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Kellie S. Jones '90
Illinois Wesleyan University

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Kellie S. Jones

Department of Biology

ILLINOIS WESLEYAN UNIVERSITY, Bloomington, IL 61701

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ABSTRACT

In this study either substance P (SP), SP 5-11, an analogue resistant to enzymatic degradation, or acidified saline was injected bilaterally into the medial preoptic nucleus (MPN) of the medial preoptic area (MPOA) in ovariectomized (ovx) estrogen-primed female rats. The lordosis quotient (LQ) and the lordosis intensity scale (LIS) were recorded using a steroid regime that produced a slow increase of sexual receptivity. In experiment 1, when receptivity was low, bilateral injections of 2 different doses of SP (100 ng,1000 ng/ cannula), had no effect on lordosis behavior when compared to those injected with acidified saline. In experiment 2, however, when receptivity was maximal, SP 5-11 (1000 ng/cannula) produced a marked decrease in lordosis responding. The results indicate that the effects of bilateral injections of SP on lordosis behavior is dependent on the level of sexual receptivity, and therefore, estrogen levels.

INTRODUCTION

It has been demonstrated that sexual receptivity (lordosis) in the female rat is dependent on circulating levels of ovarian hormones, estrogen and progesterone (28). Moreover, the hormonal induction of lordosis behavior in the female rat is dependent on the functional integrity of specific neural pathways between areas of the brain, notably the ventromedial nucleus of the hypothalamus (VMH), an area which has been repeatedly found to be facilitatory, the midbrain central gray (MCG), also facilitatory, and the medial preoptic area, an area which is involved in the inhibition of lordosis behavior (see 28 for review). Implantation of estrogen into these areas has also been shown to have a dramatic effect on sexual receptivity in ovariectomized (ovx) rats (5,18,31,35).

Accumulating evidence has shown that the concentration of some neuropeptides present in the central nervous system (CNS) are dependent on the presence of estrogen (13,17,23,24). Not surprisingly then, there have been a number of neuropeptides linked to sexual behavior in the female rat (4,7,11,33). For example, following bilateral injections of the neuropeptide, oxytocin, into the MPOA, Caldwell found a marked facilitation of lordosis behavior in ovx female rats treated with 0.5 ug of estradiol for three consecutive days (7). Dornan et al. (10) also reported a facilitation of lordosis behavior following bilateral injections of cholecystokinin (CCK) in the medial preoptic nucleus (MPN) of the MPOA.

Recently, based on the evidence which has demonstrated the presence of estrogen receptors in hypothalamic neurons (2) and the studies using the technique of *in situ* hybridization which report that estrogen regulates the expression of certain neuropeptide genes within the CNS (30,32,34), one popular hypothesis concerning the neural regulation of sexual receptivity in the female rat is that by altering the expression of certain genes, estrogen regulates mRNA levels for specific precursor proteins for neuropeptides within the VMH (6). These peptides are then transported to the dMCG where they are released to modulate descending circuits that regulate motor neurons controlling the display of lordosis behavior in the female rat.

One of these peptides is substance P (SP). Indeed, numerous SP cell bodies and fibers have been found in areas relevant to sexual receptivity in the female rat (8,19). The VMH has been documented to concentrate estrogen within its cells; these same cells have been found also to make SP (2,3). Although *in situ* hybridization studies have subsequently reported that neurons within the VMH express the gene for preprotachykinins (29), information concerning whether estrogen treatment alters preprotachykinin gene expression within the hypothalamus has been conflicting (6,29). Nonetheless, the MCG, which has SP receptors as well as cell bodies (8,19,21), shows marked changes in the number of SP immunoreactive fibers over the estrous cycle (13).

Taken together, these results suggest that SP may play an important role in the neural regulation of lordosis behavior in the female rat. Indeed, Dornan et al. (11), reported that bilateral

injections of three different doses of SP into the dMCG produced rapid and sustained (3 h) facilitation of lordosis behavior in ovx estrogen-primed female rats. Based on these results, they postulated that SP produced within the cell bodies of the VMH was transported to the MCG where it carried behaviorally-relevant information about estrogen suggesting the importance of a SP VMH-MCG pathway in the regulation of sexual receptivity in the female rat. To determine if SP immunoreactivity existed in the same cells of the VMH which projected to the dMCG, a neuroanatomical technique termed "double labeling" was used by Dornan et al. Briefly, this technique first calls for injecting Fluorogold into the dMCG. Fluorogold is a retrograde tracer, and therefore follows the path which fibers projected from to the dMCG. Brain sections are then incubated with a SP antiserum. Cells that are doubly labeled with Fluorogold and SP antiserum within the VMH indicate a VMH-dMCG SP pathway. In this double labeling study (9), however, although doubly labeled cells exist throughout the VMH, the dense population that would be expected if a major SP pathway existed from the VMH to the MCG, was absent.

