Testosterone alters membrane binding of progesterone in male rat brains

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Summary

The steroid progesterone (P) is important in control of male sexual behaviors. In this study binding sites for P conjugated to bovine serum albumin (P-BSA) were analyzed in the brains of gonadectomized male rats that were implanted with silastic capsules either filled with testosterone or empty. Frozen brains were sliced, thaw mounted on microscope slides, and incubated with radiolabeled P-BSA (P-[^{125}I-BSA]) alone or with 1000 fold P-BSA competitor. Microscope slides were then dried, dipped in photographic emulsion and placed in light-tight boxes for four days before being developed and counterstained. Grain densities over cells were then analyzed with an image analysis system. Testosterone treatment significantly increased specific binding of P-[^{125}I-BSA] in the caudal medial basal hypothalamus, the paraventricular nucleus-anterior hypothalamus, and the medial preoptic area. Testosterone treatment significantly decreased P-[^{125}I-BSA] binding in the amygdala. There was no significant effect of testosterone in the rostral medial basal hypothalamus. Testosterone treatment changes P-[^{125}I-BSA] binding in several areas that are important for male sexual behavior suggesting a function for P-[^{125}I-BSA] binding sites in endocrine systems and behavior.

Key Words: Progesterone – P-BSA – Autoradiography – Testosterone – Male sexual behavior – Amygdala – Paraventricular nucleus – Medial preoptic area – Medial basal hypothalamus

Introduction

Androgenic steroid actions have been the primary focus of research on mechanisms regulating masculine sexual behavior, yet there is limited information on the role of progesterone (P) in males. Historically, P has been characterized as a steroid with antiandrogenic properties that when administered in a high dose inhibits masculine sexual behavior. However, new findings have called into question the physiological role of P in males. The initial studies re-evaluating P showed that in two species of lizards exogenous P synergies with physiological levels of testosterone (T) and restores the full complement of courting and copulatory behavior in some gonadectomized males (Lindzey and Crews, 1986). This progestagenic effect can be blocked by concomitant administration of the P antagonist RU486, and the facilitating behavioral effects are mimicked by the nonmetabolizable P agonist R5020 (Witt et al., 1995). In our parallel studies, systemic delivery of physiological doses (low) of P enhanced androgen-dependent reproductive behavior, and RU486 completely abolished these facilitating effects of P in both gonadectomized and gonadally intact males (Witt et al., 1995). These data provide the first evidence in mammals implicating P-dependent mechanisms influencing the neurochemical pathways involved in male reproduction. Apparently, gonadectomized rats require plasma P levels (and androgens) to be maintained within physiological ranges for species-typical copulatory behavior to mimic behavior observed in gonadally-intact, sexually active males.

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Recent data using progesterone receptor (PR) knockout mice has further implicated progesterone as a modulator of androgen-dependent sexual behavior (Phelps et al., 2000). In these studies gonadectomized wild-type males were significantly more responsive to androgen replacement, exhibiting significantly higher levels of sexual behavior, than males either heterozygous or homozygous for the PR knockout after T replacement. Specifically, mice that were homozygous PR knockouts showed the most marked deficits in androgen-dependent copulatory behaviors and the heterozygous mice had moderate levels of behavioral deficits. This suggests that disruption of the P system in male mammals inhibits androgen stimulated behaviors.

Several laboratories have begun to identify non-genomic effects of steroids on neurophysiology and on reproductive behaviors. Caldwell and Moe (1999b) discovered that estradiol conjugated to bovine serum albumin (E-BSA) increased oxytocin-induced female sexual receptivity. Several studies have identified facilitative actions of P-BSA in female hamsters (Frye et al., 1992 and 1996b). Binding sites for these conjugated steroids have been identified particularly in females (Ramirez and Zheng, 1996; Zheng et al., 1996; Tischkau and Ramirez, 1993; Ke and Ramirez, 1990; Caldwell et al., 1995 and 1999). Radiolabeled P-BSA (P-[^{125}I-BSA]) has been used extensively in radioligand assays to identify binding sites in plasma membranes from the medial preoptic area and hypothalamus of females using radioligand assay techniques (Ramirez and Zheng, 1996; Zheng et al., 1996; Tischkau and Ramirez, 1993; Ke and Ramirez, 1990; Caldwell et al., 1995 and 1999). Caldwell et al., (1999) recently demonstrated a relationship between E and P binding sites in plasma membranes from females that does not exist for their intracellular receptors. Therefore, we cannot assume that the relationships developed among steroids for intracellular receptors will hold for those associated with plasma membranes. Tischkau and Ramirez (1993) used radioligand techniques to identify P-[^{125}I-BSA] binding sites in male rats where they found that gonadally-intact males had more P-[^{125}I-BSA] binding sites than castrated males, while estradiol treatment in castrated males increased the number of these binding sites to levels seen in females. All studies examining P-[^{125}I-BSA] binding have utilized radioligand techniques, whereas development of P-BSA autoradiography would provide an opportunity to better localize binding sites.

