

TESTOSTERONE MASCULINIZES THE DEVELOPMENT
OF THE SUBSTANCE P-IMMUNOREACTIVE INNERVATION
OF THE RAT LIMBIC FOREBRAIN

CENTRE FOR NEWFOUNDLAND STUDIES

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SANDRA A. BROWN, B.Sc.



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OF THE SUBSTANCE P-IMMUNOREACTIVE INNERVATION
OF THE RAT LIMBIC FOREBRAIN

by

©Sandra A. Brown, B.Sc.

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ABSTRACT

Marked sex differences in the substance P-immunoreactive (SPir) innervation of the most posterior divisions of both the medial nucleus of the amygdala (MEPD) and medial bed nucleus of the stria terminalis (BSTMP) have recently been described in adult rats. The present study has examined the effects of early postnatal testosterone exposure on the development of the SPir innervation of these regions and on certain cell groups within the medial amygdala and the BSTMP. Four groups of animals (n=4/group) were compared. Males were either gonadectomized (GM) or sham-operated (SM) on day 1, while females were injected subcutaneously with either vehicle (SF) or 500 ug testosterone propionate (TF) on days 2 and 5. All animals were killed before puberty at day 27. Alternate sections were either stained with cresyl violet or for SP using the peroxidase-anti-peroxidase method. The areas of dense SPir fiber staining and of relevant cell groups (cresyl staining) were measured using a microcomputer-based image analysis system.

Marked sex differences were seen in the medial amygdala and medial posterior bed nucleus before puberty. The areas of the discrete, darkly-staining fields of SPir fibers in MEPD (MEPD-SP) and in the most medial part of BSTMP (BSTMPM-SP) were larger in sham males than in sham females, as they are in adults. The cell groups MEPD, BSTMP, and the lateral division

of BSTMP, BSTMPL, were also significantly larger in sham males than in sham females. The early postnatal treatments completely reversed these sex differences for features of the BSTMP, i.e. measures from groups SM and TF were similar and larger than measures from groups SF and GM which were also similar. Similar results were seen for the sexually dimorphic features of the amygdala, MEPD and MEPD-SP. The areas of these features were not significantly different in groups SF and GM and were smaller than in groups SM and TF. However, these features were larger in group SM than in group TF. Thus injecting newborn females with testosterone did not completely masculinize MEPD and MEPD-SP. Unexpectedly, it was also found that certain features of the medial amygdala and medial bed nucleus were larger in the left than in the right hemisphere.

The sex differences described here are consistent with the results of recent studies which describe a sexually dimorphic system of heavily interconnected cell groups which includes the vomeronasal olfactory pathway, the most posterior parts of the medial amygdala and medial bed nucleus, and the medial preoptic nucleus. These regions have been implicated in the regulation of a number of sexually differentiated brain functions in the rat. The present data are important because they suggest that SPir neurons form an important part of this sexually dimorphic circuitry, and demonstrate that these sex differences are present before puberty.

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INTRODUCTION

Sexual differentiation of physiology and behavior is effected largely by hormones produced by the gonads. In many higher vertebrates an important part of this process is the induction of permanent and apparently irreversible sex differences in central nervous function in response to sex hormones secreted early in development.

Sexual differentiation of brain and behavior: A brief history and overview.

Over fifty years ago Pfeiffer (1936) performed experiments which are the origins of current concepts of sexual differentiation of the central nervous system (CNS). He demonstrated that masculine expression of pituitary gonadatropin release in adult rats depended on hormones secreted from the testes perinatally. He also showed that the expression of feminine patterns of gonadotropic hormone secretion occurred in males which were castrated at birth, while the expression of masculine patterns of gonadotropin secretion occurred in females that had a testis transplanted in their necks soon after birth. The idea that the testes must influence the development of certain areas of the brain was substantiated by the later demonstration that the functions of the pituitary are regulated by the hypothalamus (Green &

Harris, 1947; Harris & Jacobsohn, 1952; Harris & Naftolin, 1970).

By the late 1950's and early 1960's it was accepted widely that gonadal steroids act directly on the brain. Phoenix et al. (1959) developed the idea that steroids are able to act in two distinctly different ways: organizational and activational. For example, early in development gonadal steroids help "organize" neural pathways responsible for reproductive behavior in a permanent manner. In adulthood, sex steroids exert an "activational" effect on the previously differentiated neurons. Steroids during adulthood allow behaviors or functions (e.g., reproduction) to be activated but their effects are reversible, not permanent. Sex differences in the brain and behavior can arise from the organizational effects, while regulation of behaviors and sexually-differentiated neural circuits can be a result of the activational effects.

Besides reproductive functions such as reproductive behavior and the control of gonadotropin secretion, sex differences have been found in many other behaviors including: exercise in a running wheel and open field activity, play, intraspecies aggression, scent marking, taste preferences, feeding and body weight regulation, performance on certain types of learning tasks, and circadian rhythms (reviews from Gorski, 1979; Goy & McEwen, 1980; Hines, 1982; MacLusky & Naftolin, 1981). These sexually differentiated behaviors are also dependent on early androgen action. Therefore, the idea

has been put forth that in mammals the intrinsic pattern of development is female. In the absence of gonadal hormones in either the male or female, development proceeds according to the female pattern. Differentiation toward masculine patterns of gonadotropin secretion and behavior occurs in the male because of exposure to testicular hormones during development (Gorski, 1971). Ovarian hormones have been thought to play little, if any, role during perinatal development. However, Toran-Allerand (see 1984 review chapter) has shown a role for estrogen and its interaction with androgens in developing tissue in vitro and for ovarian hormones in the feminization of the brain.

How the effects of early androgen action on CNS development are expressed in the adult depends on several factors, such as genetic factors, hormonal effects in adulthood, and extrinsic influences from the environment including social and learning experience. Thus, this simple mechanism of gonadal hormone action may not be the only determining factor in sexual differentiation of the CNS, but there is good evidence from studies of mammals and birds (eg. MacLusky & Naftolin, 1981) that exposure to androgen early in life is a major determinant.

The direct neural effects of steroids responsible for sexual function and sexual differentiation of the brain were actively studied during the 1960's and 1970's. Critical periods during early development when the gonadal steroids were

most effective in organizing sexual function were determined in many developing organisms. The possible molecular mechanisms of steroidal action were investigated. Also, the question of which sex steroids were effective in sexual differentiation of the CNS was examined.

Although the fundamental biochemical and molecular mechanisms by which testicular androgens mediate their irreversible effects are still not completely understood (see MacLusky & Naftolin, 1981; McEwen, 1983; for reviews), important progress has been made. In many instances it appears that the masculinization of CNS physiology and behavior in rodents depends on local intraneuronal conversion of testosterone, by aromatization, to the estrogen, 17- β estradiol. The 17- β estradiol can bind to estrogen receptors present in neuronal subpopulations high in aromatase, including the medial hypothalamus, medial preoptic area (MPOA) and the medial amygdala. Further evidence for the role of aromatization in rodent sexual differentiation is that intrahypothalamic implants of either testosterone or 17- β estradiol can masculinize sexual behavior and reproductive function (Christensen & Gorski, 1978) and that testosterone and estradiol-elicited masculinization can be blocked by anti-estrogens (Luttge & Whalen, 1970). Additionally, aromatase inhibitors lessen masculinization caused by endogenous and exogenous testosterone (McEwen et al., 1977), and unlike aromatizable androgens, 5- α dihydrotestosterone (DHT), a non-

aromatizable androgen, apparently does not defeminize (prevent) female rat cyclicity (McDonald & Doughty, 1974). However, the female rat spinal cord can be masculinized by DHT but not estradiol (Breedlove et al., 1982), and the effect of DHT can be blocked by anti-androgens (Breedlove and Arnold, 1983). This demonstrates that even in the rat, not all aspects of sexual differentiation depend on aromatization. Furthermore, estrogens and non-aromatizable androgens can apparently act in concert to masculinize the CNS. For example, 17- β estradiol and DHT are effective when simultaneously delivered to male rats castrated at birth at doses which are ineffective when used alone (Booth, 1977; Hart, 1979; Van der Schoot, 1980).

Several methods have been used to identify possible sites of action of testosterone and its metabolites (i.e. 17- β estradiol and DHT) within the developing brain. One method is based on autoradiographic identification of sites of radiolabeled hormone concentration within the developing brain. Although it is not certain that sites of gonadal steroid uptake necessarily are involved in CNS differentiation, autoradiography has proved to be of considerable value in identifying hormone target cells at a high resolution. For example, Sheridan et al. (1975) injected either tritiated testosterone or 17- β estradiol into two-day-old female rats. The distribution of labeled neurons was similar regardless of which hormone had been injected, and was similar to that seen in adult males and females, including labeled neurons within

the bed nucleus of the stria terminalis (BST) and medial amygdala. Autoradiographic and biochemical assays have been used to identify regions of the developing CNS of the rat and monkey that contain intracellular androgen receptors that bind with high affinity to both testosterone and DHT. Such receptors were found within the hypothalamus, amygdala, MPOA, and septum (Fox et al., 1978; Lieberburg et al., 1978, 1980; Meaney et al., 1985; Pomerantz et al., 1985; Sheridan, 1981). Activation of intracellular steroid receptors is considered a necessary step in the organizational actions of androgens in the CNS.

Significant progress has been made in understanding the neural and hormonal basis of sex differences in behavior and reproductive function within the last 10 years. Several structural sex differences in the adult CNS have been identified. These sex differences result from gonadal steroids which greatly affect the morphogenesis and survival of specific neurons. The presence of sexual dimorphism has been demonstrated in morphological parameters such as nuclear volume (Gorski et al., 1978, 1980), dendritic field (Greenough et al., 1977) and synaptic organization (Nishizuka & Arai, 1981; Raisman & Field, 1973), as well as in the cytoarchitecture of brain regions known to control various reproductive functions (i.e., MPOA, BST, and medial amygdala, which will be discussed in the next section). These brain regions have been found to be densely-populated with steroid-concentrating neurons (Pfaff

& Keiner, 1973; Sar & Stumpf, 1975; Sheridan, 1979). Additionally, sexual differentiation of these parameters is dependent on the perinatal sex steroid environment (Raisman & Field, 1973; Gorski et al., 1978; Nishizuka & Arai, 1981; del Abril et al., 1987).

Sexual differentiation in a highly interconnected group of forebrain structures: The amygdala, BST, and MPOA.

Findings from many of the studies cited above indicate that early exposure to testosterone plays a critical role in sexual differentiation of aspects of brain organization that may underlie neuroendocrine functions and behaviors related to reproduction. Studies of the circuitry underlying reproductive function which includes the MPOA, the medial amygdala, and the BST support this conclusion (Sawyer, 1972; Scalia & Winans, 1975; Beltramino & Taleisnik, 1978, 1980). Interestingly, results show that these areas contain highly interconnected and sexually-differentiated cell groups.

The MPOA has been found to be sexually dimorphic in many species. The sexually dimorphic nucleus within the MPOA (SDN-POA) is at least twice as large in volume in the adult male rat as it is in the female, and this difference is believed to be at least partly due to a greater number of neuronal perikarya in the male SDN-POA (Gorski et al., 1980). A sex difference in opiate receptor density within the rat MPOA has also been reported. In this case there are more opiate receptors in the

female MPOA (Hammer, 1985). A cell group apparently homologous to the rat SDN-POA has been identified in the human brain. As in the rat, both the volume of the cell group and the number of neuronal perikarya were reported to be larger in males (Swaab & Fliers, 1985). Cell groups within the MPOA which are larger in the male have also been described in the gerbil (Commins & Yahr, 1982), ferret (Tobet et al., 1986), guinea pig (Hines et al., 1985), quail (Panzica et al., 1987), hamster, and mouse (Bleier et al., 1982).

Sex differences in the biochemistry and morphology of the medial amygdala have also been observed in various animals. For example, in rodents the cholinergic binding capacity, the synaptic organization, the synaptic density and the volume of the medial amygdala are sexually differentiated (Arimatsu et al., 1981; Nishizuka & Arai, 1981, 1983; Mizukami et al., 1983). Also, the size of the nuclei of individual neurons in the medial amygdala of the squirrel monkey (Bubenik & Brown, 1973) and the volume of the medial amygdala-anterior preoptic complex of the toad are larger in males than in females (Takami & Urano, 1984).

