPA and the MeA, but androgen binding in the VMH, BNST, LS, pvPOA, and ARC was not affected by E (Handa, Roselli, Horton, & Resko, 1987a). It is possible that small estradiol-induced increases in cytosolic receptor levels in the MPA and MeA were below the immunocytochemical limits of detection in the present study.

In summary, this experiment has documented that androgen influences brain AR immunoreactivity in prepubertal males, but that the magnitude of the response to androgen varies across brain region. Regional differences in the regulation of brain AR by androgen allow for the possibility that an increase in circulating T at the time of puberty could sensitize cells in some brain regions to androgen action, while not affecting cells in other regions.

Experiment IV. Regulation of AR-IR cell density by gonadal steroids in pre- and postpubertal male ferrets.

Rationale

Experiment IVa: Experiments I and II demonstrated that after the pubertal increase in T output from the testes, there are more AR-IR cells per unit area in several brain regions compared to these regions in prepubertal males, and that advancing the

onset of increased T production by using a photoperiod manipulation, also advances the time of increase in the density of AR-IR cells in these brain regions. Experiment III demonstrated that treating castrated prepubertal male ferrets with doses of androgens that result in high level of circulating steroid increases the density of AR-IR cells relative to prepubertal intact males in the POA, MeA and perhaps ARC, indicating that the pubertal rise in androgens may be responsible for the pubertal rise in AR-IR cell density in these regions. Previous experiments showed that a paradigm of androgen treatment to castrated ferrets similar to that used in Experiment III results in the activation of reproductive behaviors in adults as well as prepubertal animals, with a trend for prepubertal animals to show less behavior than adults, however differences between the groups were not statistically significant (Sisk, et al., 1992). Up to this point, Experiments I, II, and III have been performed on ferrets either in the prepubertal stage of development or in an advanced peripubertal stage, but not in fully mature adults. Indeed, the behavioral data indicating that there is a change in sensitivity to the behavioral actions of T are based on comparisons between prepubertal male ferrets and fully developed adult ferrets. This experiment directly compares the distribution, density, and intensity of androgen receptor-immunocytochemical staining between prepubertal and fully mature adult ferrets, both in gonadally intact animals, and in castrated animals treated with steroids. Differences in androgen receptor-immunocytochemical staining patterns between these two age groups may lead to insights as to why administration of androgens to prepubertal ferrets does not activate exactly the same pattern of reproductive behaviors as seen in adults. For example, it is possible that differences in behavioral capacities between prepubertal and adult male

ferrets following identical steroid treatment are because of differences in the steroid hormone induced regulation of steroid receptors themselves. Based on this hypothesis, one could predict that while steroid treatment to castrated prepubertal ferrets may increase the density of AR-IR cells above prepubertal intact levels, intact or androgen treated adults may still have a greater density than androgen treated prepubertal ferrets in certain regions of the brain involved in the control of male reproductive behaviors. Were this to be the case, it is possible that some other process occurs during pubertal maturation that modulates the way in which androgens regulate the density of androgen receptor-immunoreactive cells. On the other hand, if there are no differences between prepubertal and adult male ferrets in the ability of T to up-regulate AR-IR cells in the brain, this would indicate that other processes are at work that inhibit prepubertal male ferrets from displaying the full range of male reproductive behaviors, despite sufficient amounts of AR in behaviorally relevant brain regions.

Experiment IVb: The cellular mechanism responsible for the increase in the density of AR-IR cells observed in Experiments I, II, and III remains to be determined. However, higher concentrations of plasma T, whether endogenous or exogenous, probably contributed to the increased density of AR-IR cells in certain brain regions as suggested in Experiment III. Previous immunocytochemical studies of AR provide evidence that circulating T levels may influence AR-immunostaining by several (not mutually exclusive) mechanisms. First, the presence of ligand may increase AR-immunocytochemical staining intensity by a mechanism which does not require AR gene expression or protein synthesis. Castration leads to greatly diminished AR-immunoreactivity in rat prostate tissue, and staining intensity is increased within 15 min

of androgen replacement (Prins & Birch, 1993; Sar, et al., 1990). This effect of androgen on AR-immunoreactivity is likely too quick to be due solely to an increase in receptor protein synthesis. In brain, castration was found to reduce AR-immunoreactivity in some studies (Menard & Harlan, 1993; Wood & Newman, 1993a), but not others (Choate & Resko, 1992; Clancy, et al., 1994), however, in none of these experiments was a rapid effect of androgen on AR-immunoreactivity assessed. In principle, a rapid androgen-induced increase in AR-immunostaining could be accomplished in several ways. The presence of ligand could result in a conformational change in the receptor protein that allows an increase in the number of epitopes available for the antibody to bind. An increase in the number of epitope sites could also occur by the dissociation of heat-shock proteins in the presence of ligand. Alternatively, the presence of androgens could result in a translocation or stabilization of AR within the nucleus, effectively concentrating the receptor protein in the nucleus and increasing the intensity of nuclear staining. Indeed, some *in vitro* experiments have demonstrated an increase in androgen receptor half-life in the presence of ligand (Kemppainen, et al., 1992; Robel, et al., 1983). The increase in receptor half-life would not only result in an increase in immunocytochemical staining without requiring protein synthesis, but could have important physiological implications by allowing prolonged androgen action on the genome.

Another mechanism by which androgen influences AR-immunostaining involves an up-regulation of the receptor protein. Following 3 days of androgen replacement to castrated rats, immunocytochemical staining intensity in prostate tissue is increased over and above that seen after 15 min of replacement and approximates that observed in

gonadally intact rats, indicating that prolonged exposure to androgen results in increased expression of androgen receptor protein (Prins & Birch, 1993). Similarly, brain AR-immunostaining in hamsters and rats is reduced by castration, while chronic androgen administration for 8 hrs or more restores it (Menard & Harlan, 1993; Wood & Newman, 1993a). That the increase in AR-immunoreactivity induced by prolonged exposure to androgen is due at least in part to increased synthesis of receptor protein is corroborated by biochemical studies in tissue homogenates, which indicate that the increase in nuclear AR binding in hypothalamus and pituitary observed after 6 or more hr of androgen treatment is the result of synthesis of new receptor proteins (Bittman & Krey, 1988; Handa, et al., 1987b; Krey & McGinnis, 1990). In one of these studies (Krey & McGinnis, 1990), there was an initial rapid increase in nuclear androgen-plus-receptor complexes following administration of T that was likely due to the translocation of receptor protein into the nucleus. However, androgen-plus-receptor complexes continued to rise above this initial increase until they reached a plateau 16 hr following T administration, indicating synthesis or enhanced stability of receptor proteins (Krey & McGinnis, 1990). Other studies indicate that androgen may upregulate androgen receptor in other tissues and in cell cultures (Blondeau, et al., 1975; Grino, et al., 1990; Handa, et al., 1987b; Kemppainen, et al., 1992; Krongrad, et al., 1991; Robel, et al., 1983; Syms, et al., 1985; Verhoeven & Cailleau, 1988).

Although we cannot exclude the possibility that the increase in the density of AR-IR cells in certain brain regions of ferrets observed in the above experiments is due in part to T-dependent enhanced immunoreactivity without an actual increase in receptor protein, it seems doubtful that this could be the sole explanation of these results. First, if

it were, one would expect to observe a higher density of AR-IR cells in maturing ferrets in *all* brain regions examined, but this was not the case in the lateral septum or BNST. Second, prepubertal ferrets are not strictly comparable to castrated animals in terms of circulating T concentrations. Based on previous determinations of LH pulse frequency in prepubertal ferrets (Sisk, 1987), the demonstration that the testes of the prepubertal ferret are capable of responding to LH by secreting T (Neal, et al., 1977), and the fact that there are detectable levels of T in a small percentage of terminal blood samples obtained from prepubertal ferrets, it is likely that prepubertal ferrets secrete a few pulses of T per day. Given that it takes longer than 24 hr for nuclear AR-immunoreactivity in prostate tissue to diminish after castration, it seems improbable that the relative lack of AR-IR cells in some brain regions of intact prepubertal ferrets is comparable to the reduced AR-immunoreactivity observed by us and others after castration. Thus, we conclude that enhanced immunoreactivity in the presence of ligand is unlikely to be the sole mechanism responsible for the pubertal increase in the density of AR-IR cells, and that an increase in receptor protein expression or stability also contributes to the greater density of AR-IR cells in older animals. This experiment will examine AR immunoreactivity in brain regions of castrated adult ferrets after short term androgen replacement to determine the relative contributions of receptor translocation and/or receptor synthesis to the androgen induced pubertal increase in the density of androgen receptor-containing cells in specific brain regions.

Methods

Adult animals for Experiments IVa and IVb were raised as cohorts, thus allowing us to use castrated adult ferrets treated with oil as a control group for both the examination of long-term (10 days) exposure to gonadal steroids, as well as for the short-term (hours) exposure to T experiments. Thirty-seven male ferrets were obtained from the MSU Mink Farm at approximately 14 wks of age and placed in a short-day lighting environment (8 hr light/day). Short days were chosen so that photoperiod condition would be identical between prepubertal and adult animals at the time of sacrifice. Animals were weighed and testis measurements were taken at biweekly intervals. At approximately 33 wks of age all animals were castrated under Metofane anesthesia with the exception of one group, which was sham castrated and remained intact (n=5). Following a one-week recovery period the animals received the following steroid treatments: Daily T (5 mg/kg) for 10 days (n=5), DHT (5.0 mg/kg) for 10 days (n=5), E (10 μg/kg) for 10 days (n=5), oil vehicle for 10 days (intact and castrate animals n=5 for each group). Animals were deeply anesthetized, a blood sample was taken via heart puncture, and then perfused transcardially approximately 4 h following the final steroid injection, according to the method outlined in Experiment I. Twelve of the castrated animals were injected with oil vehicle each day for 9 days. On day 10, each animal was given an injection of T (5 mg/kg) and perfused either 30 min, 4 hr, or 8 hr after the injection (n=4 per group). Every sixth section from the above animals was processed for AR immunocytochemistry along with a set of tissue from Experiment III consisting of prepubertal males that remained gonadally intact, or that were castrated at 8 wks of age exposed to a steroid injection paradigm beginning at 10 wks of age identical to that

described for the adults in this experiment. Incorporating the prepubertal animals into this experiment enables us to make direct comparisons between pre- and postpubertal animals on the number and staining intensity of AR-IR cells using tissue immunocytochemically processed at the same time (see Appendix B for statistical analysis indicating that no significant loss of AR-IR staining occurred due to long term storage of tissue in cryoprotectant). Thus, the statistical analysis for Experiment IVa includes prepubertal and adult animals that either remained intact, or were castrated and treated for 10 days with either oil, T, DHT, or E. The statistical analysis for Experiment IVb includes only adults that were castrated and injected with oil, or with T for 30min, 4hr, 8hr or 10 days. Thus, two groups of animals will be included in the analysis for each of the experiments, adults castrated treated with oil or with T for 10 days.

Steroid Radioimmunoassays

Plasma concentrations of testosterone in the terminal blood samples from intact, castrated, and T treated animals were measured with reagents in the Coat-a-Count Total Testosterone Kit (Diagnostic Products, Los Angeles, CA). All samples were run in duplicate and the minimum detectable levels of T were 0.10 ng/ml.

Data Analysis

AR-IR Cell Density

Cell counts were recorded at a magnification of 1000x and consisted of counting all cells which fell within the boundaries of a 100 by 100μm ocular grid in the following regions: MPA, pvPOA, aBNST, pmBNST, MeA, MeP, LS, RCh, rARC, cARC, lVMH, and PMV. The pmBNST, cARC and MeP are additional brain regions that were included in this analysis in order to extend our survey of the steroid regulation of AR-IR

cells to other regions which are known to contain AR. The pmBNST and the MeP are areas which are active during male reproductive behavior based on staining for the proto-oncogene cFos (Baum & Everitt, 1992; Wood & Newman, 1993b). The PMV was included because it contains a high level of AR containing cells and shows heavy cytoplasmic staining in castrated animals (Wood & Newman, 1993b). For Experiment IVa, an overall three-way ANOVA (Steroid treatment by Age by Brain Region) was performed on the mean number of AR-IR cells/grid from two matched sections counted bilaterally for each brain region quantified. For Experiment IVb, a two-way ANOVA (Duration of T exposure by Brain Region) was performed to compare the following groups: castrate plus oil, and castrate plus T for 30 min, 4 hr, 8 hr, and 10 d. Significant effects were probed with Fisher's Protected Least Significant Difference (PLSD) test. All differences were considered significant if $p < 0.05$.

AR-IR Staining Intensity

Intact animals and those pre- and postpubertal animals that received T treatment were rated for intensity of immunocytochemical staining in the pvPOA, MPA, MeA, RCh, rARC, LS, aBNST and lVMH using a rating scale modified from DonCarlos et al., 1991. The same sections that were quantified for cell number as described above were rated for staining intensity by two investigators blind to the treatment of the animals. The category assigned to a given animal corresponded to the predominant cell type within the field of view according to the following scale: 0 = no cells stained; 1= very light: staining at threshold of detection, nucleolus not detectable within the nucleus; 2 = light: staining within the nucleus diffuse, transparent, 3 = moderate: nucleolus detectable within the stained nucleus, staining still somewhat transparent; 4 = dark:

staining dense, not transparent but can still detect individual granules of reaction product; 5 = very dark: intense stain, opaque. These estimates were made at a magnification of 1000x. All sections that contained cells stained either moderately, dark, or very dark had a high proportion of cells which, individually scored, would be considered light or very light. Thus ratings in the three darkest categories were based on the predominant cell type within the region, excluding those that were light and very light. Intensity ratings from both parts of Experiment IV were analyzed with nonparametric statistical tests. A correlation coefficient of 0.876 was calculated on the ratings of staining intensity by the two investigators. The data used in the analysis were an average of the two investigators ratings. In Experiment IVa, pair-wise comparisons between pre- and postpubertal intact animals, as well as between intact and T treated animals within the same age group, were analyzed with the Mann-Whitney U test. In Experiment IVb, the Kruskall-Wallis test was used to determine if significant differences in intensity ratings existed between treatment groups. Pair-wise comparisons were then made where appropriate with Mann-Whitney U tests. All differences were considered significant at $p < 0.05$.

Results

Circulating Testosterone

One-way ANOVA showed that there were significant differences between treatment groups in the concentration of circulating T [$F_{10,42}=52.9$; $p<0.0001$] (Figure 12). Post-hoc analysis with the Fisher's PLSD test indicated that animals treated with T for 30 min were not different than intact adults. Both of these groups, however, had significantly lower T concentrations than did animals treated with T for either 4 hr, 8 hr

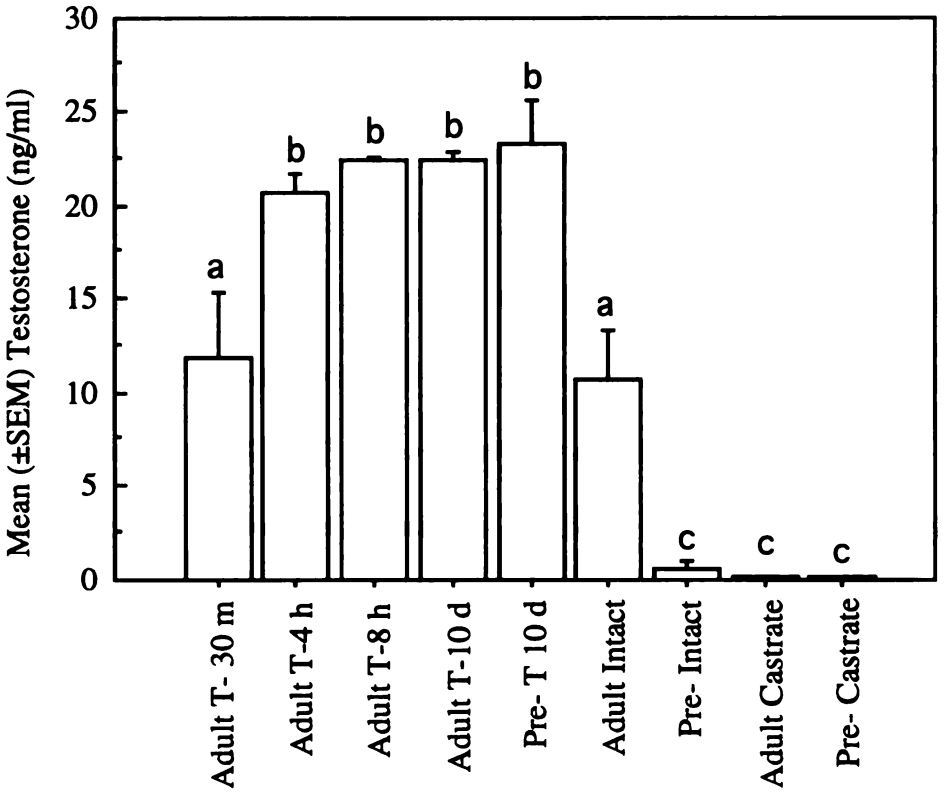


Figure 12. Mean (±SEM) testosterone concentration (ng/ml) in prepubertal and adult ferrets injected with oil or 5.0 mg/kg testosterone propionate for either 30 min, 4 hr, 8 hr, or daily for 10 days. Bars with different letters are significantly different from one another ($p < 0.05$).

or 10 days (prepubertal and adult), but both had significantly higher T concentrations than prepubertal intact animals, or castrated animals (prepubertal and adults) treated with oil. Animals treated with T for either 4 hr, 8 hr or 10 days (prepubertal and adult) were not significantly different from one another, nor were there any differences among prepubertal intact animals and animals castrated and treated with oil. All comparisons between castrated animals treated with T and those treated with oil were significantly different. Adults treated with DHT or E had undetectable levels of T (data not shown); no samples from DHT or E treated prepubertal males were processed for T levels.

Experiment IVa: Steroid Regulation of AR-IR cells in Pre and Postpubertal Male Ferrets

AR-IR cell density

The three-way ANOVA table for the number of AR-IR cells per unit area is shown in Table 1. There were significant main effects of age, steroid treatment and brain region, as well as significant interactions between age and steroid treatment, age and brain region, and between steroid treatment and brain region. The three-way interaction among age, steroid treatment and brain region was not significant. Since main effects cannot fully describe the data when interactions are present, the results of this experiment will be presented and, discussed in terms of the interactions, and therefore data will be collapsed across one factor for each 2-way interaction. However, so that all of the data from each region may be viewed, the mean number of AR-IR cells per unit area within each region for each age for each steroid treatment are illustrated in Figure 13. Photomicrographs from the pvPOA, and MeA for each treatment condition are shown in Figures 14 and 15.

