NEUROTRANSMITTER RELEASE IN THE

MALE RAT BRAIN QUANTITATED BY

IN VIVO MICRODIALYSIS.

by

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ABSTRACT

Neurotransmitter Release in the Male Rat
Brain Quantitated by In Vivo Microdialysis

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The study of synaptic transmission in the central nervous system of the living animal is of fundamental importance in the field of neuroscience. The neurotransmitters that are released induce a wide range of physiologic responses.

Research indicates that several brain areas have been implicated in playing a role in regulating male sexual behavior. Among these areas are the Medial Preoptic Area (MPOA) and the Nucleus Accumbens (NAc). The MPOA serves as an integration center for the consummatory state of the animal. The NAc functions as an interface between the forebrain areas and the motor systems. Researchers have reported that in these two brain areas Dopamine (DA) and/or Norepinephrine (NE) have been found. The precise roles of these neurotransmitters remains to be determined.

This study used microdialysis probes to measure the release of neurotransmitters during sexual behavior. The microdialysis probes were inserted into either the Medial Preoptic-Diagonal Band of Broca areas (MPOA-DBB) or the Nucleus Accumbens (NAc) of sexually experienced male rats.

Samples were collected while the male rat was exposed to an estrous female or an ovariectomized female.

In the Nucleus Accumbens, DA levels tended to increase during copulation with an estrous female. In contrast, when males were exposed to an ovariectomized female levels of DA tended to be low.

In the MPOA-DBB areas the levels of Homovanillic Acid (HVA), NE, and Epinephrine (EPI) showed a substantial increase when the males were exposed to an estrous female. Upon exposure to the ovariectomized female, the levels of these neurotransmitters were decreased below basal levels.

These results support the hypothesis that catecholamines in the MPOA-DBB and NAc play a role in mediating the complex neural mechanisms that regulate copulatory behavior in male rats.

ACKNOWLEDGEMENTS

iv

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Laboratory rats have a complex pattern of copulatory behavior. In males, three primary elements are involved; mounts, intromissions, and ejaculations (Dewsbury, 1975; Sachs and Meisel, 1988). As part of their precopulatory behavior males and females often emit ultrasonic vocalizations which may augment their excitement level. The male may also rub against or move over top of the female, often urinating (marking) as he passes over the female. An estrous female tends to respond to the male's actions by a distinct hopping and darting movement. These actions are designed to gain the attention of the male, and as the female performs these quick movements, her hind end is always directed toward the male. If the male fails to pursue, the female may approach the male and nudge his side to gain attention. Also, as an indication of receptivity, females display very rapid, ear wiggling activity during the hopping and darting sequence (Sachs and Meisel, 1988).

The male responds to the female by a series of mounts, with or without vaginal insertion. The female responds to the mount with a lordosis response: a dorsiflexion of the spine and deflexion of the tail to one side allowing vaginal access to the male (Sachs and Meisel, 1988; Noble, 1980; Bitran and Hull, 1987). The male mounts the female dorsally and from the rear. The male's forelimbs generally grasp and palpate the female's flank before and sometimes during the

initiation of pelvic thrusting. These actions of the male intensify the female's receptivity and ensure lordosis (Sachs and Meisel, 1988). Intromission patterns are mounts with vaginal insertion. These patterns are distinguished from the mounts without penetration by the presence of a deep thrust and a springing dismount (Sachs and Meisel, 1988; Dewsbury, 1972; Bitran and Hull, 1987). The male typically has eight to ten intromissions, spaced about one minute apart, before they attain their first ejaculation (Dewsbury, 1975, 1981).

Ejaculation refers to the very forceful expulsion of the ejaculate, seminal plug in male rats, from the urethra to the outside (Sachs and Meisel, 1988). This pattern is repeated for 5 to 10 copulatory series, during which the number of intromissions and latency to ejaculation at first decrease, and then subsequently increase with increasing number of ejaculations (Dewsbury, 1975; Dewsbury, 1972; Bitran and Hull, 1987; Dewsbury, 1981). Ejaculation is commonly followed by an interval of genital autogrooming, after which the male enters a period of sexual inactivity (Sachs and Meisel, 1988). This postejaculatory period, a period of 4-8 minutes when the male is refractory to further copulatory activity, is generally considered to consist of two phases; an absolute refractory period (ARP), and a relative refractory period (RRP) (Beach and Hoz-Tucker, 1949). During the ARP, the male rat is insensitive to sexual stimuli, shows little locomotion and appears to be

sleeping. The RRP follows during which arousal may occur, this being dependent upon the novelty of the partner or the presence of other stimuli (Sachs and Meisel, 1988; Toates and Archer, 1978). Male rats additionally vocalize at 22kHz beginning shortly after ejaculation and this activity continues for 50-75% of the postejaculatory interval (Barfield and Geyer, 1975). This vocalizatiion is a prominent feature of the male's postejaculatory behavior, but its function, if there is one, has not yet been determined. Because sexual behavior is very complex, it is difficult to investigate the precise neural events that are involved in the regulation of sexual behavior.

Studies investigating the neuroendocrine control of masculine sexual behavior have demonstrated that the medial preoptic area of the rostral hypothalamus forms an interface between the endocrine secretions of the testes and the neural substraits mediating sexual behavior (Sachs and Meisel, 1988; Hansen and Drake, 1984). Edwards and Einhorn (1986) found that the preoptic area contains a large number of cells which have a high affinity for radioactively labelled testosterone (T), and implantation of a small amount of T into the MPOA can restore sexual behavior in castrated male rats. These researchers suggested that testosterone may influence male sexual behavior by acting on cells in this region, and that stimulation by circulating T acting on preoptic hormone receptors may elevate sexual motivation (Edwards and Einhorn, 1986). Experiments

investigating the neural control of male copulatory behavior have also focused on forebrain pathways. The MPOA appears to be an integral component of this system (Sachs and Meisel, 1988). Damage to the MPOA produces severe copulatory deficits. The elimination of sexual behavior in male rats is a result of destroying cell bodies intrinsic to the MPOA and the nerve fibers that pass through this region. Electrolytic lesions were compared with the effects of infusions of ibotenic acid, a neurotoxin that selectively destroys nerve cell bodies (Hansen et al., 1982). Both methods eliminated copulatory behavior in a similar percentage of animals. This indicates that the intrinsic neurons of the MPOA are critical for copulatory behavior (Caggiula et al., 1973). Electrolytic lesioning has been used extensively in research involving the MPOA. For example, lesions of the MPOA eliminated copulation in sexually experienced male rats, and males did not even mount a receptive female (Arendash and Gorski, 1983). Large bilateral lesions within this region greatly impair or eliminate copulatory behavior, while electrical stimulation of the MPOA can substantially enhance this behavior (Sachs and Meisel, 1988; Caggiula et al., 1973). In other experiments, lesions of the MPOA, with additional damage extending to the rostral anterior hypothalamus, were shown to further decrease copulatory behavior in rats (Heimer and Larsson, 1966/67). The MPOA and the rostral anterior hypothalamus are closely related. They have efferent

pathways in common and they display similar binding patterns of gonadal steroids. Thus, the neurons from the MPOA and the rostral anterior hypothalamus form a continuum.

Several brain areas are involved in the integration of the response that enables the male rat to perform copulatory behavior. The MPOA appears to be an invaluable integration center for the consummatory state of the animal. Heimer and Larsson (1967) suggested that the MPOA serves as an integrative focus for afferent, particularly olfactory, impulses involved in the arousal and integration of sequential copulatory responses. The olfactory bulbs, which process olfactory cues, have an important role in the neural regulation of copulation. Subsequent damage to the olfactory system produces changes in copulation. Olfactory bulb destruction in experienced rats lengthened their ejaculatory latencies and slightly reduced the number of ejaculations (Beach, 1942; Heimer and Larsson, 1967). Bilateral destruction of the olfactory bulbs increased ejaculatory latency in experienced rats and both intromission and ejaculatory latencies in naive male rats (Bermant and Taylor, 1969). The behavior of anosmic experienced rats is remarkably similar to that exhibited by animals with stria terminalis and basomedial-cortico-medial amygdaloid lesions. Destruction of these structures results in only partial removal of olfactory input to the MPOA since contributions from the ventral pathway and medial forebrain bundle (MFB) remain undisturbed (Giantonio et al., 1970).

Neurons of the MPOA extend not only short distances, into the MFB at preoptic levels, but also send their axons significant distances outside the preoptic regions. They project to the amygdala, ventral tegmental area (Barry et al., 1973), the septum and the central grey matter (Swanson and Cowan, 1975). Millhouse (1969) observed axons of preoptic area neurons to project into the lateral hypothalamus, medial hypothalamus and the periventricular fiber system.

The bed nucleus of the stria terminalis has continuity with the preoptic area dorsally. Many projections from the nucleus of the stria terminalis are not very different from those of the MPOA. The nucleus of the stria terminalis sends axons to terminate in the ventromedial nucleus of the hypothalamus, and these projections were reported to be evenly and lightly scattered in both the internal regions and cell poor capsule (Heimer and Nauta, 1969). The projection of both the stria terminalis and its bed nucleus to the accessory olfactory bulb is of interest, particularly as the medial amygdaloid region sends efferents to and receives afferents from the accessory bulb. The bed nucleus of the stria terminalis is a redundant synaptic relay for fibers of the stria terminalis itself. It is a nuclear region in its own right, which on the basis of its efferents are similar to the preoptic area (Conrad and Pfaff, 1976). Also, lesions in the stria terminalis and, to a lesser extent, those in the basomedial-cortico-medial amygdala,

which ultimately send impulses to the MPOA, produced an increase in ejaculation latencies and a slight decrease in number of ejaculations to exhaustion (Heimer and Larsson, 1967).