The BST is also sexually differentiated. The volume of the posteromedial division of the BST is larger in male than female hamsters (Bleir et al., 1982), guinea pigs (Hines et al., 1985), and rats (del Abril et al., 1987, Hines et al., 1986; Malsbury & McKay, 1987).

Neonatal hormone manipulations cause neurochemical sex differences.

In adult animals numerous examples of changes in neurotransmitter systems related to sex hormones have been reported. Estrogen causes an increase of muscarinic cholinergic receptor density throughout the medial basal hypothalamus (Rainbow et al., 1980), of serotonin receptors in the MPOA (Biegon et al., 1982), and of striatal dopamine receptors (Hruska et al., 1980). In the adult many of the activational effects of gonadal hormones may be mediated via altered neurotransmitter function (McEwen, 1981).

Similar mechanisms may operate during development. In 12-day-old rats, brain serotonin concentrations are higher in males than females (Ladosky & Gaziri, 1970; Giulian et al., 1973). This difference appears to be the consequence of circulating perinatal androgens. Catecholaminergic neurotransmitter systems are known to sexually differentiate (Vaccari et al., 1977; Crowley et al., 1978; Demarest et al., 1981), at least in part, during the perinatal period (Goy & McEwen, 1980). Sex differences in the distribution of serotonergic fibers in the medial preoptic nucleus (Simerly et al., 1984a), of tyrosine hydroxylase-containing cells and fibers in the anteroventral periventricular nucleus (Simerly et al., 1985), and of vasopressinergic fibers in the lateral septum and habenula (De Vries et al., 1981, 1983; van Leeuwen & Caffé, 1983), have been demonstrated immunohistochemically

and appear to be affected by changes of the perinatal steroid environment (De Vries et al., 1983; Simerly et al., 1984b).

Of particular importance to this thesis is the neuropeptide transmitter substance P (SP). The size of discrete, darkly-staining fields of SP-immunoreactive (SPir) fibers within the medial amygdala and the medial BST is much larger in adult male than in female rats (Malsbury & McKay, 1987; 1989). The present thesis examines the question of whether these sex differences are determined by the organizational action of testosterone, as has been found to be the case for the neurotransmitters mentioned previously.

Substance P and reproductive behavior.

SP has been localized in brain synaptosomes and is a putative neurotransmitter or neuromodulator (Hokfelt et al., 1977; Skrabanek & Powell, 1977). Over fifty years ago, von Euler and Gaddum (1931) discovered SP. Later, Chang and Leeman (1970) identified SP as a undecapeptide member of the tachykinin family. SP is widely distributed throughout the peripheral nervous system (Pernow, 1983). It can be released from the periphery from axon collaterals of stimulated pain fibers and contributes to the inflammatory response. It has also been found to stimulate connective tissue cell growth in the periphery (see Nilsson et al., 1985). SP has been found to be extensively and unevenly distributed in the vertebrate CNS as well (Skrabanek & Powell, 1977; Brownstein et al., 1976;

Cuello & Kanazawa, 1978; Ljungdahl et al., 1978; Schults et al., 1984). High concentrations of SP immunoreactivity are found in various brain regions of adult rats including: the amygdala, the BST, the striatum, the hypothalamus, the substantia nigra, and the superficial layers of the dorsal horn of the spinal cord (Pernow, 1983). SP is present in these same regions as early as 14 days of gestation (McGregor et al., 1982). SP has been shown to be an excitatory peptide within the spinal cord, the medial amygdala (Le Gal La Salle & Ben-Ari, 1977) and the MPOA (Mayer and MacLeod, 1979).

A growing body of evidence suggests that SP may participate in reproductive functions in the CNS. Several recent studies have found that the CNS concentrations of a number of peptides, including SP, are regulated by sex hormones. For example, Frankfurt et al. (1986) reported that SP concentrations within several discrete areas of the CNS showed a fluctuating pattern over the estrous cycle of the female rat. Vijayan and McCann (1979) have found intraventricular injections of SP to facilitate release of prolactin and luteinizing hormone in the female rat, while in vitro incubation of pituitaries with SP caused only prolactin, not luteinizing hormone, to be released. The authors suggested that in the CNS, SP may release LHRH which causes the release of luteinizing hormone. Tsuruo et al. (1987) demonstrated that hypothalamic SPir neurons showed sex-dependent topographical differences and ultrastructural transformations related to

phases of the estrous cycle. Numbers of SPir neurons in the arcuate nucleus vary with the estrous cycle and subcellular alterations in these cells suggest increased ribosomal activity during proestrus and estrous. SPir content of the mediobasal hypothalamus also has been shown to vary with the estrous cycle (Micevych et al., 1988). Recently, Akesson and Micevych (1988) found evidence for co-localization of SP and estrogen receptors in neurons of the ventromedial and arcuate nuclei of the hypothalamus in female rats. SP concentrations within the male rat CNS also have an apparent dependence on gonadal hormones. For instance, Dees and Kozloski (1984) reported a reduction in SP immunoreactivity in the medial amygdala following castration in adult male rats. Also, SP concentration is greater in males than in females in the posterior part of the amygdala (Frankfurt et al., 1985; Micevych et al., 1988). Anterior pituitary concentrations of SP immunoreactivity have also been found to be greater in male than in female rats (dePalatis et al., 1985; Aronin et al., 1986; Jakubowska-Naziemblo et al., 1986) and this sex difference is determined by neonatal testosterone exposure (Yoshikawa & Hong, 1983). It has been suggested that gonadal steroids act directly at the level of the anterior pituitary to affect the synthesis and/or release of the peptide (Coslovsky et al., 1984; Vijayan & McCann, 1979). Taken together these results provide evidence for the hypothesis that discrete populations of SPir neurons may be

linked functionally to hormonal regulation of gonadotropin secretion and/or reproductive behavior.

A possible role for SP in both male and female reproductive behavior has been suggested in recent reports from this laboratory. SP injections into the midbrain central gray facilitated the lordosis response in ovariectomized, estrogen-primed female rats, while injections of a SP antiserum into this area reduced lordosis scores in highly receptive females (Dornan et al., 1987). Dornan and Malsbury (1989) have recently provided the first direct evidence that SP plays a role in the regulation of male copulatory behavior. The MPOA-anterior hypothalamic area (MPOA-AH) is an area which has been shown to be crucial for the expression of male copulatory behavior (Larsson, 1979). Following bilateral injections of 10 or 100 ng of SP into the MPOA-AH, male rats initiated copulatory behavior sooner, seemed to be more efficient copulators and ejaculated sooner. Consistent with these results, a SP antiserum injected into the MPOA-AH caused a decrease in copulatory behavior, i.e., mount, intromission, and ejaculation latencies increased significantly.

Substance P-immunoreactive (SPir) neurons within the BST and medial amygdala circuitry and reproductive behavior.

The sexually dimorphic cell groups of the BST and the medial amygdala have been shown to be interconnected. Most BST afferents originate from the amygdala, in particular, the

dorsomedial part of the caudal BST receives projections from the medial amygdala and the amygdalo-hippocampal area (Krettek & Price, 1978; Weller & Smith, 1982). These connections are interesting, because both the medial BST and medial amygdala are target sites for gonadal hormones, i.e. they contain receptors for steroids (Pfaff & Keiner, 1973; Stumpf and Sar, 1975), and both nuclei are involved in the control of testosterone-sensitive behavior such as mating (Valcourt & Sachs, 1979; Harris & Sachs, 1975; Emery & Sachs, 1976; Masco & Carrer, 1980), as well as in the control of gonadotropic hormone release in males and females (Kawakami & Kimura, 1974; Kostarczyk, 1986).

As mentioned above, SP also has been associated with reproductive functions. The MPOA, the medio-basal hypothalamus, and the midbrain central gray all contain SP and SP receptors, and have been suggested as sites of action of SP relevant to reproductive physiology and/or behavior. SPir perikarya and fibers as well as SP receptors are also present in the medial BST and the medial amygdala (Ljungdahl et al., 1978; Woodhams et al., 1983). Furthermore, the SPir innervation of the BST and medial amygdala has been shown to be sexually dimorphic. Frankfurt et al. (1985) and Micevych et al. (1988) reported that in rats SP concentrations in the posterior part of the amygdala were higher in males than in females. This is consistent with immunocytochemical observations from this laboratory. Malsbury and McKay (1989)

found that in adult rats the posterior dorsal division of the medial nucleus of the amygdala (designated MEPD in the atlas of Paxinos & Watson, 1986) stains darkly for SP and this field of SPir fibers is over twice as large in males as in females. The darkly-staining SPir field of fibers in the posterior medial BST is also much larger in males (Malsbury & McKay, 1987, 1989).

Do hormones determine the sexual differentiation of the SPir innervation of the medial amygdala and BST?

The SPir innervation of the medial amygdala is regulated by testosterone in adult male rats. Dees and Kozlowski (1984) reported a reduction in SP staining ten days after castration and Malsbury et al. (1987) found a decrease in the area of dense staining that progressively declined at 2, 4, and 8 weeks after castration. This gradual decline in the area of dense staining correlates with the gradual decline in sexual behavior following castration.

As mentioned above, previous studies conducted in this laboratory revealed that fields of SPir fibers within the BST and the medial amygdala were sexually dimorphic in adults (Malsbury & McKay, 1987; 1989). What are the origins of these sex differences? Are they determined by the action of gonadal hormones in the adult or perinatally or at both times?

The present study investigates possible early hormonal determinants of these dimorphisms. Other laboratories have

shown that testosterone treatment of neonatal female rats and gonadectomy of neonatal male rats can reverse some neuroanatomical sex differences normally found in adults (De Vries et al., 1983; Hammer, 1985; Simerly et al., 1985). In the light of these findings, the aim of the present study was to determine: a) whether or not neonatal testosterone injections can masculinize the pattern of SPir staining in juvenile females, and b) whether neonatal gonadectomy can demasculinize this pattern in juvenile males. Results of pilot work had determined that a sex difference in these SPir features was present before puberty. Thus all subjects were killed at 27 days of age to minimize any activational influences of gonadal hormones.

METHODS

Neonatal treatments.

Sprague-Dawley rats from Charles River (Quebec) were mated in our laboratory. Two litters of 8 pups from 2 mothers provided the 16 brains used in this study. Rats were maintained on a 12/12 light/dark cycle with lights on at 0800 hrs. Male pups were randomly assigned to one of two groups: gonadectomized (GM) or sham operated (SM). Group GM males were castrated on day 1, the day of birth. Pups were anesthetized by hypothermia, induced by covering them with crushed ice. A small horizontal incision was made midway between the naval and

the genital tubercle, the testes were removed with the aid of fine forceps, and the skin incision was sealed with collodion. The pups were warmed on a heating pad maintained at approximately 37 degrees Celsius before reunion with their mothers. Group SM males were sham gonadectomized on day 1. They underwent the same procedure as Group GM except for the removal of their testes. Female pups were also assigned randomly to one of two groups: testosterone-treated (TF) or oil-treated (SF). Group TF females were injected subcutaneously with 500 μ g testosterone propionate (TP) in 25 μ l ethyl oleate on days 2 and 5. Injections were administered using a 100 μ l syringe and a 30 gauge needle inserted at least 1.5 cm horizontally under the skin to minimize leakage. Using the same procedure as used for Group TF, Group SF rats were injected only with 25 μ l ethyl oleate. The litters were culled on day 2 so that each mother raised only 8 of her pups (2 from each group). Tattoos (India ink injected in the foot pad) were used to identify the group membership of each pup. In cases where the ink marks began to fade as the pups grew, ear notches were used to maintain pup identity. Rats were weighed and weaned on day 26 and reweighed and sacrificed on day 27.

To help ensure the mothers' acceptance of their pups after they had been treated, the mothers were familiarized with the experimenter before the pups were born. From the 13th day of gestation until the pups were born, the mothers were gently

handled by the experimenter for 2 to 5 minutes daily. None of the pups were cannibalized.

Immunocytochemistry.

Brains were processed for immunocytochemistry (ICC) using the peroxidase anti-peroxidase (PAP) method (Sternberger, 1986). Alternate sections were stained with cresyl-violet. Brains were processed in sets of four (one from each treatment group). Four rats from the same litter were perfused on the same day at the same time (within 1 hour of each other). A set of four brains was sectioned on the same day and carried through the staining procedures together, to ensure that any variability in procedures was distributed equally among groups (see Appendix A for ICC and perfusion solutions).