It has been documented that the MPOA does contain SP immunoreactive fibers and receptors in the rat (19,21). There is evidence from Akesson et al. (3) that estrogen-concentrating cells within the hypothalamus project to the MPN, but whether these cells are the same which produce SP is as of yet, unknown. Malsbury (20) recently reported that lesions of the VMH produced a significant reduction of SP immunoreactivity in the MPOA. Based on the above, it is not inconceivable that the SP innervation of the MPOA may play an important role in the neuroregulation of sexual receptivity in the

female rat. Presently, nothing is known about the importance of the SP innervation of the MPN in regard to the regulation of lordosis behavior in the female rat. The purpose of this study was to determine if SP injected directly into the MPN would affect sexual receptivity in estrogen-primed rats.

GENERAL METHODS

Animals and Surgery

Adult Long-Evans female rats weighing between 210g and 250g were housed in groups in steel mesh cages in a controlled environment at 21C, with an extended light cycle (on at 0700, off at 2100). Food and water were available ad lib. Animals were ovariectomized under ether.

One week after ovariectomy, each animal was anaesthetized with sodium pentobarbital at a dosage of 50 mg/kg and received a pair of stereotaxically implanted 22-gauge stainless steel guide cannulae with inner stylets (28-gauge) aimed 1mm above the MPN using the coordinates that were empirically derived from the Paxinos and Watson atlas (25): AP +2.2, ML -0.5, DV -5.3 from bregma. The cannulae were cemented into place with cranioplastic.

Intracerebral Injections

Each 28-gauge inner cannula injector was connected to a 2 ul syringe by a 12" plastic tube. Distilled water was drawn up the tubing until 1 ul of water could be drawn and expelled reliably. Air was then drawn into the inner cannula at a volume of 0.5 ul, whereupon the inner cannula was placed in the peptide solution and 1.5 ul of peptide was drawn. Movement of the air bubble during injections ensured actual peptide injection into the brain. The injector cannula were 1 mm longer than the guide cannula to decrease the original amount of lesioning when the guide cannulae were placed. Animals received no anaesthesia, but were restrained manually during injection. Aliquots of all peptide solutions were prepared on the day of behavioral testing and stored in small plastic centrifuge tubes in a freezer until used. Substance P was dissolved in 0.01 N acetic acid, 0.9% saline (pH 4.7). Acidified saline prevents adsorption to glass, and may make the peptide more stable. In addition, Hall and Stewart found that behavioral effects were stronger in those animals receiving acidified SP as opposed to those receiving SP in a normal saline media (15). Solutions were injected manually at a volume of 0.5 ul (each side) over a period of 60 s. The cannula was left in place 30-60 s before withdrawal.

Behavioral Testing

Mating tests were conducted in a dimly lit room in rectangular test boxes containing wood chip bedding after animals had been allowed to recover one week from stereotaxic surgery. Test boxes were constructed with three wooden sides and a Plexiglass front. To measure sexual receptivity, the lordosis quotient (LQ) and the lordosis intensity scale (LIS) were used. The LIS was determined by pairing a sexually vigorous male with the female and recording the intensity of the lordosis reflex following ten mounts by the male. These responses were rated on a scale from 0-3: where 0 is no lordosis; 1 is a mild response consisting of very little arch of the back; 2 equals the normal lordosis response--back arching and tipping of head back; 3 being an exaggerated response with extreme flexion of the back and elevation of the nose (16). The LQ is an all or none evaluation of the lordosis response which ratios the number of lordosis responses to the number of male mounts. The presence or absence of proceptive behaviors such as hopping, darting, and ear wiggling were also recorded during the test session as indication of overall receptivity. Since there was no a priori indication whether SP injections would inhibit or facilitate lordosis, the behavior was assessed on a paradigm which recorded the effects of SP injections following repeated estrogen treatment and mating tests. This behavioral paradigm produces a slow increase in sexual receptivity (4,10). Lordosis was scored without knowledge of the injection's content.

