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The Effects of Bilateral Injections of
Neuropeptide K into the Medial Preoptic Area
on Male Rat Copulatory Behavior

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ABSTRACT

The first mammalian neuropeptide to be characterized was substance P (sP), and it is now recognized that sP is a member of a structurally related family of peptides, the tachykinins. Extensive studies have demonstrated that sP and some related tachykinin peptides play key roles as neurotransmitters and neuromodulators. The synthesis of different members of the tachykinin family is in part due to the modifications of three sP-encoding preprotachykinin (PPT) mRNA's that are derived from a single sP gene. At least four tachykinin peptides can be synthesized as a result of these differential modifications including sP, neurokinin A, neuropeptide γ , and neuropeptide K (NPK). Whereas the behavioral significance of sP has been extensively studied, there has been very little examination of the behavioral significance of NPK. This is especially true of the examination of male reproductive behavior. Dornan and Malsbury (1) reported that bilateral injections of sP into the medial preoptic area (MPOA) facilitated male rat sexual behavior. High concentrations of PPT mRNA's are found within the MPOA, and as previously mentioned, NPK is derived from PPT mRNA's. At present, however, it is not known whether NPK plays a role in the neural regulation of male copulatory behavior. In the following experiment, we examined the role of NPK within the MPOA in the regulation of male rat copulatory behavior.

Sexually experienced adult male Long-Evans rats were used. Chronic cannulae implants were stereotaxically placed 2mm above the MPOA, and following 7-12 days of recovery, the animals were behaviorally tested to obtain a baseline measure of copulatory behavior. One week following baseline testing, the animals were randomly placed into 4 groups and were bilaterally injected with .5 μ l solutions of either saline, 100ng NPK, 1000ng NPK or 2000ng NPK and the effects of these injections on copulatory behavior were determined. Following testing, brains were histologically analyzed to confirm the placements of the cannulae. Analysis revealed that bilateral injections of 1000ng NPK into the MPOA produced an abolishment of copulation in a significant number of animals, whereas injections of 100 and 2000ng NPK produced no significant effect on the copulatory behavior. It was noted that injections of 2000ng NPK produced an abolishment of copulation in 50% of the animals, but due to the small number of animals in the group (n = 4), the result was not significant. Although the complete role of the tachykinins in the regulation of copulatory behavior has yet to be elucidated, it seems that sP and NPK, two tachykinins synthesized from the sP gene, exert opposite effects in the regulation of male copulatory behavior.

INTRODUCTION

The first mammalian neuropeptide to be biologically characterized was substance P (sP) (2), and it is now recognized that sP is a member of a structurally related class of peptides, the tachykinins. Each of the members of the tachykinin family have the defining characteristic of a similar carboxyl terminal region, Phe-X-Gly-Leu-Met-NH₂, where the X residue is either an aliphatic or aromatic amino acid, usually either Phe or Val (3). Extensive studies have demonstrated that sP and some related tachykinin peptides play key roles as neurotransmitters and neuromodulators in both the peripheral and central nervous system (4). The tachykinins have a wide range of implicated functions, ranging from nociception to sexual regulation (1,5).

The production of the tachykinins can be the result of several regulatory processes including differential RNA splicing and differential precursor post-translational processing. From a single gene, three preprotachykinin (PPT) mRNAs can be produced. The three PPT mRNAs produced are α , β , and γ . Via differential modifications, at least four tachykinins can be produced from these three PPT mRNAs including sP, neurokinin A (NKA), neuropeptide δ (NP δ), and neuropeptide K (NPK). From the α -PPT mRNA, sP can be produced. From the β -PPT mRNA, sP, NKA, and NPK can be produced. From the γ -PPT mRNA, sP,

NKA, and NP γ can be produced (6). Thus, from a single sP-gene, four different tachykinin peptides can be produced.

