

Research report

# Concurrent androgenic stimulation of the ventral tegmental area and medial preoptic area: synergistic effects on male-typical reproductive behaviors in house mice

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## Abstract

Cannulae containing testosterone propionate (T) were bilaterally implanted into the medial preoptic area (MPO), the ventral tegmental area (VTA), or both areas concurrently (MPO/VTA) of castrated male house mice. A fourth group of castrates in which the intracranial implants missed their targets (MIMP) served as controls. In addition, other castrates were implanted subcutaneously with empty silastic capsules (BSIL) or silastic capsules containing T (TSIL). All subjects were examined for the following male-typical behaviors: mounting, attraction to female urine, ultrasonic mating vocalizations, and urinary scent marking. In addition, the males were tested for activity levels to insure that they were not motorically impaired. In general, TSIL implants restored all male-typical behaviors to normal levels, whereas BSIL and MIMP implants were generally ineffective. Similar to previous findings, MPO implants alone completely restored ultrasonic vocalizations, partially restored urine marking, and had little or no effect upon mounting or urine preference. In contrast, VTA implants alone were ineffective at restoring any of these male-typical behaviors. However, the combined MPO/VTA implants were the most effective in restoring male-typical behaviors. In fact, a synergism between concurrent hormone action in the MPO and VTA was seen for mounting and urine preference. We interpret these data to indicate that androgen may act simultaneously in the MPO and VTA for more complete expression of some male-typical reproductive behaviors.

*Keywords:* Androgen; Brain; Marking; Preference; Sexual behavior; Ultrasonic vocalizations; Urine; Mouse

## 1. Introduction

Intracranial implants of crystalline gonadal hormones have been used in a variety of species to elucidate the neural sites at which male-typical reproductive behaviors are activated by gonadal hormones. Most studies have focussed on single sites in the mediobasal forebrain. Although a variety of neural sites show some hormone responsiveness, implants into the medial preoptic (MPO)/anterior hypothalamic (AH) continuum have consistently been the most effective in restoring male reproductive behavior [28]. Some reproductively-related behaviors demonstrated to exhibit MPO/AH androgen responsiveness include copulatory behavior (rats: [12,14,33,35], gerbils: [62], mice: [43], quail: [3,57], and ring doves: [4]).

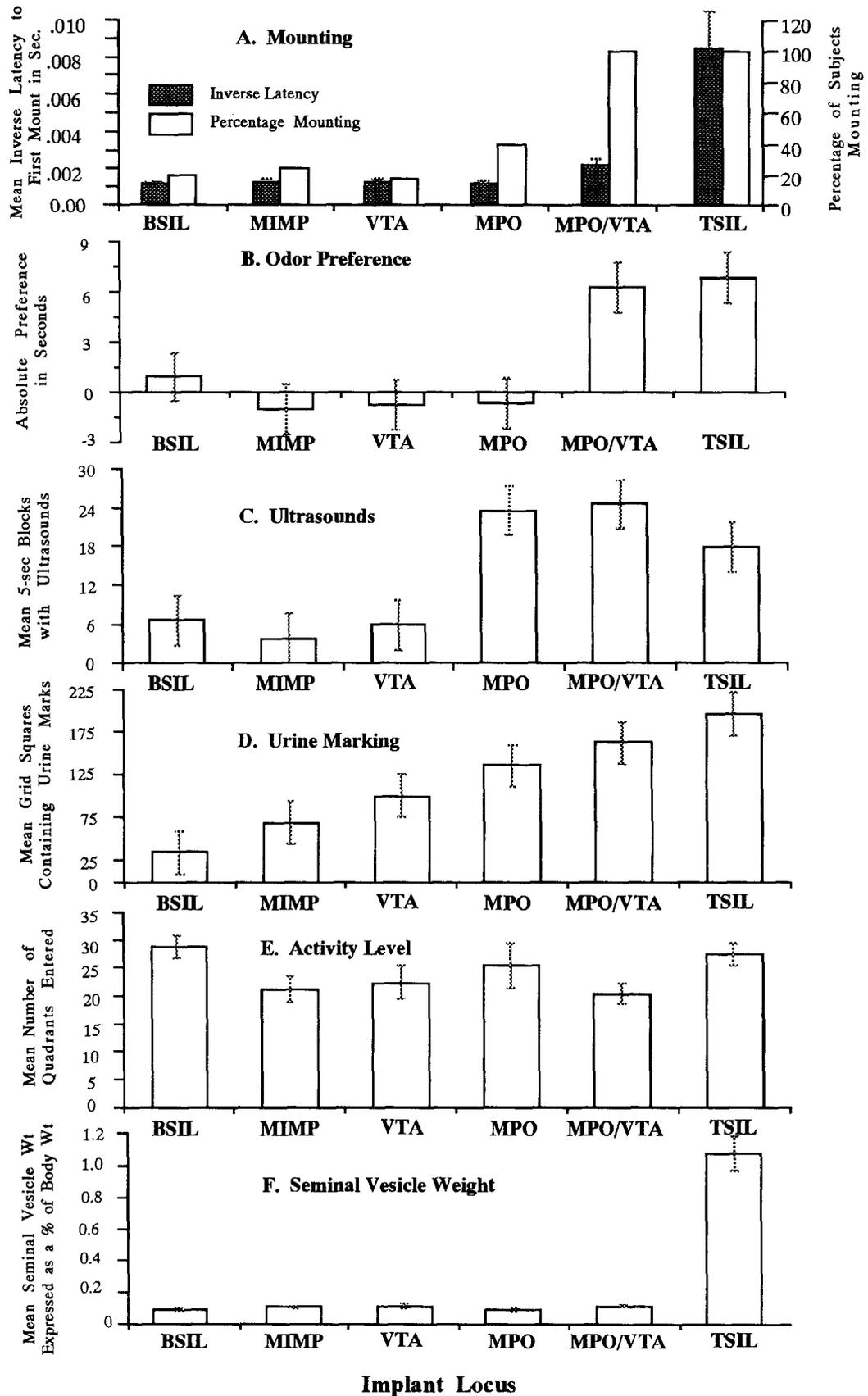
scent marking (mice: [43], and gerbils: [62]), ultrasonic vocalizations in mice [43] and preference for females in mice [39].

While many investigators have demonstrated the effectiveness of intracranial hormone implants in restoring reproductive behavior, a consistent observation is that restoration is often incomplete. For example, castrated rats receiving androgen implanted into the rostral hypothalamus ejaculated in only 50% of post-implantation tests as compared to 100% of the systemically treated rats [14]. Similarly, only 22% of the male mice implanted with testosterone propionate (T) into the MPO attempted to mount as compared to 77% of the males with systemic treatment [43]. These findings would be expected if other androgen-sensitive sites in addition to the MPO/AH area need to be stimulated for the complete expression of male sexual behavior.

Several different lines of evidence support the MPO interacting with the ventral tegmental area (VTA) in the expression of male-typical behaviors. As would be ex-

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pected of an interaction between these two brain areas, the MPO and VTA are connected via the medial forebrain bundle [52]. Furthermore, studies in which connections between the MPO and VTA were purportedly destroyed resulted in decrements in male sex behavior [9,19,29,30,37]. Moreover, electrically stimulating the medial forebrain bundle increased sex behavior in male rats [11].

The VTA, in turn, is known to provide dopamine (DA) input into the nucleus accumbens (ACC) via the tegmento-striatal branch of the mesolimbic pathway [18,60]. Research has implicated this pathway as being centrally involved in mediating the reward and incentive motivation associated with food, water, sex, and drugs (see [50] for review). For example, several laboratories have confirmed that this dopaminergic pathway is activated during male sexual behavior and by sex-related olfactory stimuli [13,36,38,47,49,58]. In addition, novel odors that are associated with sexually receptive females acquire the property of enhancing single unit responses of ACC neurons [59]. Thus, VTA projections to the ACC appear to be important in sexual reward. Also consistent with this interpretation, electrical stimulation of the VTA of male rats [20] and monkeys [45] facilitated several aspects of male-typical, reproductively related behavior and, in rats, resulted in an increase in DA transmission in the ACC [22]. And finally, apomorphine microinjections into the VTA interfered with the performance of male-typical reproductive behavior [31,32]. In addition to the possible rewarding effects associated with activating this pathway, this pathway has also been suggested to help integrate male-typical behavioral organization [32]. Perhaps these two functions are not mutually exclusive.

