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Induction of maternal behavior in virgin rats after intracerebroventricular administration of oxytocin

(neurohypophysis/vasopressin/peptides/estrogen dependence)

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ABSTRACT **Oxytocin produces uterine contractions and milk ejection, functions related to parturition and nurturing. Studies were conducted to determine if this peptide, native to the brain and the posterior pituitary gland, plays a role in the induction of maternal behavior. Intact virgin female rats were given 0.4 μg of oxytocin, 0.4 μg of [Arg^8]vasopressin, or saline through lateral ventricular cannulae. Forty-two percent of intact rats receiving oxytocin displayed full maternal behavior towards foster pups. None of the saline- or vasopressin-treated animals displayed full maternal behavior. Criteria in five behavioral categories had to be fulfilled by an animal within 2 hr of injection for its behavior to be considered fully maternal. When partial maternal responses were considered, oxytocin was significantly more effective than saline and marginally more effective than vasopressin. Five animals responding fully maternally after oxytocin injection were allowed to stay with pups for 10 days. All five continued to display full maternal behavior during this time. Nearly all animals that responded fully maternally to oxytocin injection were in the last day of diestrus or in proestrus or estrus. This suggested that elevated or recently elevated levels of estrogen may be necessary for the induction of full maternal behavior by oxytocin. Twenty-seven virgin female rats were ovariectomized and given either 100 μg of estradiol benzoate per kg in oil subcutaneously or oil alone immediately after operation. Forty-eight hours later, all animals received 0.4 μg of oxytocin intracerebroventricularly. Eleven of 13 estrogen-primed animals became fully maternal; none of 14 nonprimed animals became fully maternal.**

Rosenblatt (1) demonstrated that male and female rats, including intact virgin females, manifest maternal behavior after contact with foster pups for 5–7 days. Since then, several attempts have been made to shorten the latency for this phenomenon by manipulations of estrogen, progesterone, or prolactin. When these hormones were administered on selected schedules to ovariectomized, nulliparous females over either an 11-day (2) or 27-day (3) period, latency was reduced to as little as 35 hr.

Pharmacologic blockade of prolactin release has no effect on the onset of postpartum maternal behavior (3–5) or estrogen-induced maternal behavior (6). Similarly, induced elevation of prolactin in ovariectomized virgin females does not alter the onset of pup contact-induced maternal behavior (7). Progesterone has been found by most investigators to inhibit the onset of maternal behavior under various experimental conditions (8, 9), though dosage and timing of progesterone administration seem to be important (10).

Of the hormones studied singly, estrogen appears most potent in inducing maternal behavior. Hysterectomy in late pregnancy, which probably causes a rapid rise in plasma estrogen (11) as judged by examination of vaginal smears, results in a high incidence of maternal behavior 48–72 hr after pup contact initiated 48 hr after surgery (12–14). Ovariectomy simultaneous with hysterectomy prevents this effect. The estrogen dependence of the induction of maternal behavior is further shown by estradiol administration at the time of hysterectomy and ovariectomy in either late gestational or virginal female rats. Animals thus treated show a high rate of maternal response within 24 hr after first presentation of pups 48 hr after surgery (12, 15, 16).

Klopfer is the only investigator who has not focused exclusively on late gestational steroids or prolactin as endocrine determinants of maternal behavior. He has suggested a role for the posterior pituitary hormone, oxytocin (17–19), which is released in high concentration during the expulsive phase of labor (20–26). A small body of experimental evidence is consistent with Klopfer's concept. Terkel and Rosenblatt (27) established a vascular connection between parturient and virgin female rats. Blood exchange induced a shortened latency (14.5 hr) for maternal behavior in virgin females if the exchange began 30 min after completion of parturition. When established 24 hr before or after birth, blood exchange was not effective. However, oxytocin injected intravenously in a dose that probably transiently produced plasma concentrations well above those attained during the expulsive phase of labor has failed to reinstate maternal behavior in goats separated from their young at birth (28).