After the animals were deeply anesthetized with 0.1 ml sodium pentobarbital (Somnotol) they were injected with 0.25 ml sodium nitrite intravenously and after at least 2 minutes they were perfused using a pressure perfusion system via the heart with 250 ml Vaughn's fixative (4% buffered paraformaldehyde) for approximately 5 minutes but never for more than 10 minutes. Brains were removed and kept in the refrigerator overnight in the fixative. The next day, the brains were weighed after the olfactory bulbs and brainstem were removed. The brainstem and cerebellum were removed with a coronal-plane cut beginning just rostral to the cerebellum. Brains were then frozen by gradually submerging them in isopentane that was maintained near its freezing point using liquid nitrogen. Brains were

sectioned at 35 microns in the coronal plane in a cryostat at -6 degrees Celsius. Sections were mounted on slides coated with chrome-alum-gelatin. After the sections were dried on the slides, they were rinsed in phosphate buffered saline (PBS) for one half hour.

All antisera were diluted in a solution of PBS containing 10% normal goat serum and 0.25% Triton X-100. The slides were immersed in a rabbit antiserum to substance P (obtained from Immunonuclear, now IncStar) at a dilution of 1:3000. The slides were gently agitated using a rotary-action shaker and left in the primary antibody solution overnight in the refrigerator. Four washings with PBS during a 1 hr period were conducted between each of the next three steps. First, the slides were immersed in goat anti-rabbit IgG (obtained from Boehringer-Mannheim) at a dilution of 1:150 for 2 hrs on the shaker. Second, the slides were immersed in a solution of PAP (obtained from Sternberger-Meyer) at a dilution of 1:300 for 2 hrs on the shaker and left in the PAP solution overnight in the refrigerator. Third, the reaction product was developed by immersing slides in a solution of diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide. The DAB reaction product was intensified by the addition of cobalt chloride and nickel ammonium sulfate to the DAB solution.

Pre-absorption control experiments have been repeated over a period of years in this laboratory to check for possible artifactual (non-specific) staining. In these experiments,

which used the identical PAP procedure as used here, alternate sections of the brains of adult and newborn Sprague-Dawley rats were exposed either to the primary antibody solution or the same solution in which the anti-SP antibodies had been preabsorbed with SP. SP was added to the diluted primary antiserum solution at a concentration of 100 μg SP/ml. This solution was left overnight in the refrigerator before applying it to the sections. No staining was seen on sections incubated with the preabsorption solution. These results are consistent with the assumption that the staining reported here represents the presence of SP-like molecules.

Incstar has provided information on the specificity of this antiserum as determined using the paper spot method of Larsson (1981). With 2 μl of 100 pmole concentrations the following antigens did not react with the SP antiserum diluted 1:500 using the PAP method: neurokinin A, neurokinin B, eledoisin, CCK-8, serotonin, somatostatin, leu enkephalin, met enkephalin, neurotensin and vasoactive intestinal peptide.

Morphometry.

An image analysis system which included software (MCID) by Imaging Research Inc. of St. Catharines, Ontario, a computer (XT) by IBM, a frame grabber board (PC Vision) by Imaging Technology, and a video camera (SC505) by VSP Inc., was used to calculate all area measurements. All measurements were made without knowledge of the sex or treatment group of the animal.

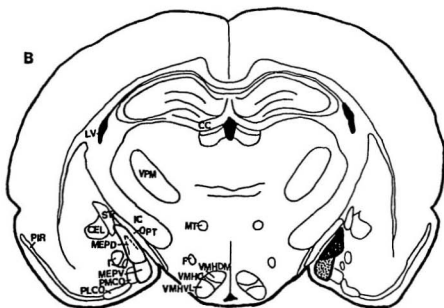
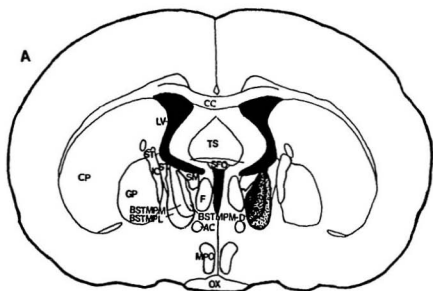
Using a video camera mounted over a light box, the image of the section was digitized and then displayed on a monitor. Using a mouse key, the area of interest was outlined and overlaid on the image monitor. The image analysis system was calibrated each time it was used. Each region of interest was measured three times and values were averaged. The perimeter of each feature was traced bilaterally for each cresyl violet- and corresponding SP-stained section, and the total cross-sectional area was calculated for each hemisphere in each animal. There were from 8 to 13 sections in which a feature appeared and the areas of each feature were summed from the total number of sections for each hemisphere.

Most reports in the literature which compare the sizes of neuronal cell groups have reported their data in units of volume, rather than area, as is done here. However, in all studies which use histological material, the actual measurements are of surface area. Estimates of volume are then derived from area by simply multiplying the total surface area of the feature by the thickness of the tissue section (eg. Hines et al., 1985). In the present report the data have been presented as total area. If desired, volumes (mm^3) can be calculated by multiplying area by .035 mm (section thickness).

An atlas (Paxinos & Watson, 1986) of the adult rat brain was used to define the general areas of interest within the bed nucleus of the stria terminalis and medial amygdala (see Figure 1). Most of the areas measured differed slightly from the

atlas. The definitions of the areas were based on both the cresyl violet- and SP-stained tissue (see photomicrographs with drawings for definition of borders, Figures 1-8). The areas of the suprachiasmatic nucleus (SCN) and whole coronal sections of the forebrain (starting at the anterior commissure and measuring every other cresyl section for a total of 8 sections per brain) were measured from the cresyl series. These two features were considered to be controls, in the sense that sex differences in their areas were presumed to be absent or minimal.

Figure 1. Schematic drawings of coronal sections of the rat forebrain (top schematic is rostral relative to bottom) modified from Paxinos and Watson (1986). Brain regions measured in the current study are shaded, A) bed nucleus of the stria terminalis, B) medial amygdala. Abbreviations: AC, anterior commissure; BSTMPM, medial division of the bed nucleus of the stria terminalis, posteromedial; BSTMPM-D, densely-packed cell portion of BSTMPM; BSTMPL, medial division of the bed nucleus of the stria terminalis, posterolateral; CC, corpus callosum; CEL, central amygdaloid nucleus, lateral; CP, caudate putamen; F, fornix; GP, globus pallidus; I, intercalated nuclei amygdala; IC, internal capsule; LV, lateral ventricle; MEPD, medial amygdaloid nucleus, posterodorsal; MEPV, medial amygdaloid nucleus posteroventral; MPO, medial preoptic nucleus; MT, mamillothalamic tract; OPT, optic tract; OX, optic chiasm; PIR, piriform cortex; PLCO, posterolateral cortical amygdaloid nucleus; PMCO, posteromedial cortical amygdaloid nucleus; SFO, subfornical organ; SM, stria medullaris; ST, stria terminalis; TS, triangular septal nucleus; VMHC, ventromedial hypothalamic nucleus, central; VMHDM, ventromedial hypothalamic nucleus, dorsomedial; VMHVL, ventromedial hypothalamic nucleus, ventrolateral; VPM, ventral posteromedial thalamic nucleus.



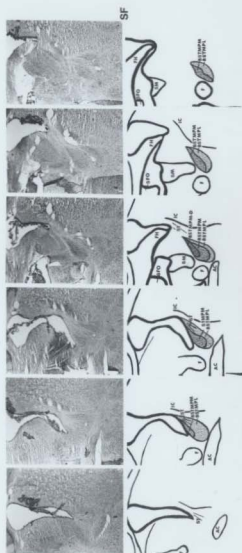
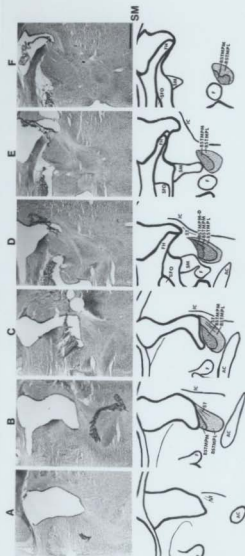
Four features of the most medial posterior BST were examined. From the SP series, the discrete and darkly-stained field of SPir fibers in the most medial BSTMP (BSTMPM-SP) was measured. From the cresyl series, the following divisions of the BSTMP were measured: the densely-packed group of small cells in the medial BSTMP which receives the SPir innervation, BSTMPM; a very densely-packed group of cells within the BSTMPM (BSTMPM-D); and the area adjacent and lateral to BSTMPM which contains less densely-packed, somewhat larger cells, the BSTMPL. This differs slightly from the atlas of Paxinos and Watson in two respects. That atlas divides the BSTMP into 3 adjacent areas; a medial division (BSTMPM), an intermediate division (BSTMPI) and a lateral division (BSTMPL). Since the border between the intermediate and lateral divisions was not apparent in the present material, these two divisions were included together into what I have called BSTMPL. Also, the Paxinos and Watson atlas does not designate a BSTMPM-D.

The BSTMPM and the BSTMPL were contained within an easily distinguishable capsule of fibers rostrally. However, caudally the capsular borders were not as easily discernable. Thus, it was necessary to verify the borders by referring to the corresponding SP-stained sections in which the area of the BSTMPM was more darkly stained and the area of the BSTMPL was more lightly stained than the surrounding areas. These borders could then be identified and traced in the cresyl sections. Within the capsule, the borders between BSTMPM and BSTMPL

remained distinct because the BSTMPM contained much more densely-packed cells (see Figure 2).

Four features of the medial nucleus of the amygdala were measured. Measurements were made from the SP series of the discrete and very darkly-stained area in the most posterodorsal part of the medial nucleus (MEPD-SP). From the cresyl series, area measurements were taken of the cell group which corresponds to this dense field of SPir fibers, MEPD, plus the cell group rostrally adjacent to MEPD (ME), as well as the posteroventral medial amygdala (MEPV). The ME and MEPV cell groups begin just caudal to where the densely-packed cell group of the bed nucleus of the accessory olfactory tract (BAOT) ends. As De Olmos et al. (1985) observed "... rostrally, [the ME] is not clearly outlined as it merges with the AA [anterior amygdaloid area] and, laterally, with the deep layer of ACO [anterior cortical amygdaloid nucleus] ..." (p. 245). At rostral levels the border between ME and MEPV (MEPV is ventral to ME) was also difficult to discriminate. The SP series showed the area of MEPV staining less for SP than most of the surrounding areas at this level. The borders seen in the corresponding SP series were used in cases where it was difficult to distinguish the borders from the cresyl series alone. Caudally the distinction between ME and MEPV was much more apparent. Another feature that was difficult to discriminate in the cresyl series was the border between ME and MEPD. Because the densest SPir staining covers all of MEPD,

Figure 2. Cresyl violet-stained sections (rows 1 and 3) and corresponding drawings (rows 2 and 4) illustrate the cell groups of the medial posterior BST in a male, SM, (top 2 rows) and a female, SF, (bottom 2 rows). Photographs and drawings are arranged in rostral to caudal order (i.e., A-F). Horizontal bar in top row, column F, equals 1 mm. The same scale is present in Figures 3 through 8. Sections from the medial amygdala of these same brains are seen in Figures 5 and 6. Shaded areas in drawings indicate the areas measured. Level A shows sections just rostral to the BSTMP. B is the level 1 or 2 sections caudal to the rostral pole of the BSTMP cell group. Level C is just rostral to where BSTMPM-D appears. At level D, the BSTMPM-D is very apparent and it is gone by level E. Level F is a few sections rostral to the caudal pole of the BSTMP. Abbreviations: AC, anterior commissure; BSTMP, medial posterior division of the bed nucleus of the stria terminalis; BSTMPL, BSTMP, lateral; BSTMPM, BSTMP, medial; BSTMPM-D, most densely-packed cell portion of BSTMPM; FH, fimbria hippocampus; IC, internal capsule; SFO, subfornical organ; SM, nucleus stria medullaris; ST, stria terminalis.



the first section of MEPD-SP was used to define the corresponding rostral boundary of MEPD (see Figures 5-8).

Statistical analysis.

All data analysis utilized the four treatment groups: GM, SF, TF, SM. Brain weight and body weight were analyzed by a 1-way analysis of variance. All histological measures were examined by 2-way analysis of variance for repeated measures designs (Winer, 1962). The analysis utilized one independent variable for group effects and the other repeated measures variable for hemispheric asymmetries.