In this study we used autoradiography, for the first time to identify P-[^{125}I-BSA] binding sites in the medial basal hypothalamus (MBH), the paraventricular nucleus (PVN), the medial preoptic area (MPOA), and the amygdala of males. P-BSA stimulated cFos immunoreactivity *in vitro* within 60-90 minutes in the PVN and the MBH of male rats (Witt et al., 1997). This identified the PVN as a potentially important area to study with regard to P-[^{125}I-BSA] binding sites in males. The MBH is important in steroid control of female sexual behavior (Barfield and Chen, 1977; Pfaff and Schwartz-Giblin, 1988; Schumacher et al., 1993) and has been found to have receptors for oxytocin that show important differences between rostral and caudal sites (Johnson et al., 1989; Schumacher et al., 1990; Caldwell et al., 1994). Since oxytocin receptors have been found to be modulated *in vitro* by P-BSA (Caldwell et al., 1994; Grazzini et al., 1998) and we have identified P-[^{125}I-BSA] binding sites in the MBH of females (Caldwell et al., 1995), we will examine the caudal versus rostral MBH for P-[^{125}I-BSA] binding sites in the current study. Copulatory performance in rats is primarily regulated by testicular androgens acting on neurons in the MPOA, while motivational aspects (sexual arousal) are modulated by olfactory and other inputs into the corticomedial amygdala (Baum, 1992) which is why we examined the amygdala for P-[^{125}I-BSA] binding sites.

Materials and Methods

A. Gonadectomy and testosterone treatment for male rats

Six adult Sprague-Dawley rats (250-300 grams) were maintained in individual cages on a 14-h light: 10 h dark cycle with lights out at noon. Rats were gonadectomized under ketamine anesthesia and then a 2-week recovery period was allowed for baseline testosterone to drop to undetectable levels in these rats. After the recovery period rats were again anesthetized and implanted subcutaneously with either a blank silastic capsule (10 mm, i.d., 0.04 in, o.d., 0.85 in) or a capsule filled with crystalline testosterone. This silastic treatment has been demonstrated to deliver 6 ng/ml blood testosterone, which is within the normal intact range (Witt et al., 1995). After one week rats were decapitated under CO_2 anesthesia, and their brains quickly frozen on dry ice and shipped on dry ice.

B. Materials

Sesame oil, isopentane, and estradiol benzoate were from purchased Sigma (St.Louis, MO), P-BSA (4-pregnen-3, 20-dione 3-CMO: BSA, Batch R163) was obtained from Steraloids (Wilton, NH), paraformaldehyde and emulsion were obtained from Kodak Co. (Rochester, NY), radiolabeled sodium iodide [Na ^{125}-I] was purchased from ICN Pharmaceutical, Inc. (Costa Mesa, CA), Clearing agent Histo-clear was from National Diagnostics (Atlanta, Georgia), Permount was from Fisher Scientific (Pittsburgh, PA).