The role of the tachykinin peptides as regulators of various behaviors has recently gained significant interest. But, whereas the behavioral significance of sP has been well studied (1,7,8,9,10), the roles of the other tachykinins is for the most part unknown. This relationship is particularly true in relation to the study of male reproductive behavior. Dornan and Malsbury (1) have studied the function of sP in the neural regulation of male rat copulatory behavior and have found that bilateral injections of 10, 100, and 200ng of sP into the medial preoptic area (MPOA) of male rats reduced the latencies to initiate copulation. Injections of the lower two doses also reduced ejaculation latencies, thus indicating a facilitory effect of sP in the MPOA in relation to copulatory behavior. At present, no other tachykinin has been implicated in the neural regulation of male rat copulatory behavior.

Copulatory behavior in the male rat is regulated via a sexually dimorphic, steroid accumulating circuit that includes the medial amygdala, the bed nucleus of the stria terminalis, and the medial preoptic area (28). Behaviorally-relevant information from a sexually receptive female is relayed from receptors in the vomeronasal organ to the accessory olfactory bulb (11). Two areas that receive

direct input from the accessory olfactory bulb (AOB) are the medial amygdala (MeA) and the bed nucleus of the stria terminalis (BNST), which in turn provide major inputs to the MPOA (11,12). Indeed, the MeA, BNST, and MPOA are considered components of the vomeronasal olfactory system and are known to participate in the neural regulation of male sexual behavior (11). For example, lesions of either the MeA (13,14,15) or the MPOA (16,17) create a severe disruption in the expression of male rat copulatory behavior.

High concentrations of PPT mRNAs are found within these cell groups (18,19). Interestingly, neurons within the bed nucleus of the olfactory tract, the BNST, the MeA, and the MPOA express PPT mRNA (18). Collectively, this widespread but highly localized distribution of PPT mRNAs within the accessory olfactory system strongly suggests that products derived from the sP gene may be at least partially responsible for the transmission of olfactory information relevant for the expression of copulatory behavior in the male rat. As previously mentioned, NPK is derived from the sP gene. Presently, it is not known whether NPK plays a role in the neural regulation of male copulatory behavior. Consequently, there is a need for research examining the effects of NPK on reproductive behavior before the role of various tachykinin peptides within this circuit becomes elucidated.

In the following experiment, NPK was bilaterally injected into the MPOA of sexually vigorous male rats in order to delineate the functional significance of NPK within the sexually dimorphic olfactory path that has previously been shown to be of crucial importance in the expression of male sexual behavior.

METHODS

Animals

Thirty adult male Long-Evans rats bred in the Illinois Wesleyan (IWU) colony were used in this study. The animals were housed individually with food and water available *ad lib* throughout the experiment and maintained in a controlled environment on a light/dark cycle (lights on at 600 and off at 2100).

Eight adult female Long-Evans rats bred in the IWU colony were used as lures in each of the behavioral tests of the experiment. The females were ovariectomized under ether. To insure proper receptivity in the behavioral tests, seventy-seven and fifty-three hours prior to testing, the females were injected subcutaneously (sc) with 10 micrograms estradiol benzoate, and five hours prior to testing the females were injected sc with 1 microgram progesterone. Prior to all behavioral testing, the lures were screened with a vigorous male to insure full receptivity.

Prior to surgery, each male was given several screening tests in which the male was placed with a lure in a testing arena until one ejaculation was achieved. Only males that ejaculated at least three times during the screening tests were subsequently used in the study.

Surgery

At the time of surgery, the males weighed between 464g and 587g. Each animal was anesthetized with Nembutal (50mg/kg) and received a pair of stereotaxically implanted 22-gauge stainless steel guide cannulae 12mm in length. The cannulae were aimed 2mm above the MPOA (from bregma, AP = +1.7mm, ML = \pm 0.6mm, DV = -6.2mm) as determined using the atlas of Paxinos and Watson as a guide (20). The cannulae were secured with cranioplastic cement.

Behavioral Testing

Seven to twelve days following surgery, the males were given a postoperative baseline test in rectangular mating arenas (20" x 15" x 14") with standard bedding. A lure was placed in an arena with each male, and various parameters of male sexual behavior were recorded. Only males that completed an ejaculatory sequence in the testing session were subsequently used in the study.