Table 1. Summary table for the three-way analysis of variance from Experiment IVa.

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Age	1	853.419	853.419	17.369	<.0001
Steroid	4	58092.620	14523.155	295.586	<.0001
Age * Steroid	4	1091.933	272.983	5.556	.0002
Region	11	39198.722	3563.520	72.528	<.0001
Age * Region	11	1241.082	112.826	2.296	.0097
Steroid * Region	44	10108.314	229.734	4.676	<.0001
Age * Steroid * Region	44	2549.209	57.937	1.179	.2077
Residual	449	22060.876	49.133		

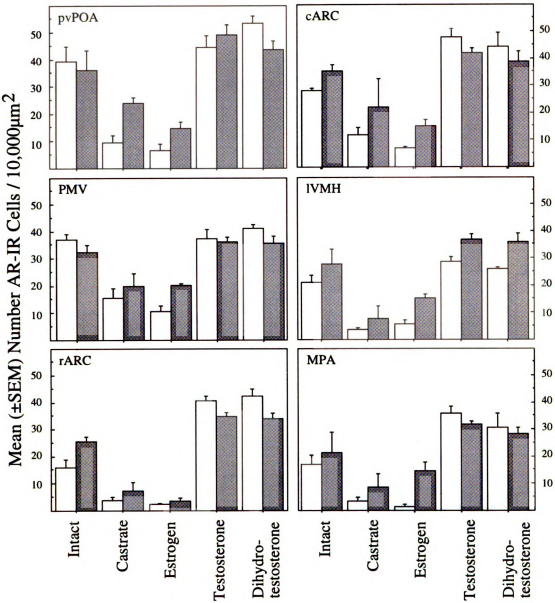


Figure 13. Mean (±SEM) number of AR-IR cells / 10,000 μm2 in individual brain regions from prepubertal (open bars) and adult (closed bars) male ferrets injected daily for 10 days with either oil (intact and castrate), 10 μg/kg E, 5.0 mg/kg T, or 5.0 mg/kg DHT.

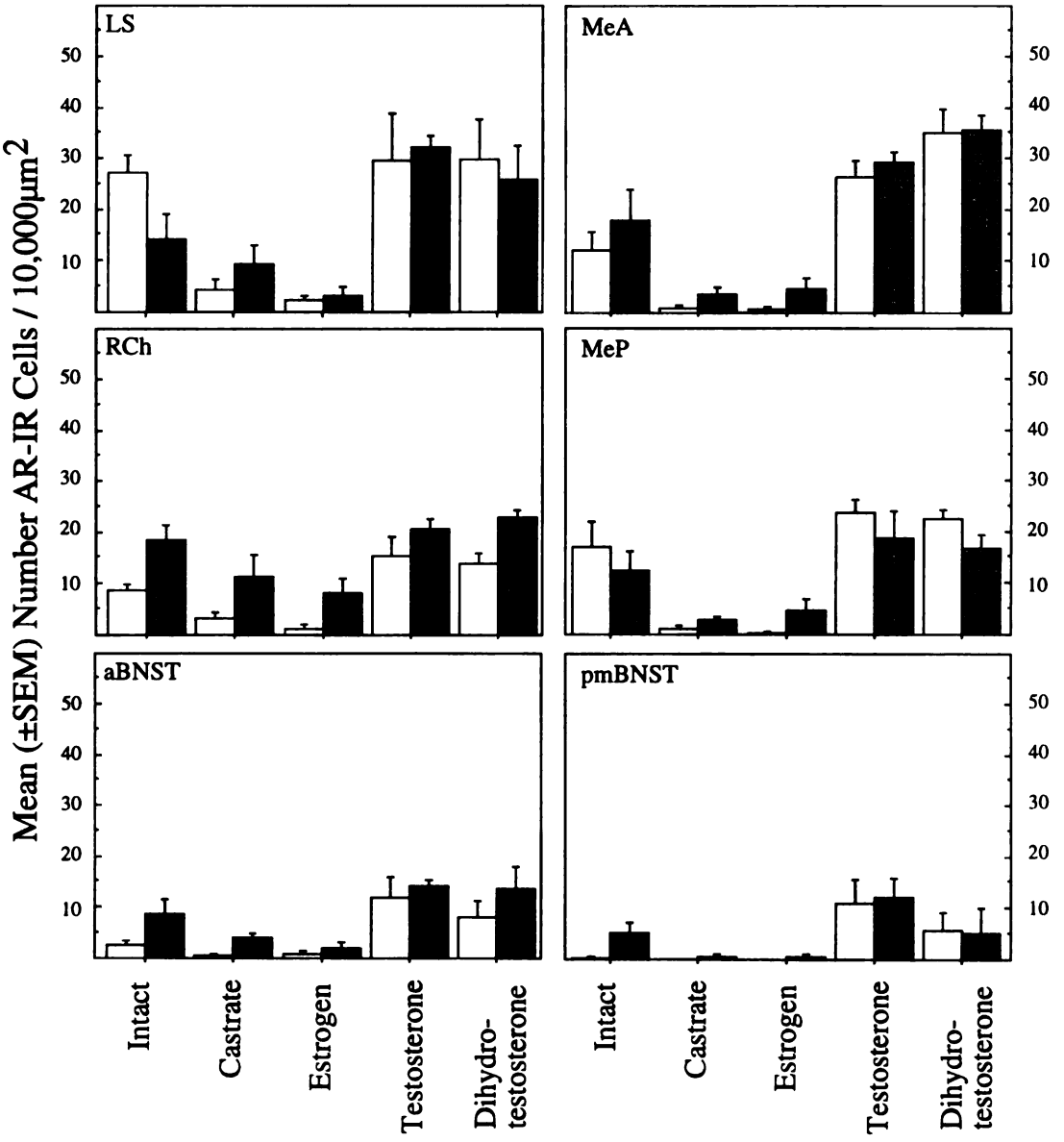


Figure 13 (continued).

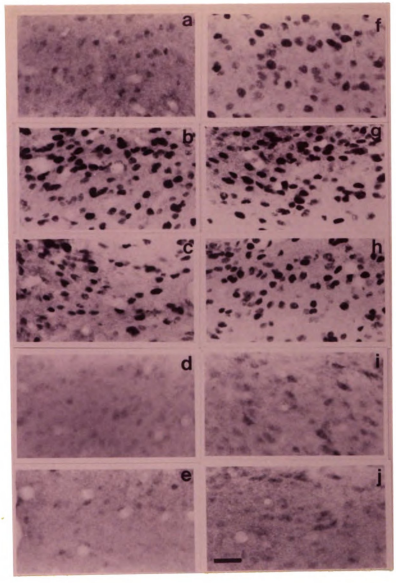


Figure 14. Photomicrographs of the pvPOA from prepubertal (A-E) and adult (F-J) male ferrets that remained intact (a, f) or were injected daily for 10 days with either 5.0 mg/kg T (b, g), 5.0 mg/kg DHT (c, h), oil (d, i), or 10 ug/kg E (e, j). Magnification bar = 30μm.

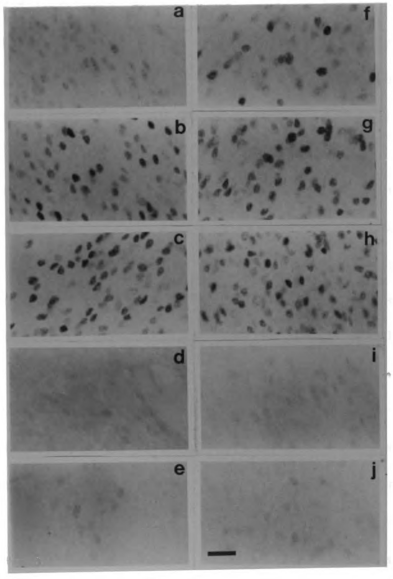


Figure 15. Photomicrographs of the MeA from prepubertal (A-E) and adult (F-J) male ferrets that remained intact (a, f) or were injected daily for 10 days with either 5.0 mg/kg T (b, g), 5.0 mg/kg DHT (c, h), oil (d, i), or 10 μg/kg E (e, j). Magnification bar = 30μm.

Interaction Between Age and Steroid Treatment: Figure 16 shows the mean number of AR-IR cells for each steroid treatment of pre- and postpubertal male ferrets collapsed across brain region. Fisher's PLSD tests examining the effects of steroid treatment within each age group showed that both adult and prepubertal ferrets treated with either T or DHT had a significantly greater density of AR-IR cells than did their age matched counterparts in any other steroid treatment group. Furthermore, intact animals in each age group had a significantly greater density of AR-IR cells than did those treated with oil or E. These differences in the density of AR-IR cells among steroid treatment groups represent the main effects of steroid treatment. The source of the interaction between age and steroid treatment lies in the differences between prepubertal and adult male ferrets that either remained intact or were castrated and treated with oil or E. Adult animals that remained intact, as well as those that were castrated and treated with either oil or E, had significantly more AR-IR cells than did similarly treated prepubertal ferrets. There were no differences between adult and prepubertal male ferrets castrated and treated with either T or DHT.

Interaction Between Age and Brain Region: Figure 17 shows the mean number of AR-IR cells in each brain region for pre- and postpubertal male ferrets collapsed across steroid treatment. Fisher's PLSD tests between pre- and postpubertal male ferrets within each brain region showed that in the RCh, lVMH, pvPOA, and the aBNST adult animals had a significantly greater density of AR-IR cells than did prepubertal animals. In all other regions there were no significant differences between pre- and postpubertal male ferrets.

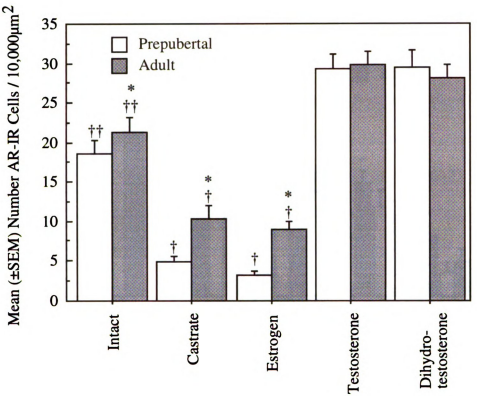


Figure 16. Mean (±SEM) number of AR-IR cells / 10,000 μm^2 collapsed across brain region from prepubertal (open bars) and adult (closed bars) male ferrets injected daily for 10 days with either oil (intact and castrate), 10 μg/kg E, 5.0 mg/kg T, or 5.0 mg/kg DHT. Asterisk indicates significant difference from prepubertal animal in same steroid condition, double cross indicates significant difference from all treatment groups within an age group, single cross indicates significant difference from I, T and D within an age group.

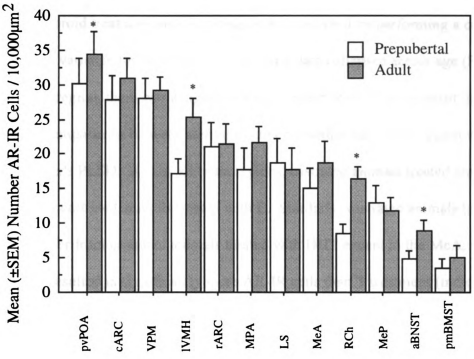


Figure 17. Mean (±SEM) number of AR-IR cells / 10,000 μm2 collapsed across steroid treatment from prepubertal (open bars) and adult (closed bars) male ferrets. Asterisk indicates significant difference from prepubertal animals within the same brain region.

Interaction Between Steroid Treatment and Brain Region: The interaction between steroid treatment and brain region was analyzed by performing a one-way analysis of variance for each brain region using data collapsed across age (Figure 18). In each brain region, there was a significant main effect of steroid treatment, and thus pairwise comparisons between steroid treatments within each brain region were made using Fisher's PLSD. Across all brain regions, castrated animals treated with oil did not differ from castrated animals treated with E. Similarly, castrated animals treated with T did not differ from castrated animals treated with DHT except in the MeA, where DHT treatment resulted in significantly more AR-IR cells than T treatment (indicated by the cross). Animals treated with either oil or E had significantly fewer AR-IR cells (indicated by the single asterisk) than did those animals treated with T or DHT in all regions except in the pmBNST, where those animals treated with oil or E did not differ from those animals treated with DHT. In the pvPOA, MPA, MeA, MeP, rARC, cARC, LS, lVMH, and PMV, intact animals had a significantly greater density of AR-IR cells compared to castrated animals treated with oil or E, while treatment with T or DHT resulted in a significantly greater density of AR-IR cells compared to intact animals in all of these regions except in the MeP, where intact animals did not differ from DHT treated animals, and in the PMV where intact animals did not differ from those treated with either T or DHT. Brain regions which deviated from this general pattern were the aBNST, pmBNST, and RCh, where the density of AR-IR cells in intact animals fell in between animals treated with oil or E, and those treated with T or DHT. This resulted in significant differences only between intact animals and T treated animals in the aBNST and pmBNST, and between intact animals and those treated with E in the RCH.

Figure 18. Mean (±SEM) number of AR-IR cells / 10,000 μm^2 within individual brain regions collapsed across age group. Significant differences only apply to comparisons within a given brain region. Double asterisk indicates significant difference from T and D, single asterisk indicates significant difference from T, D and I, cross indicates significant difference from T, solid circle indicates significant difference from E within brain region.

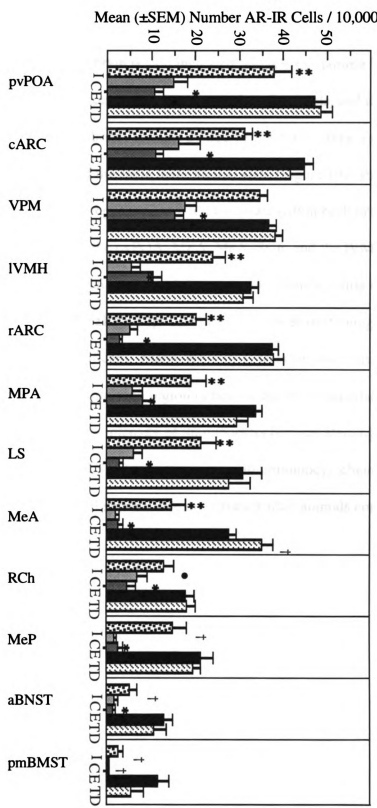

Mean (±SEM) Number AR-IR Cells / 10,000μm2
10
20
30
40
50
pvPOA
cARC
VPM
lVMH
rARC
MPA
LS
MeA
RCh
MeP
aBNST
pmBMST
ICETD

Figure 18

Intensity of AR-IR Staining: Mann-Whitney U tests between groups using data collapsed across brain region indicated that AR-IR staining in adult intact animals was significantly darker than in intact prepubertal animals, and that AR-IR staining in both adults and prepubertal animals treated with T for 10 days was significantly darker than intact animals of the same maturational state (Figure 19). Pair-wise comparisons between intact prepubertal and adult animals within each brain region (Figure 20) indicated that in the pvPOA, MPA, MeA, RCh, and the lVMH adults had significantly darker immunostaining than did prepubertal animals, while in the rARC ($p=0.083$), LS ($p=0.99$) and the aBNST ($p=0.35$) the differences in staining intensity were not statistically significant. Mann-Whitney U tests between intact and T treated animals of the same age within each region indicated that in prepubertal animals, T treatment for 10 days increased the intensity of immunocytochemical staining in all regions except the aBNST and the LS. However increases in immunocytochemical staining intensity failed to reach statistical significance in T-treated adult animals compared to intact adults, except in the LS.

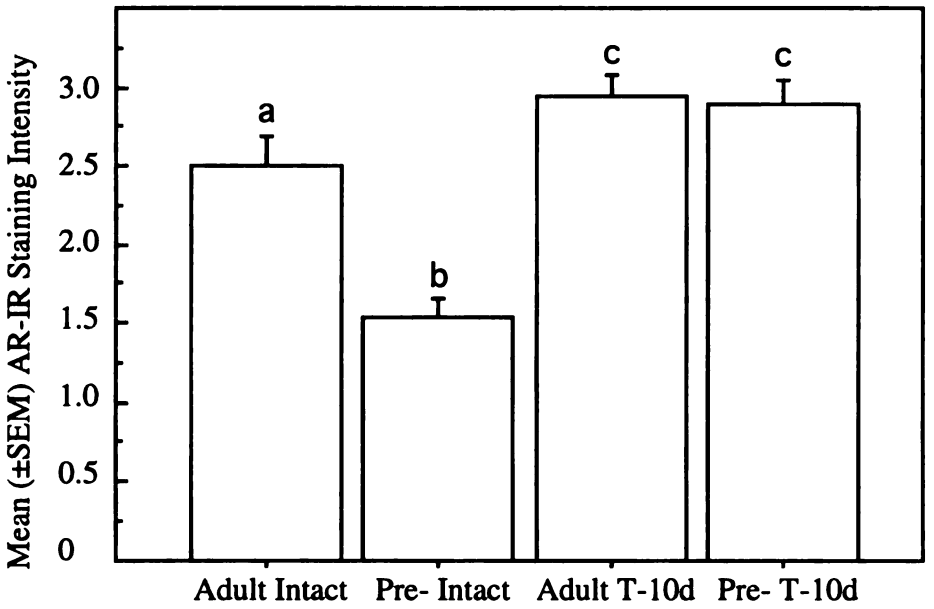


Figure 19. Mean (±SEM) immunocytochemical staining intensity from intact and T treated prepubertal and adult male ferrets collapsed across brain region. Bars with different letters are significantly different from one another (Mann-Whitney U test, $p < 0.05$).