The MPOA sends primary inputs to the medial forebrain bundle. The MFB processes the neural transmissions and sends them to the tegmental area. Bilateral lesions in the MFB just caudal to the preoptic area virtually abolished mating behavior in male rats without apparent effects upon neuroendocrine function or energy balance (Hitt et al., 1973). Hitt et al. (1970) also reported that masculine sexual behavior was effectively eliminated by large and parafornical MFB lesions, suggesting that the MFB is part of a system which is necessary for the expression of masculine sexual behavior in the rat. The primary effect of the MFB lesion was to inhibit the animal's ability to initiate the mating sequence during a given session. MFB lesions either just caudal to the preoptic area or at the level of the premammilary nuclei both produced disruption of masculine behavior. This is consistant with the notion that the role of the MFB in the mediation of male sexual behavior is that of a projection pathway connecting androgen-sensitive units to other motivational or motor systems (Hitt et al., 1973). Other lesion studies of the MFB at the level of or posterior to (but not anterior to) the MPOA abolish male sexual behavior (Brackett and Edwards, 1984).

Bilateral lesions of the dorsolateral tegmentum, placed to destroy one region of the midbrain tegmentum which receives direct projections from the cells in the MPOA, also eliminated sexual behavior (Brackett and Edwards, 1984). Also, in this study they reported that a unilateral preoptic lesion combined with a dorsolateral tegmental lesion on the contralateral side of the brain also eliminated male sexual behavior. Since this combination of lesions is only effective in abolishing male sexual activity when the separate manipulations are performed on opposite hemispheres, the result is not due to the additive effect of a unilateral preoptic lesion and a unilateral preoptic tegmental lesion. Combining a unilateral preoptic lesion with a contralateral tegmental lesion produces common bilateral destruction of neural elements which are part of one of the major connecting pathways between the MPOA and the midbrain tegmentum, which produces sexual behavior deficits (Brackett and Edwards, 1984). Both the MPOA and the dorsolateral tegmentum are integratedly involved to control the display of male sexual behavior. Brackett and Edwards (1984) concluded that this integration is via axons which originate in the MPOA and pass laterally into the MFB to descend to the tegmentum. Sagittal preoptic knife cuts which sever the lateral connections between the preoptic area and the MFB impair the initiation of male sexual behavior (Szechtman et al., 1978).

The medial preoptic-anterior hypothalamic (MPOAH) neurons respond to electrical stimulation of the lateral portion of the fimbria (Kendrick, 1982). The pathway for this response was identified as the amygdala/stria terminalis system. The majority of MPOAH neurons which responded to ipsilateral fimbria stimulation had outputs to or received projections from the MFB. Half the MPOAH neurons which are initially excited by the ipsilateral fimbria stimulation project directly into the MFB, thus this pathway via the cortico-medial amygdala to the MPOAH is involved in control of sexual behavior in the male rat. It is also significant that a quarter of the MPOAH neurons which respond to ipsilateral fimbria stimulation receive projections from the MFB, since the ascending MFB input to the MPOAH may be important for inhibitory influences on sexual behavior in the male rat (Kendrick, 1982).

The nucleus accumbens and its dopaminergic innervation plays a role in adaptive behaviors. This brain area has been regarded as a functional interface between the forebrain structures assessing motivational status, such as the hypothalamus and limbic areas, and the motor systems. Studies have indicated that sexual behavior increases the extracellular concentrations of dopamine (DA) in the nucleus accumbens and to a lesser extent the striatum of male rats (Mas et al., 1990; Pfaus et al., 1990). In an in vivo study, Ahlenius et al., (1987), found an accumulation of

Dihydroxyphenyl-alanine (DOPA), a DA precursor, in the NAc during sexual activity and treadmill locomotion.

Regarding male copulatory activity, research indicates that information about the animal's endocrine state, external environment (including the presence of an estrous female) and previous experience, is integrated in the MPOA and conveyed to the ventral tegmental area. The subsequent activation of the dopaminergic neurons innervating the NAc would enable the access of information to the motor system.

Integration of input into the forebrain areas results in the release of neurotransmitters. The specific neurotransmitters released function to induce both neural and physiologic changes. Central dopaminergic systems have been reported to play an important role in the mediation of male sexual behavior. It has been shown that dopamine receptor agonists enhance copulatory behavior (Britran and Hull, 1987; Sachs and Meisel, 1988), whereas dopamine receptor antagonists disrupt or eliminate sexual activity (Pfaus and Phillips, 1989/1991). These dopamine receptor agonists/antagonists were directly infused into the MPOA. The MPOA plays a large role in the central dopaminergic systems. Microinjections of the dopamine antagonist cis-flupenthixol into the MPOA reduces male sexual behavior, whereas copulation, penile reflexes, and seminal emission are facilitated by microinjections of dopamine receptor agonists (Hull et al., 1989; Warner et al., 1991). In vitro data collected by Mas et al., (1987), also suggested that

copulatory behavior increased dopamine activity in the MPOA. The results indicated that dopamine levels increased when a male was exposed to a receptive female compared to another male.

Dopaminergic transmission in the nucleus accumbens was investigated in male rats exposed to sociosexual olfactory stimuli using a voltammetric approach (Louilot et al., 1991). Exposition to receptive female odors induced a marked and selective increase in DA release compared to control values. Exposition to non-receptive female odors or to male odors produced no significant change. Thus, this study indicated that the mesencephalic dopaminergic neurons reaching the nucleus accumbens appear to be involved in the perception of behaviorally significant olfactory cues.

Pfaus et al. (1990) used in vivo brain microdialysis to determine central DA transmission in the nucleus accumbens and striatum of sexually experienced male rats during mating behavior. Their results indicated an increase in DA transmission in both the nucleus accumbens and the striatum of sexually experienced male rats during various phases of sexual behavior. Similar increases were seen in homovanillic acid (HVA), a metabolite of dopamine.

Immunocytochemical visualization of the nuclear products of different immediate-early genes, such as c-fos, has provided useful information about the neural pathways which are activated in response to a variety of peripheral, sensory, as well as internal, homeostatic stimuli.

Increases in dopamine neurotransmission in the MPOA, nucleus accumbens and striatum, may lead to increases in c-fos expression in these and other forebrain regions (Robertson et al., 1991). Baum and Everitt (1992) reported that increased amounts of physical contact with a female caused corresponding increments in c-fos expression in the MPOA, the caudal part of the bed nucleus of the stria terminalis, the medial amygdala, and the midbrain central tegmental field. This suggests that afferent inputs from the central tegmental field and from the medial amygdala interact to promote cellular activity, and the resultant induction of c-fos, in the ipsilateral bed nucleus of the stria terminalis and the MPOA.

Since the hypothalamus forms an interface between the neural substrait mediating sexual behavior and the endocrine secretions of the testes, the assessment of catecholamines distinct to this area would provide insight to higher brain integration. To investigate the patterns of catecholamine release in the brain, the posterior hypothalamus of conscious, freely moving rats was superfused through a push-pull cannula with artificial cerebrospinal fluid and the catecholamines; DA, norepinephrine (NE), and epinephrine (EPI), were determined in the superfusate radioenzymatically (Dietl et al., 1993). They reported that the concentrations of DA, NE, and EPI are different over a 20h basal period in the rat posterior hypothalamus and correspond to the catecholaminergic innervation of this brain region. Their

findings suggest different turnover rates of the three catecholamines in the posterior hypothalamus of the conscious rat (Dietl et al., 1993). Another interesting find was the similarity observed in the fluctuations between EPI and gamma-aminobutyric acid (GABA) in the posterior hypothalamus. Dietl suggested that, at least partially, NE and GABA, and perhaps EPI and GABA, are co-released from nerve terminals of the posterior hypothalamus. These results demonstrated the patterns of some catecholamines released in the hypothalamus of freely moving adult male rats.

NE release in the MPOA has been reported to contribute to the regulation of male sexual behavior. Clark et al. (1985) used Yohimbine systemically, which preferentially blocks alpha$_2$-adrenoreceptors, to demonstrate the effects of NE on male sexual behavior. The data suggested that the blockade, resulting in increased excititory (and/or decreased inhibitory) adrenergic activity, resulted in an increased state of sexual arousal in the intact male. In a subsequent study, Clark et al. (1985) used another pharmaceutical systemically, Clonidine, which stimulates the alpha$_2$-adrenoreceptors. Results of this study indicated that the stimulation of the alpha$_2$- adrenoreceptors resulted in diminished sexual motivation. In an intracranial study, Clark (1991) infused either Yohimbine or Clonidine through a cannula implanted into the MPOA. The pharmacuticals evoked dose-dependent increments or decrements respectively in the

number of mounts, intromissions, and ejaculations attained by the adult male rats tested.

A variety of stimuli, including those from the external environment as well as the animal's internal physiologic state, are transmitted through the forebrain areas. The main olfactory bulbs and the accessory bulbs are the initial interpreters of olfactory cues. Impulses travel to the amygdala and then project to the bed nucleus of the stria terminalis. The information is then integrated at the medial preoptic area, and hypothalamus. This integration provides for the motivational aspects of the animal. The information then projects to the ventral tegmental area and is relayed to the nucleus accumbens. The nucleus accumbens forms the interface for assessing the animals motivation and motor systems that permit the animal to execute behavior.

The integration of information is accomplished by neural transmission. The catecholamines released in the specific brain areas provide a conveyence of this information. The information is processed, integrated and finally displayed as behavior.