Histological Analysis

After all data had been collected, all animals were anaesthetized with an overdose of sodium pentobarbital. Brains were removed subsequently and 46 um sections were taken (see fig 1). The sections were stained with cresyl violet and the cannulae placements in each brain were located using microscopy.

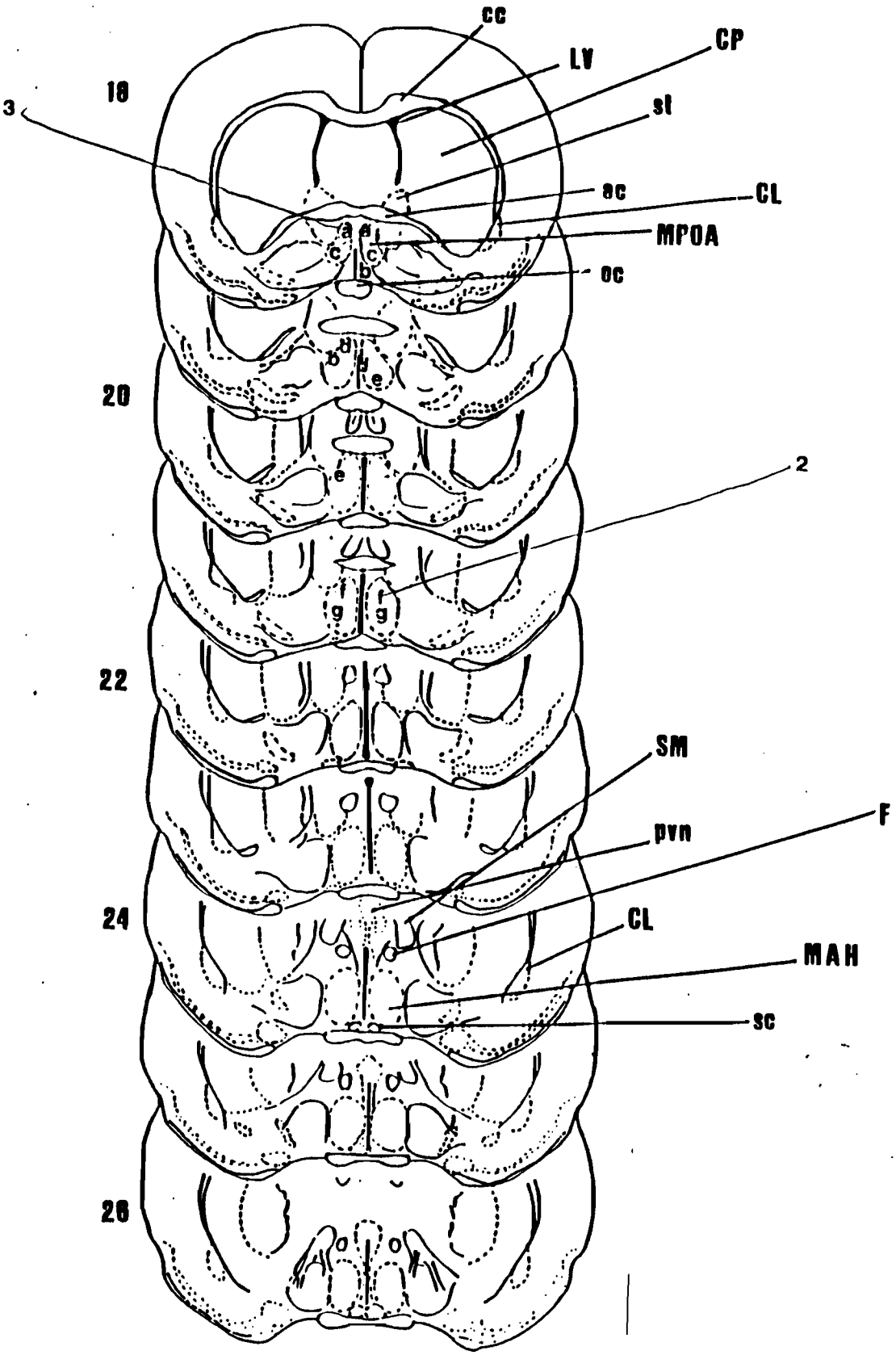
Statistical Analysis

A one way analysis of variance with repeated measures was applied to the data. In experiment 2, a two-sample independent t-test was used to determine the effect of the SP analogue on lordosis responses.