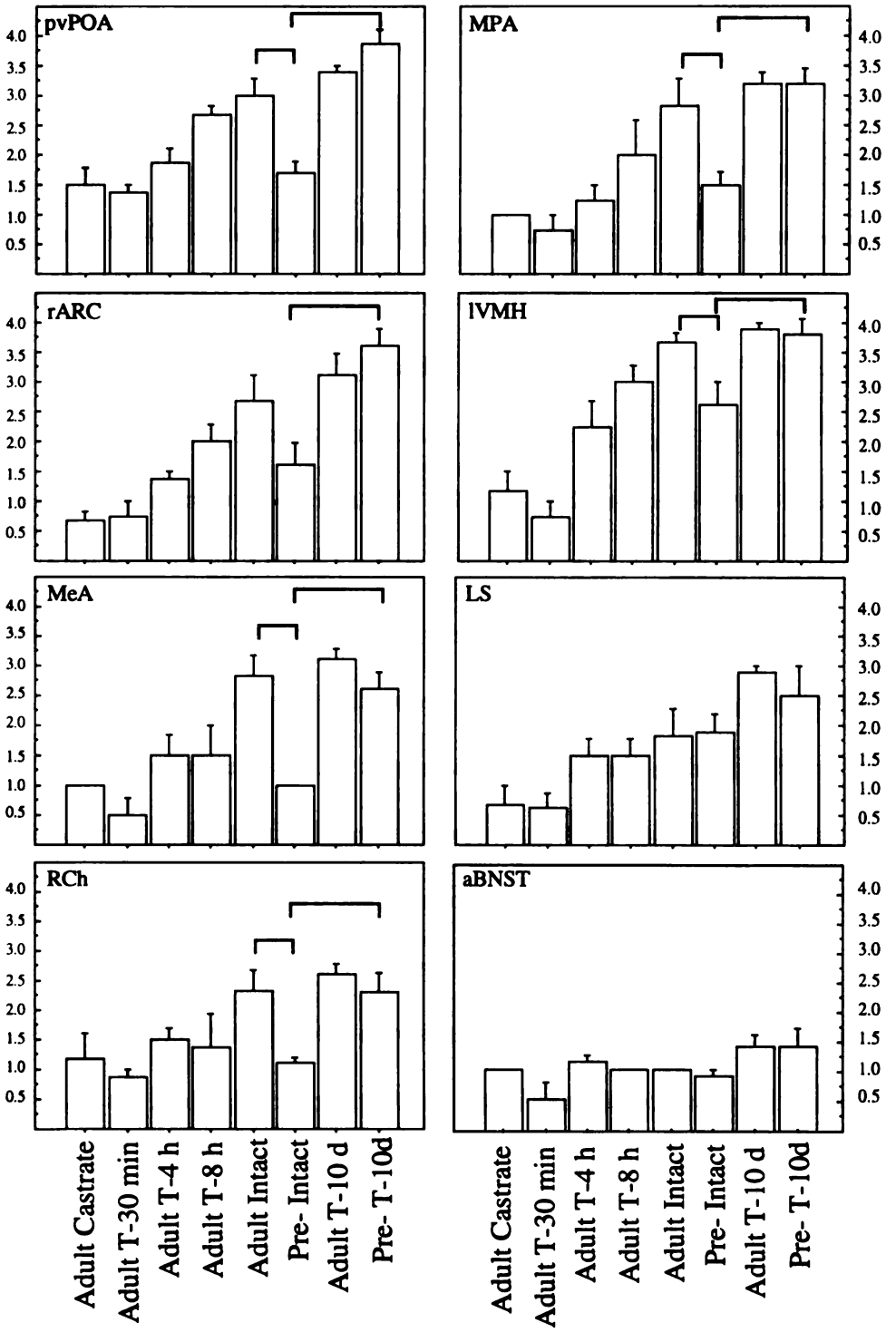


Figure 20. Mean (±SEM) immunocytochemical staining intensity in individual brain regions from prepubertal and adult intact animals, and those castrated and injected with oil or 5.0 mg/kg T for either 30 min, 4 hr, 8 hr, or 10 days. Bars joined by arches are significantly different from one another (Mann-Whitney U test, $p < 0.05$), however not all comparisons were analyzed, see text.

Experiment IVb: Effects of Duration of T Exposure on AR-IR Cell Density in Adults

AR-IR Cell density

Two-way ANOVA utilizing brain region and the duration of T exposure as the independent variables showed significant main effects of duration of T exposure [$F_{4,180}$=4395.9; p<0.0001], and brain region [$F_{11,180}$=1541.1; p<0.0001]. There was no interaction between brain region and duration to T exposure [$F_{44,180}$=1.3; p=0.15]. Figure 21 shows the mean number of AR-IR cells per grid collapsed across brain region. Post-hoc analysis showed no differences between castrated adults and those treated with T for 30 min, even though circulating T was significantly higher in the latter group (Figure 12). All other pair-wise comparisons were significant indicating that as the duration of T exposure increases, there is an associated increase in the number of AR-IR cells per unit area. Examination of individual brain regions (Figure 22) indicated that only two regions deviated from the general pattern of a steady increase in AR-IR cell number following longer exposure to T. In the PMV, AR-IR cells reached their maximum level after only for 4 hr of T exposure, while in the pmBNST, AR-IR cells were virtually absent in all groups except those which had been treated with T for 10 days.

AR-IR Staining Intensity

Kruskal-Wallis tests comparing the intensity of immunocytochemical staining among treatment groups collapsed across brain region indicated that there were significant differences in staining intensity (Figure 23). Mann-Whitney U tests indicated that castrated animals and those treated with T for 30 min were significantly different

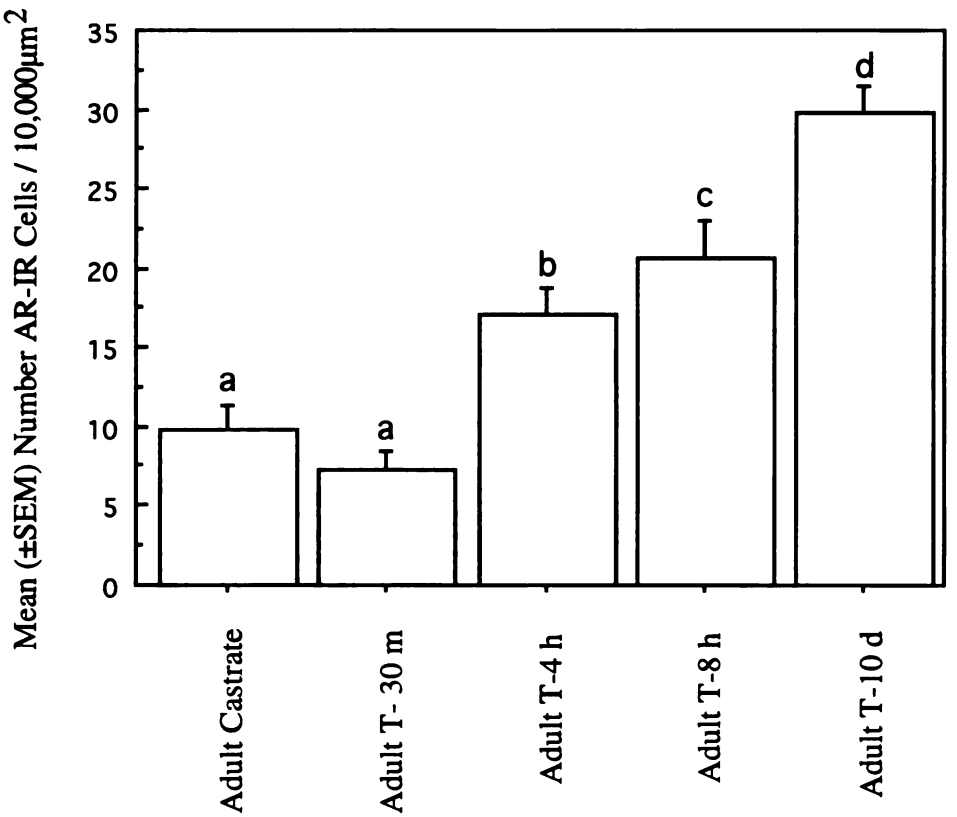


Figure 21. Mean (±SEM) number of AR-IR cells / 10,000 um^2 collapsed across brain region from adult animals castrated and injected with oil or 5.0 mg/kg T for either 30 min, 4 hr, 8 hr, or 10 days. Bars with different letters are significantly different from one another ($p < 0.05$).

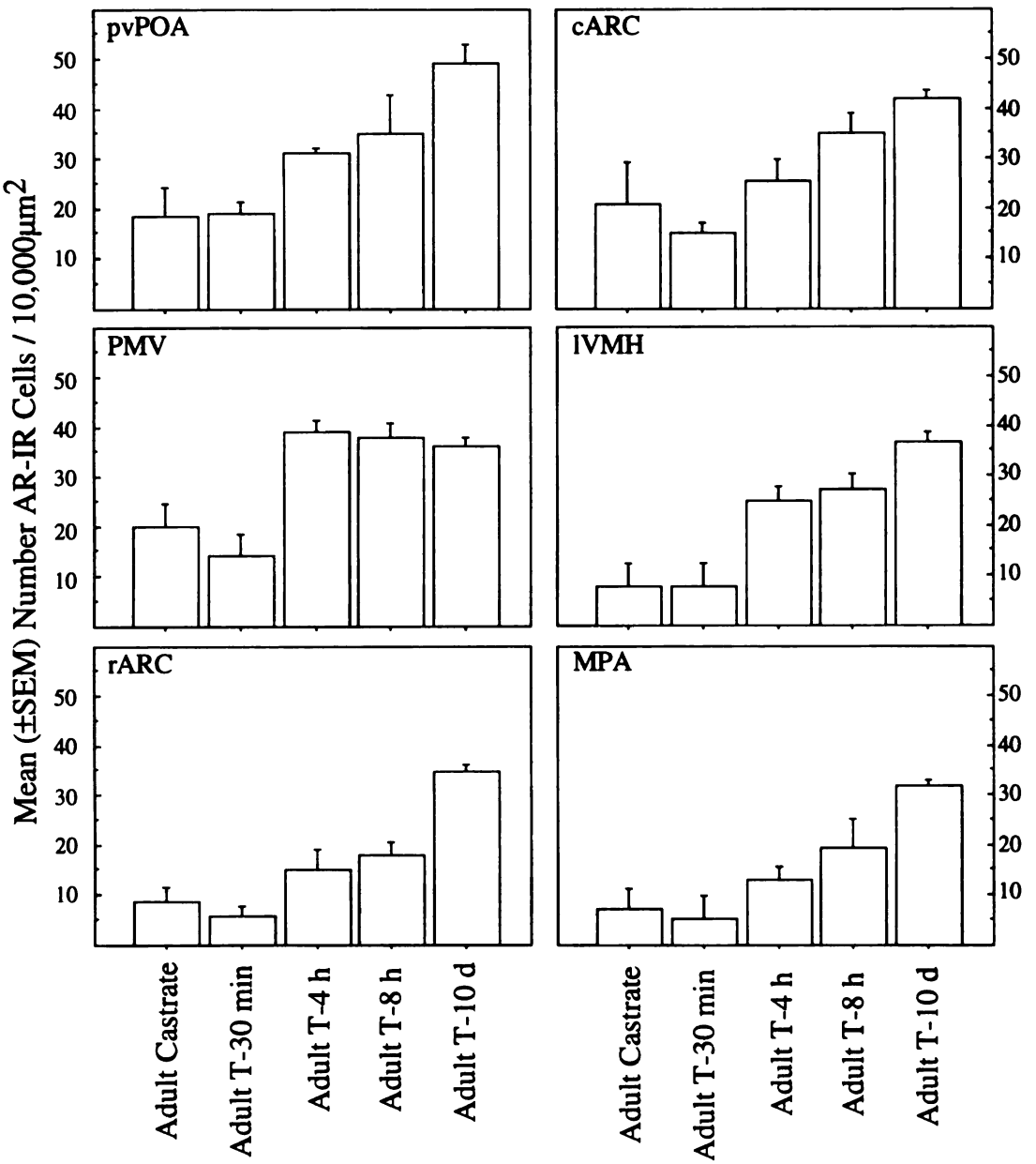


Figure 22. Mean (±SEM) number of AR-IR cells / 10,000 um2 in individual brain regions from adult animals castrated and injected with oil or 5.0 mg/kg T for either 30 min, 4 hr, 8 hr, or daily. Statistical analysis was not performed in individual regions.

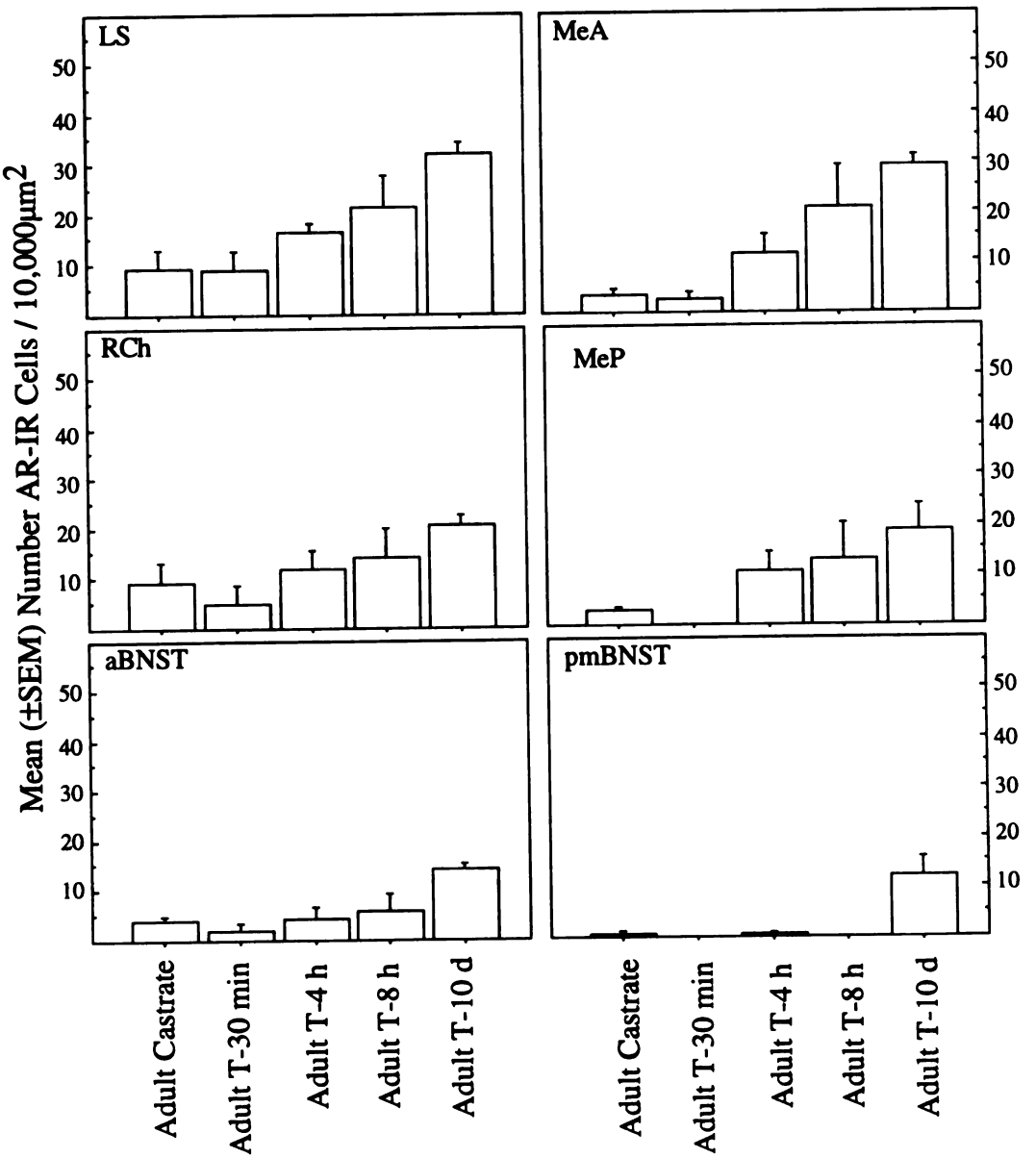


Figure 22 (continued).

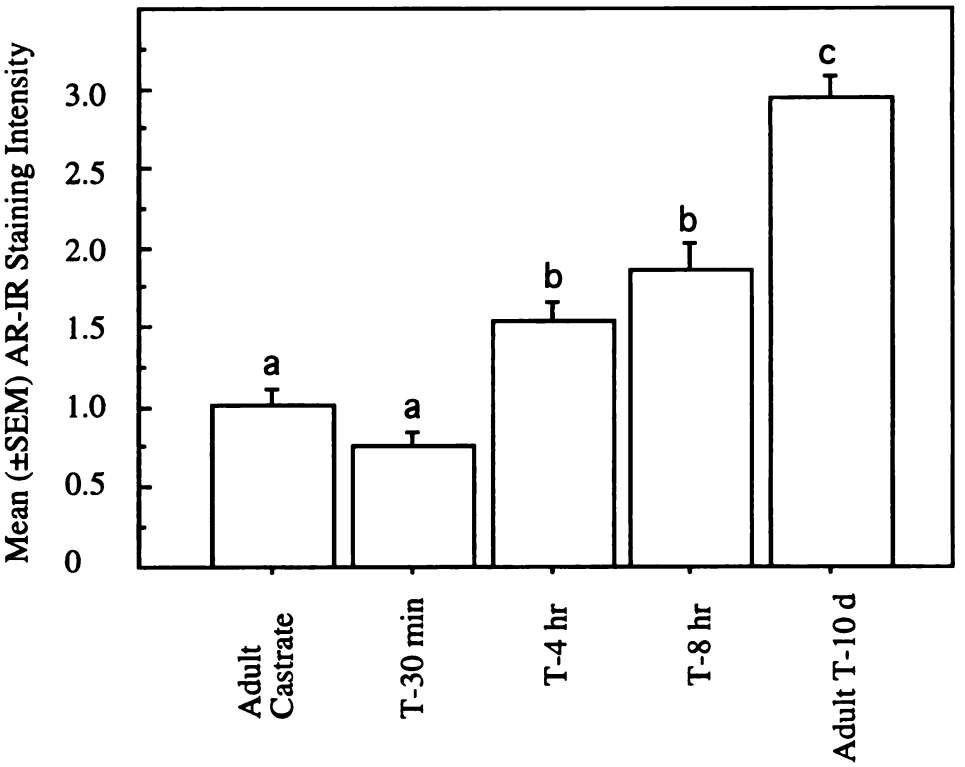


Figure 23. Mean (±SEM) immunocytochemical staining intensity, collapsed across brain region. Bars with different letters are significantly different from one another (Mann-Whitney U test, $p < 0.05$).