Given the evidence supporting a role for the VTA in the performance of male-typical sexual behavior, it was surprising to find that investigations of the neural location of intracellular steroid receptors do not mention the VTA as a site containing such receptors [40,51,56]. Unpublished mapping work in our lab using immunohistochemistry has also been unable to demonstrate the existence of intracellular androgen receptors in the VTA although receptors were seen in nearby structures such as the central tegmental field (Sipos, unpublished observations). This finding might suggest that the VTA is not a primary site of androgen action for the activation of male-typical behaviors. However, work by DeBold, Frye and their collaborators [23–27] has shown that progesterone activity in the VTA promotes lordosis in female hamsters despite the fact that very few intracellular progesterone receptors exist in

the VTA. These workers have provided evidence that progesterone acts in the VTA via nongenomic membrane receptors. Perhaps androgens also work in the VTA in this fashion.

In this paper, we examine the role the MPO and VTA play in the regulation of ultrasonic courtship vocalizations, urine marking, urine preference, and mounting following the implantation of T independently and concurrently into these brain sites.

## 2. Materials and methods

### 2.1. Animals

Three different categories of animals were used. ‘Subjects’ were adult C57BL/6J X AKR/J (‘CK’) hybrid male mice bred in our laboratory. ‘Social experience animals’ were adult male (CFW from Charles River Laboratories, Wilmington, MA) and female (CK) mice that were systematically placed in the subject’s home cage. ‘Stimulus animals’ were adult male (CFW) and female (CK) mice that were used in urine marking, ultrasound, urine preference, and mounting tests. The CFW male social experience animals received bilateral olfactory bulbectomies (OBX) under sodium pentobarbital (Nembutal) anesthesia prior to use. OBX males were used as social experience animals, because they reliably elicit aggression comparable to intact males, while neither initiating nor responding to attack themselves [16].

The subjects were individually housed in transparent plastic mouse cages (29 × 18 × 13 cm) with hardwood chips for beddings and a wire top containing food and water available ad libitum. The social experience and stimulus animals were similarly housed in groups of two to four in cages identical to those of the subjects. All animals were maintained on a 12:12 light:dark cycle with lights on at 0700 h.

### 2.2. Stereotaxis surgery and histology

#### 2.2.1. Implant procedure

All subjects were bilaterally castrated under sodium pentobarbital anesthesia (6.5 mg/g body weight). Following one week of recovery, the animals were randomly assigned to the following treatment conditions: (1) MPO: implants into the medial preoptic area ( $n = 5$ ); (2) VTA: implants into the VTA ( $n = 6$ ); (3) MPO/VTA: concurrent implants into the MPO and VTA ( $n = 8$ ); (4) TSIL:

Fig. 1. A: the percentage of subjects mounting and mean ( $\pm$ S.E.M.) inverse mount latency scores. B: mean ( $\pm$ S.E.M.) preferences for female urine over male urine. C: mean ( $\pm$ S.E.M.) number of ultrasounds. D: mean ( $\pm$ S.E.M.) number of urine marks. E: mean ( $\pm$ S.E.M.) activity levels. F: mean ( $\pm$ S.E.M.) seminal vesicle weights. The groups tested were: castrated males with empty silastic implants (BIMP), testosterone propionate filled silastic implants (TSIL), intracranial implants of testosterone propionate into the ventral tegmental area (VTA), medial preoptic area (MPO), both the MPO and the VTA concurrently, or in sites that missed their targets (MIMP).



castrates receiving subcutaneous silastic implants (length = 10 mm, i.d. = 1.57 mm, o.d. = 3.18 mm) of T ( $n = 9$ ); and (5) BSIL: castrates receiving empty silastic implants ( $n = 10$ ). Castrates receiving systemic T (TSILs) would be expected to exhibit normal male-typical levels of behavior, while castrates receiving no hormone (BSILs) represented low baseline controls. In addition, males whose intracranial implants missed their targets were removed from the groups to which they were initially assigned and placed into a sixth group called missed implants (MIMP,  $n = 8$ ).

The subjects were stereotaxically implanted bilaterally with 27-gauge cannulae using the following Slotnick and Leonard (1975) atlas coordinates (in mm): (1) MPO: AP = 0.2, DV = -5.1, ML = 0.4; and (2) VTA: AP = -3.0, DV = -4.4, ML = 0.9. All coordinates were measured on a flat skull in relation to bregma. A correction factor for individual animals was calculated by dividing the lambda-bregma distance of the subject by the lambda-bregma distance in the stereotaxic atlas [54]. This correction factor was then multiplied by the above AP, DV, and ML coordinates to obtain the actual coordinates used.

Cannulae were prepared from 27-gauge stainless steel hypodermic tubing. Cannulae were filled by tamping crystalline T into the lumen to a depth of approximately 1 mm. Testosterone propionate remaining on the outside of the cannulae was removed with a lint-free tissue prior to implantation. Animals were implanted under sodium pentobarbital anesthesia supplemented with methoxyflurane inhalant (Metofane; Pittman Moore, Washington Crossing, NJ) in a Kopf stereotaxic frame fitted with a custom built mouse head holder. Cannulae were secured to the skull with acrylic cement and the skin sealed with cyanoacrylate adhesive. Animals recovered for 7 days before beginning behavior testing.

### 2.2.2. Histological verification

Within 48 h following the last behavioral test, the animals were overdosed with sodium pentobarbital and perfused intracardially with phosphate buffered saline followed by 4% paraformaldehyde. The seminal vesicles were removed, cleaned, and weighed wet as a measure of peripheral androgenic stimulation.

The brains were removed, placed in formalin for 1–3 days, and coronal sections taken at 40  $\mu\text{m}$  through the

extent of the cannulae tracks. The sections were then placed on glass slides and stained with Cresyl violet. Cannulae placements were defined in terms of Slotnick and Leonard (1975) coordinates and plotted on coronal sections for graphical presentation.

### 2.3. Behavioral protocols

All tests were conducted in the mouse colony room during the light portion of the light:dark cycle.

#### 2.3.1. Social experience

Approximately 10 days before castration all males were provided with a social experience regimen over an 8 day period. Each day the subject sequentially encountered a male and a female social experience animal for 3 min each. The order in which the social experience animals were presented was alternated daily. Previous unpublished observations had indicated that such experience shortens latencies for the display of many male-typical behaviors. Following social experience, the animals were screened for ultrasonic courtship vocalizations. Nine animals with vocalization scores less than 12 were dropped from the study.

#### 2.3.2. Ultrasonic vocalizations

Subjects were tested for ultrasounds to female urine 10 days following implantation. Vocalizations were monitored with an ultrasonic receiver (Ultrasound Advice, model S-25) tuned to a center frequency of 70 kHz. A subject in his home cage was taken from the cage rack and placed on a table under the ultrasonic microphone. A 1-min habituation period preceded the test to ensure that males were not vocalizing to random aspects of the test situation. If a vocalization occurred during the habituation period, the animal was retested 10–15 min later.