Seven days following the postoperative testing, the males were injected bilaterally with either 2000ng, 1000ng, or 100ng of NPK in 0.5ul of a 0.9% saline solution, or saline alone. The NPK was purchased from BACHEM (Lot # ZC402, and # QC402). The injection was performed using the air bubble technique. Five minutes following the injection, the testing session began. To avoid experimenter bias during the testing session, the person grading the animals was blind as to what dosage the animal received. During the baseline and experimental testing, the following parameters of male sexual behavior were recorded: mount latency (ML), the interval from the introduction of the female to the first mount by the male; intromission latency (IL), the interval from the introduction to the first intromission; ejaculation latency (EL), the interval from the first intromission to the first ejaculation; post-ejaculatory interval (PEI), the interval from the first ejaculation to the next mount; mount frequency (MF), the number of male mounts without intromission between the introduction and the ejaculation; intromission frequency (IF), the number of intromissions between the introduction and ejaculation. The intercopulatory interval (ICI), the mean interval between intromissions, was obtained by dividing the EL by the IF. All data was collected with the aid of the computer program CADA (Computer Assisted Data Acquisition), as developed by Scott Meade. The testing session ended when one of the following events occurred:

the male achieved his PEI; the male failed to intromit within 15 minutes following the introduction of the female; or the male failed to ejaculate within 30 minutes of the first intromission.

Histological Analysis

Following testing, all animals were anesthetized with an overdose of Nembutal, and their brains were removed. The brains were sectioned into 40 micron slices using a cryostat. The sections were then stained using cresyl-violet, and the location of each cannula was determined. Animals with an injection site outside of the MPOA were eliminated from the study.

Data Analysis

The proportion of animals completing an ejaculatory sequence during the behavioral tests was assessed by the McNemar's Change Test (21). Unless otherwise stated, all other data were analyzed using a one-way unweighted means-analysis of variance (ANOVA) in a repeated measures split-plot design. Post-hoc comparisons of treatment means were assessed using a modified Tukey's ratio, required when an unweighted-means analysis is employed (22).

RESULTS

Of the thirty animals that began the study, four had both cannulae located outside the MPOA, one had only one cannulae located within the MPOA, and five never satisfied criterion at the postoperative baseline test. (See Figure 1 for a representative placement site.) In addition, one animal which will not be included in the study received bilateral injections of 10ng NPK. That left nineteen animals for the data analysis (controls, $n = 6$; 100ng, $n = 3$; 1000ng, $n = 6$; 2000ng, $n = 4$). As illustrated in Figure 2, bilateral injections of 1000ng NPK into the MPOA significantly decreased the expression of copulatory behavior in sexually vigorous male rats when compared to an injections of saline alone ($X^2 = 3.20$, $p < 0.05$). In contrast, bilateral injections of 100 and 2000ng of NPK into the MPOA failed to produce a statistically significant effect on the expression of male copulatory behavior (see figure 2) ($X^2 = 0.00$, $p > 0.05$; $X^2 = 0.50$, $p > 0.05$, respectively). As can be seen from Figures 3 and 4, bilateral injections of 100ng of NPK had no appreciable effect on ejaculation latency ($F = 0.82$, $p > 0.05$), the PEI ($F = 1.17$, $p > 0.05$) or mount and intromission latencies ($F = 0.91$, $p > 0.05$; $F = 0.21$, $p > 0.05$, respectively) when compared to the saline injected controls.

Figure 1 Diagram of a representative bilateral injection site in the MPOA.