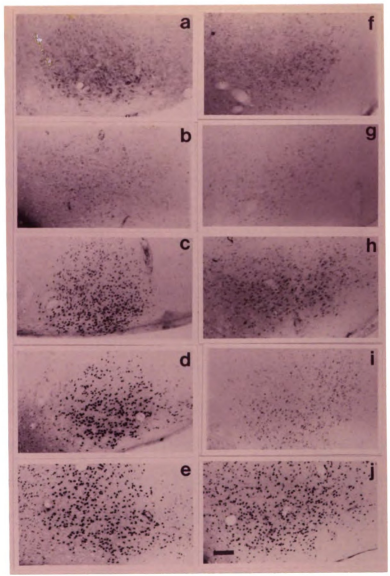


Figure 24. Photomicrographs from the PMV, and lVMH from adult animals castrated and injected with oil (a, f) or 5.0 mg/kg T for either 30 min (b, g), 4 hr (c, h), 8 hr (d, i), or once daily for 10 days (e, j). Magnification bar = 100 μm.

from those treated with T for either 4 hr or 8hr, while those animals treated with T for 4 hr were not different from those treated for 8 hr. Additional tests comparing the staining intensity between animals treated with T for 10 days and animals treated with a single dose of T for either 30 min, 4 hr or 8 hr indicated that AR-IR cells in 10d animals were significantly darker than cells in any of these groups. Photomicrographs of AR-IR cells in the PMV and lVMH following T injections are shown in Figure 24.

Discussion

Regulation of AR-IR cell density following 10 days of steroid administration

The results of this experiment demonstrate that some changes in the density of AR-IR cells following alterations in the steroidal milieu are dependent upon the maturational state of the animal. When data are collapsed across brain regions, there is a modest but significant increase in the number of AR-IR cells per grid in gonadally intact adult male ferrets compared to intact prepubertal ferrets. The magnitude of this difference between gonadally intact pre- and postpubertal male ferrets is not as great as observed in our previous experiments comparing prepubertal ferrets and those in the process of pubertal maturation (Experiments I and II). However, this is likely due to modifications in the immunocytochemical staining procedure that enhanced our ability to detect more of the lightly stained cells in the prepubertal animals. For example, in the MeA the number of cells in intact prepubertal animals is approximately 2 times greater in Experiments III and IV compared to Experiments I and II. The increase in AR-IR cell detectability across experiments did not eliminate differences between pre- and postpubertal male ferrets. This difference is clearly evident when the intensity of the

staining is examined (Figures 14, 15, and 19). In most regions where intensity of immunocytochemical staining was examined, AR-IR cells in adult ferrets were darker than in prepubertal male ferrets. Assuming that intensity of immunocytochemical staining is a function of the quantity of the antigen within the cell, it is likely that adult ferrets have more AR proteins per cell than do prepubertal ferrets. This however cannot be confirmed until other experimental methods, such as quantitative androgen receptor binding, are utilized. Based on other experiments which have quantified androgen receptor binding under various hormonal conditions it seems likely that adult ferrets would have increased androgen receptor binding relative to prepubertal ferrets. For example, in brain tissue, androgen treatment has been shown to increase androgen binding in both the hamster and the rat (Bittman & Krey, 1988; Krey & McGinnis, 1990). Thus, higher circulating androgen concentrations in adult ferrets would presumably lead to increased androgen receptor binding. Again, it has not been unequivocally determined if this increase in binding is a result of an increase in AR protein synthesis, an increase in the half-life of the AR protein, or a combination of these. An experiment where protein synthesis inhibitors are concurrently administered with androgen treatment would help to clarify this issue.

The differential response to changes in steroidal milieu between pre- and postpubertal male ferrets is also apparent when castrated animals treated with oil or estrogen are examined. There is a significantly greater density of AR-IR cells in both oil and E treated adult ferrets compared to prepubertal ferrets (Figures 14-16). The reason for this difference cannot be determined based on data from this experiment. These differences may be due to differences in the output of adrenal androgens from pre- and

postpubertal castrated animals, or it could simply be due to the fact that adult ferrets had a higher level of androgen prior to castration, and that the decline in AR-IR cell density normally seen after castration takes longer than the 17 day post-castration period examined in the present experiment. This explanation is unlikely based on data indicating that the decline in androgen receptor binding occurs within several hours of steroid removal (Krey & McGinnis, 1990). It is also possible that exposure to androgens during pubertal maturation has permanently altered a subset of AR-IR cells that retain a higher degree of AR protein in the absence of circulating androgens. It would be interesting to determine if these additional AR-IR cells are present in prepubertal animals following withdrawal of exogenously administered androgens. Evidence for permanent effects of T on the CNS during pubertal maturation does exist. Specific environment-related social interactions characteristic of adult male rats are dependent upon the presence of T during pubertal development, and not on the presence of T during adulthood (Primus & Kellogg, 1990). Further research has indicated that the GABA receptor may be involved in mediating these social interactions. The presence of the gonads during pubertal maturation influences the anxiolytic effect of diazepam on environment-related social interactions in the adult rat (Primus & Kellogg, 1990).

The most striking effects of the present experiment are the many similarities between adult and prepubertal ferrets in response to treatment with gonadal steroids. On the one hand, relative to oil treated castrated animals, there was no effect of E treatment on the density of AR-IR cells in either age group (Figure 16). Similarly, both pre- and postpubertal male ferrets castrated and treated with 5 mg/kg androgens (T or DHT) for 10 days display essentially identical patterns of AR immunocytochemical staining. This

effect is evident in terms of both cell density, and in the intensity of immunocytochemical staining. Examination of the data with regard to the age by steroid interaction (i.e., irrespective of brain region), indicates that androgen treatment resulted in a clear up-regulation in the density of AR-IR cells (Figure 16). Furthermore, immunocytochemical staining intensity in ferrets treated with androgens is greater than in castrated or intact animals (Figure 20). Thus, following 10 days of androgen treatment, prepubertal and adult ferrets display similar AR-IR staining parameters. However since adult castrated ferrets have a greater density of AR-IR cells than do castrated prepubertal animals, it is clear that prepubertal animals required a greater increase in AR-IR cell density to reach this point. Exactly when this particular level of AR-IR cell density is reached, or if prepubertal and adult animals reach it at the same time point following the beginning of androgen treatment is unclear, though based on data from the short term T-treatment to adult ferrets, it apparently takes more than 8 hr.

The underlying rationale for the present experiment was to identify anatomical substrates that may correlate with changes in behavioral responsiveness to T during pubertal maturation. It has been demonstrated that prepubertal male ferrets require a higher dose of T to engage in male reproductive behaviors compared to adults that undergo pubertal maturation in either short days or long days (Sisk, et al., 1992). At higher doses (5.0 and 10.0 mg/kg), the amount of time that prepubertal animals engage in mounting and thrusting while paired with a female compared with that of adults raised in short days also tended to be lower, however differences were not statistically significant (Sisk, et al., 1992). The evidence from this experiment indicates that any possible differences in responsiveness to the effects of 5.0 mg/kg of T are not due to a failure of T

to upregulate the expression of AR in the brains of the prepubertal ferrets treated with T. In all respects, the distribution of AR-IR cells in prepubertal animals treated with 5.0 mg/kg of T for 10 days is identical to adults treated in a similar manner. On the other hand, it is clear from the behavioral experiment (Sisk, et al., 1992), that treatment of prepubertal animals with 5.0 mg/kg of T will induce a significant amount of male sexual behavior in most of the animals. While no causal link between AR-IR cell density and behavioral capacity can be formed based on the correlational data provided in this experiment, the increase in the density and staining intensity of AR-IR cells in the brains of prepubertal animals treated with T, and their increased likelihood of engaging in male sexual behaviors is consistent with the hypothesis that an up-regulation of AR-IR cells in the brains of male ferrets as they undergo pubertal maturation is a step which precedes the induction of male reproductive behavior patterns. An experiment where both reproductive behavior and a measure of AR, either immunocytochemical staining or androgen-binding capacity, are analyzed following treatment with a lower dose of T - one capable of activating reproductive behavior in adults, but not in prepubertal animals - would help to clarify this issue. If an identical pattern of AR-IR staining were found in this case, it would indicate that an upregulation of AR could not be the limiting factor in the activation of reproductive behavior in T treated prepubertal male ferrets. On the other hand, another outcome of such an experiment could be that the AR staining patterns of prepubertal males was different, specifically that there are fewer AR-IR cells in brain regions regulating reproductive behavior patterns in steroid treated prepubertal animals compared with adults. Were this the case, it would provide support for our initial hypothesis, that pubertal changes in responsiveness to the behavioral effects of T

are due to pubertal changes in the ability of T to upregulate the AR itself. However, given that a dose of 5mg/kg of T to pre- and postpubertal animals resulted in the same pattern of AR-IR staining, it is unlikely that a lower dose of T would result in differential staining. We know that the pattern of AR-IR staining found in the present experiment is not required for the activation of reproductive behavior because intact adults, which have a lower density of AR-IR cells in many regions compared with T treated adults, will display the full range for reproductive behaviors (Sisk, et al., 1992). A more plausible explanation would be that, if responsiveness to the behavioral actions of T is a function of a differential response to circulating androgens, then this difference is not at the level of steroid induced increases in androgen receptor levels. This does not eliminate possible pubertal differences in the regulation of estrogen receptors. Pubertal changes in estrogen receptor immunocytochemistry, and estrogen receptor binding have been reported in female Guinea pigs, and rats within specific brain regions (Brown, Hochberg, Naftolin, & MacLusky, 1994; Olster, 1994). Specifically, there is an increase in the number of estrogen receptor-immunoreactive cells in the rostral portion of the ventrolateral hypothalamus in adult female compared to prepubertal female guinea pigs (Olster, 1994), consistent with the increase in responsiveness to the behavioral effects of E in these animals as adulthood is reached. Similarly, there is an increase in nuclear estrogen receptor binding in the POA and amygdala of female rats at the time of pubertal maturation (Brown, et al., 1994). Whether similar changes in brain estrogen receptors occurs during pubertal maturation in males remains to be determined.

The significant steroid by region interaction indicates that there are differences across brain regions in the way in which animals, irrespective of age, respond to changes

in steroid hormone concentrations. The source of this interaction is partly attributable to differences in the magnitude of the response to androgen treatment. In some regions, including the pvPOA, MPA, cARC, rARC, MeA, LS, and lVMH, androgen treatment resulted in a significant increase in the density of AR-IR cells above the level seen in intact animals, while in the PMV and the RCh, androgen treatment did not produce a significant increase in AR-IR cell density compared with intact animals. In our previous experiment in which we analyzed the steroid regulation of steroid receptors in prepubertal male ferrets (Experiment III), we were left with the impression that in some brain regions androgen treatment resulted in an increase in the density of AR-IR cells above the level seen in intact animals while in other brain regions this treatment only resulted in a restoration of AR-IR cell density to intact levels (Experiment III; (Kashon, Hayes, Shek, & Sisk, 1995)). We interpreted those data as representing a regional difference in the ability of androgens to upregulate AR-IR cells. However, in light of the current data, a reinterpretation seems necessary. In every brain region examined, T treatment resulted in a significant increase in AR-IR cell density relative to castrated animals, thus it is thus clear that cells in all regions are capable of increasing AR in response to increasing levels of T regardless of the maturational state of the animal. Furthermore, there are no differences in the density or the staining intensity of AR-IR cells in any brain region between prepubertal and adult animals treated with 5.0 mg/kg of androgen, indicating that the response to the dose of T used in the present experiment is the same at each maturational state. Thus differences among brain regions regarding the ability of androgens to increase the density of AR-IR cells relative to intact animals does not seem to be due to a region specific ability for androgens to increase AR, but due to a

regional differences in the steady-state levels of AR under physiological levels of androgen secretion. An increase in the density of AR-IR cells above intact levels may thus be dependent upon the number of cells within a nucleus that express AR under physiological conditions. In other words, endogenous androgen secretion may lead to a near maximal level of AR-IR cells in some regions, and thus a ceiling is reached whereby additional androgenic stimulation is not effective in increasing cell number, while in other regions endogenous androgen secretion results in a low number of AR expressing cells relative to the number possible. For example, in the PMV, androgen treatment failed to increase the density of AR-IR cells above the level seen in intact animals. The PMV contains the most intensely stained AR-IR cells within the brain, and it appears that under physiological conditions it contains its maximum density of AR-IR cells since androgen treatment failed to induce an upregulation in AR-IR cell number relative to intact animals. At the other extreme is the BNST where AR-IR cell number and staining intensity are low. Only prolonged androgen treatment (10 days) was able to produce an increase in AR staining, thus under physiological conditions, staining for AR-IR in the BNST also appears to be near its maximum density. The factors that contribute to regional differences in AR-IR cell number and intensity of staining under experimental and physiological conditions are unknown. The particular 'phenotype' of a given brain cell is likely a function of developmental processes, as well as the particular afferent and efferent connections it possesses.

The interaction between age and brain region shown in Figure 17 indicated that there are significant differences in some brain regions between prepubertal and adult male ferrets on the number of AR-IR cells when collapsed across steroid treatment.

Although this analysis is a necessary component of the 3-way ANOVA, it may have very little meaning in a physiological sense. Given that circulating androgen concentrations are one of the primary factors which influences the density of AR-IR cells, it is difficult to interpret the significance of age differences within a region when such diverse steroid treatments contribute to the means that are being compared. Figure 17 should not be confused as equivalent to Figure 5, since Figure 5 is based on data from intact animals only.

Short-Term Administration of T

Following a single injection of T to castrated adult male ferrets, a gradual increase in the number of AR-IR cells occurred between 30 min and 8 hr of exposure. This increase in AR-IR cell density relative to castrates was even greater in animals that had been exposed to daily injections of T for 10 days. These data support the notion that androgens are capable of inducing an upregulation of the AR protein, and that a translocation of receptor proteins into the nucleus, or a conformational change in the AR receptor protein that allows the antibody to bind more readily, is unlikely to fully account for the increase in the number of AR-IR cells in animals exposed to T over many days. If the increase in AR immunoreactivity were merely an artifact of immunocytochemical staining in the presence or absence of androgens we would have expected to see a single increase in AR-IR cell number and intensity of staining. In most brain regions there was a steady increase in AR-IR cell number and intensity of staining up to 8 hours following steroid treatment. There was an additional increase in these parameters seen in those animals treated for 10 days with T in most brain regions.

Although no statistical differences exist between castrated adults and those treated

with T for 30 min, there are differences in immunocytochemical staining patterns that warrant discussion. Figure 24 shows photomicrographs from the PMV and lVMH of adults treated with T for various periods of time. In the castrated animal (panels A and F) AR-IR staining is diffuse and sometimes extended into the cytoplasmic compartment of the cell. However, 30 min following a single injection of T, all staining is restricted to the nucleus (panels B and G). Not all regions displayed cytoplasmic AR staining in castrated animals. Indeed, only in those regions that display high levels of AR staining under physiological conditions, such as the VPM, lVMH, and occasionally in the POA, was cytoplasmic staining visible. Other experiments have reported rapid effects of androgen treatment on AR staining patterns, however quantification of AR-IR cells was not performed. These experiments reported an upregulation in AR-IR staining in prostate tissue within 15 min of an injection of T in an ethanol vehicle (Prins & Birch, 1993; Sar, et al., 1990), and a rapid increase in AR-IR staining was reported in hamster brain 15 min following an injection of T although the vehicle was not specified (Zhou, Blaustein, & De Vries, 1994). Thus it is possible that route of administration could partially explain these discrepancies since T administered in an ethanol vehicle may reach target cells more rapidly than T in an oil vehicle.

It is interesting that the number of AR-IR cells in those animals exposed to T for 8 hr was approximately double the number seen in castrated animals. This is consistent with the view that androgens are able to increase the half-life of receptor proteins from approximately 1.5 hr to 6 hr as reported by (Kemppainen, et al., 1992; Robel, et al., 1983). The additional increase seen following 10 days of T exposure cannot be explained in terms of an increase in the half-life of the receptor proteins without

additional information regarding transcriptional activity of the AR gene. Transcriptional activity of the AR gene in many tissue types is complex and under the influence of several factors including second messenger systems and autoregulation by androgens themselves (Lindzey, Kumar, Grossman, Young, & Tindall, 1994). The autoregulatory effect of androgens is cell and tissue specific, and appears to occur at the transcriptional, translational and posttranslational levels (Lindzey, et al., 1994). With respect to transcriptional regulation, the most common experimental finding is that androgens reduce the steady state levels of AR-mRNA in kidney, brain, coagulating gland, epididymis, and LNCaP cells, while cAMP increases AR-mRNA (Blok, Themmen, Peters, Trapman, Baarends, Hoogerbrugge, et al., 1992; Krongrad, et al., 1991; Quarmby, Yarbrough, Lubahn, French, & Wilson, 1990; Tan, Joseph, Quarmby, Lubahn, Sar, French, et al., 1988). However, some experiments have shown increases in the level of AR-mRNA in response to androgen treatment (Burgess & Handa, 1993; Gonzalez-Cadavid, Vernet, Navarro, Rodriguez, Swerdloff, & Rajfer, 1993; Shan, Hardy, Catterall, & Hardy, 1995). A decrease in mRNA levels is not necessarily accompanied by a decrease in protein levels (Krongrad, et al., 1991), which suggests that changes in protein or mRNA stability may offset changes in transcriptional activity. This is further supported by the finding that androgens induce a two-fold increase in the half-life of AR-mRNA in LNCap cells (a prostate carcinoma cell line), and reduce the initiation of new AR-mRNA transcripts (Blok, et al., 1992; Wolf, Herzinger, Hermeking, Blaschke, & Horz, 1993). Thus a general assumption that high mRNA levels lead to high protein levels is not warranted. In a functional sense, it may not be important if increased levels of AR protein following androgenic stimulation are due to an increase in

protein synthesis or an enhanced receptor stability. The bottom line is that there is more AR present to act on the genome and either enhance or repress gene transcription.

The data on the short term effects of T on AR-IR staining also indicate that steroid history is an important factor. A single blood sample, taken at the time of sacrifice, is not necessarily a good predictor of the density or the staining intensity of AR-IR cells. For example, animals treated with T for only 4 hours have fewer AR-IR cells than those treated with T for 10 days, yet there are no differences in T levels taken at the time of death. Based on data from this experiment, a longer duration of exposure to high levels of T leads to an increase in the density of AR-IR cells and to a greater staining intensity. It is thus clear that the effects of long-term steroid administration are to a certain extent cumulative in nature, thus correlations between T levels and AR-IR staining parameters could be misleading if steroid history is unknown.