The present study examined the release of neurotransmitters in the male rat brain, specifically the MPOA-DBB and the NAc, quantified by in vivo microdialysis. The study investigated the release of neurotransmitters in sexually experienced males during sexual behavior.

CHAPTER II

MATERIALS AND METHODS

Animals: Adult male and female Long-Evans rats were bred from an existing colony at Youngstown State University from rats originally purchased from Charles River Laboratories, N.Y. Animals were housed by sex in polypropylene cages (19in x 10.5in x 8in) with stainless steel lids. The colony room was maintained at approximately 21 degrees Celcius on a reversed 12h light/12h dark cycle (lights off at 1000h). Food and water were available ad libitum in the home cages. After implantation of the guide cannula, (see description below), males were housed singly.

Males were given three trials of sexual behavior at five day intervals prior to the microdialysis experiment. By the end of the training phase, 19 males included in this study exhibited consistant parameters of sexual behavior, including ejaculation within 10 min of the first intromission, and reinitiation of copulation within 15 min after the first ejaculation.

Microdialysis Probes and Guide Cannulae: Microdialysis probes were constructed with modifications according to methods described by Yamamoto (personal communication). A diagram of a constructed microdialysis probe is shown in figure 1. The development of the brain microdialysis technique has made it possible to study the chemical composition of the interstitial fluid in discrete brain regions of awake, freely moving animals (Johnson and

Justine, 1983; Ungerstedt, 1984). The use and validation of brain microdialysis to monitor neurotransmitters and metabolites has been documented extensively (Imperato and DiChiara, 1984; Johnson and Justine, 1983; Ungerstedt, 1984; Westerink et al., 1987; Zetterstrom, 1986).

Preparation of Materials: Step One (Day 1): Stainless steel tubing (Small Parts, Inc.) 26 gauge was cut to a final length of 23mm. The ends were smoothed, and one end was roughened with emory cloth. The stainless steel tubing (SST) was sonicated in 95% EtOH for 5 min. and heat dried.

PE 20 tubing (Clay Adams) was cut to a 5cm length and half its length was roughened with emory cloth.

A 30 gauge syringe needle was inserted into the roughened end of the PE 20 tubing and the side of the tubing was punctured approximately 5mm down the tubing.

A piece of silica tubing (Polymicro Technologies) was cut to 5cm, and the ends must be free of burrs. The silica tubing was sonicated in 95% EtOH.

With the needle still inserted in the PE 20 tubing, the silica tubing was threaded through the needle and into the PE 20 tubing. The syringe was then removed.

The roughened end of the 26 gauge SST was inserted into the roughened end of the PE 20 tubing so that 1.9cm of the SST remained exposed.

A piece of dialysis membrane (Spectrum) was cut to 1cm and insert 25% of the way (2.5mm) into the non-roughened end of the 26 gauge tube.

This junction was sealed with "tacky" 2-ton epoxy (Devcon). The dialysis tubing was pushed in slightly to ensure a tight seal. The junction between the PE 20 and the 26 gauge SST was glued at the same time.

Step Two (Day 1): The end of the dialysis tube was cut at 2.25mm, and the cut end was plugged with "tacky" 2-ton epoxy (0.25mm) and allowed to dry over night.

Step Three (Day 2): The silica tubing was positioned inside the dialysis membrane approximately 0.25mm from the epoxied end. The tubing was sealed in position with very tacky 2-ton epoxy and allowed to dry for 4 hours.

Step Four: The excess silica tubing was cut 1cm from the glued junction. The silica tubing was then threaded through a 4.5cm long Medline tubing (Micro-Line) and roughened about 2cm at one end. The junction of the Medline tubing and the PE 20 tubing was joined with 2-ton epoxy and allowed to dry.

The end of a 1cc syringe was cut to the 0.1cc mark. The uncut end was covered with masking tape. A hole was made in the center of the tape large enough to insert the SST portion of the probe so that 18mm of SST extended beyond the taped end. The syringe was filled from the cut end with 2-ton epoxy and allowed to dry for 4-8 hours.

Two types of connectors were made. The first connector was constructed from 26 gauge SST at a final length of 10mm and placed into the PE 20 line of the probe. The second connector was constructed from 28 gauge SST at a final length of 10mm which was placed into the Medline tubing of

the probe. Both connectors were cut, smoothed, and sonicated as in step one.

Peristaltic pump tubing was connected to the PE 20 end, and flushed through with filtered deionized water (d-H$_2$O) while the probe was immersed in 70% EtOH for 30 min. at a flow rate of 0.5ul/min. This was followed by flushing with filtered d-H$_2$O in d-H$_2$O for 24 hours at a flow rate of 0.5ul/min. The probes were stored and refrigerated in d-H$_2$O. Once the membrane is hydrated it cannot be permitted to dry.

Guide Cannulae: A 21 gauge SS needle was cut and smoothed so that the final length extended 8mm. This served as the guide cannula. The tip from a 1cc syringe was cut at the 0.1cc mark and tape was placed over the uncut end of the syringe tip.

A stylet obturator, a 26 gauge SS wire, was pushed through the cut syringe and glued with 2-ton epoxy, allowing 4 hours to dry. The new stylet was placed into the guide cannula, and the exposed portion of the 26 gauge SS wire was cut even with the end of the 21 gauge SS needle.

Surgical Procedures: Four females were anaesthetized with Xylazine (4mg/Kg) and Ketamine (50mg/Kg) and ovariectomized, via lumbar incisions. These ovariectomized females were used as control stimulus females during the experiments.

Sexually experienced male rats were anaesthetized with Xylazine (4mg/Kg) and Ketamine (50mg/Kg) and implanted stereotaxically with a vertically oriented guide cannulae

directed toward the MPOA-DBB and nucleus accumbens at 7.8mm anterior to bregma, 0.8mm lateral to midline and 6.8mm below the surface of the brain according to the atlas of Pellegrino et al. (1979). Each guide cannula was secured with dental cement to 3 anchoring skull screws and the skin was sutured.

In Vitro tests: The microdialysis probes were tested in vitro for percent recovery of catecholamines prior to use in in vivo experiments. Peristaltic pumps were used with Fisher-brand Pump Tubing (size=0.010cm). The flow rate was adjusted to 2.5ul/min. The pump tubing was plugged into the inlet line of the microdialysis probe. The outlet medline tube was attached to the probe to collect the dialysate. A modified Ringer's solution was prepared as follows: Stock molar solutions of NaCl, $CaCl_2$, and KCl were prepared. The working salt solution was prepared from the molar stock solutions as follows: the following amounts of each stock solution were placed in a 100ml volumetric flask and the final volume was adjusted to 100ml using $d-H_2O$. $CaCl_2$=0.23ml; NaCl=14.7ml; KCl=0.4ml. The pH was adjusted to 7.4-7.6 using 0.1N HCl or NaOH. This working salt solution was used as the perfusion solution for the microdialysis probes during the in vitro tests. The dialysis membrane of the probe was immersed in the same modified Ringer's solution described above with the addition of the catecholamines. The final concentration of catecholamines in the modified Ringer's solution were as

follows: DOPA=94.8pg/ul; NE=109.0pg/ul; EPI=96.8pg/ul; DA=96.6pg/ul; and HVA=97.0pg/ul. A 25ml beaker was filled with the Ringer's solution containing the catecholamines and placed in a water bath which was maintained at 37 degrees Celcius. The water bath was covered to prevent light from breaking down the catecholamines. The probes were held in a modified plastic petri dish, equiped with three holes, so that the dialysis bags were immersed into the catecholamine mixture. The probes were covered with aluminum foil to shield the catecholamine mixture from light. The outlet medline tube from the probe was placed into a microvial (100ul) located outside of the water bath. The perfusion solution was pumped through the probes for an equilibration period of one hour. Samples were then taken every 20 min (about 4 samples total for each probe). The samples (microvials) were frozen (-20 degrees Celcius) until analyzed by HPLC. Once the samples had been analyzed by HPLC the percent recovery could be obtained for each probe. The probes with a percent recovery between 5-12% were used for in vivo testing. The data was corrected for the percent recovery.

Preliminary experiment: This experiment validated the use of in vivo microdialysis probes. Six adult males were implanted with guide cannulae stereotaxically directed toward the Corpus Striatum at 7.8mm anterior to bregma, 2.5mm lateral to midline and 2.5mm below the surface of the brain. After six baseline control samples, the males were

perfused with modified Ringer's solution containing either 60mM KCl or 120mM KCl.

Experimental procedure: In the present study, the sexually experienced males that were cannulated were given at least seven days to recover from the neurosurgery.

Day One: The males were then lightly anaesthetized with metophane and the microdialysis probe was inserted at 1000 hours. This was done prior to the experimental day to allow for the equilibration of the dialysis bag in the brain area. The implanted male was then transfered to a glass observation chamber (30cm X 26cm X 50cm). The light in the testing room was synchronized with the colony room light cycle. (Modified Ringer's solutions were made fresh the day of implantation.)

Day Two: A single channel liquid swivel with a tether system was secured to a ring stand. At 0930 hours the implanted males were lightly anaesthetized with metophane and the inlet and outlet lines were connected to the microdialysis probe. A protective wire spring, (42cm X 4.6mm I.D. X 5.5mm O.D.), was then placed over the two tubes down to the top of the microdialysis probe. The inlet line was then placed through the tether and connected to the liquid swivel, which was connected to a peristaltic pump. A microvial was placed at the end of the oulet line to collect the dialysate. The modified Ringer's solution was perfused through the probe (2.5ul/min) at 1000 hours for a one hour equilibration period. At 1100 hours baseline samples were

taken every 20 min. At 1300 hours an estrous or ovariectomized female rat was placed with the male. Sexual receptivity was induced in the female rats by injections of 50ug/.1ml estradiol benzoate 48 h before, and 500ug/.1ml progesterone 4 h before each test. While sampling every 20 minutes, the male was permitted to have access to the female until 1500 hours. The samples were frozen until analysis by HPLC. Observations of sexual behavior were observered with the estrous females.