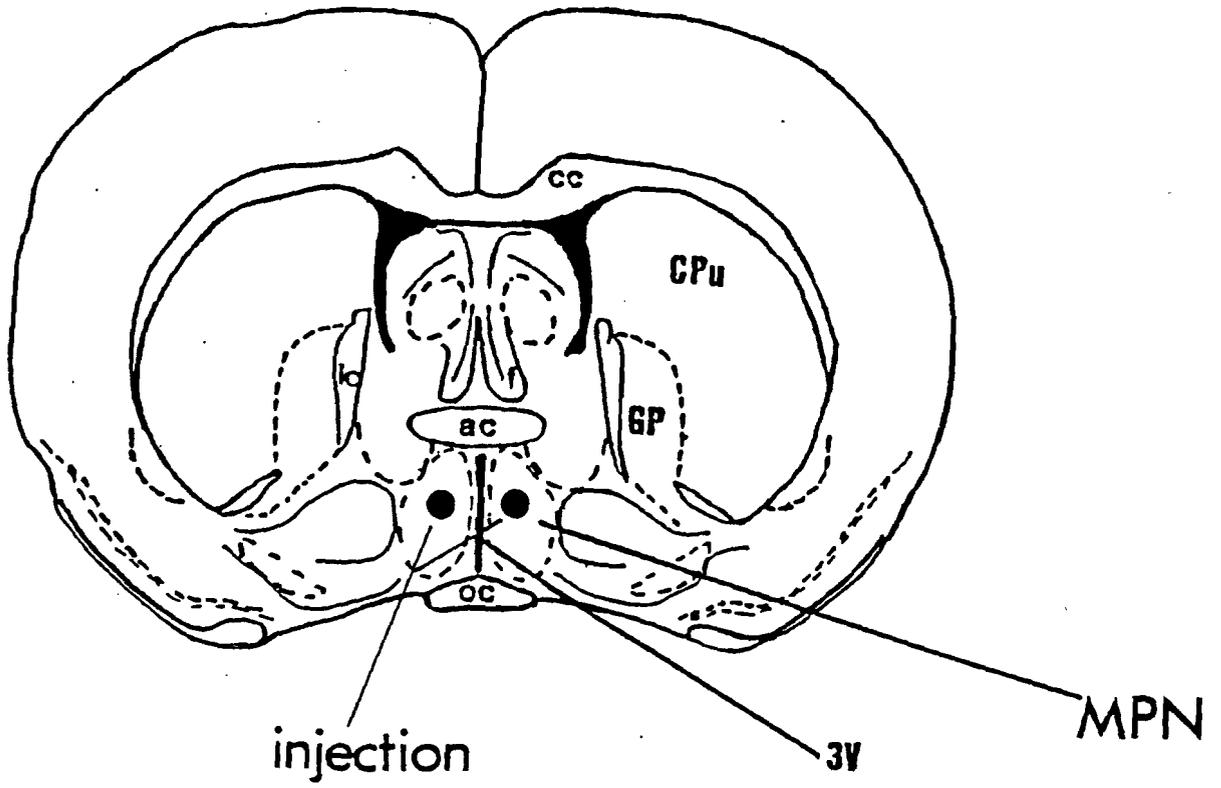


Figure 2 Comparison of the effects of bilateral injections of three different doses of NPK or saline on the percentage of animals ejaculating in the testing session.

Asterisk (*) denotes significance at $\alpha = 0.05$.

