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Intracerebral Injections of Substance P Fragments (5-11) and (1-7) into the Dorsal Midbrain Central Gray Have No Effects on Lordosis Behavior in the Female Rat

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INTRACEREBRAL INJECTIONS OF SUBSTANCE P FRAGMENTS (5-11) AND (1-7) INTO THE DORSAL MIDBRAIN CENTRAL GRAY HAVE NO EFFECT ON LORDOSIS BEHAVIOR IN THE FEMALE RAT

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ABSTRACT

Substance P (sP), a neuroactive peptide, has now been implicated in a wide range of behaviors. One in particular is female rat sexual behavior. Recently, it has been shown that bilateral injections of sP into the dorsal midbrain central gray (dMCG) of estrogen-primed female rats facilitate sexual behavior (lordosis). Uncertainty remains, however, concerning the mode of action of synaptically released sP. Indeed, most of our understanding of sP activity has revealed that its full structural sequence is not needed for biological activity. This has led to speculation that sP may be processed into one or more types of fragments before it can exert its behavioral effect. In view of these unusual properties, we must first develop a thorough understanding of the effects of sP fragments before we can fully appreciate the significance of this peptide on the expression of female sexual behavior. In a series of behavioral experiments, we assessed the effects of injecting two different fragments (sP 1-7 and sP 5-11) into the dMCG of ovariectomized, steroid primed, female rats. In the first experiment, sexual receptivity was measured following injection of sP(1-7), (5-11) or acidified saline directly into the dMCG of maximally receptive (estrogen and progesterone primed) females. At five and thirty minutes post injection, an assessment revealed that neither sP(5-11)

nor (1-7) had an effect on sexual behavior when compared to saline injections (controls). In the second experiment, sP(5-11) or acidified saline were injected into the dMCG of estrogen primed female rats showing a minimal lordosis response, and the effects of sP(5-11) injections were compared to those of controls (saline). Again, no significant difference in sexual behavior was observed following injection of sP(5-11) or saline. In conclusion, our data suggest that the effect of sP on lordosis behavior following injection into the dMCG is not due to the activity of biologically active fragments.

INTRODUCTION

Sexual receptivity in the female rat is dependent on ovarian hormones. Studies using intracerebral implants show that estrogen action restricted to the ventromedial nucleus of the hypothalamus (VMH) is sufficient to induce receptivity when this treatment is combined with systemic progesterone administration, and recent data indicate that progesterone may also facilitate lordosis by action on VMH neurons (16). Increasing evidence suggests that the hormonal induction of receptivity in rodents is mediated at least in part by projections from the region of the VMH to the midbrain central gray (MCG) (16,2). It also seems likely that substances, perhaps peptides, synthesized in the VMH region and transported to terminals in the midbrain central gray (MCG), are acting at this site to facilitate lordosis. For example, disruption of axonal transport by injection of colchicine into the VMH disrupts lordosis in rats (10).

One popular hypothesis concerning neural regulation of sexual receptivity in the female rat is that by altering the expression of certain genes, estrogen regulates mRNA levels of specific precursor proteins for neuropeptides within the VMH (4,1). 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). Numerous sP cell bodies and fibers have been found in areas relevant to sexual receptivity in the female rat (1,2,3). The VMH has been documented to concentrate estrogen within its cells; these same cells have been found also to make sP (1,2). As well, the MCG, which has sP receptors and cell bodies (12), shows marked changes in the number of sP immunoreactive fibers over the estrous cycle (7).

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. (5), reported that bilateral injections of sP in the MCG facilitated lordosis in response to male and manual stimulation in ovariectomized estrogen-treated 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 sP VMH-MCG pathway in the regulation of sexual receptivity in the female rat. This was the first report to suggest that sP-containing neurons may play a role in mediating reproductive behavior.

Although much attention has been given to sP, even today little is known definitively concerning its physiological functions. Of its function as a neurotransmitter, major uncertainties remain concerning the nature of sP receptors. It has been shown that at some synapses sP can facilitate or inhibit detonation of the post-synaptic neuron without itself producing detonation. A remarkable feature of this modulation was that opposite effects (facilitation or inhibition) were seen at different doses of sP. Several of the effects of sP on intact animals show an "inverted U" dose-response curve (6).