As described for long term (10 day) treatment with T, there are some regional differences in the manner in which short term administration of androgens can affect AR-IR staining. For example in the PMV, an area which has some of the darkest staining AR-IR cells within the brain, the number of AR-IR cells visible 4 hr after a single injection of T to a castrated animals was equivalent to the number seen in an animal which had been treated daily for 10 days with androgens. On the other hand, in the pmBNST, there were virtually no AR-IR cells present at any time point following the single injection of T, and were only present after 10 days of T administration. The significance of these differences is unknown, however it seems likely that these regional differences in the response to short term T administration are related to regional differences in AR-IR staining under physiological conditions and thus would impact the

ability of high concentrations of androgens to induce an upregulation relative to intact animals.

In summary, this experiment has demonstrated that prepubertal male ferrets are fully capable of responding to androgen treatment with an upregulation in the number and/or staining intensity of AR-IR cells. The increase in AR-IR staining parameters following androgen treatment in prepubertal ferrets is identical to what is observed in fully mature adult males. Thus it is unlikely that changes in the responsiveness to the behavioral actions of T which occur during pubertal maturation are due to differences in the steroid hormone induced regulation of the androgen receptor. This does not rule out the possibility that the transcriptional activity of the AR protein is different between prepubertal and adult male ferrets, and indeed the possibility exists that changes in responsiveness to steroid hormone treatments are due to pubertal changes in the ability of the steroid receptor complex to either initiate or repress specific gene transcription.

GENERAL DISCUSSION

The present experiments have demonstrated that as animals undergo pubertal maturation there is an increase in androgen receptor-immunoreactive staining in many regions of the brain including those known to be involved in reproductive behavior patterns. This increase in AR-IR staining is correlated with the pubertal increase in responsiveness to the behavioral effects of T, and is tightly linked to the increase in circulating androgens that occurs during puberty. Advancing the onset of pubertal maturation, and the associated increase in T, advances the age at which AR-IR staining begins to increase. Treating prepubertal male ferrets with androgens was able to increase the number of AR-IR cells in several brain regions relative to intact prepubertal ferrets, providing further support for the hypothesis that the pubertal increase in AR-IR staining is linked to the pubertal rise in T. Since prepubertal male ferrets are less sensitive to the behavioral effects of T than are adults (Sisk, et al., 1992), we then tested the hypothesis that the differences in sensitivity between prepubertal and adult male ferrets may be due to differences in the ability of androgens to induce an upregulation in AR-IR staining. Specifically we proposed that even though androgen treatment was capable of producing an upregulation in AR-IR staining in prepubertal males, it was possible that the magnitude of this response to androgens would be even greater in adults treated with androgens. However, the pattern of brain AR immunoreactivity in prepubertal males treated with a relatively high dose of androgens was indistinguishable from adults treated with androgens. Therefore, a failure of androgen treatment to induce a sufficient upregulation in AR-IR containing cells in prepubertal male ferrets is an unlikely

mechanism for the differential behavioral responsiveness to T. However, the effect of lower doses of androgen that yields a greater difference in behavioral activation in pre- and postpubertal males, and other methods, such as androgen binding which are more sensitive than immunocytochemistry, should be utilized before this hypothesis is completely ruled out.

There are several other means by which differential sensitivity to testosterone could be mediated. Data from the experiments in this dissertation do not address the cellular effects of androgen treatment outside of its apparent autoregulatory role on androgen receptor expression. It is conceivable that the activated steroid receptor complexes in prepubertal males are not operating in the same fashion as in adults, and that differential responsiveness to T may be due to changes in the downstream physiological effects of the AR. One possibility is the androgenic regulation of the aromatase enzyme. It is known that aromatase activity in specific brain regions can be increased by the administration of T and that this is an androgen receptor-dependent mechanism (Roselli & Resko, 1984). Evidence from research on the effects of photoperiod on reproductive functioning in male hamsters indicates that aromatase activity is differentially active under different photoperiod conditions. Adult hamsters that have undergone gonadal regression in short days are less responsive to the behavioral actions of T on reproductive behavior (Morin & Zucker, 1978), and have fewer nuclear androgen receptors in the POA, as measured by in vitro ligand binding techniques, than reproductively active male hamsters in long days (Bittman & Krey, 1988). Furthermore, there is a significant reduction in hypothalamic aromatase activity in gonadally regressed hamsters housed in short days compared with those hamsters

housed in long days, and androgen administration to short day hamsters fails to increase hypothalamic aromatase activity compared with those housed in long days (Callard, et al., 1986). Whether this is related to the reduced level of AR in regressed hamsters is unknown. However, if a similar situation exists in prepubertal male ferrets, then it is conceivable that the reduced responsiveness to T is a function of a reduction in aromatase activity and thus a decrease in the amount of available estrogen. This could in turn, alter estrogen receptor levels in specific regions since the level of estrogen receptor mRNA (Lauber, Mobbs, Muramatsu, & Pfaff, 1991; Lauber, Romano, Mobbs, & Pfaff, 1990; Shughrue, Bushnell, & Dorsa, 1992; Simerly & Young, 1991), and estrogen receptor binding (Krey, Lieberburg, Roy, & McEwen, 1979; Lustig, Mobbs, Bradlow, McEwen, & Pfaff, 1989; McGinnis, Drey, MacLusky, & McEwen, 1981) are modulated by changes in gonadal steroids.

Another potential site of action that may be involved in mediating differential responsiveness to the behavioral effects of T is the androgenic regulation of neuropeptides. The behavioral effects of androgens are likely mediated by the gene products that are produced following androgen administration. Thus, even though androgens stimulate an upregulation of the androgen receptor in both pre- and postpubertal males, it is possible that these receptor complexes do not have the same effect on gene transcription. This could be due to differential access to steroid response elements, or the presence of other factors that prevent or facilitate androgen receptor complexes from initiating gene transcription. However, there are a large number of androgen regulated neuropeptides such as vasopressin, oxytocin, and galanin (Harlan, 1988), yet limited knowledge regarding the effects of these gene products on male

reproductive behavior (Dornan & Malsbury, 1990), and in the mediation of negative feedback on gonadotropin secretion. Future studies investigating how responsiveness to the effects of T may be mediated by differential activation of androgen responsive gene products should be firmly based on empirical research regarding the role of these gene products on the activation of male reproductive behavior and negative feedback. When examining the role of the androgen receptor in mediating differential sensitivity to testosterone, the downstream effects of steroid responsive gene products must also be addressed. If there are no differences between prepubertal and adult male ferrets in the autoregulatory effects of androgens on the androgen receptor, this does not indicate that these animals are equally sensitive to the actual gene products which are induced following the transcription of both androgen and estrogen responsive gene products. As more information becomes available regarding the biological responses mediated by steroid responsive gene products more specific hypotheses can be generated to examine this particular aspect of steroid hormone action on reproductive behavior and negative feedback.

Another possibility that has not yet been addressed is that peripheral structures such as penile musculature, or sensory pathways which provide feedback to CNS structures may be differentially sensitive in pre- and postpubertal males. For example, it is possible that androgens modulate sensory information from the penis and that in prepubertal males, the feedback obtained from copulation may not be processed in the same manner within the CNS of prepubertal males relative to adults. The same can be said for chemosensory information encountered during precopulatory anogenital investigation of the female, which is a necessary component for copulation to occur in

many species (Meisel & Sachs, 1994). It is known that interest for vaginal secretions is increased in male hamsters that are treated with androgens (Johnston & Coplin, 1979). With respect to negative feedback on gonadotropin secretion, non-neural targets such as glia cells may be involved in modulating differential sensitivity to the negative feedback actions of testosterone. Glial cells within the median eminence have been shown to contain estrogen receptors (Langub & Watson, 1992), and the ultrastructural relationship between non-neural elements and LHRH terminals changes with steroid milieu and pubertal maturation (King & Rubin, 1994; Witkin, Ferin, Popilskis, & Silverman, 1991; Witkin & Romero, 1995). Thus glial cells may participate in the regulation of LHRH release, and are a potential site for steroid modulation of gonadotropin secretion.

Multiple and interconnected mechanisms are likely to be involved in modulating changes in responsiveness to gonadal steroids. This view is further supported by the fact that changes in responsiveness to steroid hormones, either during pubertal maturation or during photoperiod changes in reproductive state, occur in both directions. That is, as responsiveness to negative feedback effects decreases, the responsiveness to the behavioral effects of steroids increases. However, these are not likely to be occurring simultaneously since the decrease in responsiveness to negative feedback in many species occurs prior to, and is responsible for, the increase in gonadal function that eventually leads to an increase in reproductive behaviors. Moreover, there is evidence that the neuroendocrine and the behavioral effects of gonadal steroids are occurring in discrete regions of the brain. For example, implantation of T directly into the MPA of male ferrets will activate reproductive behavior patterns, but not cause a reduction in circulating LH levels (Tang & Sisk, 1991). Thus, it can be assumed that the MPA is not

a brain region which is responsible for mediating negative feedback effects on gonadotropin secretion, at least in the ferret. Similar studies in which steroids were directly implanted into the mediobasal hypothalamus of rodents have shown that such implants can induce a reduction in gonadotropin secretion, however behavior was not tested in these studies (Cheung & Davidson, 1977; Kalra & Kalra, 1980). Another approach to this problem has been to produce lesions in specific brain regions thought to mediate these distinct effects of gonadal steroids. In terms of the behavioral effects, a large body of literature has determined that many regions are involved in mediating reproductive behaviors including the POA, AHA, LS, MeA, BNST and structures in the main and accessory olfactory pathways (reviewed in (Meisel & Sachs, 1994)). On the other hand, lesions in the mediobasal hypothalamus, which could include RCh, ARC and VMH, indicate that this is a likely target site for the effects of gonadal steroid induction of negative feedback (Cheung & Davidson, 1977; Plant, Krey, Moossy, McCormack, Hess, & Knobil, 1978). Again, comprehensive studies that simultaneously examine mechanisms of both negative feedback and behavior are generally not performed. The experiments in this dissertation have indicated that in all of these regions, i.e., both those known to be involved in mediating negative feedback and those involved in the regulation of behavior, increases in T, either during puberty or by exogenous administration, lead to increases in AR-IR cells. Thus, the regulation of androgen receptors in these brain regions with diverse functions is essentially the same, even though the amount of T required to induce a behavioral response, as well as to induce negative feedback, presumably by acting in these discrete regions, is different. Thus, the working hypothesis of this dissertation, that changes in responsiveness to steroid

hormones are positively correlated with changes in steroid receptors seems to be too simple to explain what actually may be occurring. Indeed, there are no empirical data to suggest that a decrease in the responsiveness to steroid hormones necessarily requires that there be a reduction in the number of steroid receptors. Other factors such as steroid metabolizing enzymes could also prevent steroids from exerting their specific physiological actions. It is also conceivable that an increase in steroid hormone receptors could lead to a decreased biological response depending on which specific gene products were induced by the interaction of the steroid hormone receptor with the DNA, and what their effects are on the particular physiological process under investigation. In conclusion, based on the immuncytochemical experiments in this dissertation, it cannot be unequivocally determined if regulation of androgen receptors is a step at which responsiveness to the effects of T is being mediated. Future studies using more sensitive techniques of androgen receptor quantification, and those examining the cellular effects of gonadal steroids in discrete brain regions will help to illuminate exactly how responsiveness to steroid hormones is mediated.

APPENDICES

Appendix A

Distribution of Androgen Receptor-Immunoreactive Cells in the Forebrain of Male Ferrets

Testosterone and estradiol are gonadal steroids that exert profound regulatory actions on reproductive function by altering developmental, behavioral and neuroendocrine processes. Many of these processes are mediated by steroid hormone regulation of gene transcription. Binding of steroid hormones to their intracellular receptor proteins, and the subsequent binding of this steroid-receptor complex to steroid response elements on the DNA, lead to an alteration of specific gene transcription (Beato, 1989; Evans, 1988; O'Malley, et al., 1986; Truss & Beato, 1993; Yamamoto, 1985). Both reproductive behavior and gonadotropin secretion are regulated by the presence of steroid hormones and functional steroid receptors across several stages of the life span, including during sexual differentiation, puberty, and in adulthood. Localization of steroid receptor containing cells within the central nervous system has contributed greatly to our understanding of fundamental neuroendocrine processes.

The localization of steroid hormone receptors has been accomplished using a variety of techniques. Their existence was initially established using biochemical methods which demonstrated that both androgens and estrogens bind specifically to cell nuclei isolated from brain tissue homogenates (McEwen, 1976). However, this anatomical specificity of this technique is limited to microdissected brain regions and

specific cell types could not be determined. Autoradiographic histological techniques were able to achieve cellular resolution by taking advantage of the specificity of steroid hormone binding to their receptors. Cells which contain specific binding proteins (presumably steroid receptors) will accumulate radio-labeled hormones such as tritiated E or T. Specific anatomical location of these cells is possible at the light microscopic level. Using autoradiographic techniques, the distribution of androgen and estrogen receptors has been described for the rodent brain (Sar & Stumpf, 1977; Sar & W.E., 1975), and has provided the anatomical basis for much of the research conducted in the field of neuroendocrinology. More recently, *in-situ* hybridization histochemistry has been utilized to extend our knowledge of the anatomical location of steroid receptor mRNA containing cells within the CNS of the rat brain (Simerly, Chang, Muramatsu, & Swanson, 1990). However, the use of monoclonal and polyclonal antibodies directed against steroid receptors has greatly simplified the detection of steroid receptor containing cells within the brain, and has been utilized in a number of species (DonCarlos, Monroy, & Morrell, 1991; Menard & Harlan, 1993; Wood & Newman, 1993a). Immunocytochemical techniques have also been utilized to determine the neurotransmitter / neuropeptide content of steroid receptor containing cells (Harlan, 1988 2108), and the activation of steroid receptor-immunoreactive cells during steroid-dependent behaviors (Wood & Newman, 1993b).

Within the brains of European ferrets, estrogen receptor-containing cells in discrete regions have been described using autoradiographic histochemistry (Sisk, 1993; Tobet & Baum, 1991), and immunocytochemistry (Sisk & DonCarlos, 1995; Tobet, Basham, & Baum, 1993). However, only androgen receptor binding techniques in

microdissected brain regions has been used to describe the distribution of androgen receptor containing cells in the ferret brain (Vito, et al., 1985) and thus a description of the distribution of androgen receptor-containing cells with cellular resolution in the ferret does not exist. This experiment was undertaken to describe the distribution of androgen receptor-immunoreactive cells in telencephalic and diencephalic structures of the male ferret brain.

Methods

Construction of AR-IR Cell Maps

The brain of an adult male ferret that was castrated and treated daily for 10 days with an injection of 5 mg/kg testosterone propionate was used to construct the atlas of AR-IR cells within the forebrain and hypothalamus. Work described in this dissertation clearly demonstrates that treatment with T maximizes the number of androgen receptor-immunocytochemically labeled cells, and the intensity of their staining. Comparisons between T treated castrated male ferrets and intact animals at the microscopic level indicated that intact animals had the same distribution of AR-IR cells than did T-treated animals, but the intensity of immunocytochemical staining was reduced in intact animals. Since AR-IR cells can be detected by microscopic examination in intact animals within all brain regions stained following T treatment, this map generated from a T-treated adult is a good representation of the distribution of AR-IR cells in the male ferret brain. All AR-IR cells visible at a magnification of 70x were plotted using a projection scope (Bausch & Lomb). These plots were scanned into a Macintosh computer using a Silverscan II scanner and Adobe Photoshop software at a resolution of 300 dots per inch.

Individual scans were then converted to Canvas files, reconstructed, and reduced to a magnification of 15x. Two adjacent sets of tissue from the mapped brain were also utilized in the construction of these maps. One set was thionin stained, while the other was stained for AR-IR cells and counterstained with thionin. These maps do not accurately reflect the regional variation in staining intensity, therefore these differences are described in the text.

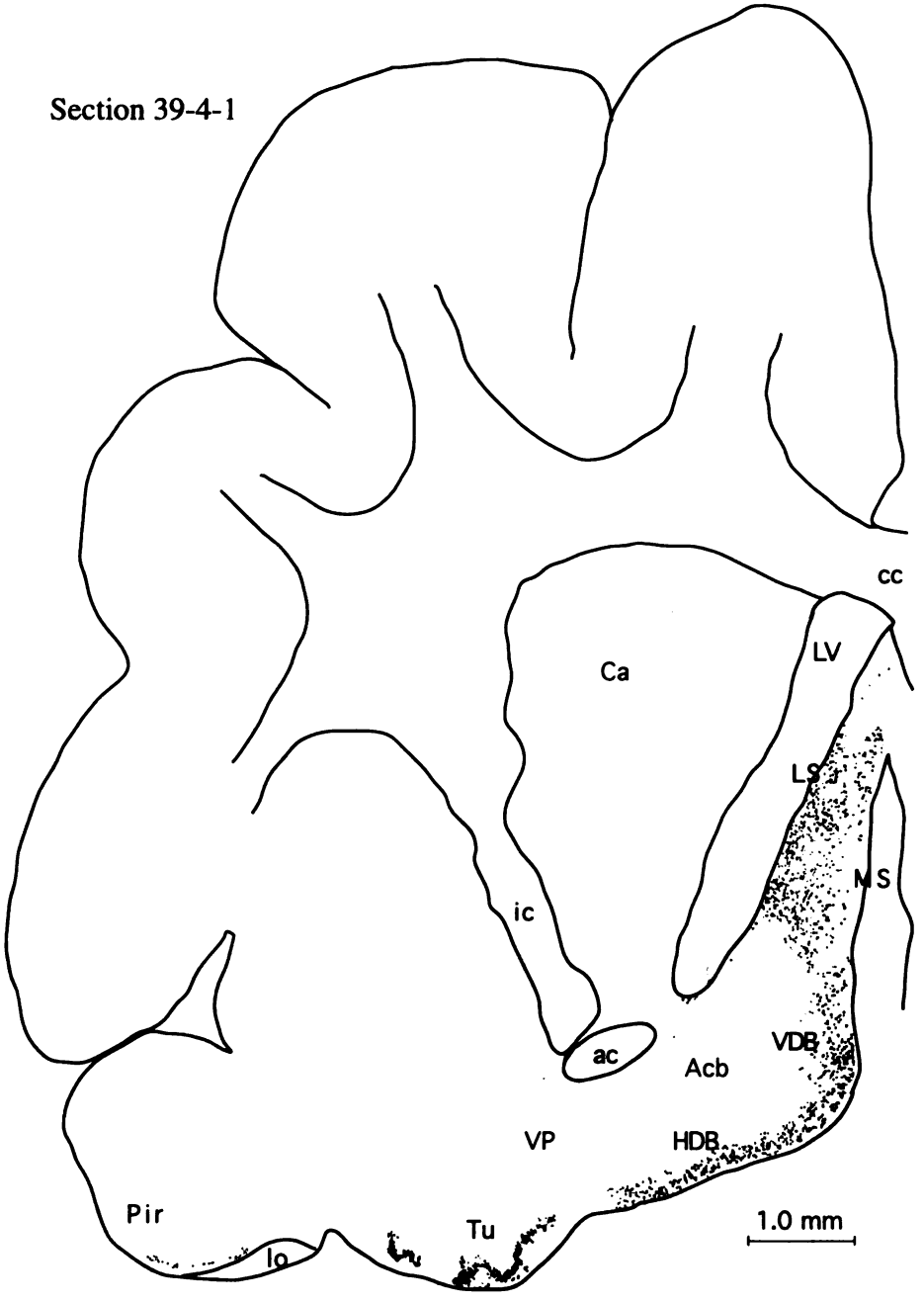
Anatomical Nomenclature

The distribution of AR-IR cells in the ferret brain is essentially a continuum of cells running throughout the hypothalamus, septum, and amygdala. AR-IR cells do fall within specific nuclei, however they do not remain within these cytoarchitectonically defined regions. Thus the boundaries of classically defined brain nuclei are not included in these maps, however their location was identified by comparison with previous work in the ferret (Lockard, 1982; Lockard, 1985; Westwood, 1962), and from previous work describing the basal forebrain and hypothalamus of the cat (Berman & Jones, 1982; Bleier, 1961). Terminology used for the current experiment is based largely on that adopted by Paxinos and Watson (1986)(Paxinos & Watson, 1986).