EXPERIMENT 1

Each female received a subcutaneous (s.c.) injection of 5 ug estradiol benzoate (EB) in sesame oil 53 h before testing. Females were divided randomly into 3 groups which received bilateral injections into the MPN of 100 ng SP, 1000 ng SP, or acidified saline in a latinized, repeated measures design. The effects of the SP injections on sexual behavior were assessed by placing females with sexually vigorous males 30 minutes following injection.

FIGURE 1 Illustration of bilateral cannula placements in nine females which received injections of SP and SP 5-11 into or near the MPN in experiments 1 and 2. The numbers identify the figure in the Konig and Klippel (1963) atlas from which the figures were redrawn. Small typed letters within the MPOA are approximations of actual cannulae placements. Numbers following the letters indicate the number of animals with this placement. Abbreviations: ac, anterior commissure; cc, corpus callosum; CL, claustrum; CP, caudate putamen; F, fornix; LV, lateral ventricle; MAH, medial anterior hypothalamus; MPOA, medial preoptic area; oc, optic chiasm; pvm, periventricular nucleus; SM, stria medullaris thalamus; st, stria terminalis.


cc
LV
CP
18
3
sl
ac
CL
MPOA
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20
2
22
SM
F
pvn
24
CL
MAH
sc
26

RESULTS

As can be seen in figures 2 and 3, the analysis of variance revealed that at low levels of receptivity both doses of SP (100 ng, 1000 ng) injected into the MPN had no statistically significant effect on the lordosis behavior response (LQ, $F(2,16)=2.68$ $P>0.05$; LIS, $F(2,16)=1.89$ $P>0.05$). Interestingly, there was a trend towards a facilitation of sexual receptivity with the largest dosage of SP, 1000 ng (see fig. 2 and fig. 3, test 1).

EXPERIMENT 2

Because animals which received bilateral injections of the largest dose of SP (1000 ng) exhibited a trend toward an inhibition in test 2, in experiment 2 all remaining animals were divided into 2 random groups and placed on a high-dose estrogen regime of 10 ug EB s.c. for 3 consecutive days to ensure maximum receptivity. On the fourth day all animals received bilateral injections into the MPN of 1000 ng SP 5-11 (more resistant to enzymatic degradation than SP) or saline and were paired with sexually vigorous males.

FIGURE 2. Effects of SP (100ng, 1000 ng) injections into the MPN on lordosis behavior during 3 mating tests (means ±S.E.M.).