Moreover, it has been shown that the full sequence of sP is not needed for biological activity; the C-terminal pentapeptide amide is sufficient, although of a low potency (6). The potency increases with increasing chain length up to sP (4-11), which is even more potent than sP. In addition, N-terminal sP fragments (sP 1-7) have been shown to produce some effects different from those of sP itself (14,17).

The potency of the sP fragments, along with their ability to modulate opposite effects at different doses, led to the theory that the effects of sP are due to the activity of fragments of the neural peptide, enzymatically cleaved in the brain. It has been shown that sP fragments, sP(1-7) and sP(5-11) produce opposite effects

on pain perception, motor behaviors, and aggressive and nociceptive behaviors. The intact sP may mimic the effects of either of these fragments (8).

In the light of the newly demonstrated action of sP in sexual behavior in female rats (5), and these findings of the action of sP fragments, the aim of the present study was to investigate the role of sP fragments in the modulation of female sexual receptivity.

GENERAL METHODS

Animals and Surgery

Twenty-four adult Long-Evans female rats weighing between 242 g and 314 g were housed doubly in steel mesh cages in a controlled environment at 21^{0}C, with an extended light cycle (on at 0700, off at 2100). Food and water were available ad lib. Animals were ovariectomized under ether. One to six days 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, cemented into place with cranioplastic cement. The cannula were aimed 1 mm above the dMCG using the atlas of Paxinos and Watson as a guide (13). The actual coordinates used were experimentally determined as: AP -7.4, ML -.6, DV -3.6 from bregma. Immediately following surgery, all females received a subcutaneous injection of 10 ug of estradiol benzoate in sesame oil.

Intracerebral Injections

Each 28-gauge inner cannula was connected to a 1-ul syringe by a plastic tube. Distilled water was then drawn up the tube until 1 ul of water could reliably be drawn and expelled. Following this, .3 ul of air was drawn up into the inner cannula, whereupon the inner cannula was placed into the peptide solution and .7 ul of peptide was drawn. During injections, movement of the air bubble ensured that the peptide had actually been injected into the brain. Animals were not anesthetized, but were restrained manually during the injection procedure. Solutions were injected manually in a volume of .5 ul (each side) over a period of 60 s. The inner cannula was left in place for 30-60 s before withdrawal. The peptide solutions were prepared immediately prior to behavioral testing and stored in small plastic centrifuge tubes until used. In both experiments 1 and 2 the fragments were dissolved in acidified saline. The rationale for using acidified saline was as follows: (a) it helps prevent absorption to glass, and may make the peptide more stable, and (b) Hall and Stewart compared the behavioral effects of sP dissolved in saline or acidified saline injected intraperitoneally into mice. They found consistently stronger behavioral effects when sP was dissolved in acidified saline (17).

Behavioral Testing

After a recovery period of at least six days from stereotaxic surgery, mating tests were conducted in a dimly lit room in rectangular test boxes containing wood chip bedding. Test boxes were constructed of three wooden sides and a Plexiglass front of dimensions 18 in W x 18 in L x 17 in H. Sexual receptivity was measured using the lordosis quotient (LQ) and the lordosis intensity scale (LIS) (9). Five minutes after receiving intercerebral injection, females were placed in the mating arena with a sexually vigorous male, and intensity of the lordosis reflex following ten mounts by the male was recorded. These responses were rated on a scale from 0 to 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 the head back; 3 being an exaggerated response with extreme extension of the back and elevation of the nose (see figure 1) (9). The LQ is calculated by the ratio of the number of lordosis responses (1 or greater on LIS) following ten male mounts. This is an all or none evaluation of the lordosis response. The incidence of proceptive behaviors (hopping, darting and ear wiggling) and rejection behaviors were also recorded during the test session.

Before receiving an intracerebral injection, females were given a prescreening test. They were placed in the mating arena with sexually vigorous males and allowed to be mounted 10 times. If the female did not show a LQ of at least .2, she was not used in that test session and was tested at a later date. 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 and 46 um sections were taken on a cryostat. The sections were stained with cresyl violet and the cannulae placements in each brain were located using microscopy.