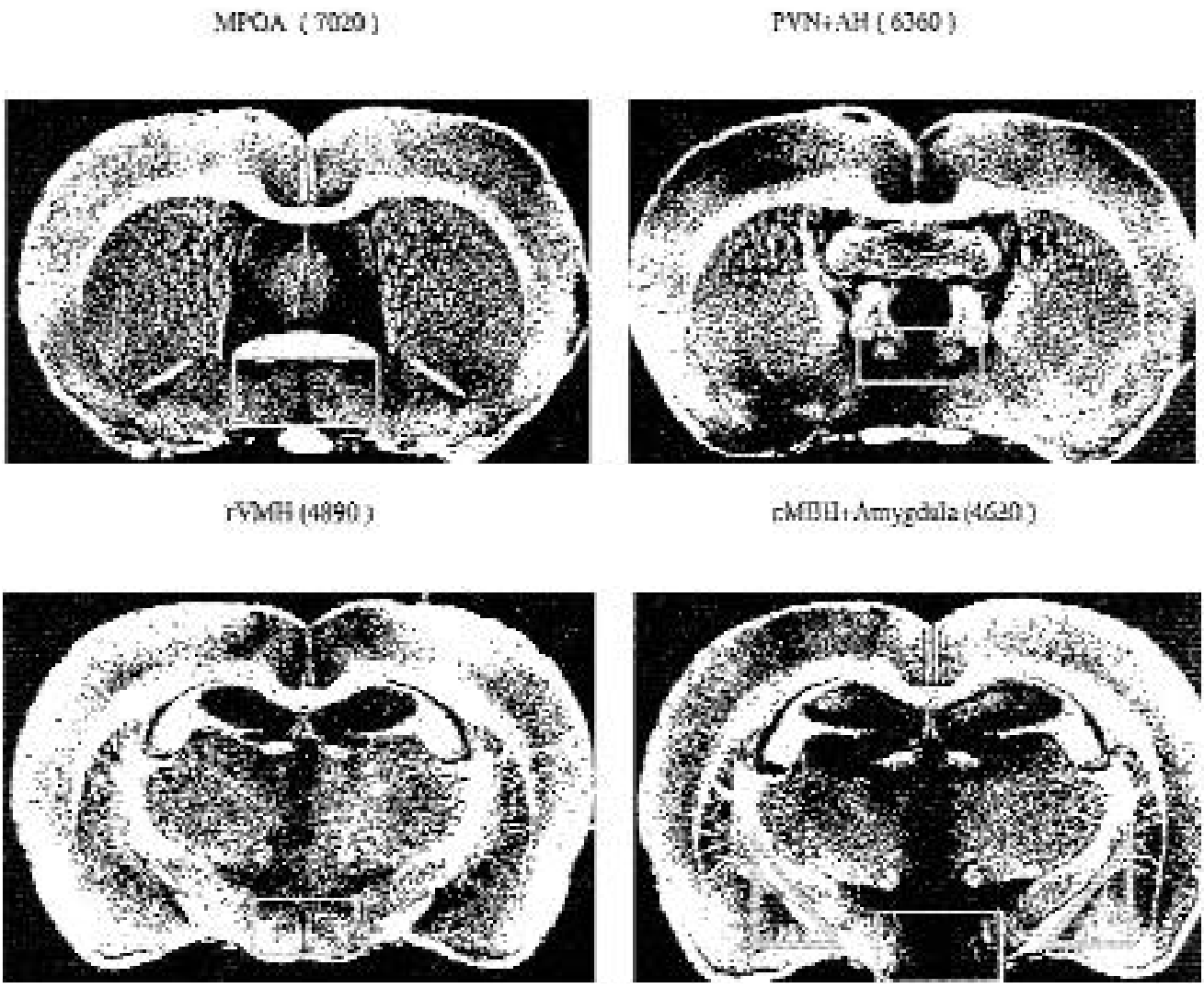

Fig. 1.- Sites of image analysis for autoradiography. The dorsal MPOA consisted of sections delimited rostrally at coronal section A7020; (Koenig and Klippel, 1963) and comprised of rectangular boxes in the dorsal MPOA. The PVN-AH encompassed the entire PVN and portions of the surrounding anterior hypothalamus at level A6360. The rostral MBH (rVMH) was found in the ventral part of section A4890. The cMBH and amygdala were found at level A 4620 with the amygdala delineated by the more lateral triangles.

C. P-3-[125I-BSA] autoradiography

1. Brain Sectioning

Brains were sliced coronally to a thickness of 10 μm in a cryostat Microtome (Leitz, Kryostat 1720) and slides were thaw-mounted on microscope slides. At least four slices were collected from each of these four areas beginning at: A) 7020, B) 6360, C) 4890, D) 4620 (see Figure 1). Then slides were dipped into 4% paraformaldehyde in 10 mM PBS for 5 minutes for fixation and washed in distilled water. After the slides were dried under a hood at room temperature, they were stored in -20˚C freezer for future use.

2. Iodination

Radioiodination of P-BSA was achieved by mixing 40 μl of a 2 mg/ml P-BSA solution in 40 mM Tris-HCl with 1 mCi Na-^{125}I. The reaction was begun with the addition of 20 (l of a 0.8 mg/ml solution of chloramine T, and after 12 minutes shaking, 20 μl of saturated solution of sodium metabisulfite was added to stop the reaction. Proteins bound to radioiodine were separated from free iodine on a PD-10 (Sephadex G-75; Pharmacia Biotech.) column using 40 mM Tris-HCl (pH 7.4). 1 ml aliquots were collected and counted in a Beckman 5500B gamma counter at an efficiency of 70%. Specific activity (232 Ci / mmol) was determined from the radioactivity of the P-[^{125}I-BSA] fraction.