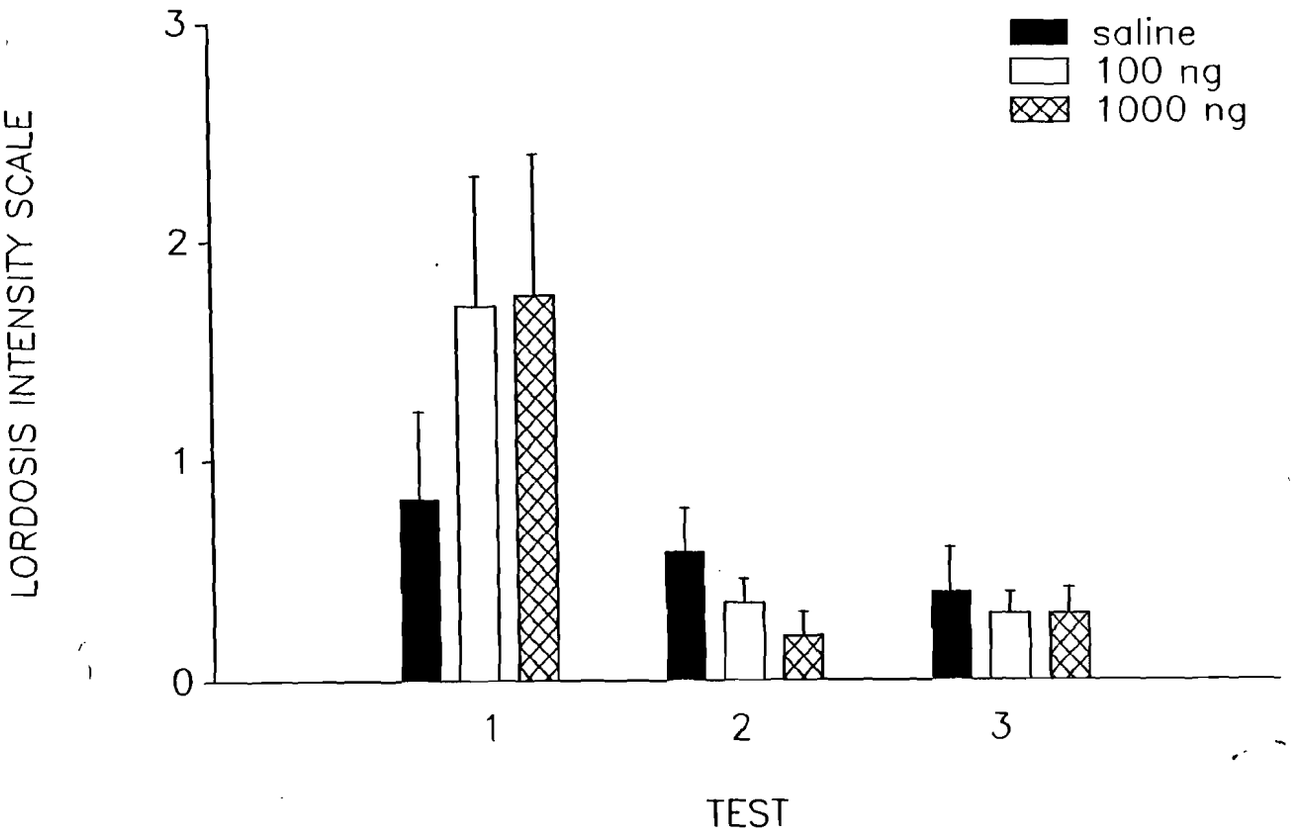
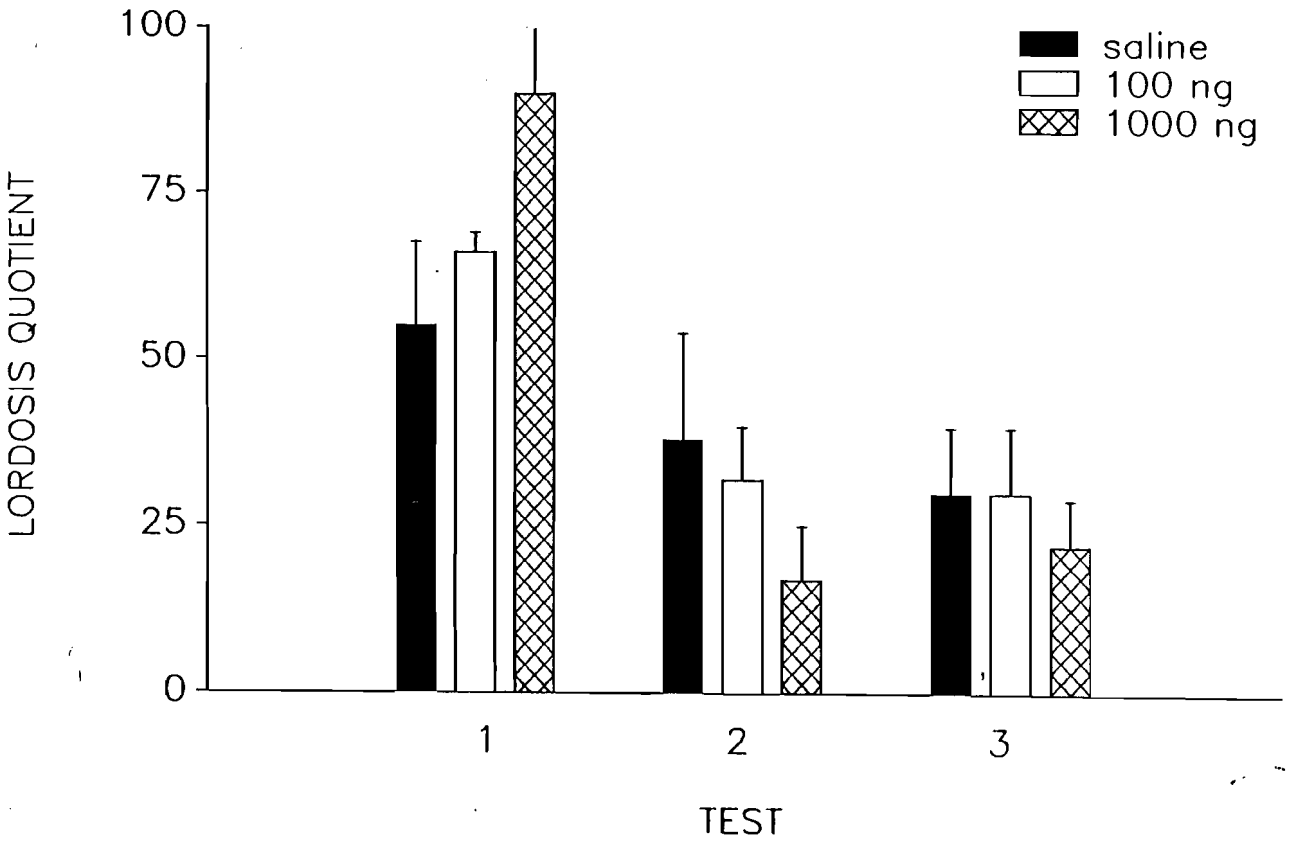