EXPERIMENT 1

Method

In order to ensure maximum sexual receptivity, all females received a series

of subcutaneous (s.c.) injections of estradiol benzoate (EB) in sesame oil. On the day of testing females received a subcutaneous injection of 1 mg of progesterone 4-6 hours before testing. Females were then divided randomly into 3 groups receiving sP(1-7), sP(5-11) or a control injection of acidified saline. The effects of the sP fragment injections on sexual behavior were assessed by placing females with sexually vigorous males 5 minutes and then 30 minutes following injection.

Results

In this experiment we were interested in examining the effects of sP fragments in animals with relatively homogeneous cannula placements. As a result, any animal which did not have both cannula within the dMCG area was excluded from data analysis. This left 18 animals which had both cannula tips within the dorsal midbrain central gray region. The distribution of cannula tips are illustrated in figure 2.

Data were analyzed using a 3x2 split-plot factorial ANOVA to assess the main effects of dose, time and any interactions. The results of this analysis revealed that bilateral injections of sP(1-7) or sP(5-11) into the dMCG had no effect

on sexual behavior when compared to controls (dose, $F(2,9)=1.52$, $p>0.05$; time, $F(1,6)=0.52$, $p>0.05$). The mean lordosis quotient (LQ) and mean lordosis intensity scale (LIS) for experiment one are presented in figure 3.

EXPERIMENT 2

Methods

In order to ensure moderate sexual receptivity each female received 5 ug 53 hr before testing or until showing a LQ of at least .2. Females were divided randomly into 2 groups which received bilateral injections into the dMCG of sP(5-11), or acidified saline. The effects of the sP fragment injections on sexual behavior were assessed by placing females with sexually vigorous males 5 minutes following injection.

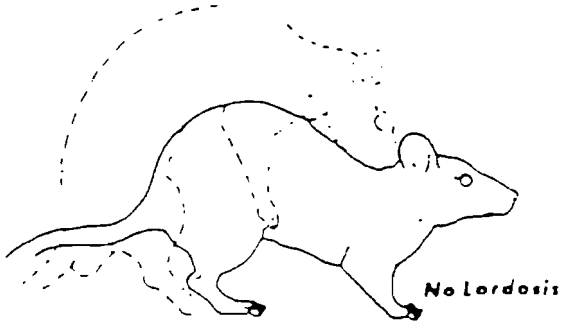
Results

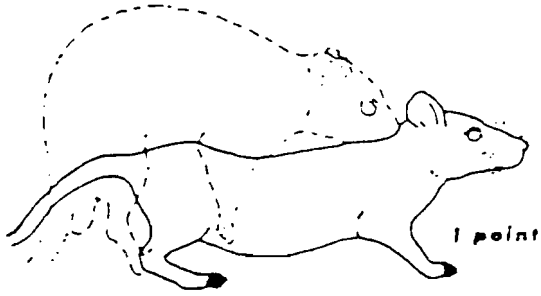
As in experiment 1, injections of sP(5-11) into the dMCG of moderately

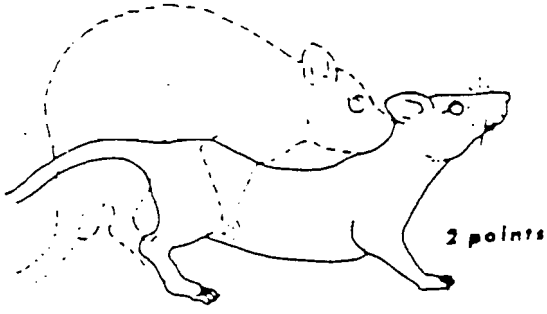
receptive females had no effect on sexually behavior when compared to controls ($t(6)=1.27$,$p > 0.05$). The mean lordosis quotient (LQ) and mean lordosis intensity scale (LIS) for experiment one are presented in figure 4.

Two of the animals with placements considered to be out of the dMCG had received injections of sP(5-11). It is interesting to note that the mean rejection occurrence for these two animals was 29 ± 3, compared to a mean rejection occurrence of 2 ± 1.38 for animals injected with sP (5-11) that had placements within the dMCG. In addition, both animals with placements out of the dMCG had placements in exactly the same area, only slightly dorsal to the nucleus.