Based on a priori expectations that the testosterone-exposed groups (TF and SM) should be equal but different in size than the testosterone-deficient groups (GM and SF) which should also be equal in size, individual mean contrasts were made by t tests rather than other methods of contrasts, when group effects were significant. First, t tests were done to determine whether, in fact, the a priori expectations of group equivalences had been met, i.e. group TF was compared with SM; group GM was compared with SF. These tests were not expected to reveal significant differences, and in most cases they didn't. In these cases data from groups TF and SM were combined, as were data from groups GM and SF, to compare testosterone-exposed with testosterone-deficient animals.

In most instances, the number of sections on which the feature appeared as well as its total cross-sectional area

within each hemisphere was calculated for each animal. The contribution of the number of sections to the group differences in area was assessed by analysis of covariance. Only when there was a group difference in the area of a feature as well as a group difference in the number of sections for that feature, were the number of sections co-varied out. The assumption tested was that if the group area difference was due entirely to the feature being longer, not necessarily wider, then the analysis of covariance, by co-varying out the effect of the number of sections, should eliminate the group area difference. Whenever a given group had a significantly greater total cross-sectional area it also had a significantly larger number of sections. In these cases if a group area difference remained after the number of sections was co-varied out, then the group area difference could be attributed to the area of the feature being wider as well as longer.

RESULTS

Control measurements.

Brain and body weights for the 4 groups are summarized in Table 1. There was no significant difference between the groups in any of the following measurements: SCN area or number of sections in which SCN was found, total area of whole coronal sections of the forebrain, brain weights, or body weights at the time of weaning (wean weight) and at the time of perfusion

one day later (sacrifice weight). The comparison of average wean weight of all the rats with the average sacrifice weight one day later showed that the rats grew a significant amount ($F(1,12)=15.155$, $P<.01$), suggesting the young rats were healthy at the time of perfusion.

Though the SCN area was not significantly affected by perinatal sex hormones, features of two additional nuclei of the forebrain, namely the BST and the medial amygdala, were.

Bed nucleus of the stria terminalis (BST).

A discrete area of dense SPir fibers was present in the region of the BSTMPM in all 4 treatment groups. This feature, designated BSTMPM-SP, is shown in Figures 3 & 4, columns B-F, rows 1 & 3. The mean of the summed cross-sectional areas of the BSTMPM-SP (Table 2 and Figure 5) show that not only is there a large sex difference ($SM>SF$) but this difference can be completely reversed by postnatal sex hormone manipulations ($TF=SM>GM=SF$). That is, the size of the BSTMPM-SP was similar in the two testosterone-exposed groups (SM and TF) and significantly larger than in the testosterone-deficient groups (SF and GM), which were also similar in size (group effect, $F(3,12)=20.70$, $P<.001$; mean contrasts all $t(12)\geq 7.686$, $P<.001$). Similarly, the number of sections in which BSTMPM-SP appeared was significantly greater in the two testosterone-exposed groups ($TF=SM>GM=SF$) as is seen in Table 2 (group effect,

Table 1. Measures of brain size and body weight.

Features of Interest	Gonadect. Males (GM)	Sham Females (SF)	Sham Males (SM)	Testosterone Females (TF)	Group Differences
SCN					
Area ^a	0.72 (0.03)	0.65 (0.05)	0.61 (0.02)	0.64 (0.01)	NS
Sections	9.75 (0.18)	10.13 (0.32)	10.00 (0.00)	10.25 (0.18)	NS
Forebrain					
Area	756.53 (22.11)	749.05 (22.61)	708.56 (7.83)	747.27 (18.10)	NS
Brain Weight					
Grams	1.25 (0.03)	1.18 (0.03)	1.20 (0.02)	1.20 (0.02)	NS
Wean Weight					
Grams	66.00 (3.38)	64.40 (2.16)	66.00 (3.81)	67.60 (3.20)	NS
Sacrifice Weight					
Grams	68.50 (4.33)	63.50 (3.30)	69.75 (4.64)	67.75 (3.99)	NS

^aSCN (suprachiasmatic nucleus) areas are the averages (SEM) of the two hemispheres.

All areas are in mm².

NS, non-significant.

N = 4 in each group.

Table 2. Features of the Medial Posterior BST (BSTMP)^a

Features of Interest	Gonadect. Males (GM)	Sham Females (SF)	Sham Males (SM)	Testosterone Females (TF)	Group Differences
BSTMP					
Area	4.78 (0.21)	5.00 (0.07)	6.63 (0.14)	6.13 (0.13)	GM=SF<TF=SM ^{***}
Sections	8.00 (0.00)	8.75 (0.34)	11.25 (0.60)	10.38 (0.54)	GM=SF<TF=SM ^{***}
BSTMPL					
Area	2.73 (0.11)	2.85 (0.17)	3.80 (0.22)	3.57 (0.16)	GM=SF<TF=SM ^{**}
Sections	8.00 (0.00)	8.75 (0.34)	11.25 (0.60)	10.38 (0.54)	GM=SF<TF=SM ^{**}
BSTMFM					
Area	2.05 (0.16)	2.15 (0.16)	2.83 (0.20)	2.56 (0.22)	NS
Sections	8.00 (0.00)	8.75 (0.34)	11.25 (0.60)	10.38 (0.54)	GM=SF<TF=SM ^{**}
BSTMFM-SP					
Area	1.59 (0.06)	1.95 (0.09)	3.17 (0.19)	2.96 (0.10)	GM=SF<TF=SM ^{***}
Sections	6.75 (0.18)	7.25 (0.18)	10.25 (0.38)	9.38 (0.54)	GM=SF<TF=SM ^{***}
BSTMFM-D					
Area	0.33 (0.05)	0.41 (0.04)	0.75 (0.13)	0.63 (0.12)	NS
Sections	2.75 (0.18)	3.25 (0.18)	3.88 (0.13)	3.38 (0.19)	GM=SF<TF=SM [*]

^aData are averages (SEM) of the two hemispheres. All areas are in mm².

*P<0.05, **P<0.01, ***P<0.001; 2-tailed T-tests.

NS, non-significant.

N = 4 in each group.

Figure 3. The pattern of SPir staining (rows 1 and 3) and corresponding cresyl violet-stained sections (rows 2 and 4) of the BST of a sham female, SF, (top 2 rows) and gonadectomized male, GM, (bottom 2 rows). Photographs of coronal sections are arranged in rostral to caudal order (i.e., A-F). At level A, at the rostral point at which the anterior commissure begins to cross the midline, dense SPir staining in the region of the BSTMP is not yet apparent. This occurs slightly more caudally at level B. B is the rostral point at which measurements of the area of SPir staining began. At Level D the capsular pattern of SPir staining seen in the testosterone-exposed groups in Fig. 4 is not as apparent in these two testosterone-deficient animals. F is the most caudal level at which the dense SPir staining is seen. At this level, a small region of dense SPir staining is confined to the most dorsal-medial perimeter of the BSTMP adjacent to the fornix (see Figure 2, F for boundaries of BSTMP at this level).

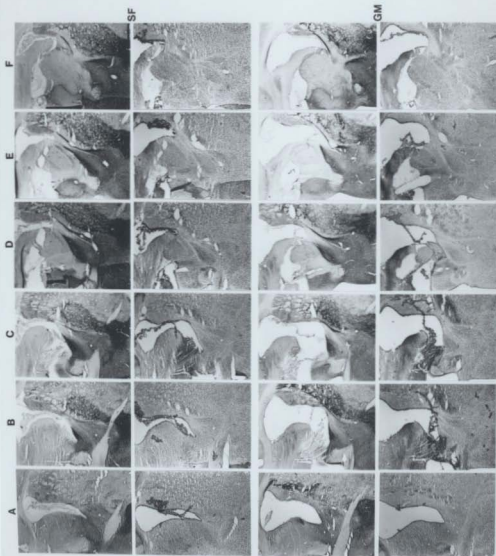


Figure 4. The pattern of SPir staining (rows 1 and 3) and corresponding cresyl violet-stained sections (rows 2 and 4) of the BST of a testosterone female, TF (top 2 rows) and a sham male, SM (bottom 2 rows). Photographs of coronal sections are arranged in rostral to caudal order (i.e., A-F). The capsular pattern of SPir staining seen in these two testosterone-exposed rats is apparent at level D (compare to Figure 3). See Figure 3 for further descriptions.

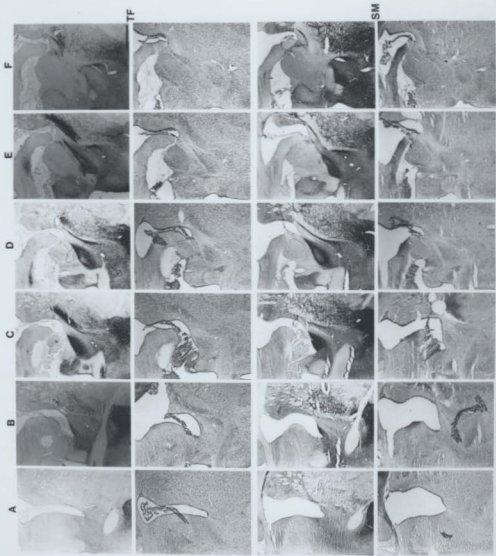
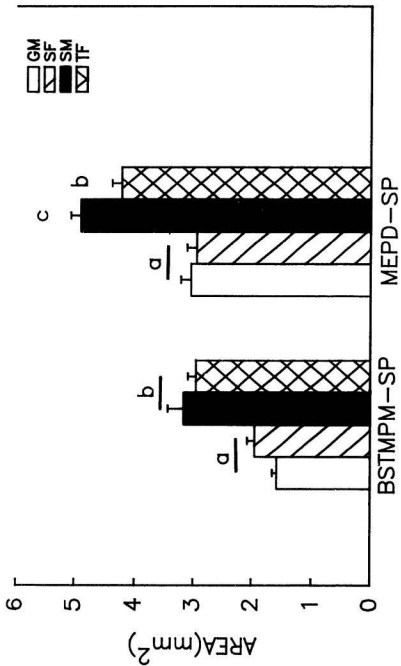


Figure 5. Area of SPir staining in the BST and medial amygdala for each group. Data are group means (and standard errors) of the average of the two hemispheres in each rat. The groups are gonadectomized males (GM, open bars, N=4), sham females (SF, hatched bars, N=4), sham males (SM, solid bars, N=4), and testosterone-exposed females (TF, cross-hatched bars, N=4). Differing letters above the bars indicate significant group differences at $p \leq .05$. Abbreviations: BSTMPM-SP, area of dense SPir staining in the medial division of the medial posterior BST; MEPD-SP, area of dense SPir staining in the posterodorsal medial amygdala.



$F(3,12)=11.78$, $P<.001$; mean contrasts all $t(12)\geq 5.762$, $P<.001$. When the influence of the number of sections was factored out with an analysis of covariance (see Table 3), the effect was still significant (group effect, $F(1,11)=6.40$, $P<.01$; mean contrasts all $t(11)\geq 6.871$, $P<.001$). This suggests that the greater area of staining was not only due to the BSTMF-M-SP being longer, but also wider, in the testosterone-exposed groups.

The pattern of SPir staining in the region of BSTMPM was similar for the two testosterone-exposed groups as illustrated by the photographs in Figure 4 column D, rows 1 and 3. These photographs show a dense SPir capsule surrounding a core which is relatively low in SPir fibers. However, the pattern of SPir staining in the testosterone-deficient groups at the same level is different, because the core that is low in SPir fibers is not observable or barely present (Figure 3, column D, rows 1 and 3). The similarity in the pattern of SPir staining between the testosterone-exposed groups and between the testosterone-deficient groups was consistent as was the difference between these pairs of groups. For example, a person, without knowledge of which brain sections belonged to which group, separated those brains with an obvious capsule/core pattern of SPir fibers in the medial BST from those without a distinct capsule/core pattern. In all cases, this distinction correctly separated the brains of TF and SM rats from those of SF and GM rats.

Table 3. Features in which group differences were seen both in area and number of sections^a. Analysis of covariance: Are group differences in area measures still present when length of the feature is co-varied out?