The same experimental procedure was applied to all implanted males. The first ten males were tested with either an estrous female or with a ovariectomized female. These males were then tested with the opposite female usually seven days later.

Perfusion and Histological Brain Sectioning: At the conclusion of the experiments, the males were deeply anaesthetized with Xylazine (4mg/Kg) and Ketamine (50mg/Kg). Each male was then perfused intracardially with 50cc of normal saline, followed by 50cc of 10% formalin. After perfusion, the rats were decapitated and the guide cannula was carefully removed from the skull. When removed, the brain was stored in 10% formalin for at least 24 h before sectioning.

The anterior and the posterior ends of the brain were blocked with a single edged razor blade at the same 5mm elevation that the rat was positioned stereotaxically. The brain was then placed on the stage of the Cryo-Histomat.

Drops of distilled water were placed on the brain to secure it to the stage. When the brain was frozen, sections were taken at a thickness of 65 microns. The sections were placed in numbered cubical trays filled with 20% EtOH. The sections were placed on albuminized slides, usually 3 sections per slide, in the order in which they were taken. The slides were placed on a slide warmer set at 40 degrees Celcius. When the slides were dry, they were placed in 70% EtOH for 24 hours. The sections were then stained via the following staining procedure.

1) 20% EtOH-3min	10) distilled water-3min
2) distilled water-3min	11) 70% EtOH-3min
3) distilled water-3min	12) 95% EtOH-3min
4) distilled water-3min	13) 100% EtOH-3min
5) basic Fuchsin-1.5min	14) 100% EtOH-3min
6) distilled water-5sec	15) xylene-5min
7) distilled water-3min	16) xylene-5min
8) acid formalin-5sec	17) cover with permount and
9) distilled water-5sec	a 50mm cover slip

The slides were viewed under 10X power to verify cannula placement. Histologically the intact male rats were divided into the nucleus accumbens group and the MPOA-diagonal band of Broca group.

DATA ANALYSIS: Differences between basal catecholamine levels during the control period and stimulated levels during the experimental period were analyzed by paired t-tests. Values during the control and experimental periods were averaged for each rat and used to calculate the percent change in neurotransmitter levels. The mean percent change

± S.E.M. was then calculated for each group and the differences between groups were analyzed by unpaired t-tests.

Figure 1. This figure depicts a diagram of the microdialysis Probe. See text for full explanation.
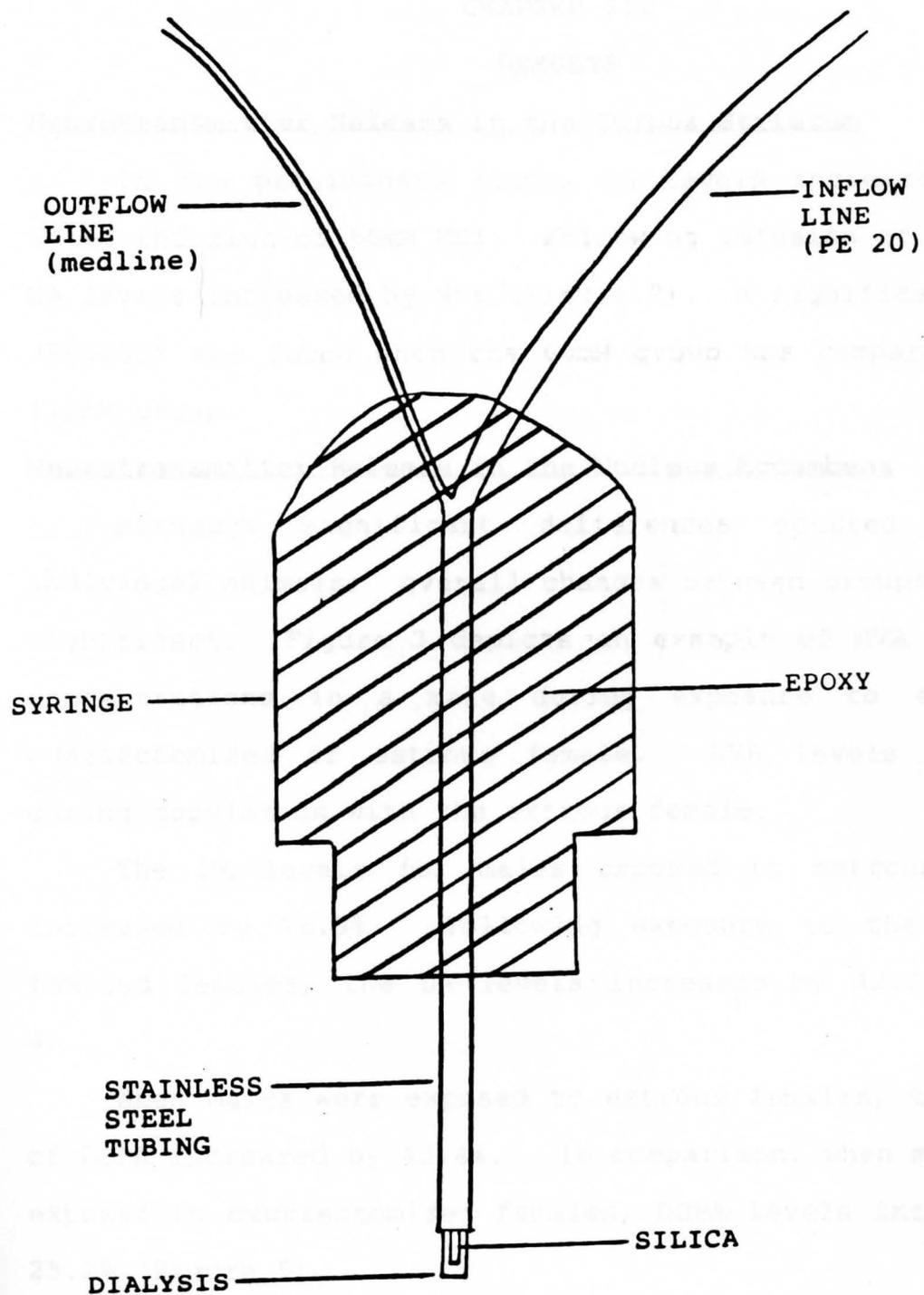
FIGURE-1

CHAPTER III

RESULTS

Neurotransmitter Release in the Corpus Striatum

In the preliminary study, DA levels increased by 23% after infusion of 60mM KCl. Following infusion of 120mM KCl DA levels increased by 90% (Figure 2). A significant change (P<0.02) was found when the 60mM group was compared to the 120mM group.

Neurotransmitter Release in the Nucleus Accumbens

Although significant differences occured in some individual animals, overall changes between groups were not significant. Figure 3 depicts an example of HVA dialysate concentrations in a male during exposure to either an ovariectomized or estrous female. HVA levels increased during copulation with the estrous female.

The DA levels for males exposed to estrous females increased by 76.9%. Following exposure to the ovariectomized females, the DA levels increased by 42.2% (Figure 4).

When males were exposed to estrous females, the levels of DOPA increased by 43.4%. In comparison, when males were exposed to ovariectomized females, DOPA levels increased by 25.2% (Figure 5).

HVA levels for males exposed to estrous females increased by 112.3%. However, when the males were exposed to ovariectomized females the levels of HVA increased by 148.6% (Figure 6).

As expected, there were only trace amounts of NE and EPI found in the NAc. Therefore these neurotransmitters were unable to be analyzed.

Neurotransmitter Release in the MPOA-DBB

In dialysate samples obtained from individual animals, significant differences were found for some neurotransmitters. However, overall changes between groups were not significant. Figure 7 depicts an example of DA dialysate concentrations in a male exposed to either an ovariectomized or an estrous female. The levels of DA were elevated upon exposure to the estrous female.

NE levels in males exposed to estrous females increased by 123.4%. When the levels of NE were quantitated in males exposed to ovariectomized females, NE levels decreased by -22.3% (Figure 8).

The levels of EPI increased by 122.5% when males were exposed to estrous females. In contrast, when the males were exposed to ovariectomized females, the levels of EPI decreased by -36.1% (Figure 9).

HVA levels for males exposed to estrous females increased by 165.5%. In comparison, HVA levels decreased by -2.5% in males exposed to ovariectomized females, (Figure 10).

When the males were exposed to estrous females, the levels of DOPA were increased by 54.6%. This is compared to DOPA levels when males were exposed to ovariectomized females which increased by 26.5% (Figure 11).

DA levels in this brain area were similar for males exposed to either estrous (56.3%) or ovariectomized females (59.8%) (Figure 12).