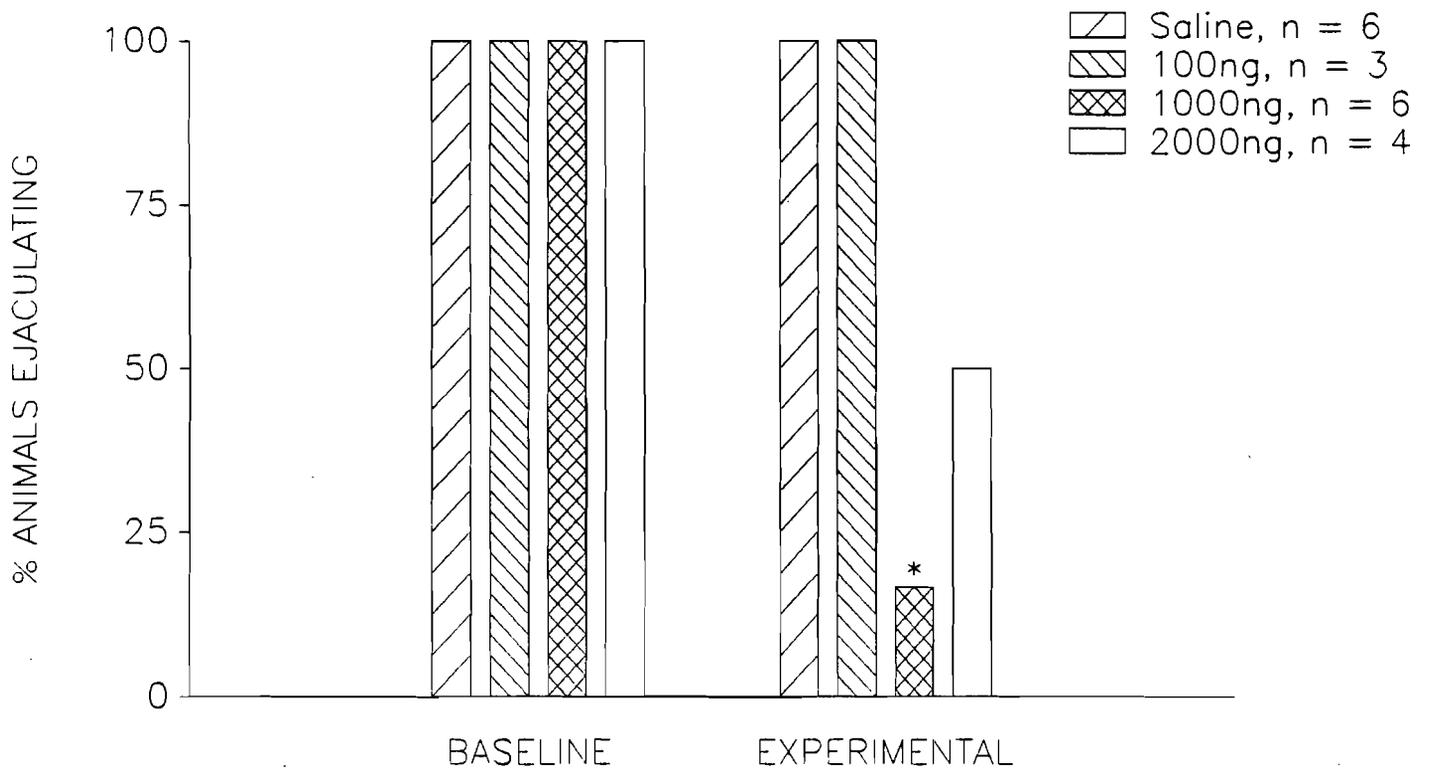


Figure 3 Comparison of the effects of bilateral injections of three different doses of NPK or saline on mount and intromission latencies. Vertical lines represent \pm standard errors of the mean.