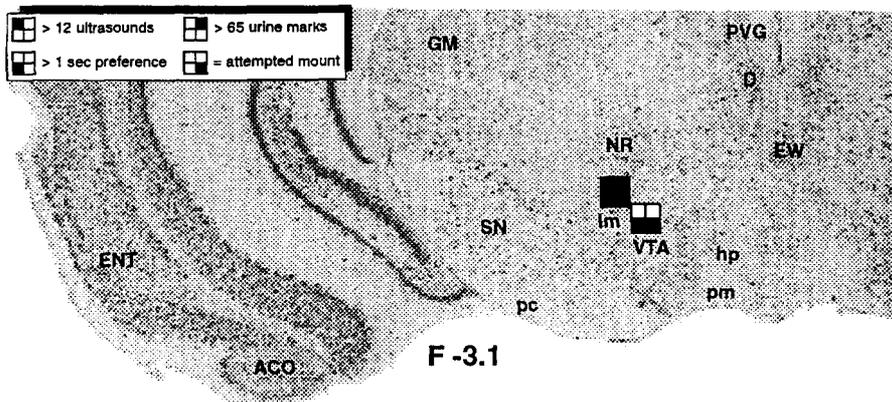
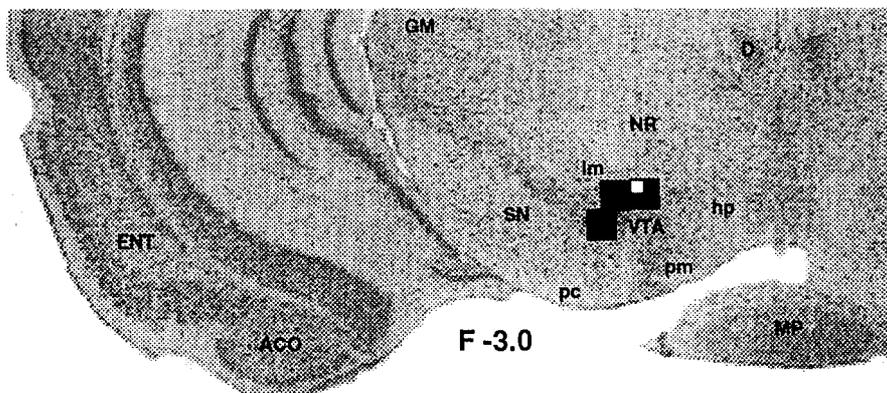
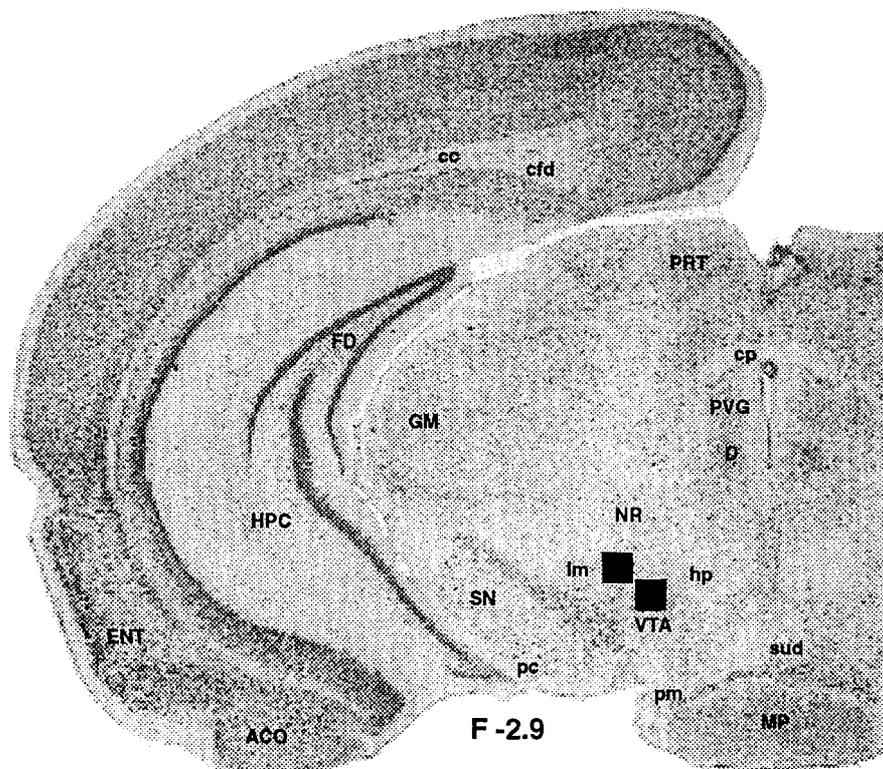
A test began with the introduction of the urine stimulus into the subject's home cage and lasted for 3 min. The 3-min test was divided into 36 5-s time sampling intervals. The number of intervals containing vocalizations was recorded yielding possible scores ranging from 0–36.

The urine of female mice was used as the stimulus. The status of the estrous cycle of the female stimulus donors was not monitored, as previous research has shown that males vocalize at high levels to females or their urine at all

Fig. 2. Three coronal sections (0.2, 0.0, and -0.1 mm in front of bregma) of a house mouse brain (adapted from [54]) showing the approximate locations of bilateral testosterone propionate implants into the medial preoptic area (MPO) of animals receiving concurrent implants into the MPO and ventral tegmental area. The mean medial/lateral and dorsal/ventral location of the implants in each animal is represented by a box indicating how effective or ineffective the implants were in restoring male-typical behaviors in castrated male mice. The animals that were above the indicated behavioral levels are represented by a blackened quadrant in the appropriate part of the square (see key in the lower left hand corner of the figure). The behavioral responsiveness of the control group getting no hormone (BSIL) and the control group getting systemic T are also pictured in the inserted table for comparison. Abbreviations: AA, anterior nucleus of the amygdala; BST, bed nucleus of the stria terminalis; ca, anterior commissure pars anterior; cap, anterior commissure pars posterior; cc, corpus callosum; CLA, claustrum; co, optic chiasm; fx, fornix; ICL, intercalatal nucleus of the amygdala; LPA, lateral preoptic area; LS, lateral septum; mfb, medial forebrain bundle; MPO, medial preoptic area; MS, medial septum; POM, magnocellular preoptic area; PP, prepyriform cortex; SF, nucleus fimbrialis septi; st, stria terminalis; sth, stria terminalis pars hypothalamica; tol, lateral olfactory tract; TS, nucleus triangularis septi; TUO, olfactory tuberculum; VL, lateral ventricle.

points in the cycle [44]. The urine was collected by grasping a female stimulus animal ( $n = 10$ ) by the loose skin of the dorsal neck. The act of handling the animal in this fashion was often sufficient to induce urination. If

urination did not occur, however, it could usually be induced by gently palpating the bladder. The donor was positioned so that its urine fell into a glass collection vial. While the amount of urine from individual animals varied,



approximately 0.05 to 0.1 ml of urine per donor was typical. After several donors urinated, the pooled urine was immediately placed in a disposable syringe and stored until use. The urine stimulus was prepared by placing 0.1 ml of urine on a cotton-tipped swab during the 1-min habituation preceding its presentation. The part of the odorized swab touched by the experimenter was broken off prior to presentation.

### 2.3.3. Urine marking

Subjects were tested for urine marking 11 days following implantation. Urine marking was measured in a transparent plastic chamber (29 × 18 × 13 cm) with the open side placed upside down on Whatman Benchkote filter paper. A cotton-tipped swab odorized with 0.1 ml of female urine collected in the fashion described above, was taped to the side of the test chamber and served as the stimulus. The test lasted 20 min. Since mouse urine fluoresces under UV light [17], an index of the number of urine marks was measured by examining the filter paper under a 15W ultraviolet (UV) light (3600 Å).

The urine marks were quantified by placing a grid that was photocopied onto transparency film over the filter paper and counting the number of squares (12 × 12 mm) containing urine marks. A correlation of  $r = 0.97$  ( $df = 12$ ,  $P < 0.01$ ) was previously found between the grid index and the actual number of marks present [43].

### 2.3.4. Mounting behavior

Subjects were tested for mounting 7 days following hormone implantation. Mounting was measured for 15 min in response to an ovariectomized female in hormone-induced estrus. Estrus was induced with subcutaneous estradiol (10 µg) injections 48 h and 24 h prior to behavioral testing and subcutaneous progesterone (500 µg) injections 4–6 h prior to behavioral testing. The estrous female was placed into the subject's home cage at the beginning of behavioral testing.