Recently, Dogterom *et al.* (29) have published evidence that oxytocin and vasopressin are released directly into the cerebrospinal fluid by pathways other than neurohypophyseal secretion. Schwarzberg *et al.* (30) have also reported that electrical stimulation of the paraventricular nucleus of the hypothalamus was followed by a significant rise of oxytocin activity in cerebrospinal fluid, whereas stimulation of the supraoptic nucleus resulted in a rise that was not statistically significant. This suggests that during the expulsive phase of labor, when neurohypophyseal secretion into blood is maximal (20–26), high concentrations of oxytocin may be released simultaneously into the ventricles of the brain, a timing and site of delivery of this hormone that could produce behavioral effects, including maternal activity.

In recent years, the concept has been proposed that at least some peptide hormones native to the central nervous system exert behavioral effects quite apart from the endocrine effects traditionally attributed to them (31). We have suggested, and critically reviewed, a concept of harmony between the endocrine and behavioral action of neuropeptides (32). Guided by this general notion and by the specific suggestion of Klopfer, we proposed to examine the possibility that oxytocin might be a potent inducer of maternal behavior. Others have investigated the influence of intraventricular administration of oxytocin on the behavior of resting mice (33) and avoidance behavior in rats (34), but not on the behavior of adult animals in the presence of offspring. After preliminary work in which this concept was supported and in which 0.4 μg of oxytocin was frequently ef-

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fective in inducing maternal behavior,* we undertook four systematic experiments.

MATERIALS AND METHODS

Sexually mature, virgin female Sprague–Dawley rats (250–350 g) were obtained from the Zivic Miller Laboratory (Allison Park, PA). Pregnant females timed to deliver at regular intervals were obtained from the same source to ensure a supply of pups. Indwelling cannulae were placed in the left lateral intracerebral ventricle of each virgin female under light ketamine anesthesia (100 mg/kg intraperitoneally). The stereotaxic coordinates used were 0.5 mm posterior and 1.8 mm left from the bregma and 4.1 mm below the surface of the skull. Each animal was allowed at least 4 days of recovery before further treatment. The animals were housed in separate cages in a room with a 12-hr light/dark cycle and given free access to food and water.

Rectangular clear plastic observation cages measuring 44 cm in length, 21 cm in width, and 20 cm in height, with wire-mesh floors, housed individual animals during behavioral observations. A metal grid frame with recesses to hold food pellets and a water bottle covered each cage. Each observation cage was placed inside a wooden observation booth that had a large, smoked Plexiglas window through which the contents of the enclosed observation cage could be fully observed from a distance as close as 16 cm. The only source of illumination was a single 7.5 W incandescent light bulb inside each observation booth. Animals were allowed 2–3 hr of habituation to the observation cages before experiments were begun.

Experimental animals were injected with 0.4 μg of oxytocin or 0.4 μg (an approximately equimolar dose) of [Arg^8]vasopressin (both obtained from Bachem, Torrance, CA) in 10 μl of normal saline (pH 5.0) delivered over 3–4 min with a 10-μl Hamilton syringe linked to the implanted cannula by a length of sterile plastic tubing. Animals were hand held during this procedure, which took place outside the observation cages. Control animals were injected with 10 μl of sterile normal saline delivered by the same technique.

In experiment 1, 13 animals received oxytocin and 12 received saline. During injection, three 2- to 7-day-old pups were placed in the center of the vacant cage, each pup approximately 10 cm distance from the others at the apices of an imaginery equilateral triangle. Immediately after injection, each animal was returned to the center of its cage. Behavior was recorded for the ensuing 2 hr by an observer ignorant of treatment assignments. Observation focused on five categories of maternal behavior. Because untreated virgin females presented with pups will occasionally display brief episodes of these behaviors, we established rigorous criteria for any given behavior to be attributed to an animal.

The minimal criteria required during 2 hr for attributing each of the five maternal behaviors to an animal are listed below:

(*i*) *Grouping.* The animal must carry in her mouth all three pups from the center of the cage and clump them together in a corner. She must also display at least one additional bout of regrouping, involving rearrangement of at least two pups, within this area.