saline
100 ng
1000 ng
LORDOSIS INTENSITY SCALE
3
2
1
0
1
2
3
TEST

FIGURE 3. Effects of SP (100 ng, 1000 ng) injections into the MPN on lordosis intensity during 3 mating tests (means ±S.E.M.).


100
75
50
25
0
LORDOSIS QUOTIENT
saline
100 ng
1000 ng
1
2
3
TEST

RESULTS

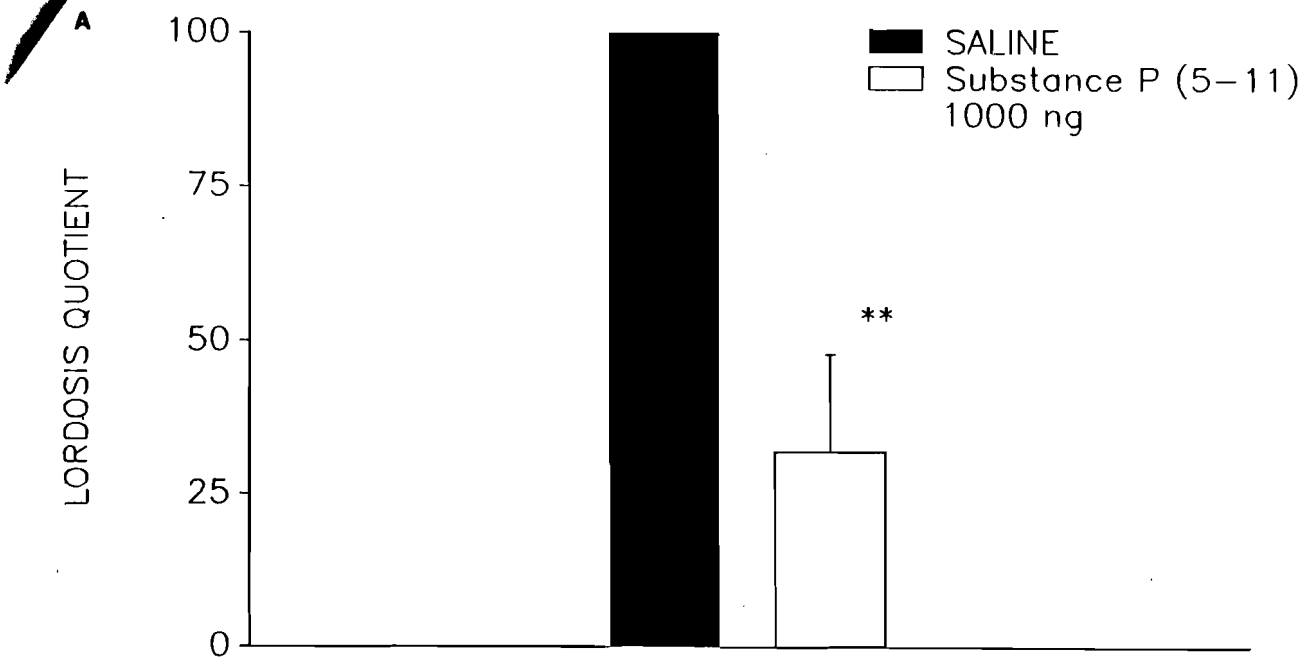
As illustrated in fig. 4, bilateral injections of 1000 ng SP 5-11 into the MPN produced a significant inhibition of lordosis when compared to saline controls reflected in both the LQ ($t(8)=3.26$ $P<0.01$) and the LIS ($t(8)=4.56$ $P<0.01$).