A mount was scored whenever the male clasped the female appropriately from the rear, regardless of whether the male was able to sustain the mount. The following measures were taken: (1) latency in seconds to first mount; and (2) the percent of subjects that mounted. Very few intromissions and ejaculations were observed during the 15 min observation period of this experiment. Thus, our measure reflected the subject's initial tendency to mount a

female and may not reflect motivations associated with the later stages of a mating bout [7].

### 2.3.5. Urine preference

Subjects were tested for their preference for female urine over male urine approximately 8–9 days following implantation. This preference was measured for 3 min in a clean cage identical to the subject's home cage in response to male and female urine. A cotton-swab odorized with 0.1 ml of female urine was taped to one end of the cage, while a cotton-swab odorized with 0.1 ml of male urine was taped to the opposite end. The subject was placed into the cage at the beginning of the urine preference test.

Two experimenters were required for the single-blind measurement of urine preference. One experimenter measured the amount of time the subjects spent sniffing each stimulus swab. This experimenter was blind with respect to the subject's treatment condition and the identity of each stimulus swab. Sniffing was operationally defined as occurring whenever the subject's nose either touched or was within 2 cm of the stimulus. The second experimenter was responsible for transporting the subjects, preparing and presenting the stimuli, and timing the trial duration.

The preference that the subject exhibited was calculated by subtracting the amount of time the males spent sniffing the male urine from the amount of time the males spent sniffing the female urine. Thus, scores above zero indicated a preference for female urine, while negative scores indicated a preference for male urine.

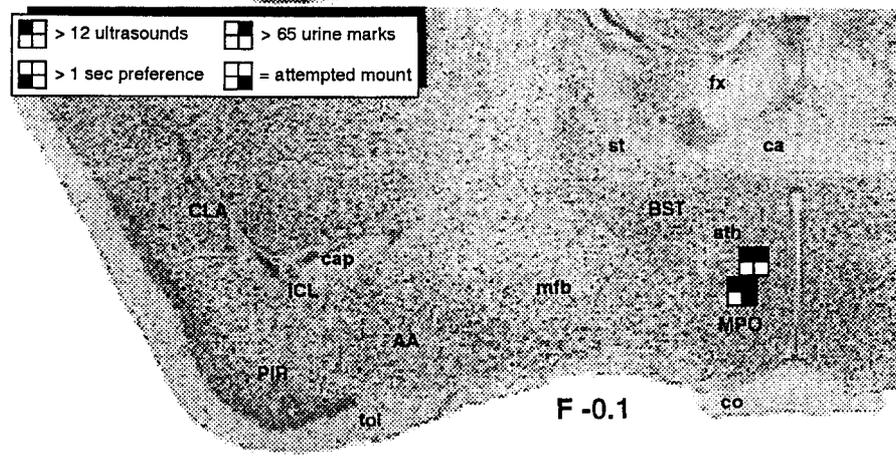
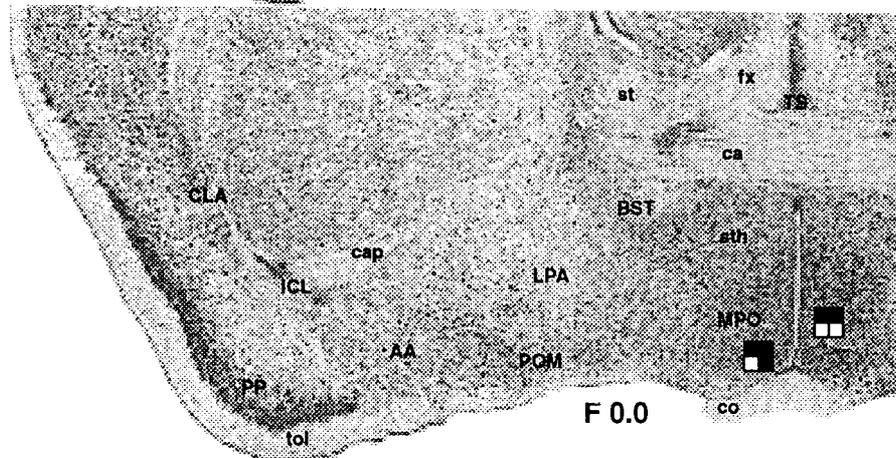
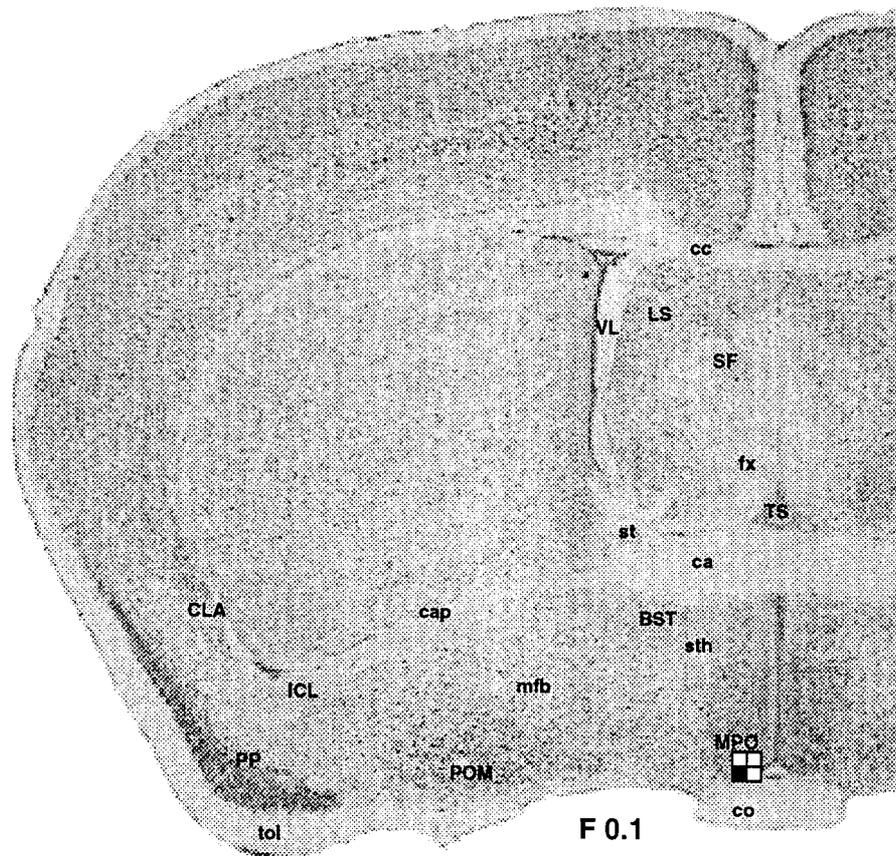
### 2.3.6. Activity levels

To insure that our brain manipulations did not indirectly affect behavioral levels through a nonspecific effect upon activity, the subjects were tested for activity levels 5 days following hormone implantation. Subjects were placed into clean cages divided into four quadrants by tape that bisected the length and width of the cage top. Looking directly down from above, an observer recorded the number of times the subject's two front feet crossed into a different quadrant during a 3 min trial.

## 2.4. Statistical analyses

Group differences in percent of males who mounted were analyzed using a chi-square test, while group differences in latency to first mount were analyzed using a

Fig. 3. Three coronal sections (2.9, 3.0, and 3.1 mm behind bregma) of a house mouse brain (adapted from [54]) showing the approximate locations of bilateral testosterone propionate implants into the ventral tegmental area (VTA) of animals receiving concurrent implants into the medial preoptic area and VTA. The mean medial/lateral and dorsal/ventral location of the implants in each animal is represented by a box indicating how effective or ineffective the implants were in restoring male-typical behaviors in castrated male mice. The animals that were above the indicated behavioral levels are represented by a blackened quadrant in the appropriate part of the square (see key in the lower left hand corner of the figure). Abbreviations: ACO, cortical nucleus of the amygdala; cc, corpus callosum; cfd, commissure fornicus dorsalis; cp, posterior commissure; D, Darkschewitz nucleus; ENT, entorhinal cortex; EW, accessory nucleus of the oculomotor nerve; FD, dentate gyrus; GM, medial geniculate body; hp, habenulo-interpeduncular tract; HPC, hippocampus; Im, medial lemniscus; MP, posterior mammillary nucleus; NR, red nucleus; pc, cerebral peduncle; pm, mammillary peduncle; PRT, pretectal area; PVG, periaqueductal gray; SN, substantia nigra; sud, supramammillary decussation; VTA, ventral tegmental area.