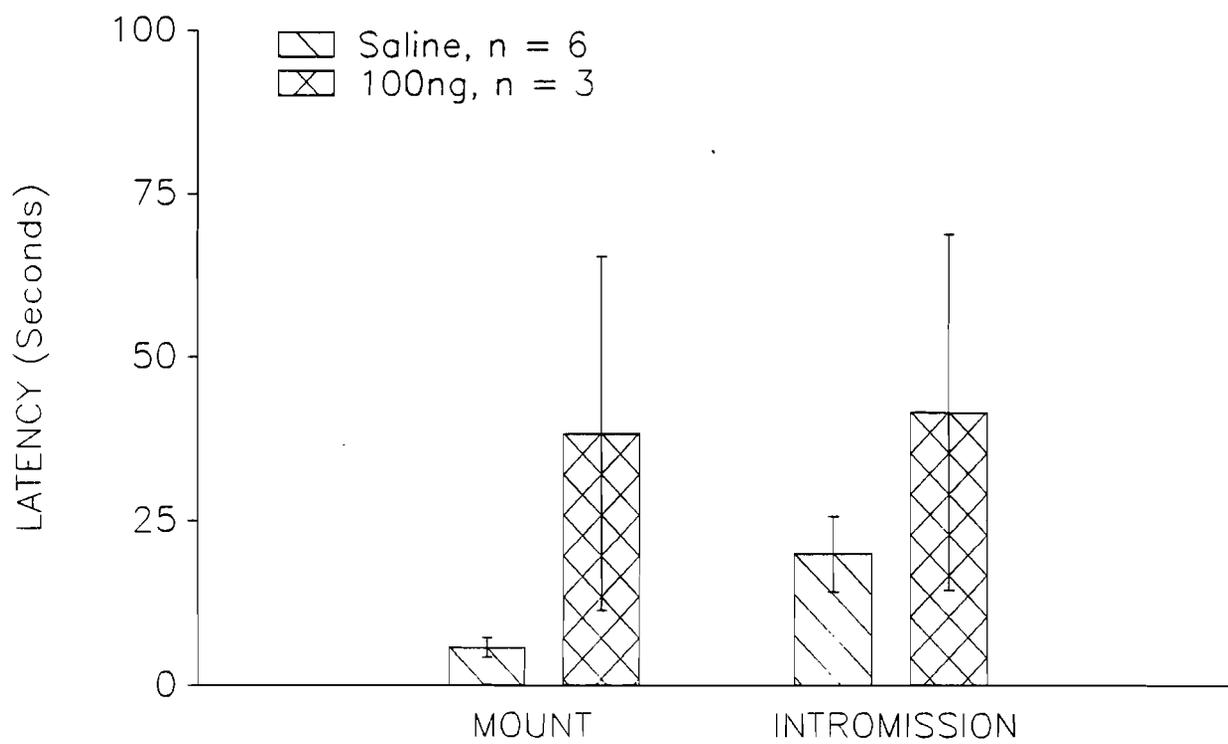
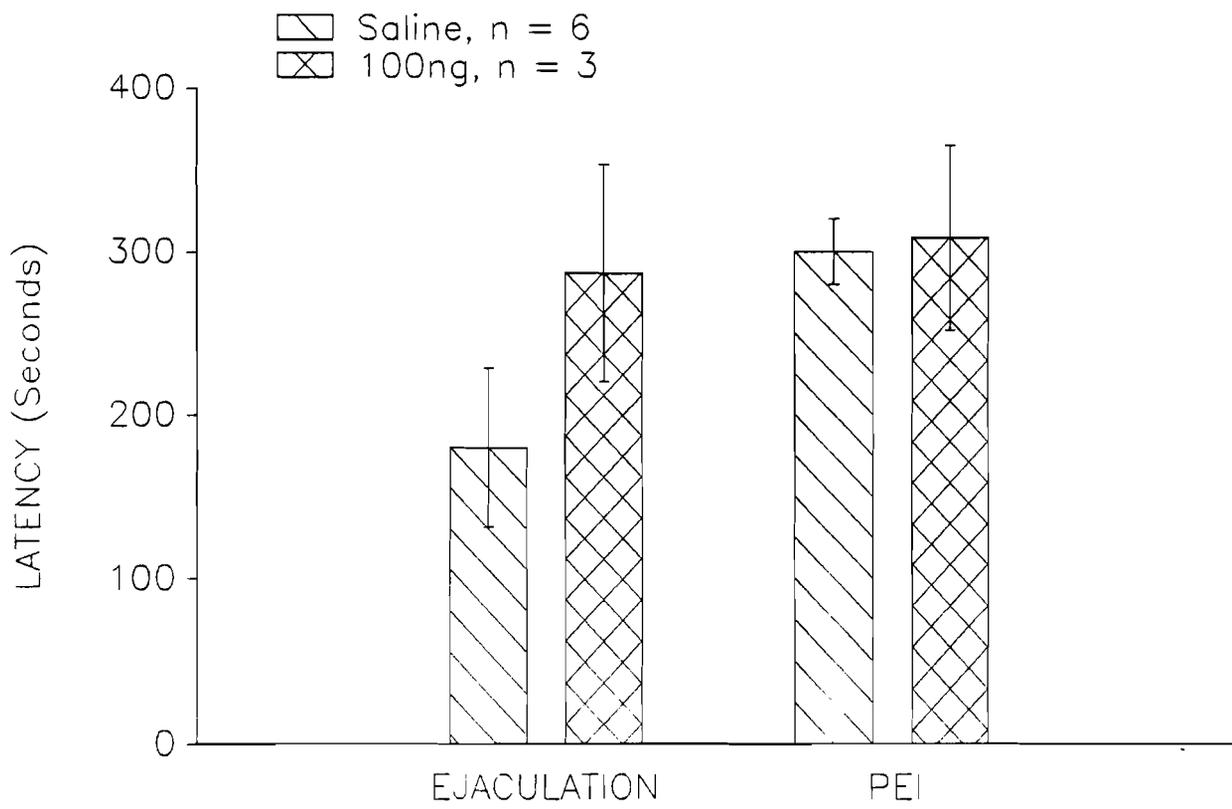