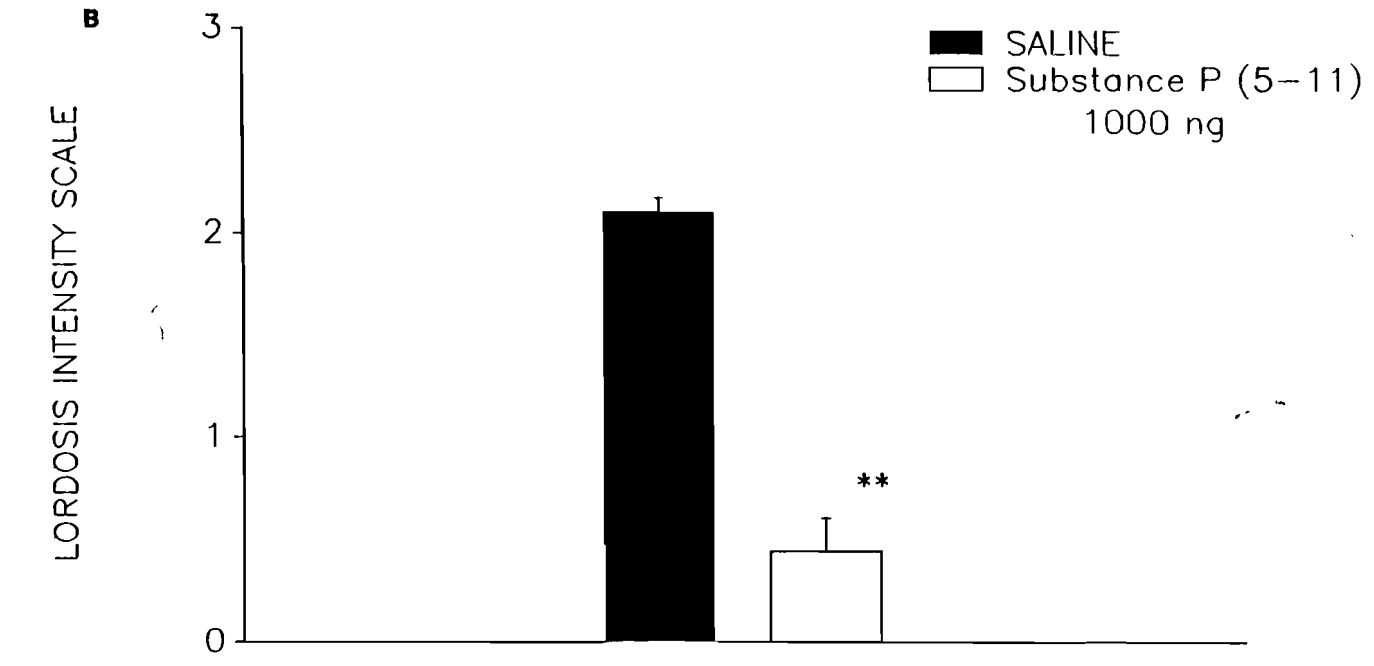
DISCUSSION

In experiment 1, bilateral injections of two different doses of SP into the MPN produced no appreciable effect on lordosis behavior in ovx estrogen-primed female rats when compared to injections of acidified saline. In experiment 2, however, bilateral injections of 1000 ng of the SP fragment, SP 5-11 produced a significant reduction in both the LQ's and the LIS's when compared to animals which received saline injections.

Using a number of different kinds of experimental procedures, results have strongly implicated the MPOA (in which the MPN is centrally located) and the VMH as important areas in the regulation of lordosis behavior. It has been found that electrical stimulation of the VMH stimulates lordosis, while stimulation in the MPOA inhibits lordosis expression (27). One explanation for the results of experiment 2 then is that the inhibition of lordosis behavior produced by an excitatory neuropeptide such as SP resulted from an activation of neurons intrinsic to the MPN.