3. Incubation

In a humidity chamber, slides were exposed to 0.3 nM P-[^{125}I-BSA] with or without 0.3 μM P-BSA at room temperature for 18 hours. Slides were then washed three times in 10 μM PBS buffer (pH=7.4) and left to air dry. Slides were dipped into Kodak emulsion in a dark room and packed in light-tight boxes for four days at 4˚C

and then they were developed in D-19 developer followed by fixer. Slides were counterstained with hematoxylin and run through a series of increasing ethanol solutions (from 70% to 100%) followed by a clearing agent and mounted with Permount™. Slides were analyzed for grain density over the MPOA, PVN and AH, rMBH, cMBH, and amygdala (Figure 1). The MPOA was analyzed at the A7020 anterior-posterior level (Koenig and Klippel, 1963) in a rectangle bordered laterally by lines ventral from the lateral edges of the anterior commissure, bordered dorsally by the ventral edge of the anterior commissure and ventrally by the dorsal edge of the optic chiasm. Therefore, in this study the MPOA will include the entire medial preoptic nucleus, the preoptic portions of the suprachiasmatic, and periventricular nuclei, the ventromedial portion of the bed nucleus of the stria terminalis and medial portions of the medial forebrain bundle and the extreme medial portion of the lateral preoptic nucleus. In an attempt to survey P-BSA binding in the rostral paraventricular and anterior commissural nuclei at the A6360 level was analyzed in a rectangle with lateral boundaries from the ventral extent of the stria medullaris, bordered ventrally by the dorsal anterior hypothalamus and dorsally 1 mm dorsal to the dorsal extent of the third ventricle. Therefore, this area included the aforementioned anterior and paraventricular nuclei as well as the fornix, the medial bed nucleus of the stria terminalis, the tractospinothalamicus, and the ventromedial stria medullaris. The rMBH was analyzed at the level of A4890 in a rectangle with the dorsal border 0.5 mm ventral to the dorsal extent of the third ventricle, lateral borders 1.2 mm lateral from the midline and the ventral border parallel to the ventral extent of the brain. Therefore, this region called the rMBH included the anterior ventromedial nucleus of the hypothalamus, the dorsal dorsomedial nucleus of the hypothalamus, the fornix, the filiform and ventral portions of the paraventricular nucleus, the periventricular nucleus of the hypothalamus, and the extreme medial portion of the medial forebrain bundle. The cMBH was measured at A4620 in a rectangle with its dorsal border a line between the midpoints of the fornices, the lateral edges perpendicular from this line at the fornices, with the ventral border the ventral edge of the brain. Therefore, the cMBH included the entire ventromedial nucleus, the ventral dorsomedial nucleus of the hypothalamus, the arcuate nucleus, and the periventricular nucleus of the hypothalamus. The amygdala was also measured at this level in two isosceles triangles with right angles ventrolateral and each with its hypoteneuse immediately lateral to and parallel with the optic tracts extending to an apex at the ventral caudate-putamen, the medial apices were located at the angle of the optic tract and the base of the brain. Therefore, the amygdala included the medial amygdaloid nucleus, and the medial portions of the central, basal, and cortical amygdaloid nuclei.

The analysis was done comparing grain densities in select areas between slides receiving only P-[^{125}I-BSA] versus P-[^{125}I-BSA] plus P-BSA using a Kontron KS-400 Image Analysis System. In this system, a special customized program was designed to analyze identifiable cells with grain densities. After establishing the background for each slide in an area of sparse grains, the observer tagged grains over densely labeled cells so that the program then detected similarly labeled cells. The program then calculated the total area circumscribed by such cells per screen. Eight screen samples (4/side) were analyzed per slide. Mean area/slide was calculated for slides exposed to P-[^{125}I-BSA]. Data for slides exposed to P-BSA competitor were subtracted from this mean for the specific binding calculation. Specific binding was then divided by the mean for radiolabeled slides (total binding) to give a relative specific binding as Bound Ratio. Significant differences were calculated between the two treatment groups using a T test for unequal variances (two-tailed; $p < 0.05$ for statistical significance).

Results

The autoradiographic technique proved capable of detecting specific binding of P-[^{125}I-BSA] in brain sections of male rats. This was evidenced by the ability of P-BSA to clearly displace P-[^{125}I-BSA] in several brain regions and was confirmed by the demonstration that such specific binding was significantly altered by testosterone treatment in several regions studied.

Testosterone treatment significantly (*** = $p < 0.001$) increased P-[^{125}I-BSA] binding in the cMBH (Figure 2A; $T_{38} = 2.797$; $p < 0.01$; n = 3 per treatment), in the PVN-AH (Figure 2B; $T_{18} = 8.9$; n = 2 per treatment), and in the MPOA (Figure 2C; $T_{36} = 7.9$; $p < 0.0001$; n = 3 per treatment). By contrast testosterone treatment significantly (** = $p < 0.01$) decreased P-[^{125}I-BSA] binding in the amygdala (Figure 2D; $T_{61} = 2.73$; n = 3 per treatment). There was no significant effect of testosterone treatment in the rostral MBH ($T_{29} = 0.31$; n = 2 per treatment).