(*ii*) *Licking.* The animal must lick one or more pups for at least a total of 1 min. Bouts of 2 sec or less are not added to the cumulative time.

(*iii*) *Crouching.* All pups must be grouped before crouching can be recorded. The animal must have its hind legs spread, its back arched, and its ventrum high enough off the cage floor to accommodate pups beneath her. There must be at least one pup beneath her during a crouch. This position must be displayed for at least a total of 1 min. Bouts of 2 sec or less are not added to the cumulative time.

(*iv*) *Nest building.* The female must pull more than half the 20 paper scraps into the corner where at least one pup has been or eventually will be carried. (Twenty scraps were put in each cage during habituation; they were redistributed while the animal was removed for injection.)

(*v*) *Retrieval.* Any time within the 2-hr observation period after grouping has taken place, one pup is separated by the observer to a distance of 15 cm from the female. Retrieval constitutes carrying the pup back to the group within 15 min of separation. Retrieval must be observed, rather than inferred, since a pup may return by its own efforts.

Animals were assigned a behavioral score at the end of the 2-hr observation period. One point was given for each behavioral category in which the animal fulfilled minimal criteria. Females were required to fulfill minimal criteria in all behavioral categories (score of 5) to be considered fully maternal.

Rosenblatt (1) used categories *ii–v* above, though without stating quantitative criteria for their fulfillment. We added "grouping" as a maternal behavior because, during pilot work, we observed that a few untreated animals initially grouped and regrouped pups but then failed to retrieve a separated pup. Thus the separate scoring of grouping and retrieval may provide a more accurate description of partial maternal responses.

In experiment 2, 10 animals were injected with 0.4 μg of oxytocin as described above with the observer cognizant of treatment. Five animals responded fully maternally (score of 5) within 2 hr by the criteria used in experiment 1. Each of these five was kept in constant contact with three pups for 10 days. Each morning, each animal was observed for 20 min. During this abbreviated observation period the fulfillment of four criteria was accepted as evidence of persistent full maternal behavior. The presence or absence of a *nest* with grouped pups was noted. Each animal was then given 15 min in which to display one cumulative minute of *crouching*. Then the three pups were replaced by three other pups, placed at the front of the cage. The animal was allowed 5 min in which to *retrieve* and also *group* these three pups within the nest.

Experiment 3 was conducted during four laboratory sessions. At each session the observer was ignorant of the treatment to which an animal was assigned. Animals were prepared, injected, and observed as described for experiment 1. At each session some animals were injected with 0.4 μg of oxytocin and some with an equal amount of [Arg^8]vasopressin. Saline was not included as a test substance because, in experiment 1, none of 12 animals receiving saline had shown more than a partial maternal response.

Prior to experiments 1–3, 44 animals, later to be randomly assigned to treatment groups, were assessed as to estrous state by daily examination of vaginal smears. A high correlation of full maternal response after oxytocin with estrous states during which estrogen levels are elevated prompted us to conduct experiment 4. We ovariectomized 38 animals that had been previously implanted with cannulae. Immediately after operation, 24 animals were injected subcutaneously with 100 μg of estradiol benzoate per kg in corn oil (0.1 ml), a dose used after ovariectomy and hysterectomy by other workers (12, 15, 16). Forty-eight hours later, estrogen-primed animals were given 0.4 μg of oxytocin or saline through their lateral ventricular cannulae. Nonprimed animals were given only oxytocin. All groups were presented with pups and observed for 2 hr by an observer ignorant of treatments as in experiments 1–3.

* Pedersen, C. A. (1979) *North Carolina Neuroscience Society Meeting*, May 11, 1979, Raleigh, NC, 11 (Abstr.).

At the conclusion of each experiment, all animals were injected with 1 μl of a concentrated solution of Evans Blue dye by the technique used to deliver experimental substances. The animals were then killed. Their brains were removed, frozen, and sectioned to confirm staining of the ventricles.