Features of Interest	Gonadect. Males (GM)	Sham Females (SF)	Sham Males (SM)	Testosterone Females (TF)	Group Differences
BSTHFM-SP					
Area	1.59 (0.06)	1.95 (0.09)	3.17 (0.19)	2.96 (0.10)	GM=SF<TF=SM
Sections	6.75 (0.18)	7.25 (0.18)	10.25 (0.38)	9.38 (0.54)	GM=SF<TF=SM
Sections Co-varied Out	1.65	1.99	3.10	2.92	GM=SF<TF=SM
BSTMP					
Area	4.78 (0.21)	5.00 (0.07)	6.63 (0.14)	6.13 (0.13)	GM=SF<TF=SM
Sections	8.00 (0.00)	8.75 (0.34)	11.25 (0.60)	10.38 (0.54)	GM=SF<TF=SM
Sections Co-varied Out	5.03	5.13	6.37	6.01	GM=SF<TF=SM
BSTMPL					
Area	2.73 (0.11)	2.85 (0.17)	3.80 (0.22)	3.57 (0.16)	GM=SF<TF=SM
Sections	8.00 (0.00)	8.75 (0.34)	11.25 (0.60)	10.38 (0.54)	GM=SF<TF=SM
Sections Co-varied Out	3.31	3.16	3.20	3.29	NS

^aData are averages (SEM) of the two hemispheres. All areas are in mm².

*P≤0.05, **P≤0.01, ***P≤0.001; 2-tailed T-tests. NS, non-significant.

N = 4 in each group

Table 3 continued on next page.

Table 3. Continued.

Features of Interest	Gonadect. Males (GM)	Sham Females (SF)	Sham Males (SM)	Testosterone Females (TF)	Group Differences
MEPD-SF					
Area	3.04 (0.13)	2.95 (0.14)	4.90 (0.13)	4.22 (0.13)	GM=SF<TF<SM ^{***}
Sections	7.38 (0.19)	7.88 (0.24)	9.75 (0.18)	9.38 (0.19)	GM=SF<TF=SM ^{***}
Sections	3.24	3.12	4.62	4.02	GM=SF<TF<SM ^{***}
Co-varied Out					
MEPD					
Area	2.49 (0.09)	2.59 (0.12)	3.86 (0.13)	3.37 (0.14)	GM=SF<TF<SM ^{***}
Sections	7.38 (0.19)	7.75 (0.25)	9.63 (0.19)	9.13 (0.24)	GM=SF<TF=SM ^{***}
Sections	2.97	2.90	3.35	3.08	NS
Co-varied Out					

^aData are averages (SEM) of the two hemispheres. All areas are in mm².

*P≤0.05, **P≤0.01, ***P≤0.001; 2-tailed T-tests.

NS, non-significant.

N = 4 in each group.

The findings above raise the question of the relation between the SPir fibers and the cytoarchitectonic boundaries of BSTMPM. In the present study, unlike in previous studies from this laboratory, all staining was done on previously mounted sections. Because of this the areas of BSTMPM-SP and BSTMPM can be directly compared. Interestingly, BSTMPM-SP is larger than BSTMPM in the testosterone-exposed groups, but not in the testosterone-deficient groups, where BSTMPM-SP is either smaller (GM) or similar in size (SF) to BSTMPM (Table 2). This is consistent with the presence of SPir fibers in the capsule of fibers surrounding BSTMPM in testosterone-exposed animals. In testosterone-deficient animals, the field of SPir fibers corresponds more closely to the boundaries of the BSTMPM cell group.

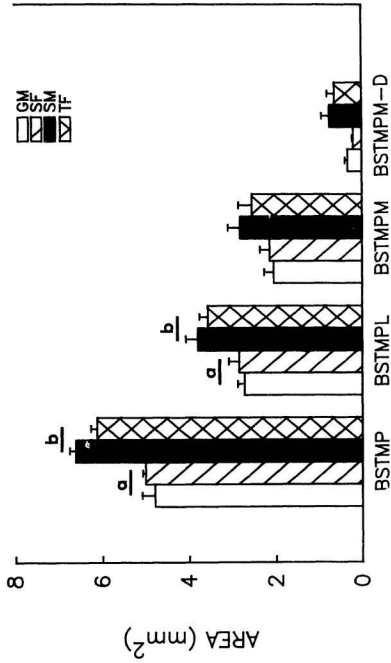
Significant differences in the size of the cell groups BSTMP and BSTMP-L were also found between the treatment groups (Table 2 and Figure 6). The areas of these features in the testosterone-exposed groups were similar in size but larger than in the testosterone-deficient groups which were also similar in size (group effect for BSTMP, $F(3,12)=23.44$, $P<.001$; mean contrasts all $t(12)\geq 8.121$, $P<.001$; and group effect for BSTMP-L, $F(3,12)=5.76$, $P<.01$; mean contrasts all $t(12)\geq 4.075$, $P<.01$). The BSTMPM and BSTMPM-D did not differ significantly between groups; however, they did show a tendency to be similar in size and larger in the testosterone-exposed groups than in the testosterone-deficient groups which were also similar in

size (Table 2, Figure 6).

There was a significant sex difference in the number of sections for all measured features of the BSTMP and this difference was reversed by sex-hormone manipulations soon after birth (i.e. SM=TF>SF=GM). Table 2 shows that in all cases there was a significantly greater number of sections on which the feature was identified for the testosterone-exposed groups compared to testosterone-deficient groups (for BSTMP, BSTMPL, and BSTMPM, group effect, $F(3,12)=5.98$, $P<.01$; mean contrasts all $t(12)\geq 4.015$, $P<.01$; for BSTMPM-SP, group effect, $F(3,12)=11.78$, $P<.001$; mean contrasts all $t(12)\geq 5.762$, $P<.001$; for BSTMPM-D, group effect, $F(3,12)=4.32$, $P<.05$; mean contrasts all $t(12)\geq 2.809$, $P<.05$).

For the two cell groups which had group differences in area, BSTMP and BSTMPL, the influence of the number of sections was factored out with an analysis of covariance. For BSTMP, significant group differences remain. Table 3 illustrates that when the number of sections was co-varied out, the areas of the BSTMP in testosterone-deficient groups were not significantly different, but a large difference was found between those groups and the testosterone-exposed groups (group effect, $F(1,11)=8.97$, $P<.01$; mean contrasts all $t(11)\geq 5.837$, $P<.001$). This suggests that the large difference in area between the testosterone-exposed groups and the testosterone-deficient groups was due to both a greater length and width of BSTMP. For BSTMPL, when the influence of the number of sections was

Figure 6. Areas of the BSTMP cell group and its components for each of the four treatment groups. Data are group means (and standard errors) of the average of the two hemispheres in each rat. The groups are gonadectomized males (GM, open bars, N=4), sham females (SF, hatched bars, N=4), sham males (SM, solid bars, N=4), and testosterone-exposed females (TF, cross-hatched bars, N=4). Differing letters above the bars indicate significant group differences at $p \leq .05$. Abbreviations: BSTMP, the total medial posterior part of the BST; BSTMPL, the lateral cell group of the BSTMP; BSTMPM, the medial cell group of the BSTMP; BSTMPM-D, the region of densely-packed cells in the medial part of the BSTMPM.



factored with an analysis of covariance (Table 3), no significant difference was found, suggesting that the difference in size was largely due to BSTMPL being longer, not necessarily wider, in the testosterone-exposed groups.

Medial amygdala.

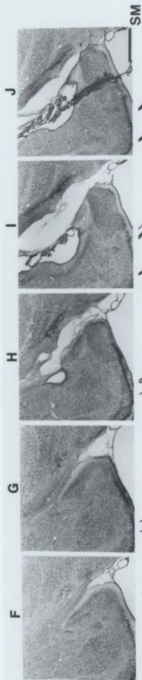
Figures 7 and 8 contain photographs of cresyl-violet stained sections of the medial amygdala for a typical SM and SF. Below the photographs are drawings which define the cell groups that were measured. No major sex differences in the cytoarchitecture of the medial amygdala are obvious, except for cell group MEPD, which looked larger in the SM group. Groups GM and TF are not represented in these photographs because GM looked very similar to SF and TF looked very similar to SM. Significant differences were found between the treatment groups in the size of MEPD (Table 4 and Figure 11; group effect, $F(3,12)=21.35$, $P<.001$; mean contrasts all $t(12)\geq 2.445$, $P<.05$). The areas of the testosterone-deficient groups were similar in size but much smaller than group TF ($t(12)\geq 4.799$, $P<.001$). The MEPD was significantly larger in SM than in TF ($t(12)\geq 2.445$, $P<.05$). The number of sections in which MEPD appeared was not significantly different between the testosterone-deficient groups and between the testosterone-exposed groups, but the number of sections was significantly larger for the testosterone-exposed groups compared to the testosterone-deficient groups (i.e. $SM=TF>SF=GM$, group effect,

$F(3,12)=14.61$, $P<.001$; mean contrasts all $t(12)\geq 6.431$, $P<.001$). When the influence of the number of sections was factored with an analysis of covariance (Table 3) no significant difference was found, suggesting that the difference in size was largely due to MEPD being longer, not necessarily wider, in the testosterone-exposed groups.

Similar group effects were seen in measures of the field of SPir fibers which appears to innervate MEPD, designated MEPD-SP. Typical examples from each treatment group illustrating MEPD-SP are shown in Figures 9 and 10. Observation of the photographs suggests that not only is the area of SPir fibers greater in the testosterone-exposed groups (best seen in Figure 9, columns -5 through -1), but the number of sections in which the dense field of fibers is present is larger for these groups as well. Table 4 and Figure 5 verify this observation by showing the group differences in the areas of MEPD-SP (group effect, $F(3,12)=20.70$, $P<.001$; mean contrasts all $t(12)\geq 2.923$, $P<.05$). The major difference was that MEPD-SP was significantly larger in the testosterone-exposed groups than in the testosterone-deficient groups ($t(12)\geq 5.964$, $P<.001$). Also, a significant difference was found between the testosterone-exposed groups; SM was found to be just significantly larger than TF ($t(12)\geq 2.923$, $P<.05$). The number of sections in which MEPD-SP appeared was not significantly different between SM and TF and between SF and GM, but was significantly greater in the testosterone-exposed than in the

Figure 7. Cresyl violet-stained sections (rows 1 and 3) and corresponding drawings (rows 2 and 4) of the medial amygdala of a male, SM, (top 2 rows) and a female, SF, (bottom 2 rows). The caudal portions of the medial amygdala are continued in figure 8. Photographs and drawings are arranged in rostral to caudal order (i.e., A-E). The drawings of SM are typical of a testosterone-exposed rat (SM and TF) and the drawings of SF are typical of a testosterone-deficient rat (SF and GM). Shaded areas in drawings indicate the areas measured. Column A for SM and column B for SF show the most caudal portion of the BAOT which is the section just before the most rostral portions of ME and MEPV are visible. After this most rostral point of ME, every other section is represented continuing to column I in Fig. 8. Abbreviations: BAOT, bed nucleus accessory olfactory tract; CEL, central amygdaloid nucleus, lateral; CEM, central amygdaloid nucleus, medial division; I, intercalated nuclei of the amygdala; ME, medial amygdaloid nucleus; MEAD, medial amygdaloid nucleus, anterodorsal; MEAV, medial amygdaloid nucleus, anteroventral; MEPV, medial amygdaloid nucleus, posteroventral; MEPD, medial amygdaloid nucleus, posterodorsal; OPT, optic tract; ST, stria terminalis.

Figure 8. Continuation from Figure 7 of the cresyl violet-stained sections (rows 1 and 3) and corresponding drawings (rows 2 and 4) of the medial amygdala of a male, SM, (top 2 rows) and a female, SF, (bottom 2 rows). Photographs and drawings are arranged in rostral to caudal order (i.e., F-J). The drawings of SM are typical of a testosterone-exposed rat (SM and TF) and the drawings of SF are typical of a testosterone-deficient rat (SF and GM). Shaded areas in drawings indicate the areas measured. Every other section continues to be represented from Figure 7 for the rest of the medial amygdala (columns F-I). Column J shows the section just caudal to the most caudal pole of MEPD. Abbreviations: AHIAL, amygdalohippocampal area, anterior; PMCO, posteromedial cortical amygdaloid nucleus; see Figure 7 for other abbreviations.