Figure 2.   Mean percent change in DA levels in rats treated
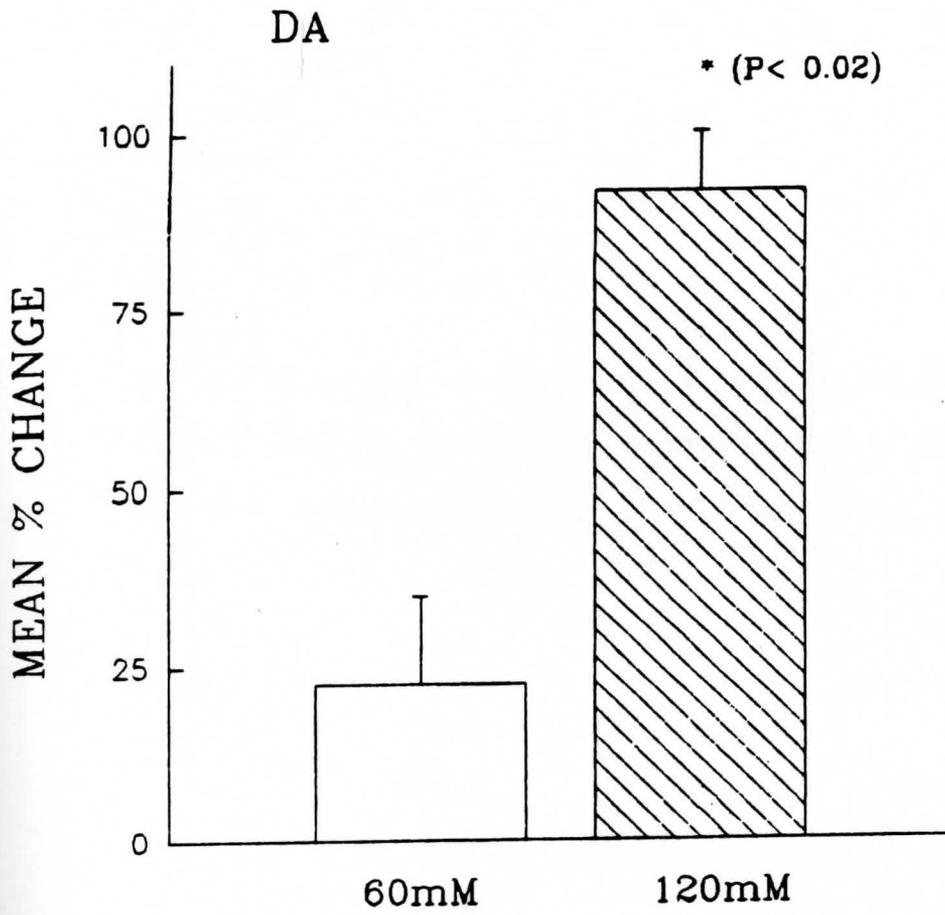with 60mM (n=3) or 120mM (n=3) KCl.   Values given are the
mean ± S.E.M.

Figure 3. HVA changes in a male when exposed to an ovariectomized (OVX) or an estrous female at 20 min sample periods. Time zero represents when the female was presented to the male.
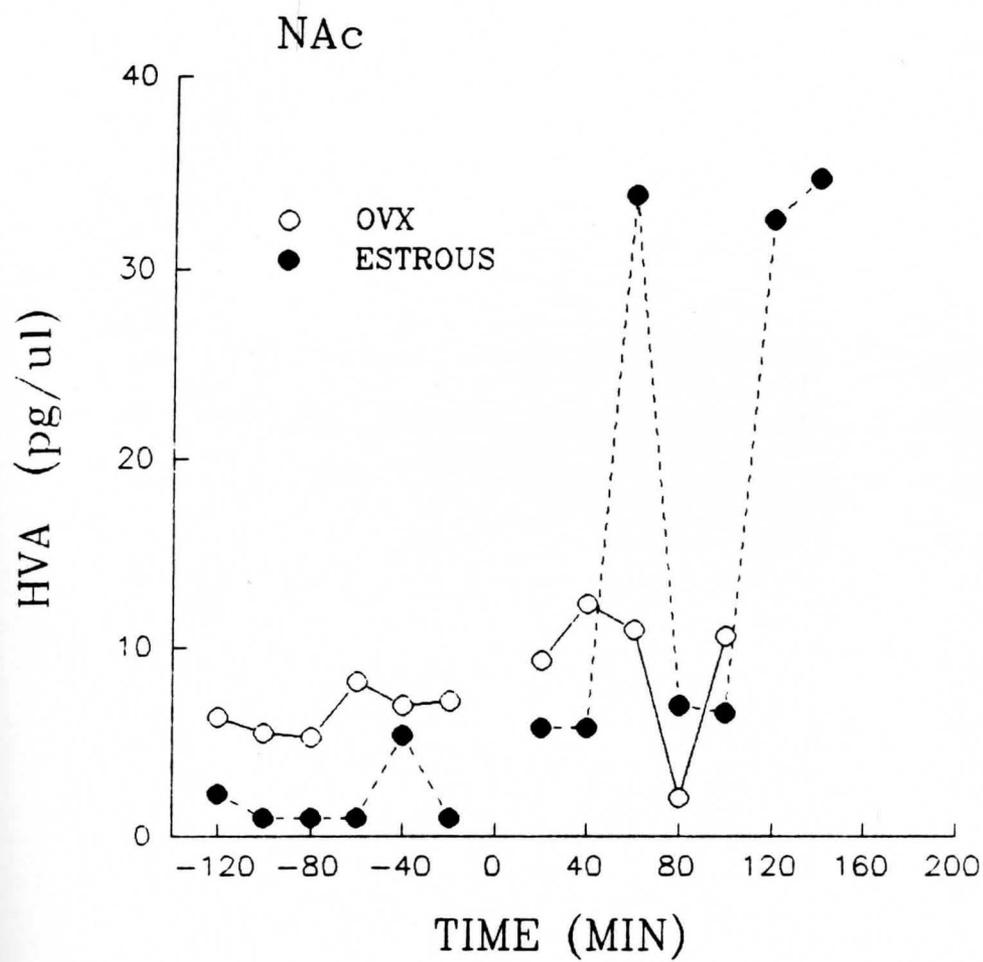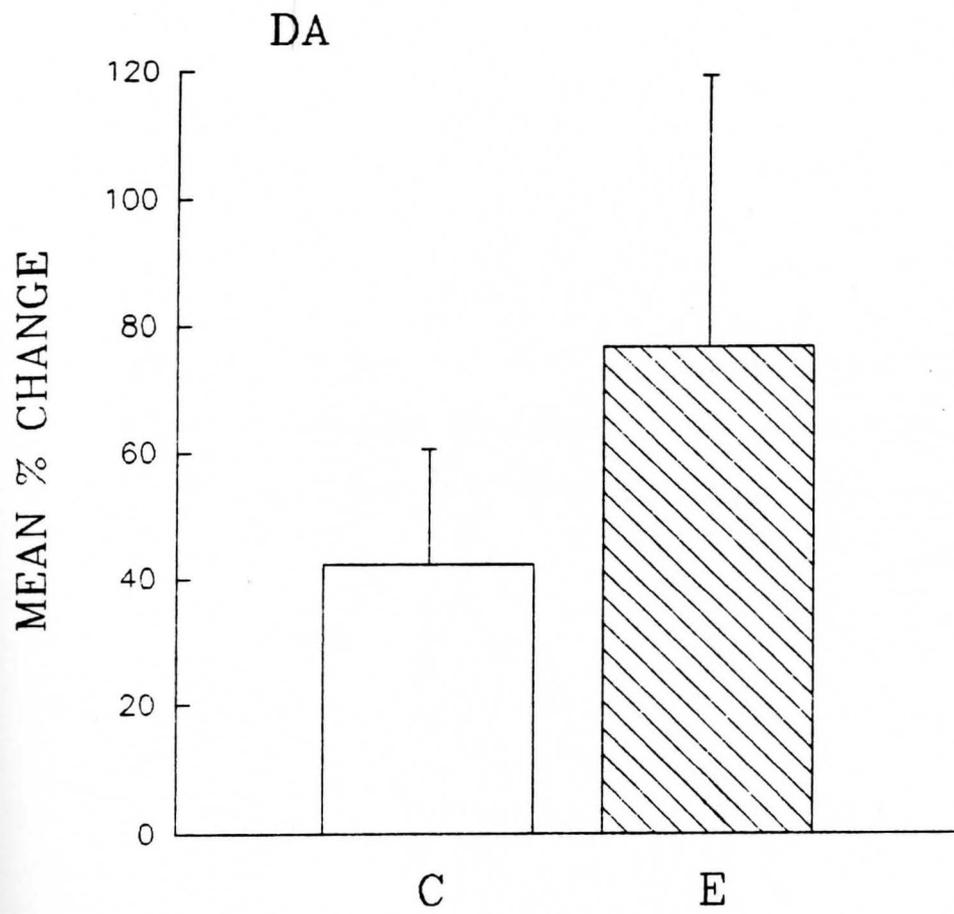
Figure 4. Mean percent change in DA levels in the NAc after males were exposed to ovariectomized females (C, n=8) or allowed to copulate with estrous females (E, n=6). Values are the mean ± S.E.M.
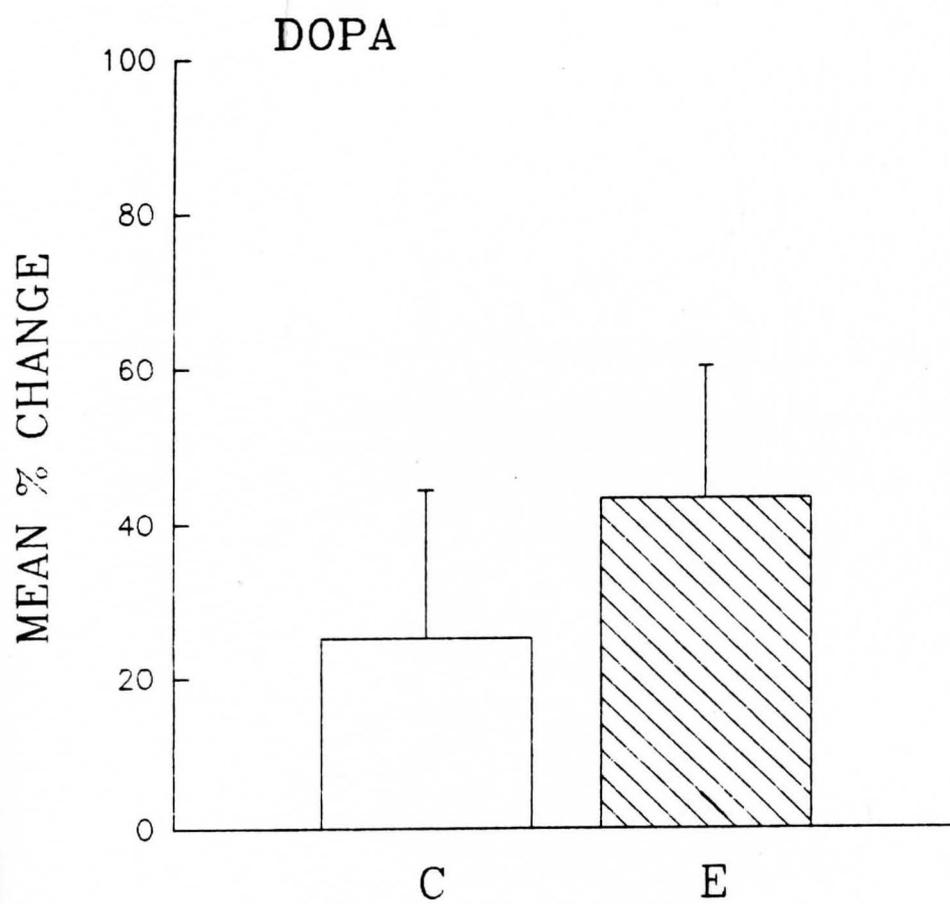
DA