Kruskal-Wallis H test for the overall analysis followed by Mann-Whitney U tests for planned comparisons. Group differences in ultrasonic vocalizations, urinary marking, and urine preference were analyzed with a one-way ANOVA, followed by planned comparisons.

### 3. Results

#### 3.1. Histology

As seen in Fig. 1F, the six groups differed significantly in seminal vesicle weights expressed as a percent of body weight ( $F_{5,40} = 67.53$ ,  $P < 0.0002$ ). Animals receiving systemic T (TSIL) had larger seminal vesicle weights than males in the other treatment groups ( $F_{1,40} = 333.16$ ,  $P < 0.0002$ ), while the seminal vesicles of the brain-implanted animals were similar in weight to those of castrated males receiving no hormone (BSILs,  $F_{1,40} = 0.115$ ,  $P = \text{ns}$ ). Thus, the T from the brain implants did not appear to leak appreciably out of the brain into circulation and so the behavioral effects of these implants can be attributed to their effects inside the brain. The cannula placements and their effectiveness for each of the male-typical behaviors are seen in Figs. 2–7.

#### 3.2. Mounting

As seen in Fig. 1A, the groups differed significantly in the percentage of males mounting ( $X_5^2 = 26.42$ ,  $P < 0.01$ ) and latency to first mount (Kruskal-Wallis,  $H(5) = 28.475$ ,  $P < 0.0001$ ). As expected, the TSIL males mounted females significantly more than the BSILs ( $X_1^2 = 12.24$ ,  $P < 0.01$ ) and had significantly shorter latencies (Mann-Whitney  $U$ ,  $z = -3.674$ ,  $P < 0.0003$ ). Intracranial implants into either the MPO or the VTA alone did not appear particularly effective in restoring mounting. In fact, males with MPO, VTA, or MIMP implants did not differ significantly from the BSIL males in the number of mounts ( $X_1^2 = 0.10$ ,  $P = \text{NS}$ ) or latencies (Mann-Whitney  $U$ ,  $z = -0.275$ ,  $P = \text{NS}$ ). In contrast, males with concurrent MPO/VTA implants mounted significantly more than males receiving either MPO or VTA implants alone ( $X_1^2 = 10.53$ ,  $P < 0.01$ ) and had shorter latencies (Mann-Whitney  $U$ ,  $z = -3.22$ ,  $P < 0.0014$ ). These data provide clear evidence of a synergism between the MPO and VTA implants. Although all TSIL and MPO/VTA males mounted, the TSIL males did have significantly shorter

latencies than MPO/VTA males (Mann-Whitney  $U$ ,  $z = -2.502$ ,  $P < 0.013$ ).

#### 3.3. Urine preference

As seen in Fig. 1B, the groups differed significantly in their preferences for male and female urine ( $F_{5,40} = 6.121$ ,  $P < 0.0004$ ). As expected, TSIL males showed significantly higher preference for females than BSIL males ( $F_{1,40} = 9.94$ ,  $P < 0.004$ ). Intracranial implants into neither the MPO nor the VTA alone appeared to be particularly good at stimulating male-typical preference for female urine. Furthermore, males with MPO, VTA, or MIMP implants did not differ significantly from the BSIL males ( $F_{1,40} = 1.062$ ,  $P = \text{NS}$ ). In contrast, males receiving combined MPO/VTA stimulation exhibited preferences that were significantly higher than males receiving either implant alone or missed implants ( $F_{1,40} = 15.454$ ,  $p < 0.0004$ ). In addition, MPO/VTA males exhibited preferences that did not differ significantly from the TSIL males ( $F_{1,40} = 0.076$ ,  $P = \text{NS}$ ). Thus, a clear synergism existed between the VTA and MPO for the expression of urine preference. With the measure used for preference, restoration appeared to be complete in males with the combined implants.

#### 3.4. Ultrasonic vocalizations

As seen in Fig. 1C, the groups significantly differed in their vocalizations to female urine ( $F_{5,40} = 4.575$ ,  $P < 0.003$ ). As expected, TSIL males vocalized significantly more than BSIL males ( $F_{1,40} = 4.284$ ,  $P < 0.05$ ), but did not differ significantly from the MPO and MPO/VTA males ( $F_{1,40} = 1.363$ ,  $P = \text{NS}$ ). Furthermore, the MPO and MPO/VTA males did not differ significantly in their ultrasonic responsiveness to female urine ( $F_{1,40} = 0.037$ ,  $P = \text{NS}$ ), but did vocalize significantly more than the VTA males ( $F_{1,40} = 9.310$ ,  $P < 0.005$ ). The VTA and MIMP males did not significantly differ from the BSILs ( $F_{1,40} = 0.129$ ,  $P = \text{NS}$ ). Thus, any synergism that might have existed was masked by the high levels of ultrasounds emitted by the MPO males. In fact, MPO implants alone appeared to cause complete restoration of this behavior using our present methodology.

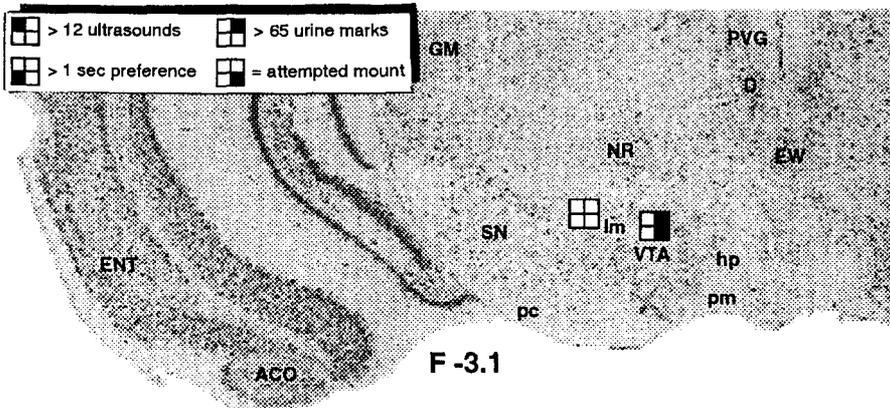
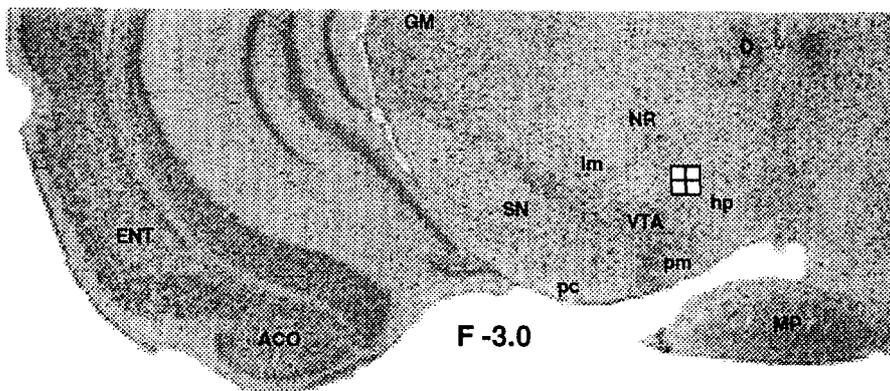
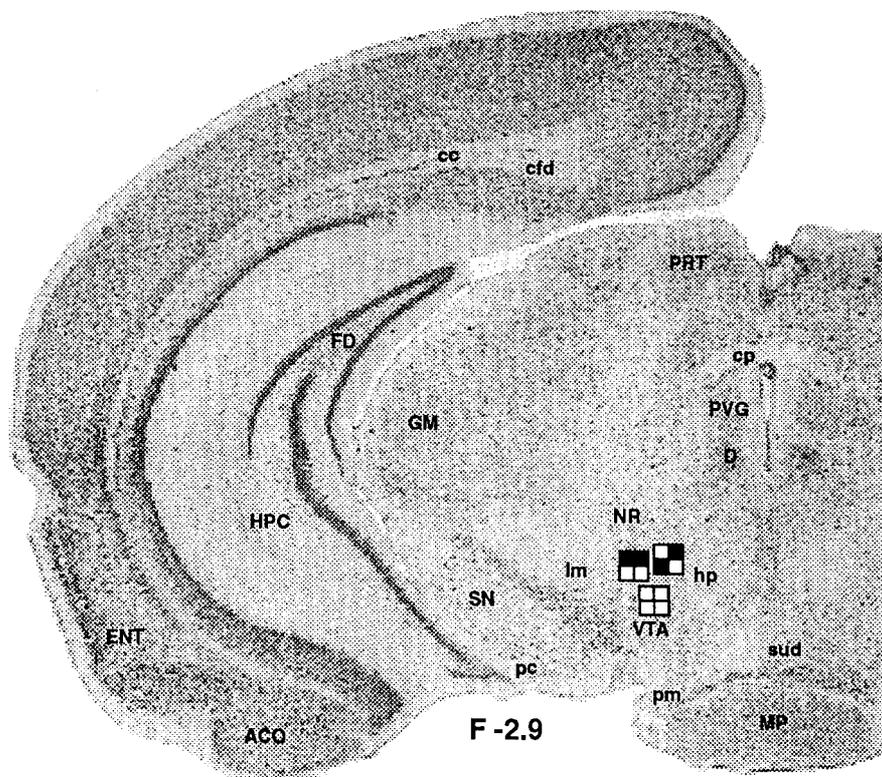
#### 3.5. Urine marking