RESULTS

The results of experiment 1 are displayed in Table 1, which summarizes the distribution of maternal scores in each treatment group and presents the mean score of each group. Six of 13 animals receiving oxytocin became fully maternal (score of 5), whereas none of 12 animals receiving saline became fully maternal. Oxytocin was more effective than saline in producing a full maternal response ($P < 0.01$ Fisher's exact probability test). In a comparison of mean scores of the two injection groups, oxytocin was more effective than saline ($P < 0.04$ Student's t test, two-tailed interpretation).

In experiment 2, 5 of 10 animals showed full maternal behavior within 2 hr after oxytocin injection. All five fulfilled the abbreviated criteria for full maternal behavior each morning of the 10-day observation period.

In experiment 3, 13 animals were injected with oxytocin and 13 with [Arg^8]vasopressin. In the oxytocin group, four showed full maternal behavior; in the [Arg^8]vasopressin group none showed this response ($P < 0.05$, Fisher's exact probability test). Table 1 classifies the maternal responses of the animals in the two treatment groups and presents the mean score of each group ($P < 0.06$, Student's t test, one-tailed interpretation).

During experiments 1–3, 44 animals received daily vaginal smears and all were found to have active estrous cycles. Table 2 shows the estrous state of animals at the time of injection of experimental substances. Inspection of the data suggests that a full maternal response to oxytocin injection may be at least partly dependent on estrous state. Estrogen begins to rise late in diestrus (D_2), peaks in proestrus (P), falls in late proestrus, and reaches its nadir in early diestrus (D_1) (35, 36). It seemed reasonable, therefore, to examine the responses to oxytocin according to estrogen state at the time of injection: rising, elevated, or recently elevated levels (late diestrus, proestrus, and estrus, respectively) as opposed to low levels furthest removed in the estrous cycle from peak levels (early and mid diestrus). Seventeen animals were in late diestrus, proestrus, or estrus at the time of injection. Eleven showed a full maternal response after oxytocin injection; six did not. Twelve animals were in early or mid diestrus at the time of oxytocin injection. One showed a full maternal response; eleven did not. The probability that this distribution of responses would occur by chance is less than 3 in 1000 (Fisher's exact probability test). Table 2 also shows that [Arg^8]vasopressin and saline were administered proportionately at least as often as oxytocin at times in the estrous cycle that are apparently propitious for the induction of a full maternal response.

Table 2. Ratio of numbers of animals showing full maternal response to total number injected in each estrous state

Estrous state	Oxytocin	Vasopressin	Saline
D_2	2/4	0/1	0/1
P	3/5	0/4	0/2
E	6/8	0/2	0/1
Total	11/17	0/7	0/4
D_1	0/3	0/1	0/1
Prolonged mid-D	1/9	0/2	—
Total	1/12	0/3	0/1

P, proestrus; E, estrus; D_1, day after estrus; D_2, day before proestrus; prolonged mid-D, diestrus between D_1 and D_2.

The results of experiment 4 are summarized in Table 1. The distribution of maternal scores and the mean score for each treatment group are presented. Eleven of 13 animals primed with estradiol benzoate displayed full maternal behavior; none of the 14 animals injected only with the oil vehicle did ($P < 0.00001$, Fisher's exact probability test). A comparison of mean scores of the two groups showed that oxytocin was conspicuously more effective in animals injected with estradiol than with oil alone ($P < 0.0000001$, Student's t-test, two-tailed interpretation). Saline injection in estrogen-primed animals produced a low level of full maternal responses. Saline, in this model, was clearly less effective than oxytocin ($P < 0.002$, Fisher's exact probability test). However, saline in this model tended to be slightly more effective than saline in intact, nonprimed animals ($P < 0.22$, Fisher's exact probability test), suggesting that ovariectomy or estrogen priming (or both) can bring a small number of animals to threshold for demonstration of maternal responses.

It was impossible to verify cannula placement in animals used in experiment 1 because other experimental uses of these animals, which were irrelevant to the present report, delayed necropsy 3–4 weeks. Cannula placement appeared to be accurate in the animals used in experiments 2–4, after which the interval before necropsy was less than 2 days (experiments 3 and 4) or 10 days (experiment 2). Seventy-four of 80 animals showed staining with Evans Blue in both lateral and third ventricles of their brains. Data pertaining to the other six animals were deleted from analyses.