Figure 1 Is an illustration of the lordosis intensity scale. The intensity of the lordosis reflex is rated on a 3-point scale: 1 point is given for a marginal lordosis, 2 points for a nominal lordosis, and 3-points for an exaggerated lordosis response. (Taken from Hardy and Debold, 1971.)


No Lordosis


1 point


2 points

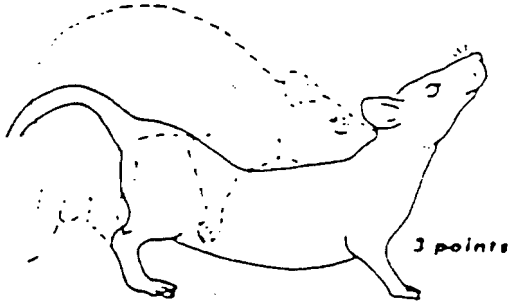

3 points

Figure 2 An illustration of the distribution of cannula tips considered to be in the dMCG.

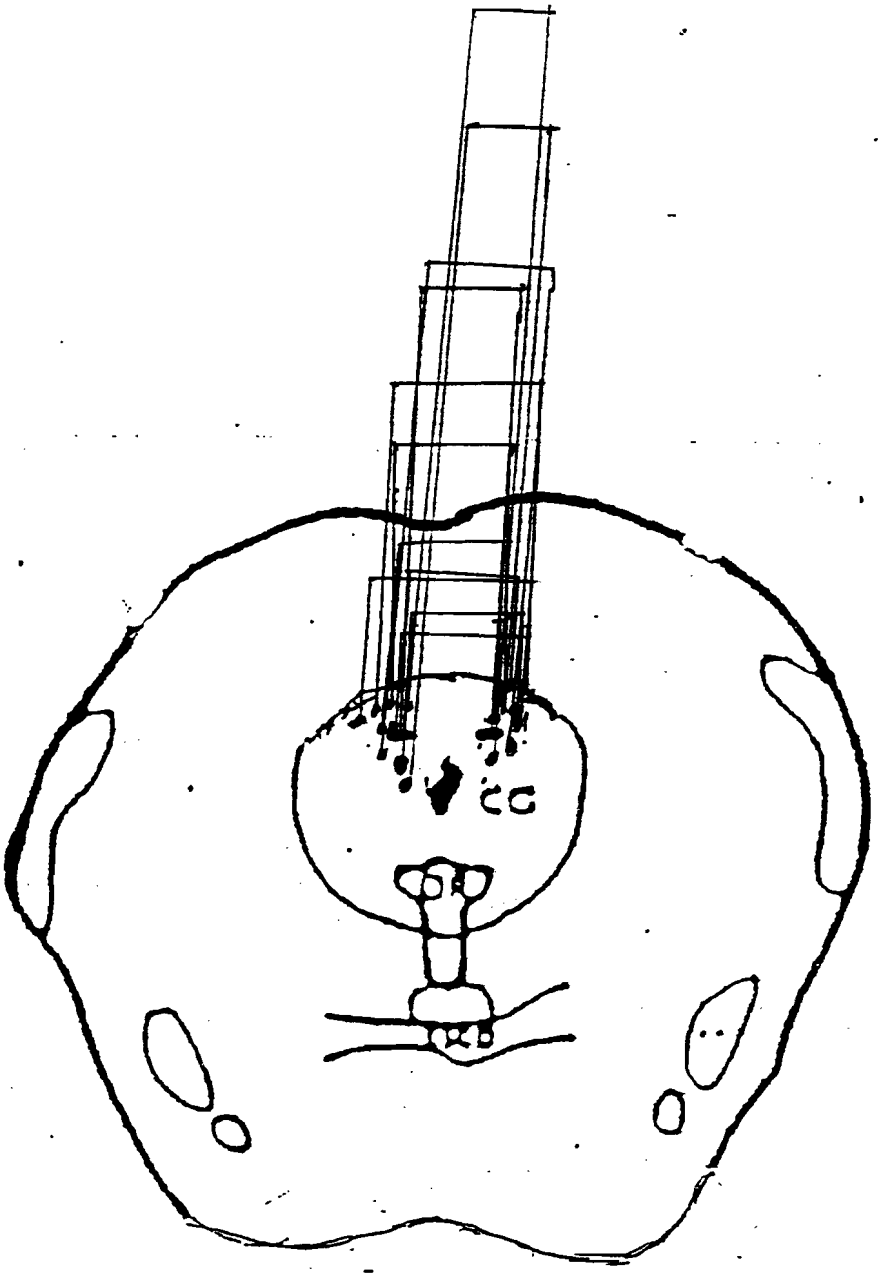
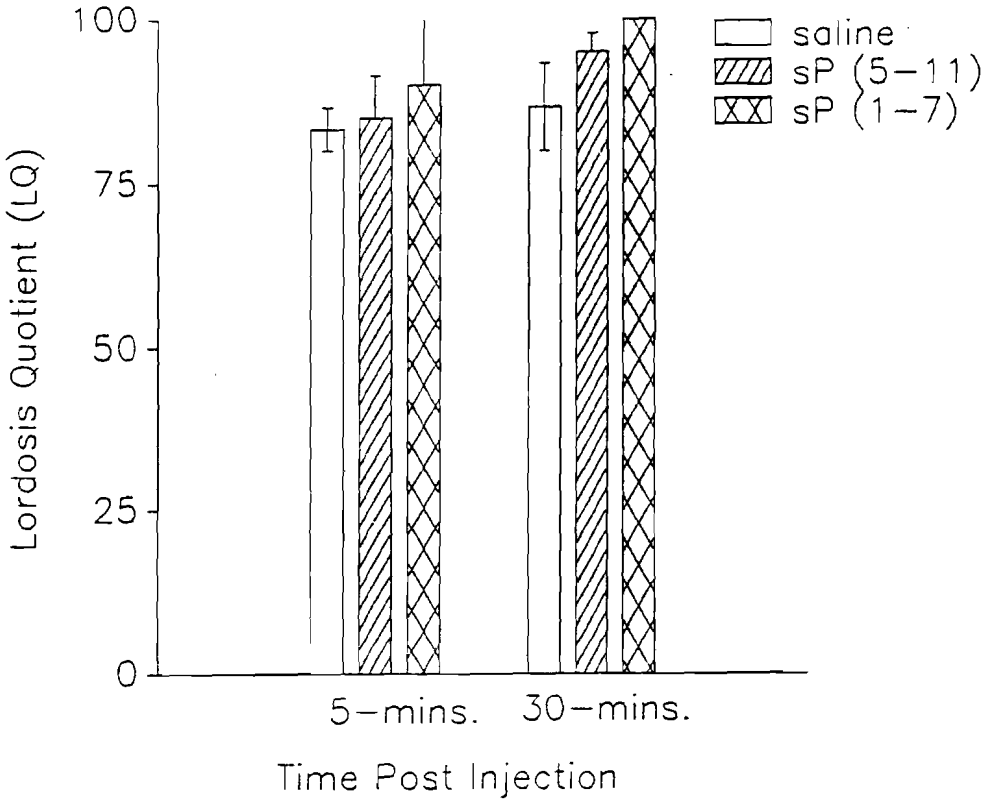