FIGURE 4. Effects of SP 5-11 (1000 ng) into the MPN on lordosis behavior (A) and on lordosis intensity (B) during one mating test (means ±S.E.M.). Asterisk indicates significantly different from controls ($p<0.01$).


A
100
75
50
25
0
LORDOSIS QUOTIENT
SALINE
Substance P (5–11)
1000 ng
**


B
3
2
1
0
LORDOSIS INTENSITY SCALE
SALINE
Substance P (5–11)
1000 ng
**

This explanation, however, does not explain the increase in lordosis scores seen in experiment 1. Alternatively, since rats that received only 5 ug EB did not respond to the doses of SP and only the highest dose (1000 ng) in test two produced a trend toward an inhibition, something very similar to the action of CCK injections into the MPN may be occurring in the SP circuit. Although a specific mechanism is yet unknown, it seems that an increase in sensitivity occurs in the CCK circuit, producing effects parallel to the increase in LQ with repeated estrogen doses (10). If SP acts much in the same way as CCK, only during high levels of receptivity, when estrogen concentrations are at their highest, does the MPOA-VMH-MCG circuit become sensitized so that the SP can produce an effect. This could have very profound effects on the lordosis regulatory circuit. It has been thus far a plausible hypothesis that neuropeptide input from the VMH to the dMCG ultimately regulates the descending motor neurons related to the expression of sexual receptivity. Since it has been demonstrated that the estrogen-concentrating cells of the VMH with inherent SP immunoreactivity do not by and large extend to the dMCG, it can be speculated that regulation produced by SP may originate from the MPOA and extend to the MCG. Following this line of reasoning, estrogen sensitizes the circuit to SP. When estrogen concentrations reach a certain level, SP is released from the VMH and excites the inhibitory MPN. The MPN then affects the dMCG by turning off the lordosis circuit and inhibiting the behavior. Future studies may focus on this hypothesis by using a high-dose estrogen regime on female rats, injecting SP into the MPOA to determine if it will have a similar effect as did the SP analogue, SP 5-11.

Although the MPOA has been shown to have a net inhibitory action on lordosis expression, it must be noted that this area in not of uniform cell type throughout. The MPN itself has three subdivisions with different distributions of steroid-concentrating cells (22). Integration of these distinct cellular areas requires that signals of one type be augmented over others during certain situations. The SP signal may, therefore, be amplified when estrogen concentrations reach high levels.

It can be also speculated that SP 5-11 is acting as a chemical signal for the release of a classic neurotransmitter which then directly produces the inhibitory response in the female rats. Dornan et al. (10) hypothesized that CCK injected into the nucleus accumbens acts to facilitate lordosis by decreasing the inhibitory action of the dopamine on lordosis behavior (1,12). In fact, it has been demonstrated in other areas such as the substantia nigra and striatum that SP induces a release of newly synthesized dopamine (26). Therefore, it is not inconceivable that the inhibition produced by MPN-injected SP may be produced by an increase in dopamine release within the MPOA.

It is interesting to note, although rejection behaviors were not recorded, it seems that the SP analogue-injected females were far more aggressive towards the males than were the saline-injected controls. The test animals often times turned and reared at the males. When females ran from the males, they would often kick the male in the face with their hind legs. These kicks were reported to be as often as 30 times per mating session. These females fought viciously with the pursuing males; males usually being forced onto their backs as a sign

of submission. In fact, some testing sessions were terminated before the prerequisite ten mounts due to the aggressiveness of the female in preventing male mounts. In fact, a similar finding has been reported by Hall and Stewart (14). They found that a SP terminal analogue, SP 7-11, injected intraperitoneally (i.p.) in rats significantly increased the duration of isolation-induced aggression and the number of these aggressive attacks. This finding of SP 7-11 increasing aggression correlates with the effect produced by the SP 5-11 used in experiment 2. A larger percentage of these mating sessions were terminated before the ten mounts than occurred with the SP. The inhibition produced by SP 5-11 may, therefore, be more of motivational then motor.

In summary, the results of experiment 2 support the idea that the SP innervation of the MPOA plays an important role in the neuroregulation of sexual receptivity in the female rat. Obviously, however, further studies are necessary before the role of SP neurons within the MPOA on sexual receptivity in the female rat is fully understand.

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