Figure 5. Mean percent change in DOPA levels in the NAc after males were exposed to ovariectomized females (C, n=5) or allowed to copulate with estrous females (E, n=5). Values are the mean ± S.E.M.

Figure 6.   Mean percent change in HVA levels in the NAc after males were exposed to ovariectomized females (C, n=8) or allowed to copulate with estrous females (E, n=6). Values are the mean ± S.E.M.
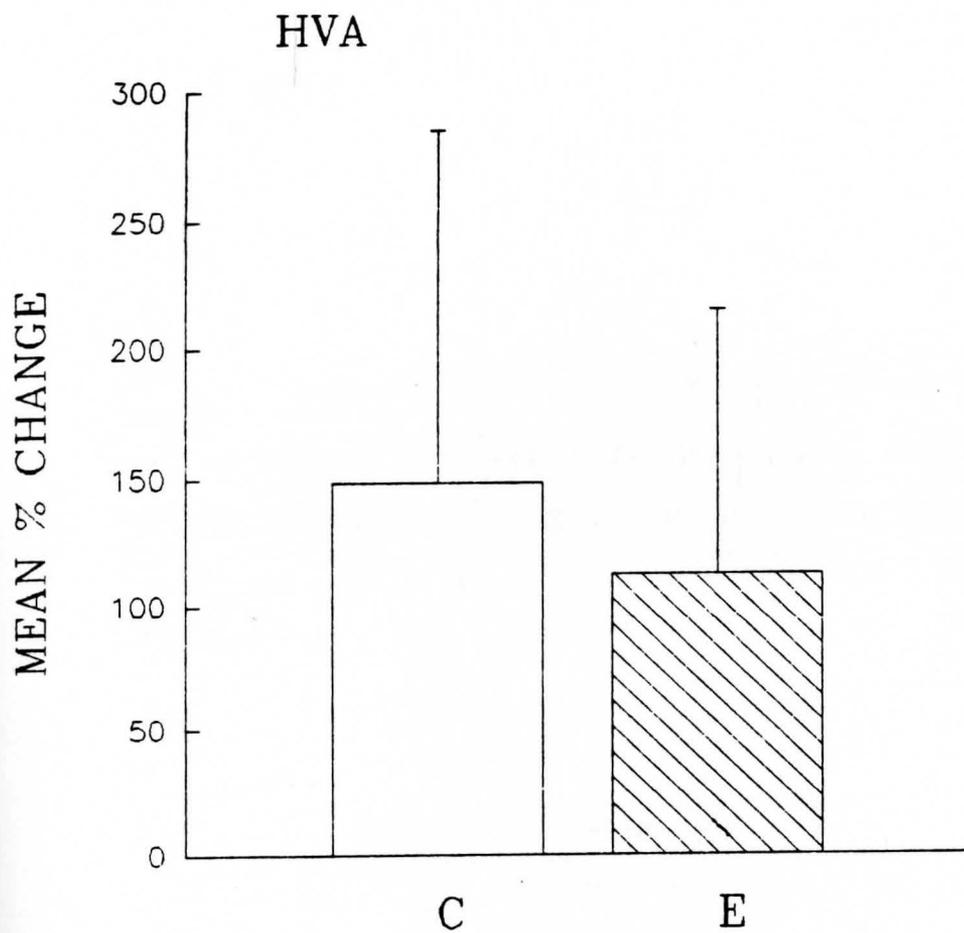
HVA