SM



SF

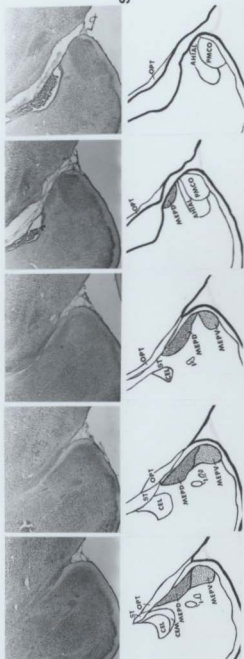


Table 4. Features of the Medial Amygdala.^a

Features of Interest	Gonadect. Males (GM)	Sham Females (SF)	Sham Males (SM)	Testosterone Females (TF)	Group Differences
ME					
Area	2.09 (0.15)	1.83 (0.11)	1.87 (0.19)	1.72 (0.09)	NS
Sections	6.13 (0.54)	5.50 (0.29)	5.38 (0.47)	4.75 (0.57)	NS
MEPV					
Area	2.65 (0.10)	2.87 (0.11)	2.85 (0.11)	2.78 (0.11)	NS
Sections	11.13 (0.32)	11.00 (0.34)	11.00 (0.18)	10.38 (0.54)	NS
MEPD					
Area	2.49 (0.09)	2.59 (0.12)	3.86 (0.13)	3.37 (0.14)	GM=SF<TF<SM ^{***}
Sections	7.38 (0.19)	7.75 (0.25)	9.63 (0.19)	9.13 (0.24)	GM=SF<TF=SM ^{***}
MEPD-SF					
Area	3.04 (0.13)	2.95 (0.14)	4.90 (0.13)	4.22 (0.13)	GM=SF<TF<SM ^{***}
Sections	7.38 (0.19)	7.88 (0.24)	9.75 (0.18)	9.38 (0.19)	GM=SF<TF=SM ^{***}

^aData are averages (SEM) of the two hemispheres. All areas are in mm².

*P≤0.05, **P≤0.01, ***P≤0.001; 2-tailed T-tests.

NS, non-significant.

N = 4 in each group.

testosterone-deficient groups (i.e., $SM=TF>SF=GM$, group effect, $F(3,12)=22.47$, $P<.001$; mean contrasts all $t(12)\geq 8.004$, $P<.001$; Table 3). Thus, MEPD and MEPD-SP show very similar group differences in area and number of sections. However these two features differed in one respect. When the influence of the number of sections was factored out with an analysis of covariance, significant group differences remained for MEPD-SP (group effect, $F(1,11)=4.76$, $P<.05$; mean contrasts all $t(11)\geq 2.558$, $P<.05$). Table 3 illustrates that when the number of sections was co-varied out, the areas of MEPD-SP in the testosterone-deficient groups were not significantly different, but a large difference was found between those groups and the testosterone-exposed groups ($t(11)\geq 20.829$, $P<.001$). In addition, MEPD-SP was significantly larger in group SM than in group TF ($t(11)\geq 2.558$, $P<.05$). This suggests that the large difference in the area of SPir fibers between the testosterone-exposed groups and the testosterone-deficient groups was due to both a greater length and width of the area. The other cell groups measured in medial amygdala, MEVD and ME, showed no significant group differences in area or number of sections (Table 4, Figure 11).

As observed in the adult (Malsbury & McKay, personal communication) there is clearly a close correspondence between the cytoarchitectonic boundaries of MEPD and the SPir field of fibers, MEPD-SP. However SPir fibers also extend beyond MEPD into the cell-sparse neuropil surrounding it and into a

relatively small area of the dorsal and medial borders of the caudal MEPV. Consistent with this, the areas of MEPD-SP are larger than the areas of MEPD in all 4 treatment groups (Table 4).

Hemispheric asymmetries.

As shown in Table 5, the left hemispheric area was significantly greater than the right for the cell group MEPV ($F(1,12)=64.66$, $P<.001$). The large hemispheric asymmetry for MEPV is illustrated for all four treatment groups in Figure 12. This difference was quite consistent, as the left MEPV was larger than the right in 15 of the 16 animals. Smaller, but significant asymmetries were also seen for three other features; the cell groups ME and BSTMP, and BSTMPM-SP. In each case, the feature was larger in the left than in the right hemisphere: ME, $F(1,12)=4.91$, $P<.05$; BSTMP, $F(1,12)=6.52$, $P<.05$; and BSTMPM-SP, $F(1,12)=5.24$, $P<.05$. It was also true for each of the three features, ME, BSTMP, and BSTMPM-SP, that the four treatment groups varied in the degree of asymmetry. Even though the overall hemispheric effect showed that each of these features was significantly larger in the left than in the right hemisphere, in each case the feature was larger, but not significantly so, in the right hemisphere for one of the testosterone-exposed groups (either TF or SM). Thus, as with MEPV, the left over right asymmetry was more pronounced in the testosterone-deficient groups.

Table 5. Hemispheric asymmetries in the BST and Medial Amygdala^a.

Features of Interest	Left Hemisphere	Right Hemisphere	Hemispheric Differences
BSTMPM-SP			
Area	2.45 (0.09)	2.38 (0.08)	L>R*
Sections	8.44 (0.24)	8.38 (0.26)	NS
BSTMP			
Area	5.77 (0.12)	5.50 (0.09)	L>R*
Sections	9.63 (0.31)	9.56 (0.30)	NS
ME			
Area	1.95 (0.11)	1.80 (0.09)	L>R*
Sections	5.88 (0.34)	5.50 (0.36)	NS
MEPD			
Area	3.17 (0.10)	2.98 (0.07)	NS
Sections	8.38 (0.15)	8.56 (0.16)	NS
MEPV			
Area	3.06 (0.09)	2.52 (0.06)	L>R***
Sections	10.75 (0.24)	11.00 (0.28)	NS

^aAll areas are in mm².

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; 2-tailed T-tests.

L, left hemisphere; R, right hemisphere; NS, non-significant.

Figure 9. SPir fibers within the medial amygdala are shown in typical examples from the four treatment groups. Rows 1 and 3 represent the testosterone-deficient groups, gonadectomized males (GM) and sham females (SF). Rows 2 and 4 represent the testosterone-exposed groups, sham males (SM) and testosterone females (TF). Photographs of consecutive coronal sections are arranged in rostral to caudal order (i.e., -6 through -1) and continued in Figure 10 (i.e., 0 through 5). The sections were aligned so that the SPir caudal pole of the CEL was at column 0 in Figure 10. The most rostral section of each group is lightly stained for SP in the region of MEPD but it is the following section which is more densely stained for SP that is considered the most rostral section of the cell group MEPD and the section on which measures of MEPD-SP begin. In the testosterone-exposed groups there are not only more sections but larger areas of SPir staining (especially in columns -3 through -1) than in the testosterone-deficient groups.

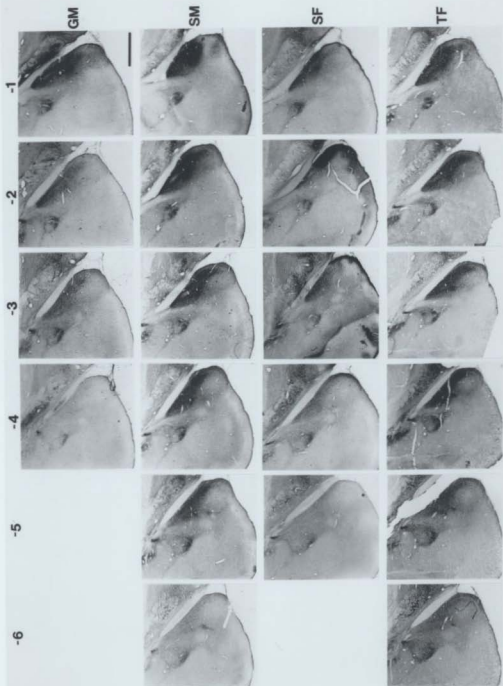


Figure 10. Continuation from Figure 9 of the SPir staining of the medial amygdala shown in typical examples from the four treatment groups. Rows 1 and 3 represent the testosterone-deficient groups, gonadectomized males (GM) and sham females (SF). Rows 2 and 4 represent the testosterone-exposed groups, sham males (SM) and testosterone females (TF). Photographs of consecutive SP-stained coronal sections are arranged in rostral to caudal order (i.e., 0 through 5) continued from Figure 9. The most caudal section for each rat does not contain dense SPir staining. The preceding section contains the caudal pole of the dense field of SPir fibers innervating MEPD (i.e. MEPD-SP). At this level the SPir fibers are in the neuropil surrounding MEPD, as the MEPD cell group ends just rostrally to this point.

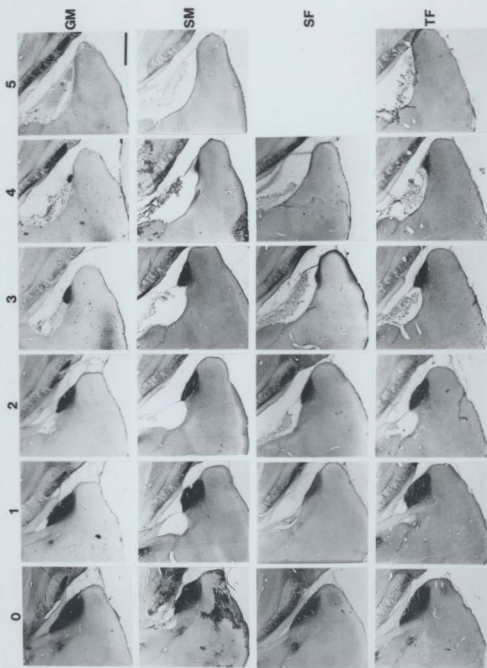


Figure 11. Areas of cell groups of the medial amygdala for each group. Data are group means (and standard errors) of the average of the two hemispheres in each rat. The groups are gonadectomized males (GM, open bars, N=4), sham females (SF, hatched bars, N=4), sham males (SM, solid bars, N=4), and testosterone-exposed females (TF, cross-hatched bars, N=4). Abbreviations: ME, the cell group within the medial nucleus of the amygdala which is rostrally adjacent to MEPD; MEPD, posterodorsal medial amygdala; MEPV, posteroventral medial amygdala.

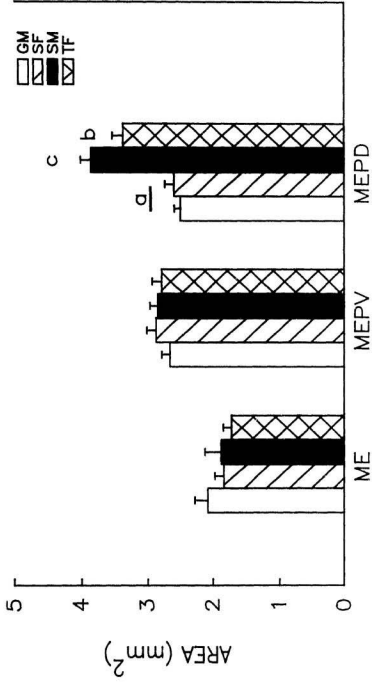
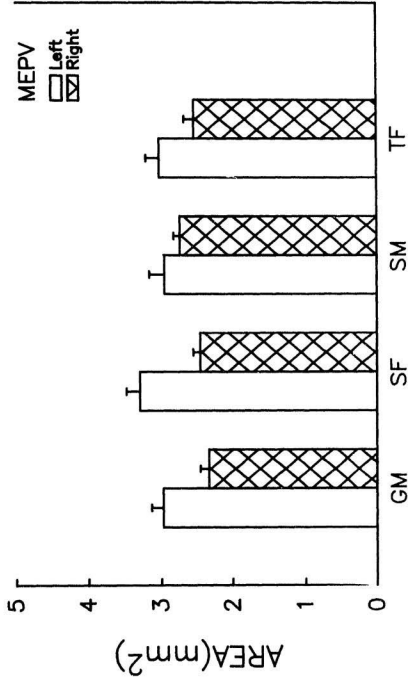


Figure 12. Mean (and standard error) of the area of the cell group MEPV in each hemisphere for each group. The left hemispheric area is represented by open bars and the right hemispheric area is represented by cross-hatched bars. Abbreviations: GM, gonadectomized males (N=4); SF, sham females (N=4); SM, sham males (N=4); TF, testosterone-exposed females (N=4).



DISCUSSION

Sex differences in the SPir innervation of the posterior medial divisions of the BST and medial amygdala.