The computer-assisted image analysis program was critical for quantification of P-[^{125}I-BSA] binding. While there were clear differences between sections exposed to P-[^{125}I-BSA] alone and those with P-BSA added (see Figure 3), the program was essential in identifying quantitative changes in specific binding with treatments.

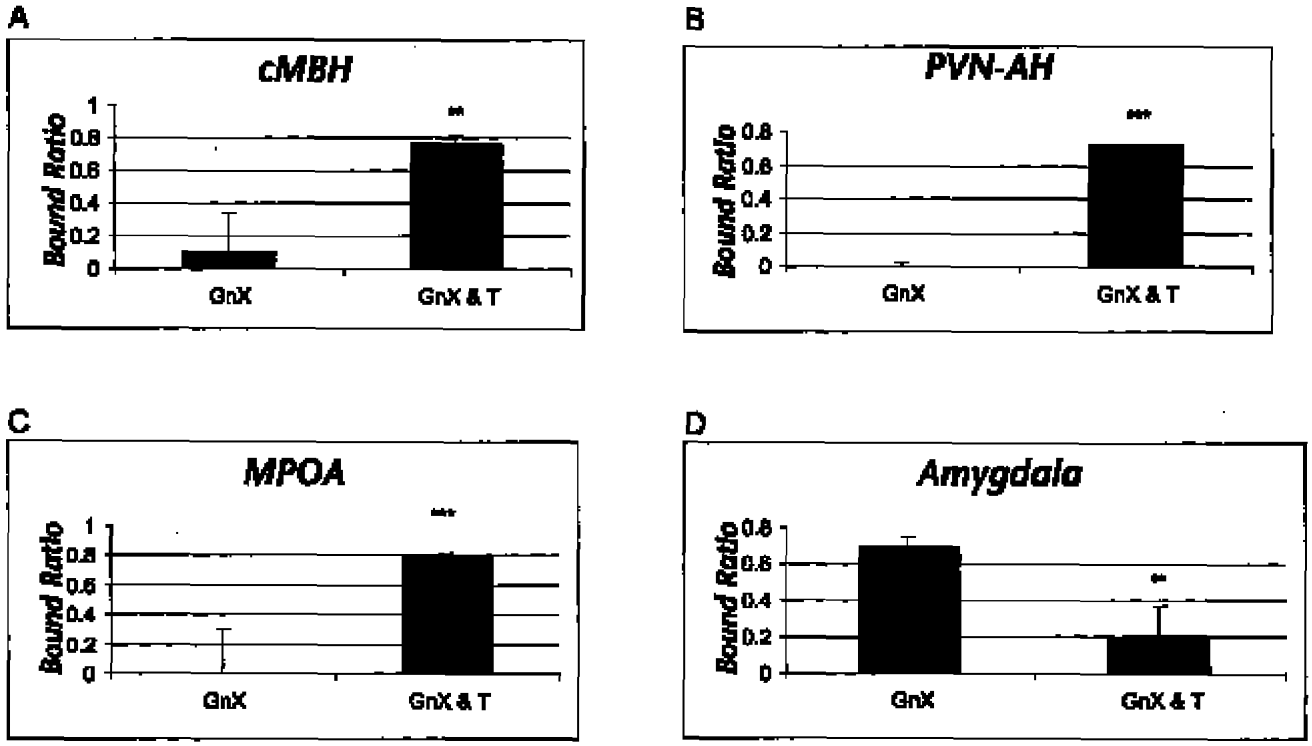

Fig. 2.- P-[^{125}I-BSA] specific binding increased significantly in the cMBH (A), the PVN-AH (B), and the MPOA (C), while it decreased significantly in the amygdala (D). Testosterone treatment (GnX and T) significantly increased P-[^{125}I-BSA] binding in the cMBH, in the PVN-AH, and in the MPOA (** = $p < 0.01$; $T_{38} = 2.797$, n = 3 per treatment; *** = $p < 0.001$, $T_{18} = 8.9$, n = 2 per treatment; *** = $p < 0.0001$, $T_{36} = 7.9$, n = 3 per treatment respectively) over specific binding seen in gonadectomized males with empty implants (GnX). Testosterone treatment significantly (** = $p < 0.01$) decreased P-[^{125}I-BSA] binding in the amygdala (D; $T_{61} = 2.73$; n = 3 per treatment). By contrast there was no significant effect of testosterone treatment in the rostral MBH ($T_{29} = 0.31$; n = 2 per treatment).