Results

General Description

Figures 25-34 show the distribution of plotted AR-IR cells, visible through a projection scope at a magnification of 70x, from a point just anterior to the OVLT through the mammillary bodies. The most dense aggregations of AR-IR cells were located in the preoptic area, hypothalamus, septum and amygdala. Indeed there was a continuum of AR-IR cells which extended uninterrupted along the midline from a point



Figures 25-34. Maps of the distribution of androgen receptor-immunoreactive cells in the forebrain of an adult male ferret castrated and injected daily with 5.0 mg/kg testosterone for 10 days. Each dot represents one AR-IR cell visible at a maginification of 70 x.

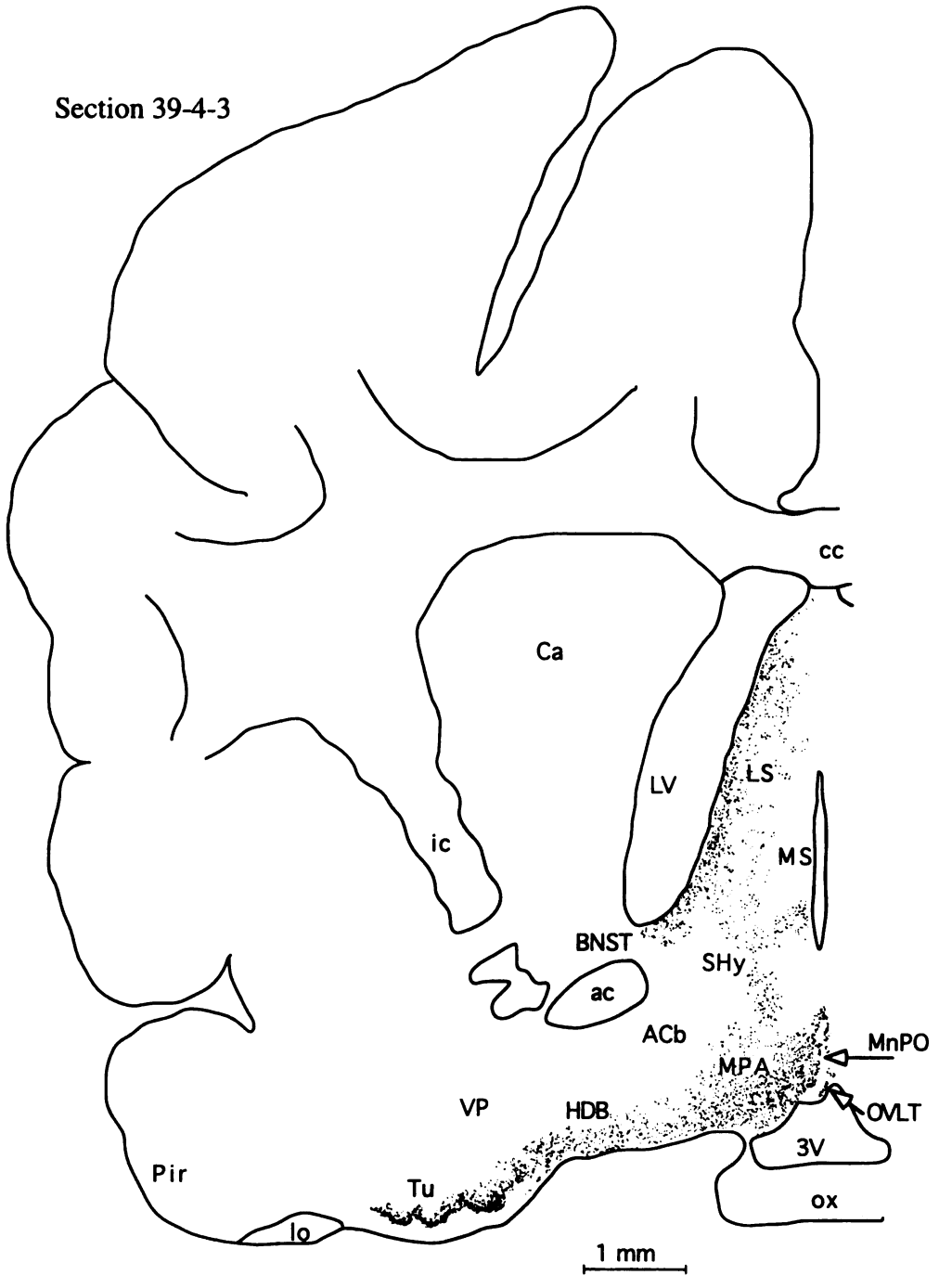

Section 39-4-3
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Figure 26

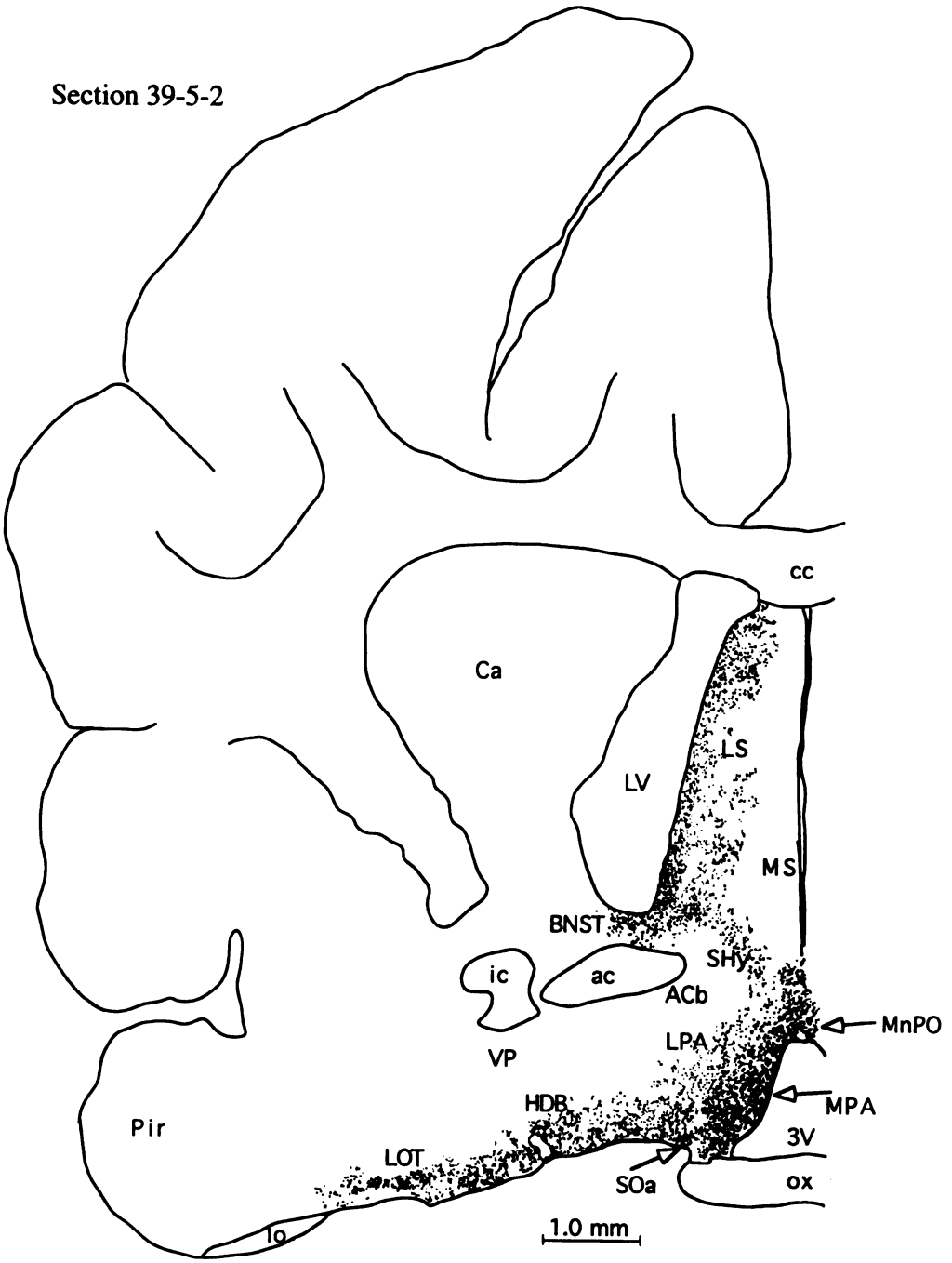
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Figure 27

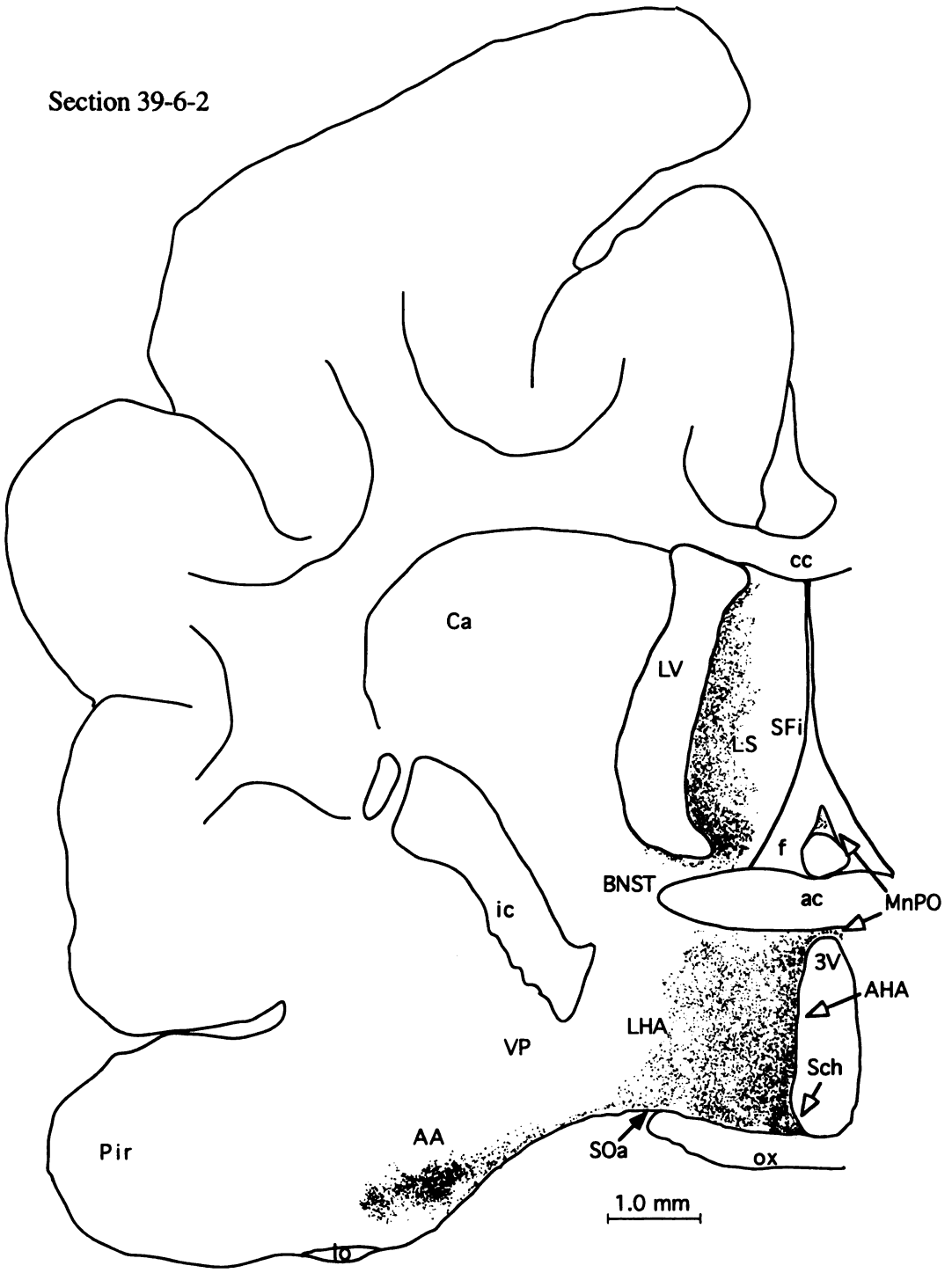
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Figure 28

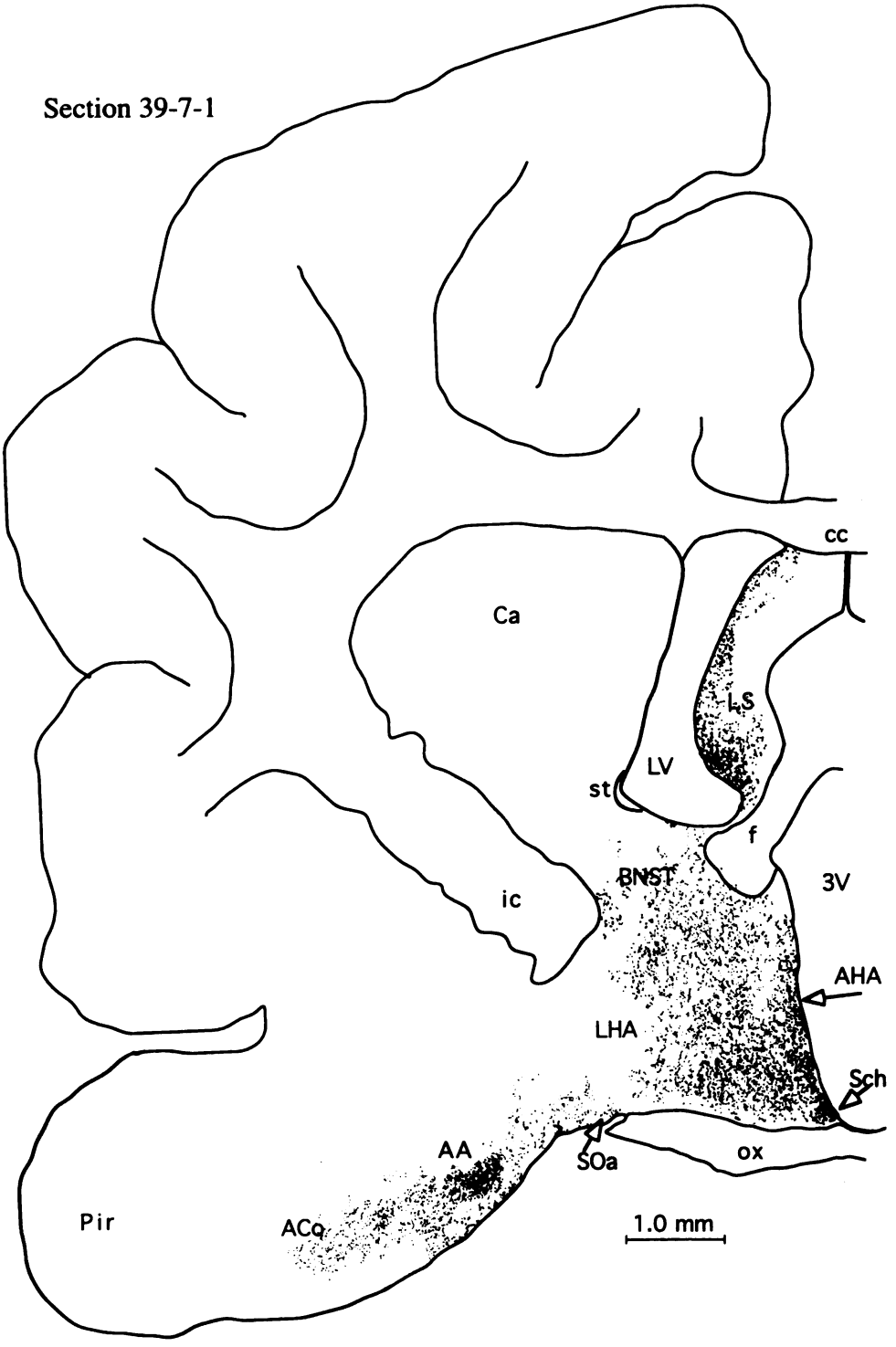
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Figure 29