Previous studies have found sex differences in the cytoarchitecture of two forebrain nuclei, the BST and the medial nucleus of the amygdala, in adult rats (discussed below). Two cell groups within these nuclei, the BSTMPM and MEPD, are innervated by dense fields of SPir fibers. The areas of this innervation are highly dimorphic in adult rats, as in both nuclei the area occupied by the dense field of SPir fibers is more than twice as large in males as in females (Malsbury & McKay, 1989). Consistent with these results, two recent studies using radioimmunoassay methods have shown the concentration of SPir material to be higher in males than in females in the posterior portion of the medial amygdala which includes the MEPD (Frankfurt et al., 1985; Micevych et al., 1988). Cytoarchitectonic and neurochemical sex differences in adults raise the question of whether they are determined by the organizational or activational effects of gonadal steroid hormones.

It has been generally accepted that the gross morphological features of the adult brain are not sensitive to the activational effects of gonadal steroids. For example, the size of sexually dimorphic cell groups within the preoptic area seems relatively unaffected by gonadectomy in adult rodents

(Gorski et al., 1978; Hines et al., 1985; cf Bloch & Gorski, 1988; Commins & Yahr, 1984). However, it is clear that certain neurochemical measures are greatly affected by sex hormones in adult rats. For example, castration produces a dramatic decrease of vasopressin fiber staining in a number of limbic regions (De Vries et al., 1985), and this laboratory and others have demonstrated that castration of adult males can reduce SPir staining in MEPD in rats (Dees & Kozlowski, 1984; Malsbury et al., 1987) and hamsters (Swann & Newman, 1987). This laboratory has found that by 8 wks post-castration the size of the field of SPir fibers in MEPD was reduced by 42% in castrated males versus intact males. However, this was not a complete reversal of the sex difference, as the reduced area of innervation was still larger than in normal adult females. It was also found that testosterone capsules inserted at the time of castration maintained the size of the field of SPir fibers (Malsbury et al., 1987).

The present study investigated the possible organizational actions of testosterone on the development of the SPir innervation and on the size of certain cell groups within BSTMP and the medial amygdala. The results show for the first time, in 27-day-old prepubescent rats (i.e. just prior to the period when the activational effects of hormones are present) that; a) sex differences in the areas of the dense SPir innervation of BSTMP and MEPD are already present, but are not as great as in the adult, and b) that these dimorphisms are largely reversible

by postnatal hormone manipulations. With regard to point a), the areas of BSTMPM-SP and MEPD-SP are more than twice as large in adult males as in females. In the prepubertal animals of the present study however, the size of BSTMPM-SP is 63% larger, and MEPD-SP is 66% larger in males than in females. These sex differences are highly significant, but clearly not as great as in the adult. With regard to point b), castration of one-day-old male rats (GM) caused a complete sex reversal of the BSTMPM-SP and the MEPD-SP. A complete sex reversal was also found in female rats injected with a total of 1 mg TP within 5 days of birth (TF) in the BSTMPM-SP and a partial sex reversal was found in MEPD-SP (i.e. MEPD-SP was significantly larger in group TF than in the testosterone-deficient groups, SF and GM, but not as large as in SM). As discussed above, the SPir innervation of BSTMPM and MEPD was previously shown to be influenced by the activational effect of hormones in adult male rats and is now shown to also be controlled by the organizational effect of testosterone in the early postnatal period. Thus sex differences in the SPir innervation of these areas in adults depend on both the organizational and activational effects of testosterone.

Not only the area, but also the pattern of SPir fibers in the region of the BSTMPM was found to be sexually dimorphic. At a particular level, a distinct capsule/core pattern of staining was seen in the testosterone-exposed groups, TF and SM. In the testosterone-deficient groups, SF and GM, the

relatively less densely-stained core was not observable or barely present. This sex difference in the pattern of SPir fibers in the BSTMPM region was observed previously in adult rats (Malsbury & McKay, 1987). The present results show that this dimorphic pattern is also present in prepubescent rats and reversible by hormone manipulations early after birth.

Sex differences in cytoarchitecture.

A sex difference was present in the area of BSTMP as a whole. This includes medial (BSTMPM) and lateral divisions (BSTMPL). There were significant group differences in the area of BSTMPL, but not BSTMPM, although similar trends were present for BSTMPM. For BSTMP as a whole and BSTMPL, total areas, and the number of sections in which these two features appeared, were larger in males than in females. Male gonadectomy on day 1 and postnatal female androgenization reversed these sex differences thus demonstrating that they are controlled by sex steroids acting early after birth. A sex difference and similar postnatal treatment effects have been reported for the adult BSTMP by Del Abril et al. (1987).

In following the nomenclature of the atlas of Paxinos and Watson (1986), we have used BSTMPM to denote the cell group within BSTMP which receives the dense SPir innervation. It has also been called the small-celled division of the BSTMP (De Olmos et al., 1985; Del Abril et al., 1987) or the encapsulated part of the BST (Malsbury & McKay, 1987; McDonald, 1983). As

mentioned above, at day 27 the area of BSTMPM was larger, but not significantly so, in the testosterone-exposed groups than in the testosterone-deficient groups. For example, it was 32% larger in group SM than in group SF. The number of sections in which the BSTMPM appeared was significantly greater in group SM than in group SF and this sex difference was completely reversed by neonatal castration (GM) or TP treatment (TF). The area of BSTMPM is sexually dimorphic in adult rats (Del Abril et al., 1987; Malsbury & McKay, 1987) and guinea pigs (Hines et al., 1985). In addition Del Abril et al. studied the organizational effects of testosterone on several cell groups of the rat BST using neonatal manipulations very similar to those used here. They killed their animals at 90 days of age and found that the areas of both BSTMP and BSTMPM were reduced by castration of newborn males and increased by testosterone injections into newborn females (Del Abril et al., 1987).

It is possible that increased androgen secretion at puberty contributes to the sex differences seen in the adult BST and medial amygdala. This might explain the lack of significant group difference in the area of BSTMPM in prepubescent rats in the present study. Perhaps the BSTMPM is not fully developed at day 27. However, it should be noted that the magnitude of the sex difference in the area of BSTMPM reported here is nearly identical to the magnitude of the sex difference in the same feature in adult brains as reported from this laboratory (Malsbury & McKay, 1987).

The idea that cytoarchitectonic differentiation of sexually dimorphic brain regions may continue during puberty is suggested by the findings of Roos et al. (1988) who studied the development of sex differences in the accessory olfactory bulb in rats. They found that the size of the AOB continues to increase in males, but not in females, between days 40-60. This late period of growth in males was prevented by castration on day 20 or 30. This result suggests that an increase might also be occurring in the size of closely-related structures, the BSTMP and the MEPD, in response to an increase in testosterone secretion occurring in males at this time. Whether pubertal growth actually occurs in features other than the AOB remains to be seen. Interestingly, a single testosterone injection (1.25 mg) into females on day 1 did not completely masculinize the size of the adult BSTMPM (their small-celled component of medial BST) in the study of Del Abril et al. (1987). Obviously, these females did not experience the second, pubertal, increase in androgen secretion seen in males. Thus this incomplete masculinization of BSTMPM in neonatally androgenized females is consistent with the concept of continuing differentiation of cytoarchitectonic features at puberty. However, no support for such continuing differentiation is present in the results of Mizukami et al. (1983) who studied the sexual differentiation of the volume of the entire medial nucleus of the amygdala of rats. They killed their animals at days 1, 5, 11, 21, 31 and 91. The medial

nucleus was larger in males at day 21, but not at earlier time points. Of particular relevance here is the fact that although the magnitude of the sex difference may not have been quite as large at day 21 as at day 31, it did not increase between days 31 and 91.

As with BSTMPM, the region of densely-packed cells in the BSTMPM (BSTMPM-D) tended to be larger in testosterone-exposed groups, but these differences did not reach significance. The number of sections in which the BSTMPM-D appeared was significantly greater in testosterone-exposed groups and these differences could be reversed by either gonadectomy (GM) or testosterone treatment (TF).

Two of the three cell groups measured in the medial amygdala, ME and MEPV, were not found to be sexually dimorphic. However, the area of MEPD, the cell group innervated by the dense field of SPir fibers, was sexually dimorphic and controlled by sex hormones perinatally. MEPD was much larger in group TF than in the testosterone-deficient groups, SF and GM, and larger in SM than in TF. This is consistent with an abstract by Hines et al. (1986) who found MEPD to be much larger in adult males than in females.

Hemispheric Asymmetries.

In the medial amygdala and the BST, hemispheric asymmetries were seen in MEPV, ME, BSTMP, and BSTMPM-SP. These features were all larger in the left than in the right

hemisphere. The hemispheric difference was marginally significant in ME, BSTMP, and BSTMPM-SP, but a relatively large and consistent difference was found in MEPV. Several areas of the brain, including the cortex and hippocampus, are known to be larger in one hemisphere or the other (reviewed by Carlson & Glick, 1989). However, this is the first report of hemispheric asymmetry in the medial amygdala and BST. It is also significant that the testosterone-deficient groups were more asymmetrical than the testosterone-exposed groups. This is clearly seen in the data for MEPV (Fig. 12). In fact, with respect to the asymmetries of the ME, the BSTMP, and the BSTMPM-SP, one of the two testosterone-exposed groups had an asymmetry, although not significant, in the opposite direction (right > left, see Table 5).

It would be interesting to find out whether the asymmetries in the medial amygdala and BST seen here at day 27 are present in the adult brain. Van Eden et al. (1984) have found that hemispheric asymmetries in the prefrontal cortex change during development, and that an asymmetry during development does not necessarily mean an asymmetry will remain in adulthood. In fact, the relatively small asymmetry in BSTMPM-SP seen here at day 27 is not present in adult males or females (Malsbury, personal communication). It remains to be seen whether the greater asymmetry in MEPV persists into adulthood, especially in females, where it is more pronounced.

Turning, or rotational behavior, has been used as an index of functional brain asymmetry (Carlson & Glick, 1989). Rotational behavior is sexually dimorphic within some populations of adult Sprague-Dawley rats in the sense that females show a greater degree of asymmetry. That is, females show a stronger tendency to circle in one direction or the other than do males. A related behavioral asymmetry has been observed in newborn rats. Ross et al. (1981) observed that on the first 3 postnatal days nearly all individuals directed their tails in one direction or the other (tail bias). Furthermore, this neonatal tail bias predicted the direction of rotational asymmetry in the adult. That is, when Ross et al. compared the direction in which a newborn rat turned its tail to the direction of amphetamine-stimulated rotation in a rotometer in the same animal when it was 85 days old, the direction was the same in 15/16 males and 13/17 females. In the neonatal animals, as a group, females had a significant right-sided bias in tail direction. In males, a left-sided tail bias was more frequent, but this asymmetry was not significant. Thus, as in the adult, the females showed a greater degree of behavioral asymmetry. The fact that this behavioral asymmetry is sexually dimorphic in newborn animals and persists in adults suggests that it results from the organizational effect of testosterone. This idea receives further support from an analysis conducted by Ross et al. which found a significant inverse correlation between the percentage

of females in each litter with a right-sided tail bias and the number of males in the litter. This is consistent with the idea that testosterone produced by males in utero can masculinize certain features of the brain and behavior in their female littermates (Meisel & Ward, 1981). Ross et al. also looked for asymmetries in brain metabolic activity in newborn rats using the 2-deoxy-D-glucose method. Left-right asymmetries were seen in cortex, hippocampus, diencephalon, and medulla-pons, but only in females. As with the present results female brains were more asymmetric than male brains. However, this is not always the case, as in the adult cortex, males show a greater asymmetry than females (Diamond, 1987; Van Eden et al., 1984).

Sex differences in the limbic brain: Which hormone receptor mediates differentiation?

A major conclusion of this study is that the presence or absence of testosterone during the early postnatal period determines sexual differentiation of the SPir innervation of the BSTMPM and MEPD. Testosterone injections into newborn females masculinize, while the absence of testicular androgens beginning shortly after birth feminizes. What mechanism(s) mediate this differentiation? During the first week of life the amount of circulating testosterone is significantly higher in males than in females (Lieberburg et al. 1979; Resko et al., 1968; Weisz & Ward, 1980). At that time androgen receptor

concentration in the cytosol appears equal between the sexes (Lieberburg et al., 1978; Meaney et al., 1985). However, there was a sex difference, favoring the male, in nuclear-bound androgen receptors (i.e. receptor-occupancy) during the first week of life, and the region with the largest sex difference was the amygdala (Meaney et al., 1985). The amygdala and the BST in the developing brain contain intracellular androgen receptors which bind with high affinity to testosterone and non-aromatizable androgens (Fox et al., 1978; Lieberburg et al., 1978; 1980; Meaney et al., 1985; Pomerantz et al., 1985; Sheridan, 1981). These receptors are considered a plausible mechanism for the organizational actions of androgens on the CNS.