Discussion

The autoradiographic technique proved effective in identifying P-[^{125}I-BSA] binding sites in male rat brains. We found testosterone increased binding in the MPOA, PVN-AH, and the cMBH while it decreased P-[^{125}I-BSA] binding in the amygdala. The image analysis system was critical for quantification of P-[^{125}I-BSA] binding. Autoradiography has typically analyzed cytoplasmic or nuclear binding sites, and thus has focused on binding, if not near a cell nucleus, at least in cells demonstrating a visible nucleus. Since we are attempting to examine binding sites associated with the plasma membrane we chose not to limit our analysis to neurons with visible nuclei. Still, we also wanted to be sure only to count silver grain concentrations in excess of background and over identifiable cells. Therefore, the image analysis system was devised to identify counterstained cells with greater than background concentrations of silver grains. The area of such cells was then calculated per screen and used to analyze P-[^{125}I-BSA] binding.

The success of this autoradiographic method indicates that it is possible to identify membrane-associated steroid binding sites with autoradiography. However, this technique requires some alterations from the autoradiographic techniques that have been used for cytoplasmic receptors. One important difference is that when analyzing silver grains, it is not necessary to require visualization of cell nuclei. It is, however, important that the image analysis program be capable of detecting silver grains over cells and distinguishing these based on concentrations over a selected background concentration. The advantage of using steroids conjugated to BSA is that the presence of the BSA moiety ought to limit penetration of the ligand into cells (Ke and Ramirez, 1990). Thus, binding seen in association with intact cells is likely to be due to association of the ligand to membrane receptors. However, this is more problematic where fragments of the cell are seen. Hopefully, the requirement that silver grains appear in association with stained cells assures that cellular fragments are not measured.

These findings are in agreement with the work of Tischkau and Ramirez (1993) who demonstrated using radioligand techniques that P-[^{125}I-BSA] binding was higher in intact males than in castrated males in the MPOA. These authors found that estradiol treatment in castrated males also increased P-[^{125}I-BSA] binding as measured by radioligand assay. Therefore, it appears that autoradiography confirmed the