Section 39-8-2

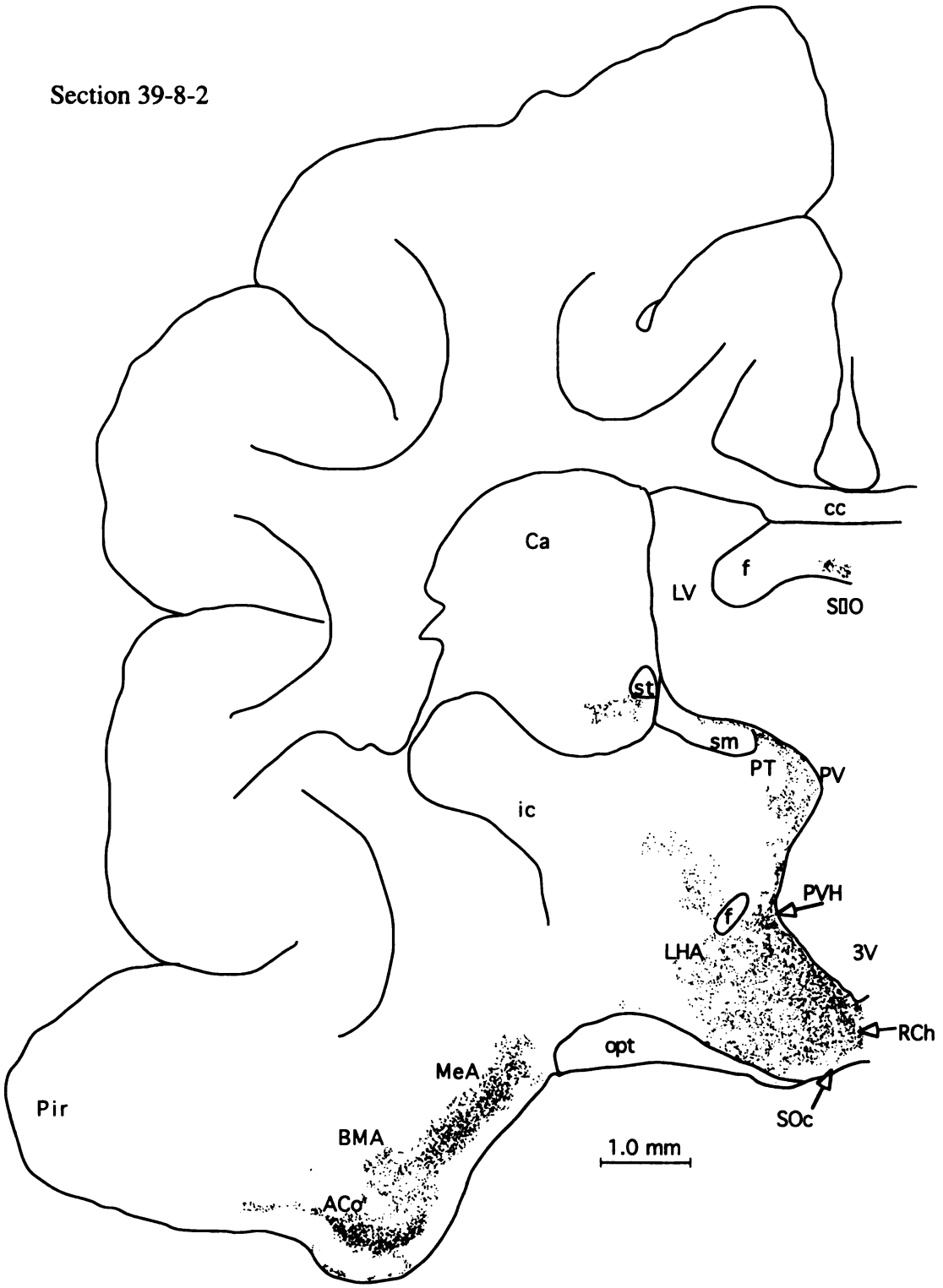

Figure 30

Section 39-9-2

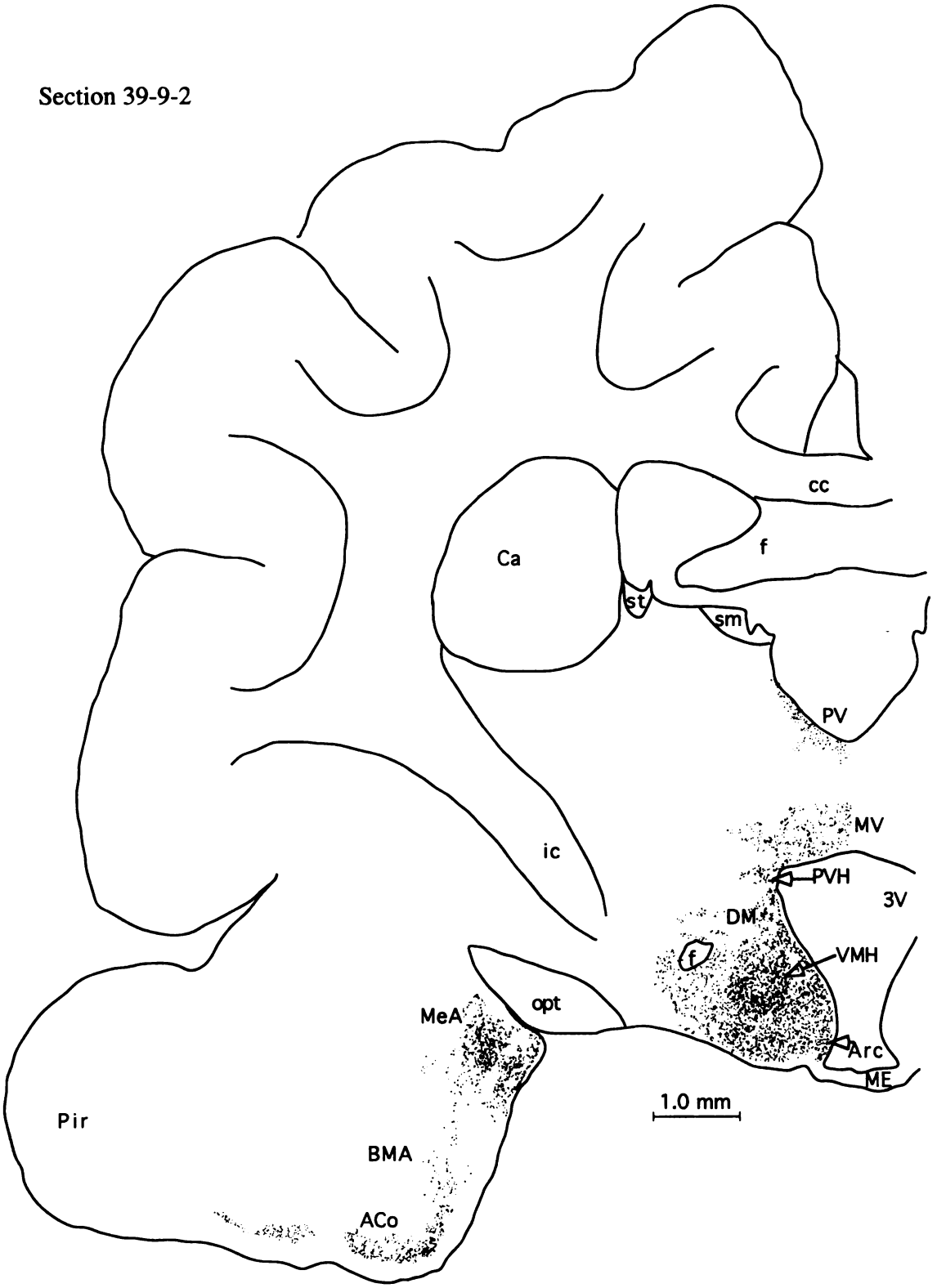

Figure 31

Section 39-10-2

Figure 32

Section 39-11-3

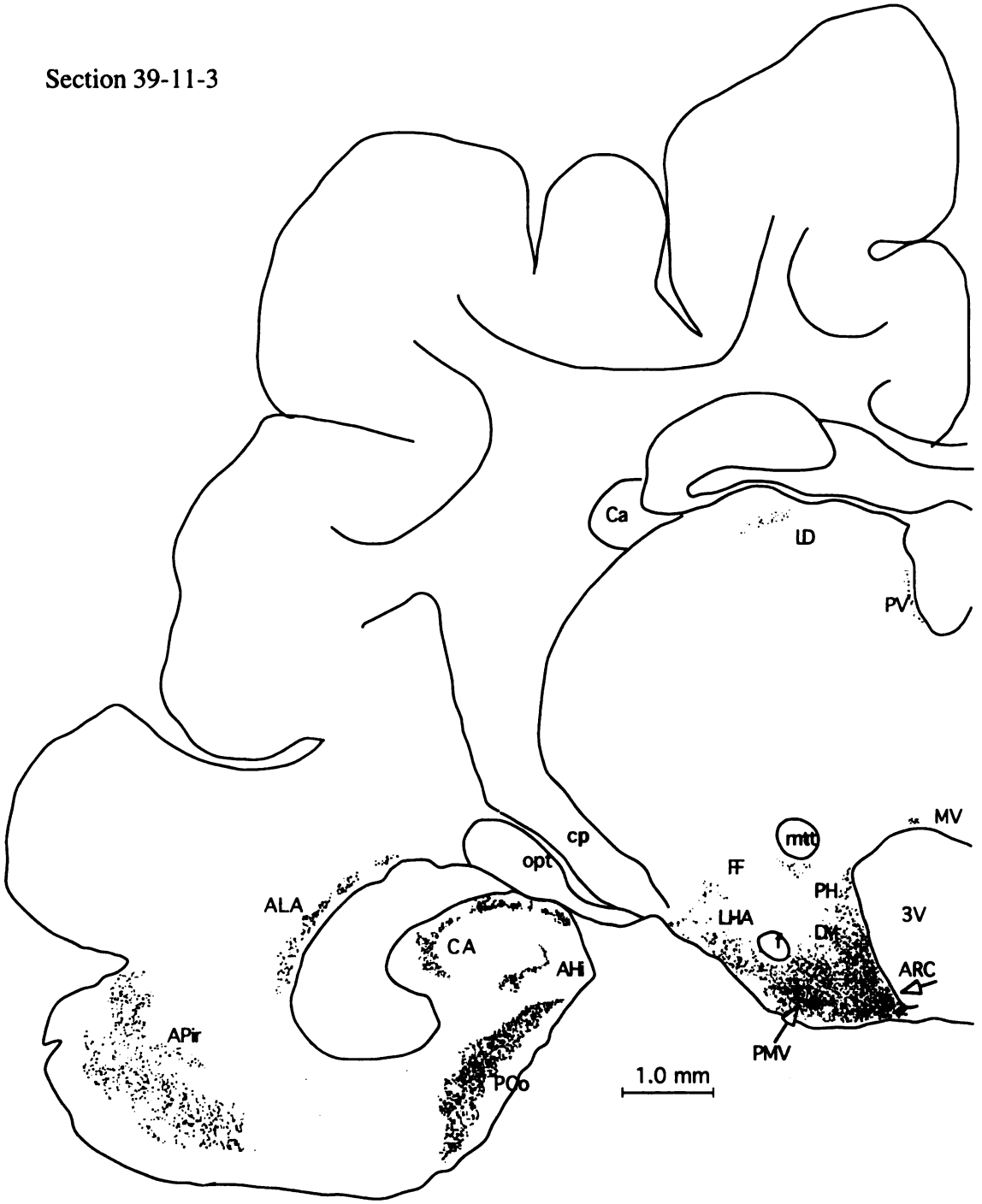

Figure 33

Section 39-12-3

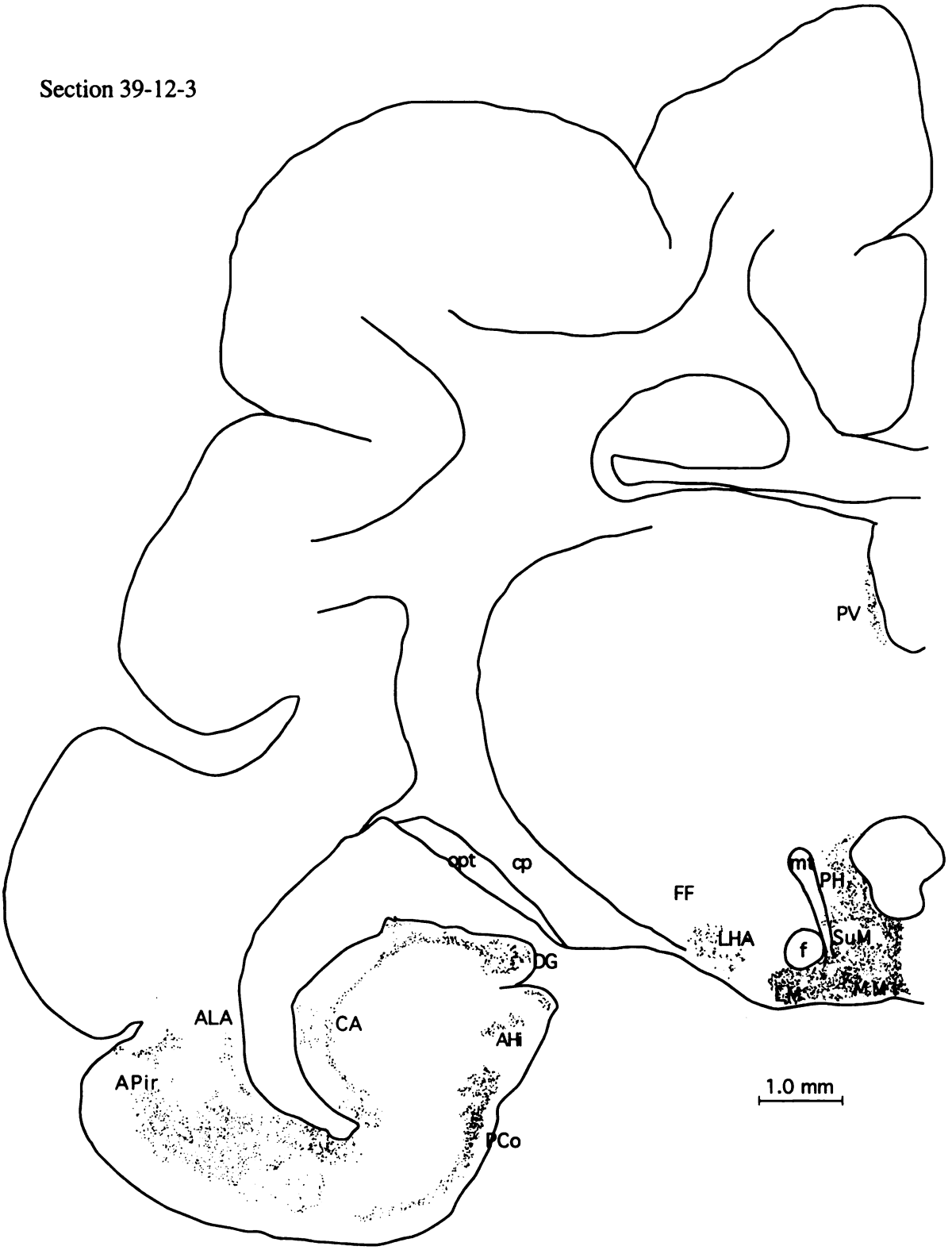

Figure 34

rostral to the OVLT through the mammillary bodies, and in lateral regions from the olfactory tubercle and the nucleus of the diagonal band, caudally through the amygdaloid complex and amygdalo-hippocampal transition area. AR-IR cells were located throughout the lateral septum, but were not prevalent in the medial septum. Lightly stained cells, detectable under microscopic examination but not visible as individual cells under the projection scope, were found throughout the piriform cortex primarily in lamina II and III, but also a few scattered cells in the molecular layer. Additional lightly stained cells were visible in the cingulate cortex. Ependymal cells lining the rostral-caudal extent of the third ventricle, as well as most portions of the lateral ventricle were heavily stained for the AR protein. AR-IR cells were also present in the circumventricular organs examined including the median eminence, OVLT, SFO and choroid plexus (Figure 35).

Telencephalon

Noncortical Nuclear Groups: The olfactory tubercle (Tu) is bounded medially by the nucleus of the diagonal band (HDB), and laterally by the lateral olfactory tract (lo). The Tu contains a dense collection of light to moderate staining AR-IR cells within the pyramidal and polymorphic cell layers. The anterior portions of the Tu are in close association with the Islands of Calleja which are composed of tightly packed granule cells containing a small proportion of lightly stained AR-IR cells. The lateral septal nuclei, including the dorsal, intermediate and ventral subdivisions all contain AR-IR cells. These cells are lightly stained in sections rostral to the anterior commissure, but are stained moderate to dark proceeding caudally to the fimbria of the fornix. Moderately stained AR-IR cells are located in the subfornical organ (Figure 35). Dorsal

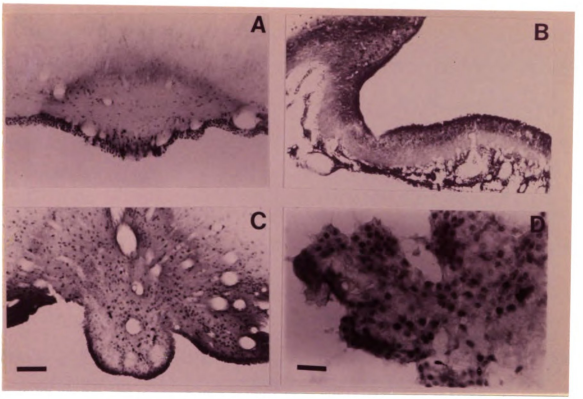

Figure 35. Photomicrographs of AR-IR cells within circumventricular organs of the male ferret brain. (A), subfornical organ; (B), median eminence; (C), vascular organ of the lamina terminalis; and (D), choroid plexus. Magnification bar = 100 μm (A, C) and 30 μm (B , D).

and medial to the ventral subdivision of the LS and rostral to the anterior commissure, AR-IR cells are found in the septohypothalamic nucleus (SHy). Cells in SHy are lightly stained and scattered. The darkest staining cells in the LS are in the ventral subdivision. A few lightly stained AR-IR cells are located in the medial septum, primarily where it is in continuation with the vertical limb of the nucleus of the diagonal band. Both nuclei associated with the diagonal band (horizontal and vertical limbs) contain a large number of AR-IR containing cells . These cells are lightly stained and are more diffuse than cells in the nearby MPA.

Cells stained for AR in the bed nucleus of the stria terminalis were very light throughout its rostro-caudal extent. Cells were more numerous, and slightly darker in the anterior medial and posterior medial portions of this nucleus than in lateral components, though still lighter than AR-IR cells in the surrounding structures such as the POA, AHA, and LS.

Several subdivisions of the amygdaloid complex contained AR-IR cells. A few scattered lightly stained AR-IR cells were found in the nucleus of the lateral olfactory tract. The highest concentration of AR-IR cells was seen in the anterior, medial, and cortical amygdaloid nuclei (Figure 36). These cells form a sheet stretching from the anterior amygdaloid area through the posterior subdivision of the medial amygdaloid nucleus. These cells were light to moderately stained and were tightly packed. AR-IR cells were also seen in the rostral-caudal extent of the cortical nucleus, being more dense and intensely stained in the posterior region. A few lightly stained AR-IR cells were detected in the central nucleus, the lateral nucleus in posterior regions, as well as the basomedial nucleus. AR-IR cells were detected in the amygdalo-hippocampal transition area, as well as the amygdalo-piriform transition area.