Figure 7.   DA changes in a male when exposed to an ovariectomized (OVX) or an estrous female at 20 min sample periods.   Time zero represents when the female was presented to the male.
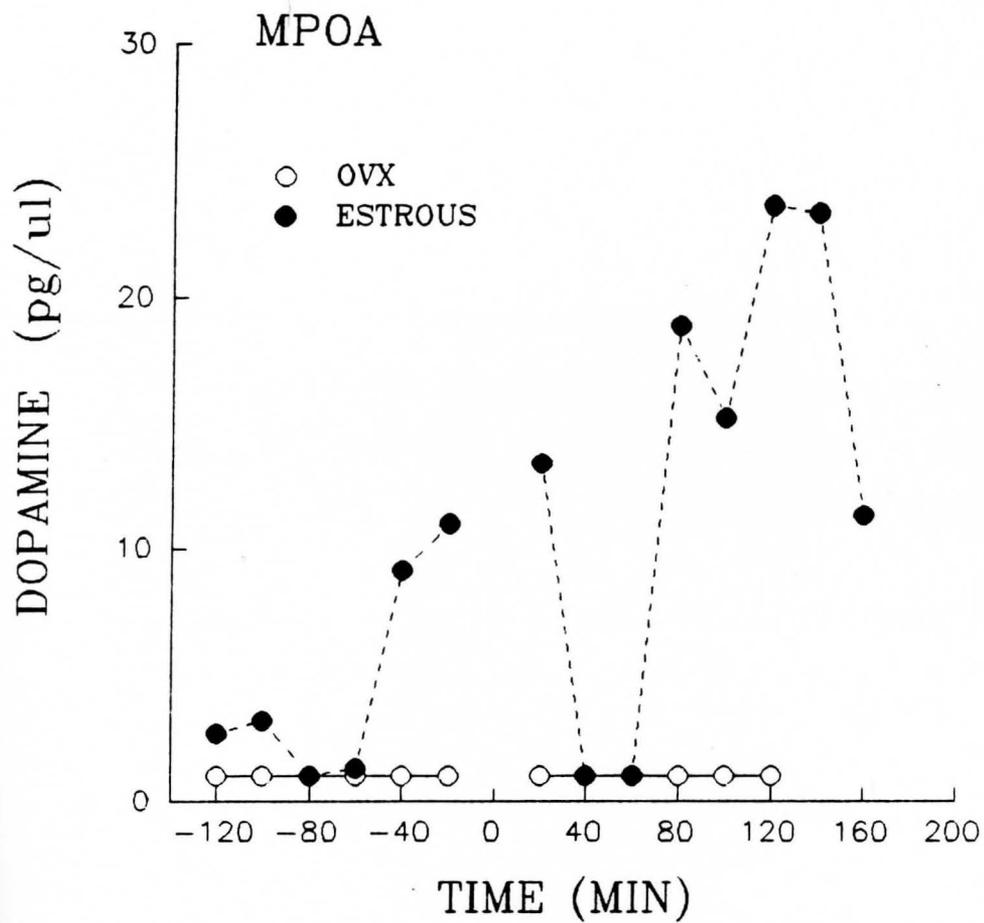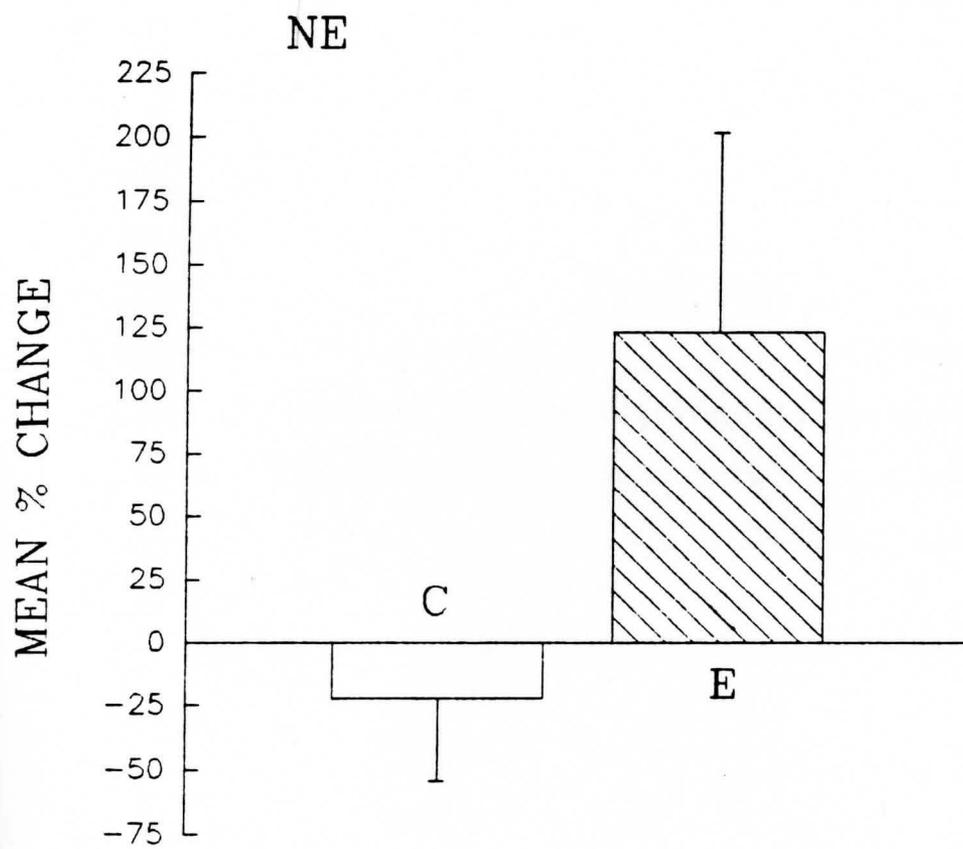
Figure 8. Mean percent change in NE levels in the MPOA-DBB after males were exposed to ovariectomized females (C, n=2) or allowed to copulate with estrous females (E, n=4). Values are the mean ± S.E.M.
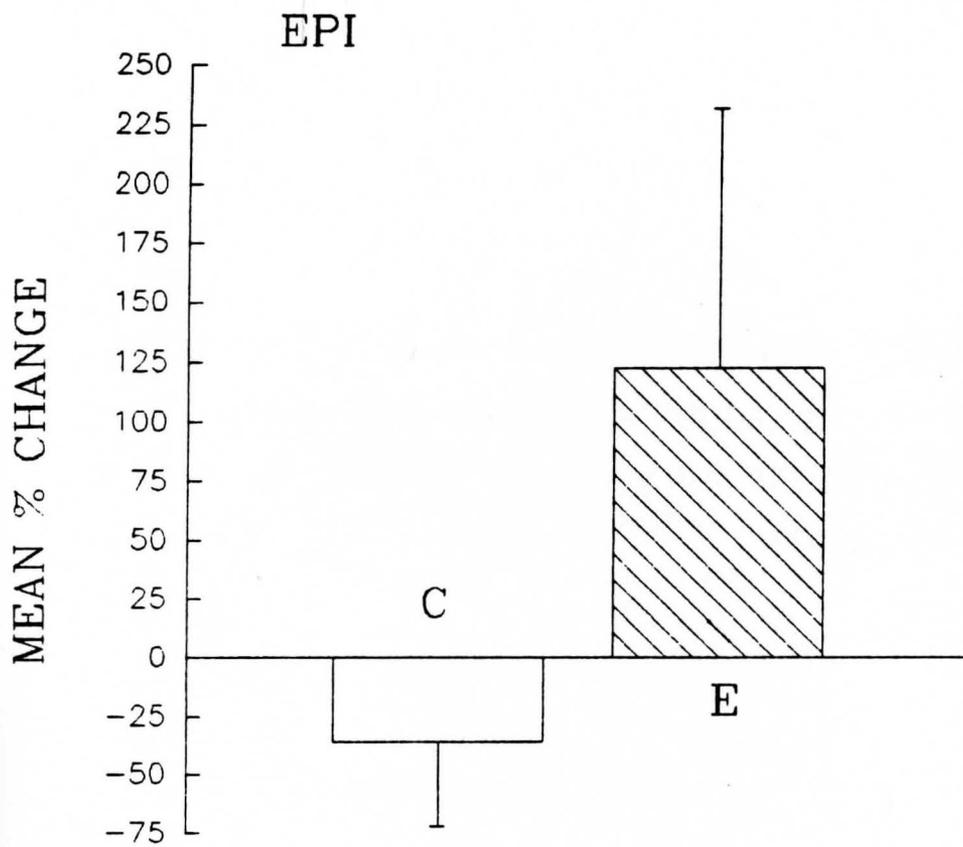
Figure 9. Mean percent change in EPI levels in the MPOA-DBB after males were exposed to ovariectomized females (C, n=2) or allowed to copulate with estrous females (E, n=4). Values are the mean ± S.E.M.

Figure 10. Mean percent change in HVA levels in the MPOA-DBB after males were exposed to ovariectomized females (C, n=2) or allowed to copulate with estrous females (E, n=4). Values are the mean ± S.E.M.
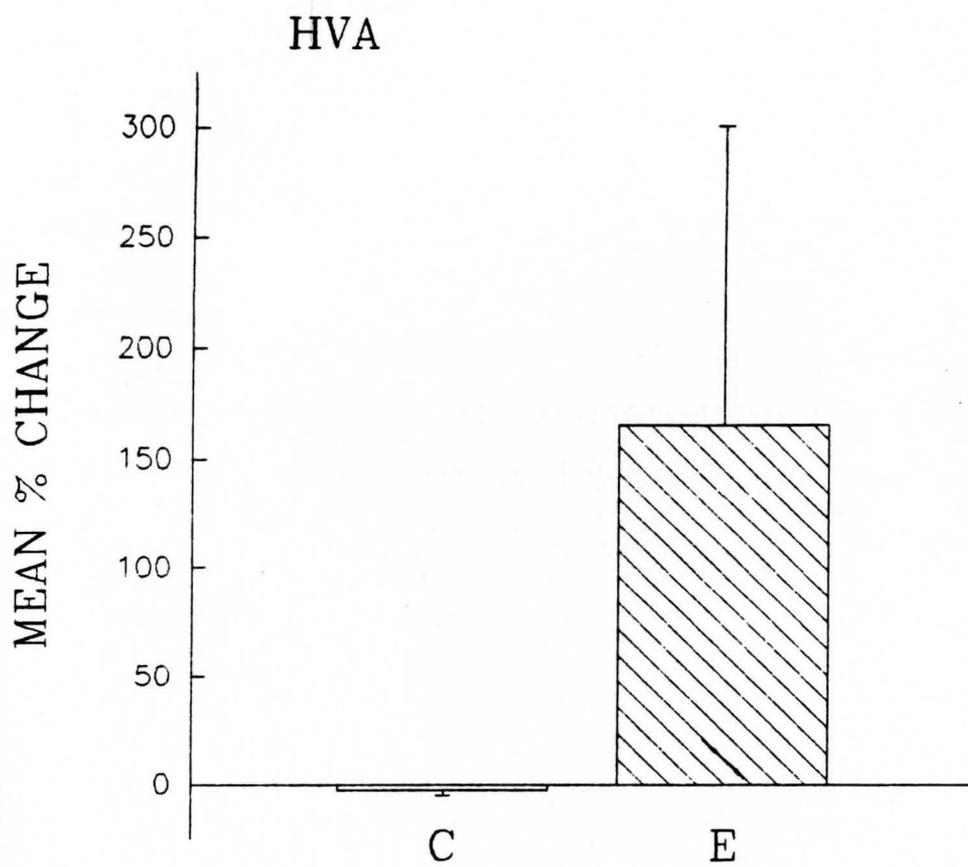
HVA