As seen in Fig. 1D, the groups differed significantly in their amount of urine marking ( $F_{5,40} = 10.754$ ,  $p <$

Fig. 4. Three coronal sections (0.1, 0.0, and  $-0.1$  mm in front of bregma) of a house mouse brain (adapted from [54]) showing the approximate locations of bilateral testosterone propionate implants into the medial preoptic area (MPO). The mean medial/lateral and dorsal/ventral location of the implants in each animal is represented by a box indicating how effective or ineffective the implants were in restoring male-typical behaviors in castrated male mice. The animals that were above the indicated behavioral levels are represented by a blackened quadrant in the appropriate part of the square (see key in the lower left hand corner of the figure). See Fig. 2 for abbreviations.

0.0002). TSIL males deposited significantly more urine marks than BSIL males ( $F_{1,40} = 40.889, p < 0.0002$ ), but did not differ significantly from MPO/VTA males ( $F_{1,40}$

$= 1.611, P = NS$ ). Although concurrent implants into the MPO/VTA were more effective in restoring urine marking than VTA and MIMP implants alone ( $F_{1,40} = 8.47,$



$p < 0.006$ ), further analysis revealed that MPO and MPO/VTA males did not differ significantly ( $F_{1,40} = 0.745$ ,  $P = \text{NS}$ ). However, MPO/VTA males marked more than VTA males ( $F_{1,40} = 4.771$ ,  $P < 0.04$ ), whereas MPO males did not significantly differ from VTA males ( $F_{1,40} = 1.224$ ,  $P = \text{NS}$ ). Finally, the VTA males marked significantly more than MIMP and BSIL males ( $F_{1,40} = 4.735$ ,  $P < 0.04$ ). While some evidence for a synergism in androgen action between the MPO and VTA can be seen graphically (Fig. 1D), the high amounts of urine marking by the MPO males prevented a statistical demonstration of this phenomenon.

### 3.6. Activity levels

As seen in Fig. 1E, the groups did not differ significantly in the number of quadrants entered ( $F_{5,40} = 2.41$ ,  $P = \text{NS}$ ). Thus, the higher levels of male-typical behaviors displayed by the MPO and MPO/VTA groups cannot be accounted for by increased levels of motor behavior.

## 4. Discussion

The major novel contribution of the present research was the finding that when androgen implants into the VTA were combined with concurrent implants into the MPO, the levels of some male-typical behaviors were higher than could be accounted for by implants into either area alone. The synergy between hormone activity in the MPO and VTA was statistically evident for mounting and for preference for female urine. Perhaps the failure to demonstrate a synergy for urine marking was related to the fact that the activation of urine marking by MPO implants alone appeared somewhat higher than in our previous work [39,43]. In contrast, MPO implants alone have, in the past, more consistently resulted in high levels of ultrasonic vocalizations and no evidence of a synergy was demonstrated for this behavior. While considerable evidence points to a role for the VTA in the performance of male typical behaviors (e.g. [20,31,32,45]), the present research clearly shows that hormonal stimulation of this area alone is not sufficient to activate any of the male-typical behaviors we examined. We conclude that while androgen action in the VTA alone does little to restore male-typical behaviors, such activity does serve to augment androgen action in the MPO for some male-typical behaviors.

Since no evidence of hormone leakage from the brain into systemic circulation was found (as measured by seminal vesicle weights), we believe that the behavioral effects

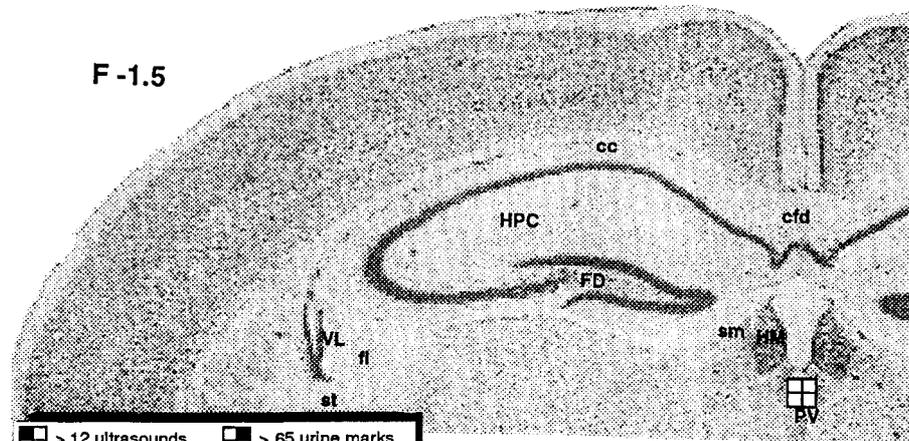
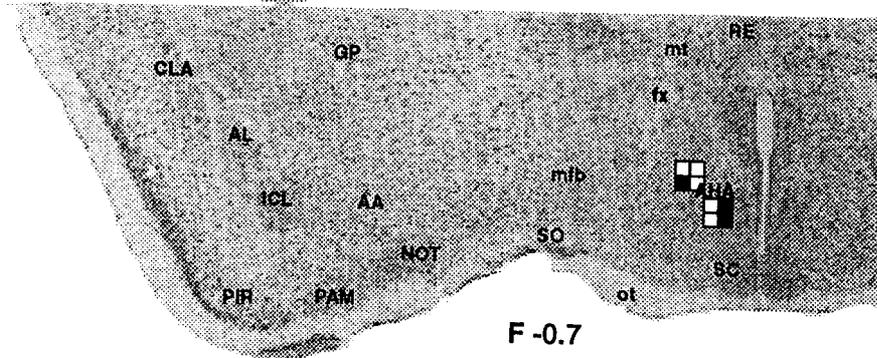
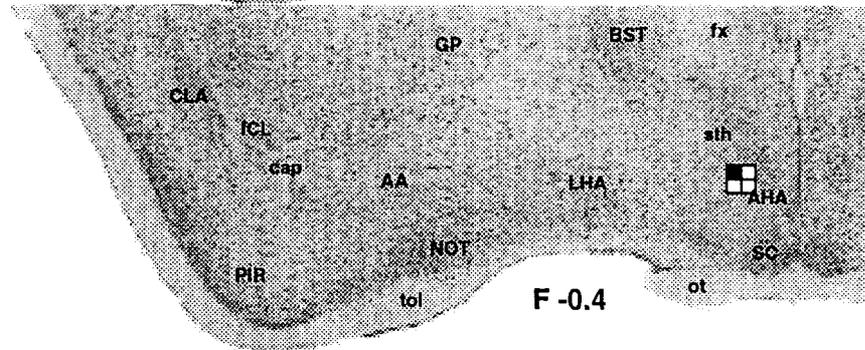
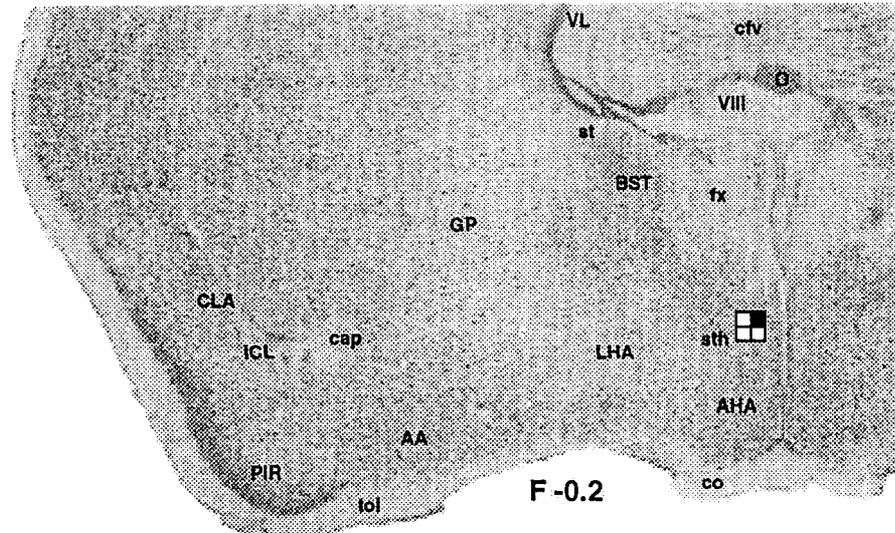
of our intracranial hormone implants must be accounted for by their effects inside the brain. We also believe that the implanted hormone was promoting behavior by acting in close proximity to the cannula tip. For example, the evidence is fairly consistent in indicating that steroid hormones implanted into the brain using our methodology, do not diffuse far. Although hormone from large radiolabeled testosterone pellets has been detected as much as 2 mm away from the implant and in peripheral circulation [55], when hormone was delivered from inside a cannula such that release occurred only from the tip of the cannula, as we did in this study, the radiolabeled hormone was generally undetectable more than 1 mm from the cannula tip [41,46]. Barfield et al. [4] similarly provided functional evidence that the degree of spread varies as a function of the amount of hormone implanted. When a small amount was implanted (inside 27-gauge cannulae identical to those in the present study) male-typical behaviors were elicited only if the tip of the cannulae was in the MPO [4].