Alternatively, it is possible that masculinization of the features measured here is mediated by estrogen receptors. Testosterone can be converted to 17- β estradiol within certain neurons, and this conversion, called aromatization, is involved in the sexual differentiation of the rat brain (McEwen et al., 1977). Aromatization has been demonstrated within limbic structures, including the medial amygdala, of newborn rats (MacLusky et al., 1985). Further studies are necessary to determine whether masculinization of the features measured here depends on activation of androgen or estrogen receptors. Perhaps both types of receptors are involved. An answer to this question would be relevant to understanding the functional significance of the sex differences reported here, as

aromatization is not necessary for all aspects of sexual differentiation (see section below on play-fighting).

SPir fibers in BSTMPM and MEPD: Anatomical and functional considerations.

De Olmos et al. (1985) have advanced the view that the BST can be considered an extension of the amygdala. Of particular relevance here is the close relation between the medial nucleus of the amygdala and the medial division of the BST. These areas are reciprocally connected, and the amygdala is the major source of afferents to the BST. Most relevant is the demonstration that the posterior medial BST receives input from the medial nucleus of the amygdala (Krettek & Price, 1978; Weller & Smith, 1982). Information on the connectivity of SPir neurons within these structures, although incomplete, suggests that the field of fibers I have called BSTMPM-SP arises from the MEPD and reaches the BSTMPM via the stria terminalis.

SPir perikarya have been reported in the MEPD (Ljungdahl et al., 1978; Roberts et al., 1982) and Warden and Young (1988) have now demonstrated the presence of many SP-synthesizing neurons within both the medial BST and MEPD using in situ hybridization. SPir fibers are present within the stria terminalis (Malsbury & McKay, 1989; Roberts et al., 1982), and when the stria terminalis is cut, most of the SPir fiber staining within the medial BST disappears (Sakanaka et al., 1981; cf Emson et al., 1978). Because of these results,

Malsbury and McKay (1989) have suggested that the sexually dimorphic field of SPir fibers within the posterior medial BST originates from MEPD. What then is the origin of the SPir innervation of MEPD? The results of Emson et al. (1978) suggest that it arises locally, as knife cuts which isolated the medial amygdala did not reduce the SPir staining within. It is possible then, that sexually dimorphic fields of SPir fibers in both BSTMPM and MEPD arise from axon collaterals of the same perikarya within MEPD.

The medial nucleus of the amygdala and the medial BST are components of a highly interconnected group of sexually dimorphic forebrain structures which also includes the accessory olfactory bulb and parts of the medial preoptic area and medial hypothalamus. Anatomically these structures may be considered part of the accessory olfactory system. The vomeronasal organ, a tubular structure within the nasal cavity, contains olfactory receptors which project to the accessory olfactory bulb. The vomeronasal organ and the accessory olfactory bulb are larger in male than in female rats (Roos et al., 1988; Segovia et al., 1984; Valencia et al., 1986). The accessory olfactory bulb is a major source of afferents to the posterior medial amygdala, including the MEPD (Scalia & Winans, 1976). Thus some MEPD neurons may be only two synapses removed from olfactory receptors. The medial preoptic area has reciprocal connections with both the medial amygdala via the stria terminalis, and via short axon connections with the

medial BST. Thus projections from MEPD to BSTMPM and to the preoptic area may carry olfactory information. The MEPD, the BSTMPM and the preoptic area contain numerous gonadal steroid target neurons, i.e. neurons which contain either androgen or estrogen receptors. This would provide a way for the hormonal state of the animal to influence processing of olfactory information. The identities of the neurotransmitters/peptides that carry olfactory information within this circuitry, however, have not been established. The presence of large numbers of SPir perikarya and fibers within the MEPD, the medial BST and the medial preoptic area, and within fibers of the stria terminalis, along with the sensitivity of SPir fibers in the MEPD and BST to castration, suggests that SPir neurons may integrate olfactory and hormonal information.

Studies too numerous to review here have demonstrated the influences of olfactory stimuli and gonadal hormones on various aspects of reproductive physiology and behavior. Many aspects of reproductive physiology and behavior are sexually dimorphic, so the neural substrates of these responses must, in some way, be dimorphic as well. The medial amygdala and medial BST have been demonstrated to play a role in reproductive responses. For example, both areas are involved in controlling gonadotrophic hormone release and sexual behavior in both male and female rats (Kostarczyk, 1986). The functional significance of the SPir innervation of MEPD and the medial BST is not known. However, SPir neurons which innervate other

brain regions have been implicated in reproductive physiology and behavior. For example, evidence is accumulating that hypothalamic SP neurons are involved in controlling gonadotrophic hormone release (Ohtsuka et al., 1987; Vijayan & McCann, 1979). Recently this laboratory has demonstrated a role for SP in both male and female sexual behavior. In the female, SP injections into the midbrain central gray can facilitate sexual receptivity (Dornan et al., 1987). In the male, SP injections into the medial preoptic area can facilitate certain aspects of male copulatory behavior (Dornan & Malsbury, 1989). The fact that lesions of the olfactory bulb, including damage to the accessory olfactory bulb, as well as lesions of the medial amygdala and BST can all disrupt male rat copulatory behavior (Larsson, 1979), makes it tempting to speculate that SPir neurons which project from MEPD to BSTMPM form part of the hormone-sensitive circuitry controlling male copulatory behavior.

Sex differences in play behavior before adulthood.

The functional significance of the prepubertal sex differences reported here are not known. At day 27 the SPir innervation of the BSTMPM and MEPD looks very much as it does in the adult. The presence of dense fields of darkly-staining fibers makes it tempting to speculate that we are looking at releasable stores of SP. If SP is releasable within these presumed terminal fields at this time, what is its function?

In the adult, SPir neurons may be involved in controlling sexual behavior (discussed above). However, in both male and female rats sexual behavior is not seen until well after day 27. For example, in one report mounting behavior was first seen at day 39, while intromissions and ejaculation did not appear until day 48 in male Long Evans rats (Sodersten et al., 1977). In contrast, play, a sexually dimorphic behavior, occurs before and during puberty. The frequency of social play, or play-fighting, is higher in male than in female rats and monkeys. Recently Meaney (1988) has reviewed what is known of the hormonal and neural basis of the sexual differentiation of social play. The following points are taken from his review.

The organizational action of androgen on play-fighting has been well-documented. The sex difference can be reversed by exposing females to androgens prenatally in the monkey (Goy, 1970) and within the first few days after birth in the rat (Olioff & Stewart, 1978; Meaney & Stewart, 1981). Castration of males at day 1 or day 6, but not later, decreases the frequency of play-fighting at puberty (Meaney & Stewart, 1981a; Beatty et al., 1981). Males with an androgen receptor deficiency (Tfm), play-fight less frequently than normal males (Meaney et al., 1983). Also, neonatal anti-androgens prevent the masculinization of play-fighting behavior in rats (Meaney et al., 1983).

The amygdala has been implicated as a neuroanatomical region involved in the sexual differentiation of play behavior. Male play-fighting decreased to the level of female play-fighting when lesions in the amygdala were made around Day 22. Female pups were not affected by the same lesions (Meaney et al., 1981). Meaney suggested that because androgen receptors are abundant in the amygdala during the early postnatal period, androgens could act directly on the amygdala to masculinize play-fighting. In a test of this idea, testosterone-filled cannulae were implanted bilaterally in the amygdala of females on day 1 and removed on day 6. Animals were tested between days 26 and 40. The frequency of play-fighting in the females with implants was no different from that of control males, and both of these groups showed a higher frequency than control females. Thus direct application of testosterone to the amygdala completely masculinized play-fighting, suggesting that sexual differentiation of the amygdala underlies the sex difference in play-fighting seen at puberty.

In both rats and monkeys, either testosterone or 5- α dihydrotestosterone (DHT) can masculinize the frequency of play-fighting. This is important because DHT is a non-aromatizable androgen. In the rat, neonatal estrogen injections are less effective than DHT injections. Thus in both rat and monkey, the masculinization of play-fighting appears to be mediated via androgen receptors activated by either T or its metabolite, DHT, while estrogen receptors may

not be involved. Thus, in contrast to sexual behavior (McEwen et al., 1977), aromatization does not seem to be critically important for the masculinization of play-fighting in the rat. In the light of these previous results concerning the neural substrates of sex differences in play-fighting, it would be interesting to know whether the SPir innervation of BSTMPM and MEPD can be masculinized by DHT. If DHT is effective, further, more direct tests of the importance of these SPir neurons could be conducted. For example, SP antibodies could be injected directly into the BSTMP or medial amygdala to block the action of SP immediately before play-fighting tests in juvenile rats.

Summary.

Recent studies have demonstrated that the SPir innervation of BSTMPM and MEPD is sexually dimorphic in adult rats, and that testosterone has an activational effect on these neurons. The present results reveal for the first time that testosterone also has a permanent, organizing action on these SPir neurons during the first few postnatal days, and that sex differences in the SPir innervation of these regions are present before puberty. In addition, prepubertal sex differences and the organizing action of testosterone have been demonstrated for certain cytoarchitectonic features of the BSTMP and the medial amygdala.

The functional significance of the SPir innervation of these regions is not known. However there is a great deal of

evidence implicating the medial amygdala and medial BST in the control of sexually dimorphic brain functions including various aspects of reproductive physiology and behavior in adult rats. It is tempting to speculate that the SPir fibers within these regions are involved in these functions. The presence of a relatively mature-looking SPir innervation of BSTMPM and MEPD at day 27 suggests that these neurons may play some functional role at this age as well. It is suggested here that they may be involved in controlling the frequency of play-fighting before and during puberty and perhaps in other sexually dimorphic responses, such as sexual behavior, after puberty.

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APPENDIX

Solutions for Immunocytochemistry

Vaughn Fixative (4% buffered paraformaldehyde).

- a. Add 40 g paraformaldehyde to 400 ml of 60 degree C distilled H_2O while stirring on a heated plate. Clear this milky suspension by the dropwise addition of 1 N sodium hydroxide.
- b. Dissolve 16.88 g monobasic sodium phosphate in 300 ml of distilled H_2O .
- c. Dissolve 3.86 g sodium hydroxide pellets in 200 ml of distilled H_2O .
- d. Combine solutions b and c, then add to solution a. Mix well and bring to 1 L with distilled H_2O . Check pH and adjust to 7.2 with the addition of 1 N HCl or 1 N NaOH.
- e. Filter through Whatman #2 filter paper using vacuum system. Cool in refrigerator before use. Can be stored for several days.

1% Sodium Nitrite.

1 g sodium nitrite.

100 ml distilled H_2O .

Store at room temperature.

Phosphate Buffered Saline (PBS).

107.2 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$.

64 g NaCl.

8 L distilled H_2O .

Mix. Adjust pH to 7.4 with 2 N HCl. Store in refrigerator.

Antibody Diluent.

45 ml PBS.

5 ml normal goat serum.

0.2 ml Triton X-100.

Mix. Store in refrigerator.

Diaminobenzidine (DAB) Solution.

Working under the hood, 1 g of 3,3'-diaminobenzidine tetrahydrochloride (DAB) is suspended in 200 ml PBS. While it is still mixing, 10 ml aliquots are transferred into screw-top centrifuge tubes and stored in the freezer. Each aliquot is mixed with 90 ml of PBS just prior to use.

1% Nickel Ammonium Sulfate.

1 g $\text{NiSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$

100 ml distilled H_2O .

Mix. Store in refrigerator.

1% Cobalt Chloride.

1 g cobalt chloride.

100 ml distilled H_2O .

Mix. Store in refrigerator.

0.3% H_2O_2 .

1 ml 30% H_2O_2 .

100 ml PBS.

Mix. Store in refrigerator.

DAB-Ni-Co.

To 15 ml of DAB add 300 μ l of 1% nickel ammonium sulfate dropwise, then add 375 μ l of 1% $CoCl$ dropwise. Filter.

DAB-Ni-Co- H_2O_2 .

Repeat DAB-Ni-Co, then add 250 μ l of 0.3% H_2O_2 . Mix. Filter.