Figure 11. Mean percent change in DOPA levels in the MPOA-DBB after males were exposed to ovariectomized females (C, n=2) or allowed to copulate with estrous females (E, n=4). Values are the mean ± S.E.M.
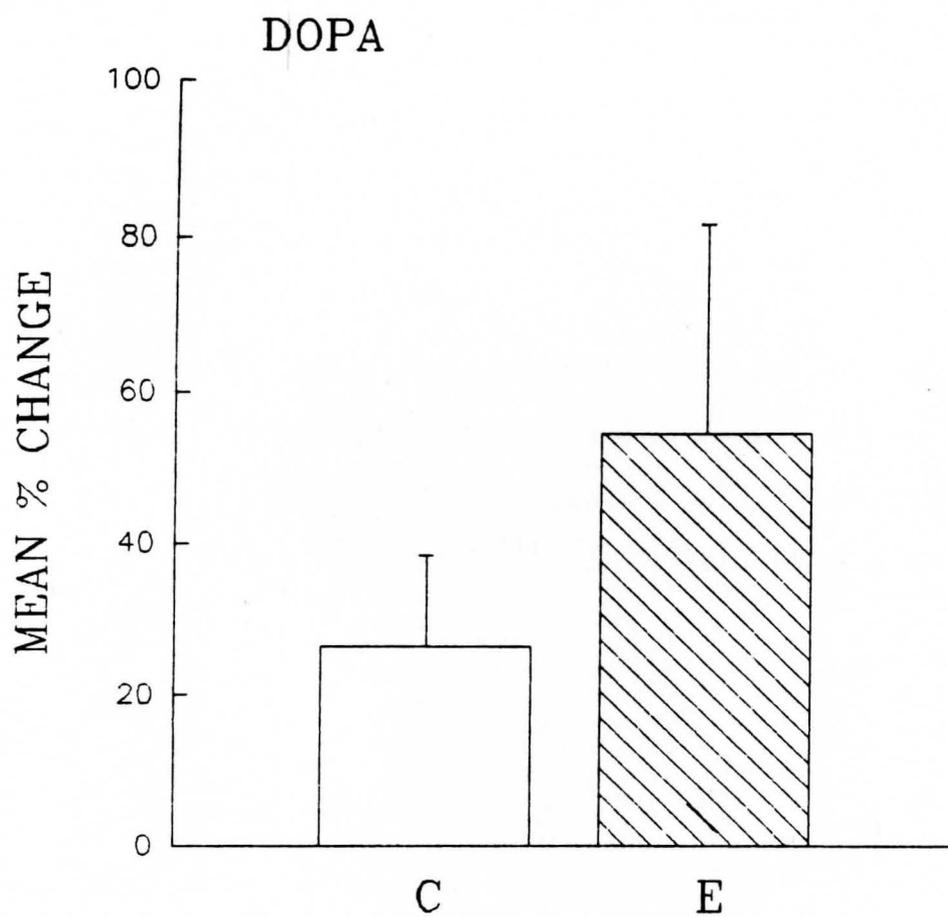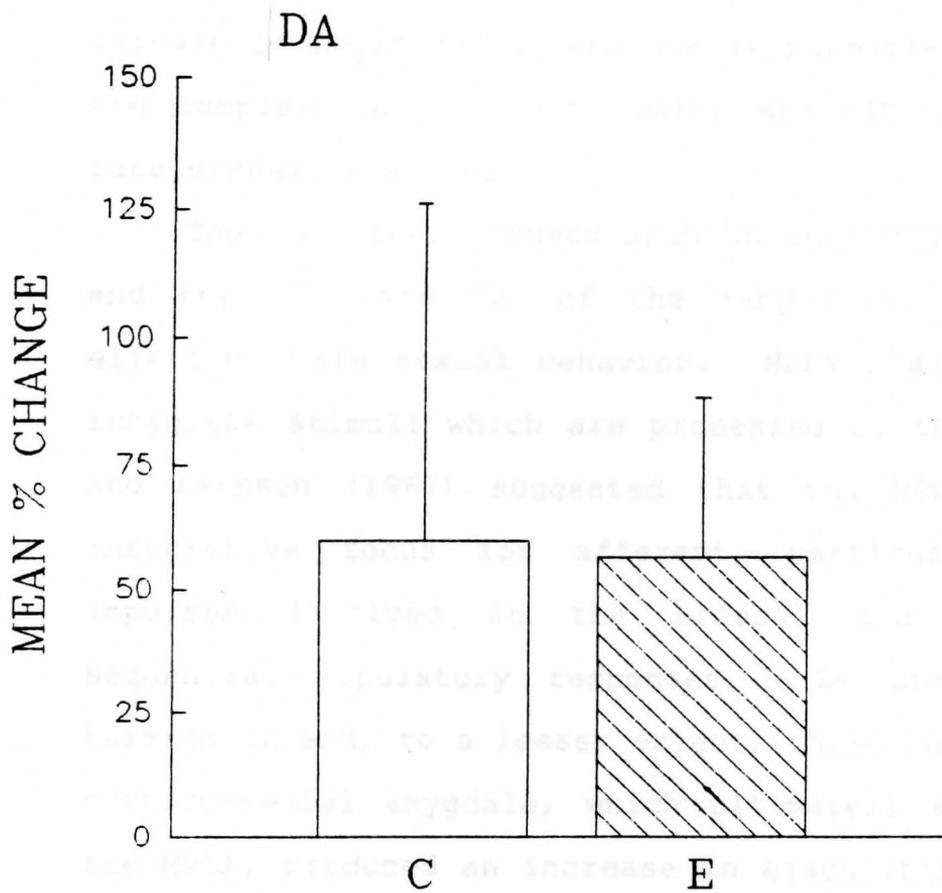
Figure 12. Mean percent change in DA levels in the MPOA-DBB after males were exposed to ovariectomized females (C, n=2) or allowed to copulate with estrous females (E, n=4). Values are the mean ± S.E.M.

DA

CHAPTER IV

DISCUSSION

Although many studies have begun to explore the neural substraits involved in regulating sexual behavior, little is known about the precise role(s) of neurotransmitters. The release of neurtransmitters are responsible for integrating the complex neural events which are ultimately translated into sexual behavior.

The two areas focused upon in this study, the MPOA-DBB and the NAc, are two of the many areas responsible for eliciting male sexual behavior. Many brain areas seem to integrate stimuli which are processed by the MPOA. Heimer and Larsson (1967) suggested that the MPOA serves as an integrative focus for afferent, particularly olfactory, impulses involved in the arousal and integration of sequential copulatory responses. Lesions of the stria terminalis and, to a lesser extent, those in the basomedial-cortico-medial amygdala, which ultimately send impulses to the MPOA, produced an increase in ejaculation latencies and a slight decrease in number of ejaculations to exhaustion (Heimer and Larsson, 1967).

Other studies investigating the neuroendocrine control of masculine sexual behavior have demonstrated that the medial preoptic area of the rostral hypothalamus forms an interface between the endocrine secretions of the testes and the neural substraits mediating sexual behavior (Sachs and Meisel, 1988; Hansen and Drake, 1984). The MPOA and the

rostral anterior hypothalamus have efferent pathways in common and they display similar binding patterns of gonadal steriods. Thus, the neurons from the MPOA and the rostral anterior hypothalamus form a continuum (Heimer and Larson, 1966/67). The neurons of the MPOA are of particular importance since they influence the hypothalamus. Destruction of the nerve cell bodies of the MPOA eliminates sexual behavior in male rats. Arendash and Gorski (1983) found that lesions to this brain area eliminated copulation in sexually experienced male rats.

The present study attempted to correlate neurotransmitter release in the medial preoptic area-diagonal band of Broca and the nucleus accumbens with sexual behavior in the adult male rat. An in vivo microdialysis approach was used to estimate the release of neurotransmitters in these brain areas.

In this study, analysis of the dialysate from the MPOA-DBB detected levels of NE, HVA, and EPI which nearly tripled when sexually experienced males were exposed to estrous females. In contrast, when ovariectomized females were used as the stimulus, the levels of NE, HVA, and EPI decreased below basal levels. Analysis of DOPA (a DA precursor) indicated levels which also increased when males were exposed to estrous compared to ovariectomized females. In contrast, dopamine levels were similar in males exposed to estrous or ovariectomized females. However, because levels of HVA, a DA metabolite, increased in males exposed to

estrous females an excititory role for this catecholamine is suggested.

The release of these neurotransmitters from the MPOA supports other published results which have suggested that NE and DA act in this area to regulate male sexual behavior. For example, in a intracranial study, Clark (1991) infused either Yohimbine (an alpha$_2$ antagonist) or Clonidine (an alpha$_2$ agonist) through a cannula implanted into the MPOA. The pharmacuticals evoked dose-dependent increments or decrements respectively in the number of mounts, intromissions, and ejaculations attained by the adult male rats tested. This study suggested that alpha$_2$-adrenoceptors are important in the control of male sexual behavior and that alterations in adrenergic mechanisms in the MPOA may underlie sexual dysfunction of various etiologies.

It has also been shown that dopamine receptor agonists enhance copulatory behavior (Britran and Hull, 1987; Sachs and Meisel, 1988), whereas dopamine receptor antagonists disrupt or eliminate sexual activity (Pfaus and Phillips, 1989/1991). These dopamine receptor agonists/antagonists were directly infused into the MPOA. The MPOA plays a large role in the central dopaminergic systems. Microinjections of the dopamine antagonist cis-flupenthixol into the MPOA reduces male sexual behavior, whereas copulation, penile reflexes, and seminal emission are facilitated by microinjections of dopamine receptor agonists (Hull et al., 1989; Warner et al., 1991).

When sexually experienced males are permitted to copulate with estrous females a number of sensory afferents are stimulated. These neural transmissions are processed and ultimately reach the MPOA. Once the processed information reaches the MPOA the catecholamines act to activate specific efferent pathways to the ventral tegmental area and the NAc. This processed information is then projected to motor brain areas where specific behavioral responses are initiated.

The NAc is an area rich in dopaminergic neurons. In the present study, levels of DA as well as DOPA in the NAc were increased when the male was exposed to estrous compared to ovariectomized females. Analysis of HVA for the NAc did not follow the trend seen with DA or DOPA. HVA had a slightly larger increase when males were exposed to ovariectomized females than when exposed to estrous females.

The levels of DA in the NAc reported in the present study is supported by other literature. For example, Pfaus et al. (1990) used in vivo brain microdialysis to determine central DA transmission in the nucleus accumbens and striatum of sexually experienced male rats during mating behavior. Their results indicated an increase in DA transmission in both the nucleus accumbens and the striatum of sexually experienced male rats during various phases of sexual behavior. Similar increases were seen in HVA. The results for HVA from the present study were contrary to the

results reported by Pfaus et al., (1990). It is possible that the turn over rate in the NAc is accentuated for DA.

Another study investigated dopaminergic transmission in the nucleus accumbens in male rats exposed to sociosexual olfactory stimuli using a voltammetric approach (Louilot et al., 1991). Exposure of males to receptive female odors induced a marked and selective increase in DA release compared to control values. In contrast, exposure to non-receptive female odors or to male odors produced no significant change. Thus, this study indicated that the mesencephalic dopaminergic neurons reaching the nucleus accumbens appear to be involved in the perception of behaviorally significant olfactory cues.

Results of these experiments support a role for the catecholamines NE and DA in the MPOA and NAc in regulating the complex neural mechanisms that regulate sexual behavior in male rats. Further studies are needed to delineate the precise roles of these transmitters in integrating the complex sensory inputs which ultimately are translated into specific behavioral patterns.

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