CG

Figure 3 Represents the mean lordosis quotient (3a) and the mean lordosis intensity scale (3b) in response to 10 male mounts in females that received bilateral injections of sP(1-7) (n=3), sP(5-11) (n=4) or acidified saline (n=3) into the dorsal midbrain central gray. All females were estrogen and progesterone primed to ensure maximum receptivity. Vertical lines represent $\pm$ standard errors of the mean.

A)


100
75
50
25
0
Lordosis Quotient (LQ)
saline
sP (5–11)
sP (1–7)
5–mins.
30–mins.
Time Post Injection

B)

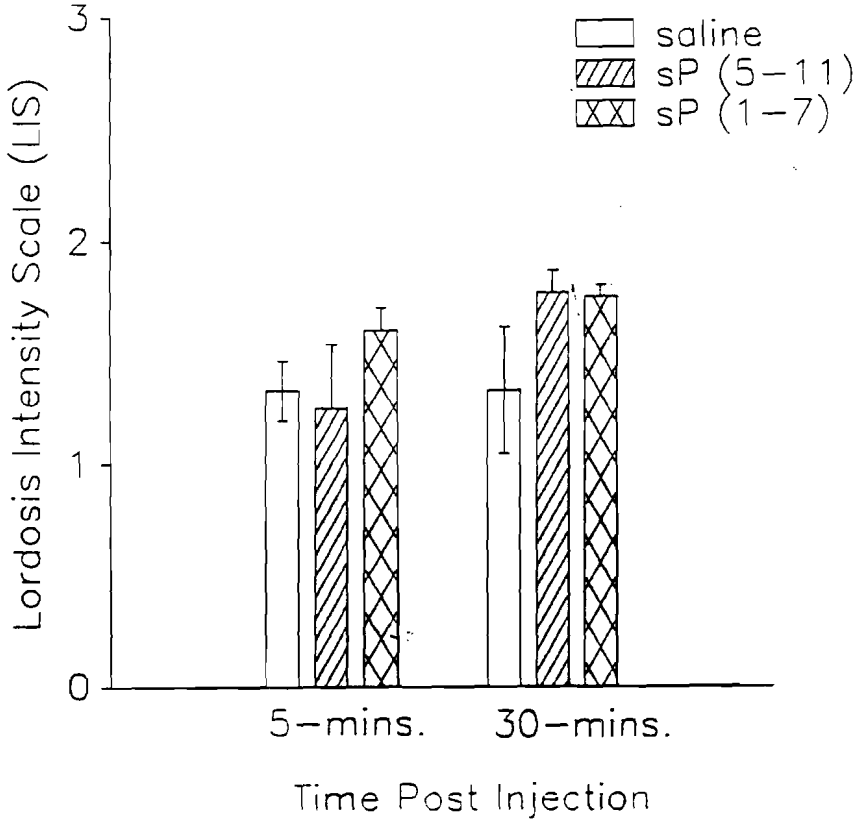
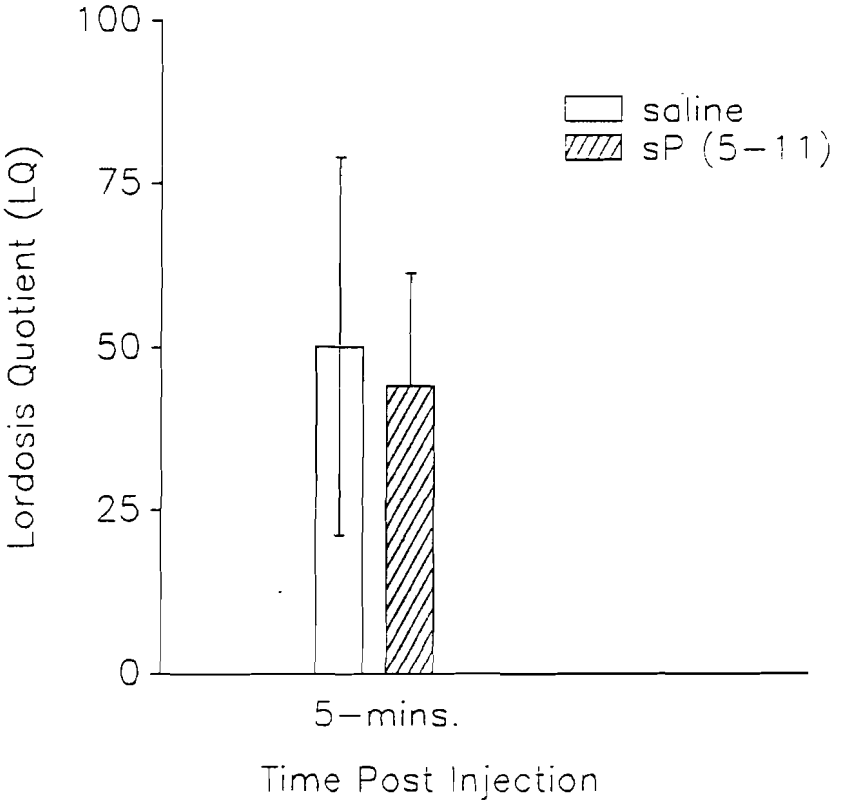
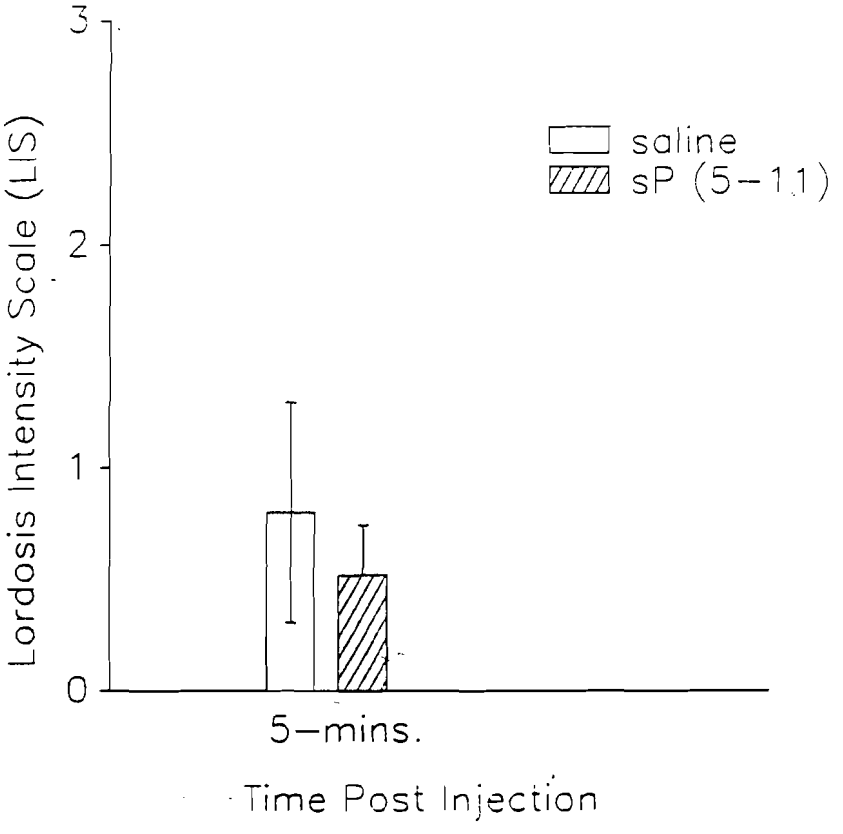