In the present work, full maternal behavior was defined as

Table 1. Numbers of virgin female rats attaining various scores of maternal behavior

	Exp. 1*		Exp. 3*		Exp. 4†		
					Estrogen-primed		Nonprimed
Scores	Oxytocin	Saline	Oxytocin	Vasopressin	Oxytocin	Saline	Oxytocin
5	6	0	4	0	11	2	0
4	0	0	0	1	0	0	0
3	0	2	1	2	0	3	0
2	0	1	0	0	0	0	0
1	0	1	3	0	0	0	1
0	7	8	5	10	2	6	13
Total	13	12	13	13	13	11	14
Mean score	2.3	0.8	2.0	0.8	4.2	1.7	0.07
(±SEM)	±0.7	±0.4	±0.6	±0.4	±0.5	±0.6	±0.07

* Intact, nonprimed animals.
† Ovariectomized animals.

the fulfillment of criteria for each of five behavioral categories. However, a descriptive sketch of oxytocin-induced full maternal behavior may provide a more vivid portrayal of the phenomenon. Full responses began as early as 5 and as late as 73 min after oxytocin injection, usually between 10 and 45 min. The initial behavior was usually sudden avid licking or grouping of pups, though a few animals started by building nests. Licking was predominantly directed towards the pups' anogenital areas. Licking, grouping, and regrouping were quickly followed by crouching and nest building. Once initiated, maternal behavior usually met all criteria for a full response quite rapidly, though a few animals appeared temporarily to lose interest in pups. All full responders displayed considerably more licking and crouching than one cumulative minute in 2 hr. Full responders, left in contact with pups for several days, often displayed repetitive tail retrieval and, when alarmed, frantic carrying of a pup about the cage. Both of these delayed behaviors were commonly seen in normal mothers during informal observations.

DISCUSSION

We have examined the data from four experiments from two points of view: the frequency with which a *full* maternal response was produced by experimental substances and the degree to which a maternal response, full or partial, occurred in animals in the various experimental groups. Oxytocin was clearly superior to both saline and [Arg^8]vasopressin in producing full maternal responses. Its effects, compared to those of the other experimental substances, were only slightly less striking when partial responses were also taken into account. In these comparisons, oxytocin was statistically significantly more effective than saline and strongly tended to be more effective than [Arg^8]vasopressin. Data pertaining to estrous cycle accumulated across experiments strongly suggested that the maternal response to oxytocin was partly estrogen dependent. This dependence was demonstrated by a high rate of full maternal response after oxytocin administration in ovariectomized estrogen-primed females (85%) as compared to a virtual absence of response in ovariectomized females not primed with estrogen. Serum estradiol levels peak just before parturition (37), the integrity of the Ferguson reflex (oxytocin release in response to vaginal distension) is estrogen dependent (38–41), and oxytocin-induced uterine contractions also require the presence of estrogen (42, 43). Thus, our present findings, like the earlier work cited, attest to the permissive effect of estrogen on oxytocin activity. Nevertheless, when in previous studies estrogen, progesterone, and prolactin have been manipulated, these procedures have never succeeded in producing maternal responses in virgin female rats so promptly and completely as produced by oxytocin.

The present studies pose a number of questions for future investigation. Preliminary data in rats suggest that even high doses of intravenous oxytocin are ineffective in producing maternal behavior. Nevertheless, a careful exploration of the possible efficacy of peripheral administration of this hormone remains to be done. In a similar manner we have not yet attempted to describe a dose–response relationship between oxytocin and induction of full maternal behavior. The specificity of the oxytocin effect also requires further examination. Nevertheless, oxytocin and [Arg^8]vasopressin are structurally closely related, and the former appears more effective than the latter in producing full maternal responses. Thus, present results suggest that the oxytocin effect is not totally nonspecific. A variety of other peptides must be examined, prominent among them the putative melanocyte stimulating hormone release inhibiting factor (melanostatin). Chemically this hormone is Pro-Leu-Gly-NH_2; this is also the side chain of oxytocin (44).

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