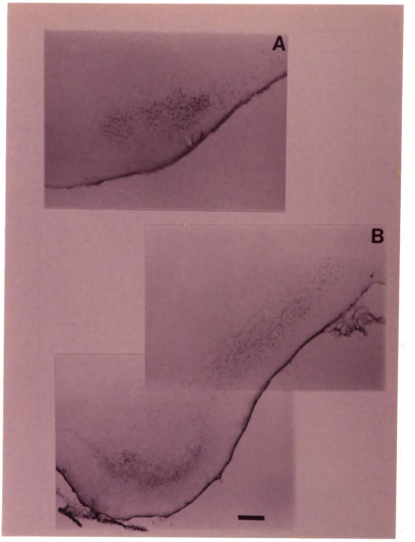

Figure 36. Photomicrographs of AR-IR cells within the anterior (A), medial and cortical (B) amygdaloid nuclei of an adult male ferret. Magnification bar = 250 µm.

Preoptic area: Within the preoptic area there was a dense collection of intensely stained AR-IR cells (Figure 37). These cells are very tightly packed adjacent to the 3V, in the periventricular nucleus of the preoptic area, and became less tightly packed in a lateral direction through the MPN into the lateral preoptic area. As described for the distribution of estrogen receptor-immunoreactive cells in the Guinea Pig (DonCarlos, et al., 1991), AR-IR cells in the ferret MPA formed columns running parallel with the 3V (Figure 37C). AR-IR cells were numerous in the MnPO (Figure 37A), a group of tightly packed small cells located on the dorsal aspect of the 3V beginning at the level of the OVLT and extending to the anterior commissure (Bleier & Byne, 1985). There were also numerous AR-IR cells within the OVLT (Figure 35C), and a large number of AR-IR cells running along the edges of the lamina terminalis itself.

Diencephalon

Hypothalamus: There is no clear demarcation between the POA and the AHA, thus it has been arbitrarily set at the rostral edge of the anterior commissure. Like the POA, AR-IR cells in the AHA are more tightly packed medially near the 3V and become less dense laterally into the LHA (Figure 38A). Within the AHA there are a number of nuclei which contain AR-IR cells including the male nucleus of the AH/POA as defined by Tobet 1986, the suprachiasmatic nucleus (SCh), and the paraventricular nucleus of the hypothalamus (PVH). Moderately stained, AR-IR cells are also found within the MN-POA/AH, a sexually dimorphic nucleus within the AH (Figure 39). Cells within the non-sexually dimorphic ventral nucleus contain fewer AR labelled cells compared to the MN-POA/AH. The SCh in the ferret is a prominent nucleus located in the ventral AHA

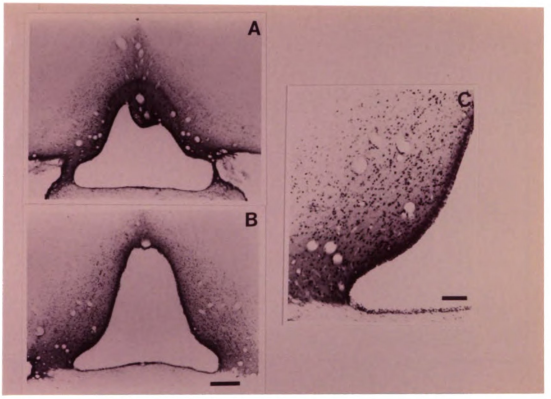

Figure 37. Photomicrographs of AR-IR cells within the preoptic area of the male ferret. Magnification bar = 250 μm (A and B), and 100 μm (C).

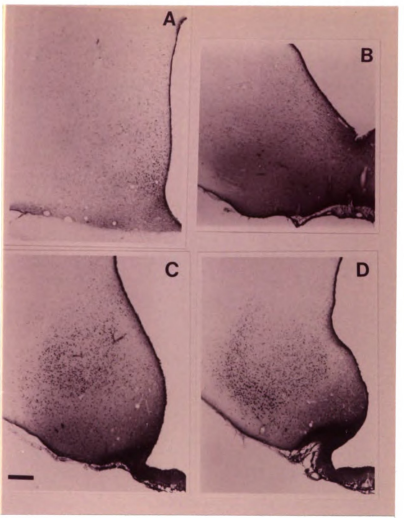

Figure 38. Photomicrographs of AR-IR cells within the anterior hypothalamus (A), retrochiasmatic area (B), ventromedial hypothalamic nucleus and arcuate nucleus (C and D) of an adult male ferret. Magnification bar = 250 μm.

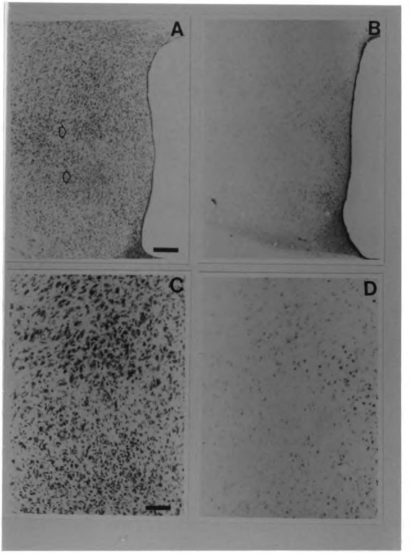

Figure 39. Photomicrographs of the male nucleus of the preoptic area/ anterior hypothalamus of an adult male ferret (upper arrow panel A). Ventral nucleus is indicated by lower arrow. A and C are nissl stained sections adjacent to the AR-IR stained sections in B and D. Magnification bar = 250 μm (A and B), 100 μm (C and D).

dorsal to the optic chiasm and adjacent to the 3V (Figure 38A). The SCh contains small, round, and tightly packed cells; AR-IR cells are distributed homogeneously within this nucleus. Within the paraventricular nucleus of the hypothalamus AR immunostaining was detected in both magnocellular and parvocellular neurons throughout its rostro-caudal extent.

In the area of the tuber cinereum, AR-IR cells are located within the retrochiasmatic area (RCh), ventromedial nucleus of the hypothalamus (VMH), the arcuate nucleus (ARC) and median eminence (ME), as well as the dorsomedial nucleus (DM) (Figure 38 B,C,D)). AR-IR cells in the RCh are moderately stained for AR, and extend from the formation of the optic tracts to the ME. Cells in the RCh are less dense than the SCh rostrally and the ARC caudally. The ventromedial nucleus contains a high density of heavily labeled cells which form an oval. AR-IR cells within the interior of the VMH are less dense than the lateral component (Figure 38 C,D). The lateral portion of the VMH extends more dorsal, and contains darker staining cells than the medial component. The dorsomedial nucleus of the hypothalamus forms between the descending column of the fornix and the PVH. Cells in the DM are small and extend in a ventromedial direction merging with the periventricular nucleus anteriorly and the arcuate nucleus posteriorly (Figure 40). AR-IR cells in the DM are lightly stained and less dense than the adjacent VMH and ARC. The ARC contains moderately stained AR-IR cells which are of approximately the same density, but not as darkly stained as AR-IR cells in the VMH. AR-IR cells become more tightly packed proceeding caudally to its terminus in the posterior hypothalamus rostral to the mammillary nuclei. Several AR-IR cells are located within the median eminence, primarily in the interior zone (Figure 35).

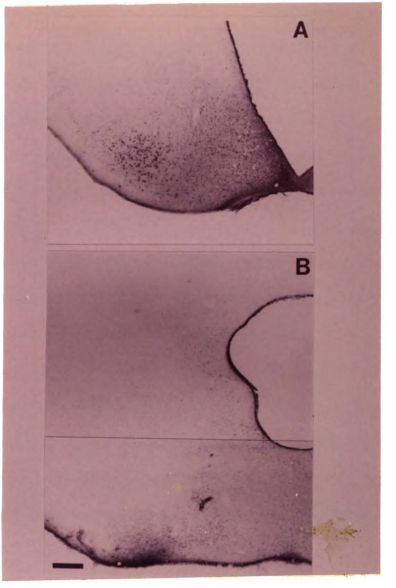

Figure 40. Photomicrographs of AR-IR cells in (A) the caudal portion of the arcuate nucleus and ventral premammillary nucleus, and (B), in the medial, and lateral mammillary nuclei, and the posterior hypothalamus. Magnification bar =250 μm.

However, background staining in the ME was too dark for cells to be detected under the projection scope and are thus not illustrated in the illustrations.

Caudal to the VMH, the ventral premammillary nucleus forms in the ventral-lateral portion of the tuber cinereum (Figure 40A). This nucleus contains some of the darkest staining AR-IR cells within the brain. The remainder of the posterior hypothalamus consists of the nuclei of the mammillary bodies (Figure 40B). The lateral and medial mammillary nuclei contain a moderate density of light to moderate stained AR-IR cells, however in the supramammillary nucleus there is are considerably fewer AR-IR cells than the surrounding LM and MM ventrally or the posterior hypothalamic area dorsally between the mammillothalamic tract and the 3V.

Thalamus: The most notable nuclear configurations within the thalamus containing AR-IR cells were the paraventricular thalamic nucleus, and the medioventral nucleus. The paraventricular nucleus contains lightly stained AR-IR cells which border the dorsal aspect of the 3V. Cells in the MV are more numerous but lightly stained. Other thalamic nuclei containing AR-IR cells were the paracentral nucleus and the lateral dorsal nucleus.

Comparisons between Intact and T-Treated Adult and Prepubertal Ferrets

Examination of intact and T treated adult and prepubertal males at the light microscope level indicated a number of differences in the distribution of AR-IR cells. These differences reflect what has been demonstrated in the above experiments, that animals with lower T levels have lighter staining AR-IR cells in many regions than do animals with higher T levels. This effect is most prominent in those regions that contained lightly stained AR-IR cells such as the piriform cortex, BNST, SHy, and the

nuclei within the thalamus. AR-IR cells could be detected in intact adults in all regions in which they were mapped in T treated adults, and prepubertal animals treated with T were identical in all respects to adult animals treated with T. However, prepubertal animals often lacked any detectable AR-IR cells in these lightly stained regions when observed under lower magnifications, and high magnification optics were often required to detect any AR-IR cells in these regions. In those regions classically thought of as containing steroid receptors, such as the POA, hypothalamus, amygdala, and septum, AR-IR cells were readily detectable in prepubertal intact animals. In no region were there AR-IR cells detectable in prepubertal intact animals that were not also detectable in adults.

Discussion

Autoradiographic techniques have demonstrated the presence of steroid receptor containing cells from every major class of vertebrate (Kim, Stumpf, Sar, & Martinez-Vargas, 1978; Morrell & Pfaff, 1978). There are many common regions across species that accumulate gonadal steroids including the preoptic area, septum, hypothalamus and amygdala (Kim, et al., 1978; Morrell & Pfaff, 1978). Furthermore, considerable overlap exists regarding the distribution of androgen and estrogen receptors across species and across techniques, including autoradiography, immunocytochemistry, and in situ hybridization (see (Blaustein & Olster, 1989; Simerly, et al., 1990)). Immunocytochemical experiments that have described the distribution of AR in mammals and birds have revealed a similar pattern within this collection of neural structures (Balthazart, Foidart, Wilson, & Ball, 1992; Choate & Resko, 1992). The

present study adds to the comparative literature by describing the distribution of androgen receptor immunoreactive cells in the European ferret. Not surprisingly, the greatest concentration of AR-IR cells was detected in the within the preoptic area, hypothalamus, septum, and amygdala. This highly conserved distribution of steroid receptors across vertebrate species reflects many of the common neuroendocrine and behavioral effects mediated by gonadal steroids across species.

The neural circuits for the activation of male sexual behavior in rodents have been well characterized (Meisel & Sachs, 1994). The display of reproductive behavior requires activation of this circuitry by both chemosensory stimuli and steroid hormones, and the information encoded by these exogenous and endogenous cues is integrated within components of the circuit. Chemosensory input is obtained during ano-genital investigation of the female prior to copulatory behavior and is transmitted through the main and accessory olfactory systems. This information travels along the lateral olfactory tract to the cortical and medial amygdala (Davis, Macrides, Youngs, Schneider, & Rosene, 1978; Scalia & Winans, 1975). The amygdala then sends olfactory information to the mPOA (Kevetter & Winans, 1981) as well as to the BNST (Lehman & Winans, 1983 4800), which in turn also projects to the mPOA (Chiba & Murata, 1985). Reproductive behavior can be blocked by disrupting either the hormonal or chemosensory input to this neural circuitry, or by disruptions to the circuitry itself. For example, either elimination of circulating gonadal steroids (Beach & Pauker, 1949; Whalen & DeBold, 1974), or blockage of the nares, which eliminates stimulation of the olfactory bulbs, (Doty, Carter, & Clemens, 1971; Murphy & Schneider, 1969) results in a decline in reproductive behavior. Reproductive behavior is also compromised by lesions in brain regions, such as the POA, BNST and amygdala, which are part of the steroid

sensitive neural circuitry, or by lesions along the olfactory pathway that supplies chemosensory information to these regions (Winans & Powers, 1974). Although the olfactory bulbs were not analyzed in the present study, AR-IR cells in the male ferret brain were found within all other components of the neural circuit mediating reproductive behavior. Interestingly, relative to what has been described for the rodent brain (Clancy, et al., 1992; Menard & Harlan, 1993; Sar, et al., 1990; Wood & Newman, 1993a), the male ferret brain contains considerably fewer AR-IR cells within the nuclei of the BNST. Unfortunately the contribution of the BNST to reproductive behavior in the male ferret is unknown and thus no conclusion can be drawn regarding the functional significance of this species difference.

Sexual differentiation of reproductive behavior patterns is a product of the steroid environment during specific stages of development (Baum, et al., 1990). In the male ferret, as in other species (Arnold & Gorski, 1984; Meisel & Sachs, 1994), there is a morphological sexual dimorphism in the preoptic area-anterior hypothalamic area continuum (Tobet, et al., 1986b). In this region there is a nucleus present only in male ferrets, the dorsal nucleus of the POA/AH (also called the male nucleus (MN) of the POA/AH. The formation of this nucleus in the male is dependent upon exposure to T, and aromatization to E within the brain, during a critical period of development (Cherry, Basham, Weaver, Krohmer, & Baum, 1990; Tobet, Zahniser, & Baum, 1986a). However, the increased size of the cells within this nucleus, is reduced in castrated adults, but can be restored by treatment with either E , T or DHT (Tobet, et al., 1986b). Thus, androgens, acting via the androgen receptor, can directly and indirectly influence cells within the MN-POA/AH. Small lesions restricted to this nucleus in the ferret produce small deficits in male reproductive behavior, however animals were still capable

of achieving intromissions (Cherry & Baum, 1990). Interestingly, when animals were castrated and treated with estradiol benzoate, the time it took to approach a stud male in an L-maze was lower than even E treated females suggesting that this region of the brain may inhibit female behaviors from occurring in males. (Cherry & Baum, 1990). Estrogen receptors in this nucleus has been identified using autoradiography (Tobet & Baum, 1991). The presence of AR-IR cells in the MN-POA/AH of the male ferret, as shown in the present experiment, is consistent with the regulatory role of both androgens and estrogens in the modulation of cell size within this nucleus, and in the inhibition of the occurrence of female reproductive behaviors. The ultimate action of T in this nucleus may thus be directly on the AR, or through estrogen and the estrogen receptor following androgen dependent aromatization to estrogen (Weaver & Baum, 1991), in either case the androgen receptor is playing a critical role.

In summary, the findings of this experiment are in agreement with those of others describing the distribution of androgen receptors within the forebrain of vertebrate species. Several techniques have been utilized to locate putative steroid receptor containing cells within the brain. The use of immunocytochemistry for steroid receptors has advantages over others with regard to simplicity of procedures, and the ability to detect multiple antigens within individual cells. Future use of double-label techniques for steroid receptors and other neurochemicals will undoubtedly lead to an increase in our understanding of the many actions of steroid hormones on brain function.

Appendix B

Effects of Long-term Storage in Cryoprotectant on the Staining of AR-IR Cells

Since separate sets of tissue from prepubertal animals under various steroid treatment conditions were processed immunocytochemically and quantified for AR-IR cells in Experiments III and IV, statistical analyses were performed to compare the replicability of these results across immunocytochemical runs. Only data from the short incubation in DAB from Experiment III was analyzed so that the comparisons would not be confounded by this experimental manipulation. A two-way ANOVA using brain region and experiment as the independent variables was performed. There was not a significant main effect of Experiment [$F_{1,332}$=1.106, p=0.29] indicating that there was not a significant loss of antigen due to long-term storage in cryoprotectant at -20°C. There was however a significant interaction between experiment and brain region. Post hoc analysis indicated that significantly more cells were detected in the lVMH and the aBNST in Experiment III than were detected in Experiment IV. There is no obvious reason for the differences between the two experiments regarding the density of AR-IR cells in these regions. In the lVMH, cell counts for Experiment III were performed by a different experimenter than in Experiment IV. However, cell counts in the BNST were made by the same experimenter in both studies. Further analyses were conducted in order to determine if our finding of brain region specific increases in AR-IR cells following androgen treatment to castrated animals, relative to intact animals, was replicated in Experiment IV since the statistical analysis in Experiment IV did not call

for these comparisons to be made. One-way ANOVA's for each of the brain regions that were analyzed in both experiments were performed. Pair-wise comparisons within each brain region were made with the Fishers PLSD. In the ARC, MPA, and MeA these comparisons indicated that a significant increase in the density of AR-IR cells relative to intact animals occurred following androgen treatment in Experiment IV as was found in Experiment III, however in the lVMH, and pvPOA these results were only partially replicated in that in the pvPOA only DHT treatment resulted in a significant increase in AR-IR cell density relative to intact animals. In the lVMH, an area in which no significant up-regulation of AR-IR cells was found in our previous experiment, T treatment again failed to statistically increase AR-IR cell number, however DHT treatment did. In the LS, and the BNST, areas in which androgen treatment did not yield an increase in the density of AR-IR cells relative to intact animals in Experiment III, the results were replicated in that there were no differences between intact animals and T or DHT treated animals in Experiment IV. Thus, although the density of cells in every brain region across the two experiments were not all replicated in terms of absolute numbers, the relative differences among groups was consistent.

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