3
2
1
0
Lordosis Intensity Scale (LIS)
saline
sP (5–11)
sP (1–7)
5–mins.
30–mins.
Time Post Injection

Figure 4 Represents the mean lordosis quotient (4a) and the mean lordosis intensity scale (4b) in response to 10 male mounts in females that received bilateral injections of sP(5-11) (n=5) or acidified saline (n=3) into the dorsal midbrain central gray. All females were estrogen primed to show moderate receptivity. Vertical lines represent $\pm$ standard errors of the mean.

A)


100
75
50
25
0
Lordosis Quotient (LQ)
saline
sP (5−11)
5−mins.
Time Post Injection

B)


3
2
1
0
Lordosis Intensity Scale (LIS)
saline
sP (5−11)
5−mins.
Time Post Injection

DISCUSSION

Despite the wide range of effects found to be due to sP fragments rather than the whole peptide (8,14,17), bilateral injection of two sP(1-7) and (5-11) into the dMCG produced no appreciable effect on lordosis behavior in OVX steroid-primed female rats when compared to injections of acidified saline. However, in experiment 2, minimally receptive females showed a greater occurrence of proceptive behavior (hopping and earwiggling), and of rejection behavior (data not shown). It has been shown that sP(7-11) attenuates passive avoidance behavior when injected into the nucleus accumbens (8). Perhaps the higher occurrence of rejection behavior is evidence that the C-terminal portion of sP has an effect of active avoidance.

These results are interesting because, other experiments have demonstrated that peptides containing only the N-terminal part of sP produce effects on other rodent behaviors (8). It has also been shown that the potent C-terminal fragment agonist sP-C produces effects opposite to those of sP-N. Intact sP may mimic the effects of either fragments: it mimics the action of sP-C on motor behaviors but mimics the action of sP-N on aggressive and nociceptive behaviors (8). These

results are consistent with the hypothesis that sP must be processed to one or the other type of fragment before it exerts its effects on behavior.

Brain enzymes which could carry out such processing of sP have been described. Enzymes have been studied which cleave sP at the 7-8 bond (and perhaps the 6-7 and 8-9 bonds as well) (11). The enzyme which cleaves sP at the 4-5 bond can yield sP(5-11), which can be transformed rapidly and spontaneously into pyroglutamyl-sP(6-11). This fragment is very similar in its effects on mouse behavior to sP-C. The sP fragment sP-C is a well-established potent sP-like agonist (8) , at least for peripheral sP receptors.