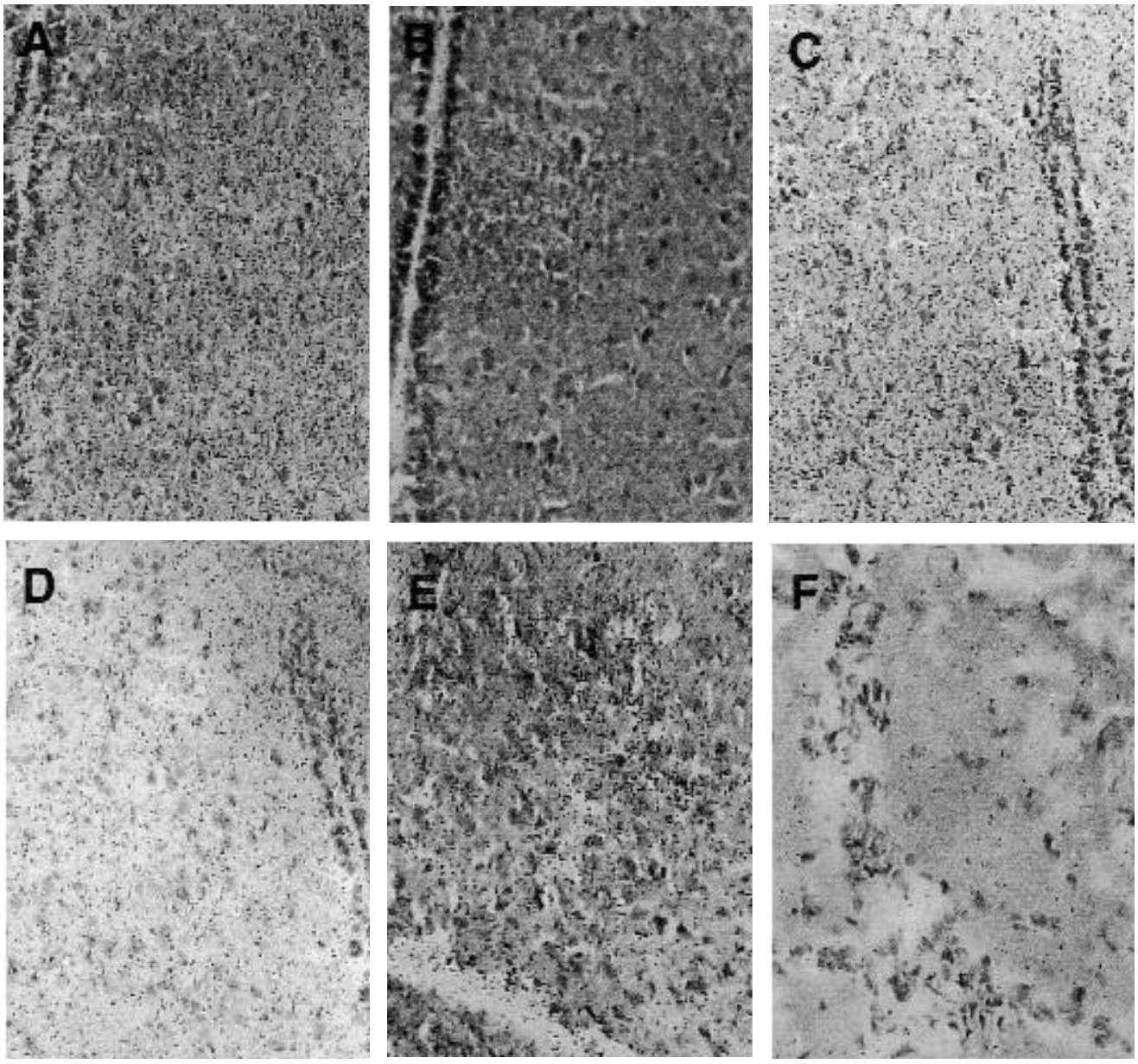

Fig. 3.- Specific binding of P-[^{125}I-BSA] could be identified as a clear-cut decrease in silver grain densities over identified cells in sections exposed to radioligand only (**A**, **C**, and **E**) versus those with radioligand and 0.3 μM P-BSA (**B**, **D**, **F**). This can be seen in photographs of the cMBH (A and B; third ventricle on left), the PVN-AH (C and D; third ventricle on right) and the MPOA (E and F). x 16.5 for all the sections.

radioligand data as best as can be determined since Tischkau and Ramirez did not treat males with testosterone. It also appears that testosterone treatment has the same effect as estradiol on P-[^{125}I-BSA] binding in males.

Testosterone treatments that restored masculine sexual behaviors also significantly elevated P-[^{125}I-BSA] binding in the PVN-AH and cMBH. In fact, there was almost no specific P-[^{125}I-BSA] binding in these two areas in gonadectomized males. This suggests that these binding sites are highly regulated by plasma testosterone levels. It also warrants another review of the role of these areas in control of androgen-dependent behaviors and physiology. The PVN and anterior commissural nuclei are sites of oxytocin production (Sofroniew and Weindl, 1978; Buijs et al., 1983; Caldwell et al., 1989) and the cMBH is an area of dense oxytocin receptors (Caldwell et al., 1994; Johnson et al., 1989, Schumacher et al., 1990 and 1993) as well as oxytocin processes (Sofroniew and Weindl 1978; Buijs et al., 1983; Caldwell et al., 1988). While, there is no evidence of direct influence of androgen on oxytocin immunocytochemistry, it may be that androgens influence oxytocinergic systems via their actions on P-[^{125}I-BSA] binding sites, which have been seen to interact with oxytocin receptors and oxytocin release (see Caldwell et al., 1994 and 1996) and Grazzini et al. (1998) showed P-binding sites on oxytocin receptors.

Lesions of the MPOA in males reduce sexual behavior in virtually every vertebrate species examined and the MPOA plays a critical role in

sexual performance in rats. Intracranial implantation of androgens into this region restores mating behavior in gonadectomized males (Baum, 1992). Recent studies in the whiptail lizard have shown that implantation of P into the MPOA of castrated, P-sensitive males completely restores sexual behavior (Crews et al., 1996). These studies implicate the MPOA as a key region involved in the expression of P-mediated copulatory behavior in lizards. In the current study P-[^{125}I-BSA] binding in the MPOA increased after testosterone treatment. It appears that androgen treatments that restore masculine sexual behavior also significantly elevate P-[^{125}I-BSA] binding in this important area for masculine sexual behaviors. This may implicate membrane-associated P receptors in androgen control of masculine sexual behaviors.

Copulatory performance appears to be correlated with androgen receptor levels in male rats (McGinnis and Dreifuss, 1989; McGinnis et al., 1996). Yet, individual variability persists despite functional androgen levels and sufficient numbers of androgen receptors. Several research groups have suggested that the key to understanding individual variations in the expression of male sexual behavior may lie in the characterization of specific P mechanisms that regulate androgen receptor responses in distinct brain regions. P and progestin receptors may play an important role in androgen sensitivity. For example, progestin receptor knockout mice are less responsive to testosterone replacement and show deficits in androgen-dependent copulatory behaviors (Phelps et al., 2000). In whiptail lizards P-sensitive males receiving P implants into the MPOA showed decreases in progestin receptor mRNA in the MPOA, and increases in androgen receptor mRNA in the MPOA, lateral septum, and amygdala (Crews et al., 1996). In our study testosterone significantly altered P-[^{125}I-BSA] binding in several brain regions. This may be a mechanism whereby androgens increase cellular sensitivity to P.