Very recently, using androgen receptor induction in castrated male hamsters to measure hormone spread, Wood and Newman [61] found that receptors were induced only within about 1 mm of 23-gauge cannulae containing testosterone and within 600  $\mu\text{m}$  of 26-gauge cannulae while we [53] found receptor induction in mice only within 400  $\mu\text{m}$  of 27-gauge cannulae placed in the septum. Thus we believe it unlikely that hormone diffused from the VTA to other nearby midbrain areas (such as the central tegmental field) which have intracellular androgen receptors.

Our results also replicated previous findings [39,43] that androgen implants into the MPO alone resulted in complete restoration of ultrasonic vocalizations, partial restoration of urine marking, but had little effect upon mounting. A new finding was that such implants were not sufficient to restore the preference for female urine over male urine shown by gonadally intact males. While T in the MPO did not restore a preference for female urine, previous research [39] demonstrated that T in the MPO did restore a preference for the female herself. Perhaps more androgenic stimulation is required when the stimulus is less salient. Thus, the extent to which androgen implants into the MPO restored male-typical behaviors depended upon the behavior. Although speculative, it appeared to us that the more sensory inputs and motor outputs that must be integrated for the performance of a particular male-typical behavior, the less likely androgen implants into the MPO alone are sufficient to restore high levels of behavior.

An alternative explanation for the increased levels of behavior of the MPO/VTA animals was related to the

Fig. 5. Three coronal sections (2.9, 3.0, and 3.1 mm behind bregma) of a house mouse brain (adapted from [54]) showing the approximate locations of bilateral testosterone propionate implants into the ventral tegmental area. The mean medial/lateral and dorsal/ventral location of the implants in each animal is represented by a box indicating how effective or ineffective the implants were in restoring male-typical behaviors in castrated male mice. The animals that were above the indicated behavioral levels are represented by a blackened quadrant in the appropriate part of the square (see key in the lower left hand corner of the figure). See Fig. 3 for abbreviations.



> 12 ultrasounds	> 65 urine marks
> 1 sec preference	= attempted mount

greater amount of T placed into their brains. Twice as much hormone was implanted into the brains of the MPO/VTA animals as into the brains of animals receiving implants into only a single site. However, two lines of evidence argue against this explanation. First, one male in the MIMP group that received a double implant did not show high levels of male-typical behavior despite having higher amounts of implanted T. Second, more compelling evidence comes from three unpublished studies [53] that were methodologically identical to the present study. In these studies, males receiving combined implants into the corticomedial amygdala and the MPO, into the periaqueductal gray and the MPO, or into the central tegmental field and the MPO generally performed male-typical behaviors at a level comparable to animals receiving implants into the MPO alone. Thus, the amount of behavior restored in all of these studies was related to the location of the hormone implant rather than the amount of hormone implanted.

We feel that our results become more impressive in view of the general observation that it is typically more difficult to elicit a behavior with brain manipulations than to eliminate one. Furthermore, the higher levels of behavior in the MPO/VTA animals were also in spite of the increased neural destruction from the additional cannulae in their brains.

At the same time, we wish to point out that the androgenic activation of male-typical behaviors can not be accounted for solely by androgen action in the MPO and VTA. For example, the latencies to first mount were significantly longer in the MPO/VTA males than in the TSIL males. This finding indicates that in spite of the synergy between concurrent hormone action in the MPO and VTA, behavioral restoration was not complete. Perhaps additional neural sites may need to be stimulated in order to completely restore copulatory behavior to pre-castration levels.

One important issue raised by this research is the mechanism of T action in the VTA since the VTA is thought to be almost devoid of intracellular androgen or estrogen receptors [40,51,56]. However, evidence exists that testosterone may also act through membrane receptors both inside [10] and outside [34] of the brain. Along similar lines, progesterone action in the VTA helps to support lordosis in female hamsters despite very few intracellular progesterone receptors in this area [15]. DeBold,

Frye and their colleagues [23–27] present compelling evidence that progesterone acts in the VTA by binding the GABA<sub>A</sub> benzodiazepine receptor complex. It is known, for example, that the sensitivity of the GABA<sub>A</sub> receptor to GABA can be modulated both up and down by steroid binding [5]. Moreover, some metabolites of testosterone, synthesized by glia cells, are known to be GABA<sub>A</sub> agonists [8]. Perhaps T, or one of its metabolites, might act nongenomically in the VTA via GABA<sub>A</sub> receptors.