In summary, our data provide no evidence that biologically active fragments are responsible for the effects of sP on lordosis behavior in the female rat following injection into the dMCG. However, the possibility still remains that sP fragments may act on other sexual behaviors such as proceptive and rejection behaviors.

REFERENCES

1. Akesson, T.R., Micevych, P.E. Estrogen concentration by substance P-immunoreactive neurons in the medial basal hypothalamus of the female rat. *J. of Neurosci. Res.* **19**:137-145, 1988.

2. Akesson, T. R., Simerly, R.B., Micevych, P.E. Estrogen-concentrating hypothalamic and limbic neurons project to the medial preoptic nucleus. *Brain Res.* **451**:381-385, 1988.

3. Cuello, A.C., Priestley, J.V. & Matthews, M.R. Localization of substance P in neuronal pathways. In: *Substance P in the Nervous System,* London: Pitman Press, 1982, pp. 55-83.

4. Cuello, A.C., Priestley, J.V. & Paxinos, G. Substance P and enkephalin containing pathways. In: *The Rat Nervous System,* Australia: Academic Press, 1985, pp. 441-469.

5. Dornan, W.A., Malsbury, C.W. & Penney, R.B. Facilitation of Lordosis by injection of substance P into the midbrain central gray. *Neuroendocrinology* **45**:498-506, 1987.

6. Frederickson, R.C.A., Burgis,V., Harrell, E., & Edwards, J. Dual actions of substance p on nociception. Possible role of endogenous opioids. *Science* **199**:1359-1361, 1978.

7. Frankfurt, M., Siegel, R.A., Simm, I., Wuttke, W. Estrous cycle variations in cholecystokinin and substance P concentration in discrete areas of the rat brain. *Neuroendocrinology* **42**:226-231, 1986.

8. Hall, M.E. & Stewart, J.M. Substance P and behavior: Opposite effects of N-terminal and C-terminal fragments. *Peptides* **4**:763-768, 1983.

9. Hardy, D.F. & DeBold, J.F. Effects of mounts without intromission upon the behavior of female rats during the onset of estrogen-induced heat. *Physiology and Behavior* **7**:643-645, 1971.

10. Harlan, R.E., Shivers, B.D., Kow, L.M. Pfaff, D.W. Intrahypothalamic colchicine infusions disrupt lordotic responsiveness in estrogen-treated female rats. *Brain Res.* **238**:153-167, 1982.

11. Lee, C., Sandberg, B., Hanley, M. & Iversen, L. Purification and characterization of a membrane-bound substance P-degrading enzyme from human brain. *Eur J Biochem* **114**:315-327, 1981.

12. Mantyh, P.W., Hunt, S.P., Maggio, J.E. Substance P receptors: Localization by light microscopic autoradiographic in the rat brain using (3H)-sP as the radioligand. *Brain Res.* **307**:147-165, 1984.

13. Paxinos, G. & Watson, C. *The rat brain in stereotaxic coordinates.* Sydney, Australia: Academic Press, 1982.

14. Pelleymounter, M.A., Fisher, Q., Schlesinger, K., Hall,M., Dearmey, P. & Stewart, J. The effects of substance P and its fragments on passive avoidance retention and brain monoamine activity. *Behavioral Brain Research* **21**:119-127, 1986.

15. Pfaff, D.W. & Schwartz-Giblin, S. Cellular mechanisms of female reproductive behaviors. In E. Knobil & J. Neill (Eds.), *The physiology of reproduction.* New York: Raven Press, 1988, pp. 1487-1568.

16. Rubin, B.S., Barfield, R.J. Induction of estrous behavior in ovariectomized rats by sequential replacement of estrogen and progesterone to the ventromedial hypothalamus. *Neuroendocrinology* **37**: 218-224, 1983.

17. Stewart, J.M., Hall, M.L., Harkins, J., Frederickson, R.C., Terenius,L., Hokfelt, T., & Krivoy, W.A. A fragment of substance P with specific central activiey: sP(1-7). *Peptides* **3**:851-857, 1982.