However, the relationship of membrane-associated steroid binding sites to steroids is not the same as for intracellular steroid receptors (see Caldwell, 2001b). Therefore, we should be prepared for new relationships among the steroids, such as testosterone significantly altering membrane-associated progesterone receptors. Because of the nascent nature of our knowledge of these membrane steroid receptors in brain, it is important to realign our thinking about how they interact.

Young et al. (1964) introduced the concept of organizational versus activational effects of steroids. Perhaps because the organizational or developmental effects of steroids were thought to be due to actions at the genome, the genome received the bulk of attention for steroid activational effects as well. The activational effects of steroids, on the other hand, are defined as those effects of steroids that more immediately alter sexual behavior (Young et al., 1964). For example, restoration of sexual behavior with steroid injections after castration of an adult animal qualifies as an activational effect of steroids. Very elegant studies were conducted, for example, showing that steroid stimulatory actions on sexual behavior could be blocked by infusing protein synthesis inhibitors into various brain regions with the effect of inhibiting sexual behavior (Barfield et al., 1984; Meisel and Pfaff, 1985). Perhaps because of this emphasis on nuclear steroid actions no one had seriously attempted to study immediate steroid effects on sexual behavior. Researchers have been content to analyze "activational' effects of steroids over periods of 1-3 days.

But what if steroids have more immediate effects on reproductive behaviors? Recent evidence indicates that not only are there many rapid steroid physiological effects, but some of them impinge on sexual behavior and perhaps sexual arousal. Frye et al. (1992) first demonstrated an effect of a steroid acting extracellularly on behavior when they infused P-BSA into hamsters and altered their sexual behavior. The presence of the BSA moiety limits entrance of the steroid into the cell (Ke and Ramirez, 1987) thus limiting its behavioral action to extracellular sites. Our laboratory also found that estradiol-BSA (E-BSA) increased female sexual receptivity when administered centrally in combination with oxytocin (Caldwell and Moe, 1999). There are numerous binding sites for both E- and P-BSA in the hypothalamus and preoptic area (Tischkau and Ramirez, 1993; Caldwell et al., 1995; Caldwell et al., 1999). However, since there is no E- or P-BSA in brain, the question arises as to what the endogenous ligand might be for these conjugated steroids. Given the high density of these binding sites, it is likely that the population of P-[^{125}I-BSA] binding sites is made of sub-populations of receptors for numerous ligands. The steroid carrier protein sex hormone binding globulin (SHBG) has been shown to mimic the effects of E-BSA in facilitating female sexual receptivity (Caldwell et al., 2000) suggesting that SHBG couples to neurosteroids and serves as a ligand for demonstrated binding sites for E- and P-BSA. Further support has been provided for this postulate with the displacement of P-[^{125}I-BSA] by SHBG and SHBG coupled to estradiol (Caldwell, 2001a). Other studies suggest that P metabolites are important for functioning at GABA receptors (Morrow et al., 1990). Steroids acting at such sites, which are likely a sub-population of P-[^{125}I-BSA] binding sites, also contribute to control of sexual behaviors (Frye et al., 1996b). It is likely that similar steroid binding

sites exist in association with other neurotransmitters (Petitti and Etgen, 1992; Schwarz and Pohl, 1994; Xiao and Becker, 1998). For example, a progesterone binding site has been demonstrated closely associated with oxytocin receptors (Grazzini et al., 1998). Therefore, it is likely that steroid receptors as well as receptors for SHBG coupled to steroids make up the P-[^{125}I-BSA] binding site population.

As new concepts emerge from the study of rapid steroid actions, we need to remain open minded about the relationships of receptors for these non-genomic steroid effects. For example, Razandi et al., (1999) inserted the gene for the estradiol receptor into Chinese hamster ovary cells and found that these receptors reside in the cell membranes where they mediated numerous physiological actions such as increased inositol phosphate activity, ERK activity, and cell proliferation. Also, laboratories of Gametchu and Watson (Chen et al., 1999a and b) have isolated and cloned a gene for the membrane-associated receptor for glucocorticoids that is unique from the intracellular form of the receptor. Therefore, we may discover that some rapid steroid actions are mediated by insertion of the intracellular receptor into the membrane, while others are due to completely unique membrane-associated receptors.

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