Figure 4 Comparison of the effects of bilateral injections of three different doses of NPK or saline on ejaculation and PEI latencies. Vertical lines represent \pm standard errors of the mean.



DISCUSSION

Bilateral injections of 1000ng neuropeptide K into the medial preoptic area of sexually vigorous male rats produce an abolishment of copulatory behavior in a significant proportion of animals while lower doses have no apparent effect. Statistically speaking, bilateral injections of 2000ng did not have a significant effect on the percentage of animals ejaculating. However, this was due to the small number of animals in this group (n = 4). Speaking generally, the abolishment of copulation in 2 of the 4 sexually vigorous animals of the group is noteworthy, and upon enlargement of the number of animals in the group, we expect to find significance. There are several important implications of these results. First of all, our data implicate NPK as being yet another neuropeptide in a continually growing list of compounds that have a role in the neural regulation of copulatory behavior in the male rat. In addition, we have also been able to presumably localize receptors for NPK to the MPOA. Previous studies have demonstrated the presence of at least three different classes of receptors for the tachykinins (23,24,25). The three receptor subtypes are the NK-1 subtype, the NK-2 subtype, and the NK-3 subtype. It appears that the three receptor subtypes interact to varying degrees with the different tachykinin peptides. Initial studies (26) indicate that NPK is the most potent ligand for the NK-2 receptor subtype and that NPK also has a lesser potency

at the NK-1 receptor subtype. Thus, it seems that either the NK-1 or NK-2 receptor subtypes are present in the MPOA of male rats, and these receptors appear to be involved in a circuit regulating the copulatory behavior of male rats. Since only high doses of NPK (1000 and 2000ng) had any effect on copulatory behavior, it may be that the receptors at which NPK has a lesser potency, the NK-1 receptor subtype, are the receptors that play a role in the regulation of copulatory behavior in the male rat in the MPOA.

The theory of the colocalization of certain neurotransmitters in secretory vesicles has recently gained popularity (6). It is possible that sP, NKA, NP ζ , and NPK may be colocalized in synaptic vesicles in the MPOA and that the neurons containing these synaptic vesicles play a role in the regulation of male rat copulatory behavior. In a rat salivation bioassay, Takeda and Krause (27) have demonstrated that coadministration of sP with either NPK or NP ζ result in a substantially greater level of salivation than do administration of any of the three alone. These results indicate a possible synergistic effect of sP and NPK in rat salivation. In contrast to the salivation study, it appears that sP and NPK play differential roles in the regulation of copulatory behavior, with sP causing a facilitation and NPK causing an inhibition of copulatory behavior.

It is possible that these opposite effects may in part be a control for the

regulation of male rat copulatory behavior. The MPOA is a site that is central to the regulation of male rat copulatory behavior, and the amount of convergence of information dealing with copulatory behavior to the MPOA is astounding. As previously mentioned, the MPOA is part of a highly interconnected, sexually dimorphic circuit that includes the MPOA, the amygdala, and the bed nucleus of the stria terminalis whose integrity is essential for the expression of copulatory behavior in the male rat (28). Neurons converging to the MPOA carry information ranging from auditory to visual to vomeronasal information (29). Thus, the opposite effects of sP and NPK in the MPOA taken together with the possibility of colocalization of the two tachykinins in synaptic vesicles lead to the hypothesis of a differential regulation of copulatory behavior due to differential production of sP and NPK in the MPOA.

The results of this study indicate that the tachykinin NPK, when injected in high doses into the MPOA of sexually vigorous male rats, causes an abolishment of copulatory behavior. This effect may be mediated via NK-1 or NK-2 receptors.

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