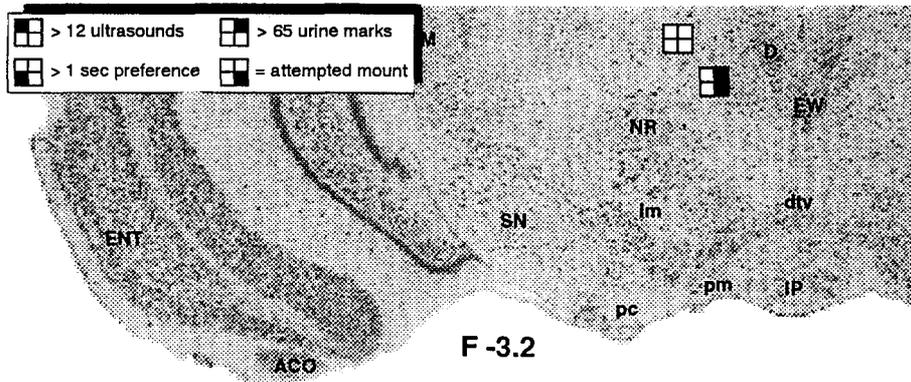
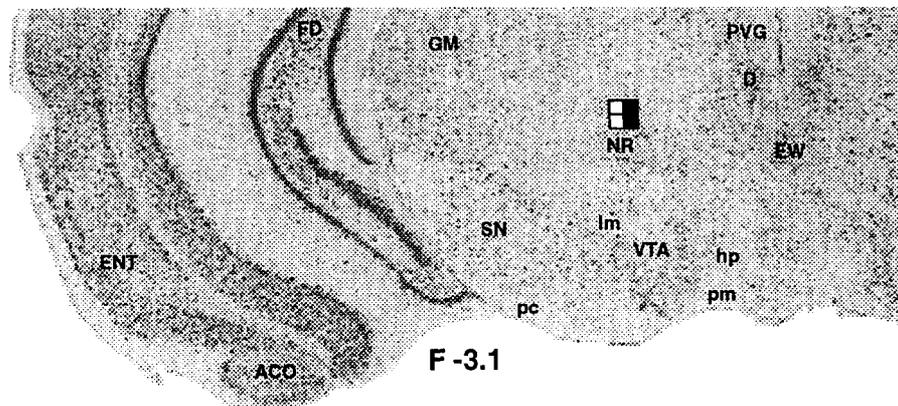
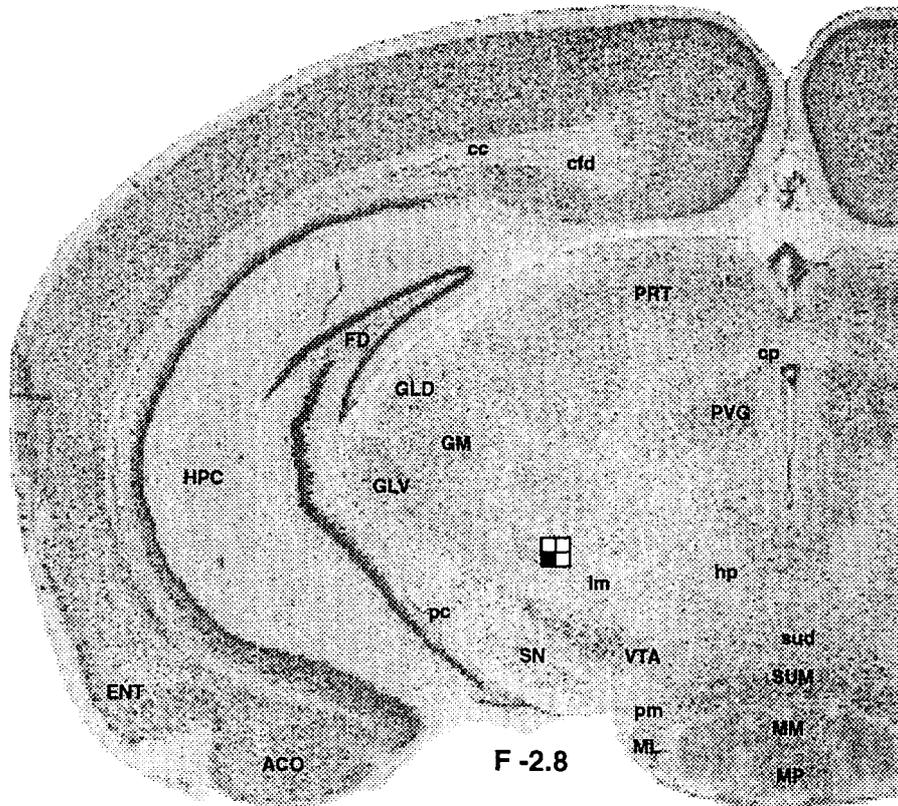
Everitt [19] hypothesized that the mesolimbic dopaminergic circuitry may mediate the reward-related aspects of sexual behavior. In support of this hypothesis, Everitt found that bilateral infusions of D-amphetamine, a DA releasing agent, into the ACC of male rats reduced mount and intromission latencies and increased the rate of instrumental responses to gain access to a receptive female under a second-order schedule of sexual reinforcement, while having insignificant effects on mounts, intromissions, and copulatory rate. West et al. [56] further supported this hypothesis by finding that odors which had been associated with sexually receptive females during training evoked significantly more single unit activity in the ACC of trained male rats than untrained rats, suggesting a role for the ACC in associating environmental stimuli with natural reward processes. The results of microdialysis and voltammetric studies (e.g. [13,38,47–49]) also support the hypothesis that both anticipatory and consummatory components of sexual behavior are rewarding based on increased DA release in the ACC. We believe that our results are consistent with the idea that while MPO androgenic action may be critical for the initiation of male-typical reproductive behaviors, but that the reward associated with sex-related behaviors, mediated by VTA action, further facilitate both behavioral initiation and maintenance.

And finally we suggest a possible mechanism by which androgen action in the VTA could support the performance of male-typical behaviors. This mechanism relates to the possibility that T maintains the DA levels in the mesolimbic pathway necessary to support sexual reward. For example, castration reduces DA concentration in the ACC, whereas T replacement restores DA concentration to pre-castration levels [1,6,42]. Furthermore, several labs have found that T has rewarding properties. Testosterone replacement increased instrumental responding in castrated male rats prior to their first contact with a female [21]. In addition, rats displayed a preference for an environment

Fig. 6. Four coronal sections (0.2, 0.4, 0.7, and 1.5 mm behind bregma) of a house mouse brain (adapted from [54]) showing the approximate locations of bilateral testosterone propionate implants directed at the medial preoptic area that missed their targets (MIMP). The mean medial/lateral and dorsal/ventral location of the implants in each animal is represented by a box indicating how effective or ineffective the implants were in restoring male-typical behaviors in castrated male mice. The animals that were above the indicated behavioral levels are represented by a blackened quadrant in the appropriate part of the square (see key in the lower left hand corner of the figure). Abbreviations: AHA, anterior hypothalamic area; cfd, commissure fornix dorsalis; FD, dentate gyrus; GP, globus pallidus; HM, medial habenula; HPC, hippocampus; mt, mammillothalamic tract; LHA, lateral hypothalamic nucleus; NOT, nucleus of the olfactory tract; O, organ subfornicale; ot, optic tract; PAM, periamygdaloid cortex; PV, paraventricular thalamic nucleus; RE, reuniens thalamic nucleus; SC, supra-chiasmatic nucleus; sm, stria medullaris thalami; SO, supraoptic hypothalamic nucleus; VIII, third ventricle. See Fig. 2 for additional abbreviations.

previously paired with T administration as opposed to an environment associated with saline treatment in a conditioned-place-preference paradigm [2]. Thus, one effect of T

implants into the VTA of castrated males might be to restore incentive motivation by restoring DA metabolism in this part of the brain.



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Fig. 7. Three coronal sections (2.8, 3.1, and 3.2 mm behind bregma) of a house mouse brain (adapted from [54]) showing the approximate locations of bilateral testosterone propionate implants directed at the ventral tegmental area that missed their targets (MIMP). The mean medial/lateral and dorsal/ventral location of the implants in each animal is represented by a box indicating how effective or ineffective the implants were in restoring male-typical behaviors in castrated male mice. The animals that were above the indicated behavioral levels are represented by a blackened quadrant in the appropriate part of the square (see key in the lower left hand corner of the figure). Abbreviations: dtv, decussation of the ventral tegmentum; IP, interpeduncular nucleus; GLD, lateral geniculate body pars dorsale; GLV, lateral geniculate body pars ventrale; ML, lateral mammillary nucleus; MM, medial mammillary nucleus; SUM, supramammillary nucleus. See Fig. 3 for additional abbreviations.

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