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Effects of Prenatal Exposure to Morphine Sulfate on Reproductive Function of Female Rats[1]

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VATHY, I. U., A. M. ETGEN, J. RABII AND R. J. BARFIELD. *Effects of prenatal exposure to morphine sulfate on reproductive function of female rats.* PHARMACOL BIOCHEM BEHAV **19**(5) 777–780, 1983.—The purpose of this study was to investigate the effect of prenatal exposure to morphine sulfate on the development of reproductive function in female rats. Female rats exposed to morphine sulfate in utero (5–10 mg/kg on days 5–12 of gestation) exhibited varying dates of vaginal opening and a partial inhibition in adult feminine sexual behavior when compared to controls. However, the estrogen binding capacity of hypothalamic cytosols from morphine- and saline-treated females was identical. While we cannot rule out the possibility that the observed changes in reproductive function were an indirect result of morphine-induced alterations in maternal physiology or behavior, these results suggest that the development of feminine sexual behavior can be influenced during the early prenatal period and that morphine sulfate can affect the process of feminization.

Morphine sulfate Development Reproductive behavior Neural estrogen receptors

IN recent years there has been increasing interest in the biological effects of opiate abuse. The special problems of drug abuse among pregnant women and potential adverse effects of opiates on the developing fetus are major concerns. Opioids interact with high affinity, stereospecific binding sties or receptors in the brain and other tissue [7]. The observation that the highest concentrations of neural opiate receptors are in the limbic system, thalamus, striatum, hypothalamus, midbrain and spinal cord [16,19] suggests that physiological mechanisms other than analgesia and pain perception may be affected by narcotics. For example, exogenous opiates, such as morphine, administered to adult male rats can partially [8] or completely [13] eliminate masculine sexual behavior. In addition, morphine and the morphinomimetic enkephalin analogue FK 33-824 reduced the frequency of lordosis responses in adult female rats [12].

It is also possible that prenatal exposure to opiates might modify the development of adult sexual behavior. There are certain critical times during brain development when abnormalities can be induced by agents that affect the central nervous system (CNS). In rats and mice this period of sensitivity extends roughly from the fifth to the fourteenth day of gestation when organogenesis takes place [6]. The possible effects of intrauterine narcotic exposure on brain development should be examined since many such compounds cross the blood-brain barrier [15]. Opiates such as morphine can penetrate the maturing CNS and interact with brain receptors, perhaps influencing later behavior. For example, prenatally morphine exposed rats exhibit a sustained period of hyperactivity [17] during the third and fourth postnatal weeks. Similarly, clinical reports show an emergence of hyperkinesis between 1 and 2 years of age in human infants born to narcotic-dependent mothers [20]. Furthermore, human infants whose mothers are dependent on narcotic drugs such as opium, morphine, heroin or methadone may be born addicted themselves and show serious withdrawal symptoms from birth to about four days [2].

It is not known, however, if adult reproductive function can be affected by prenatal morphine exposure. Therefore, in the present study the effect of prenatally administered morphine sulfate on the date of vaginal opening and on adult reproductive behavior in female rats was examined.

METHOD

General Method

Sprague-Dawley female rats were obtained from Charles River breeding laboratories (Willmington, MA). They were housed two per cage in standard rat cages and maintained on a 14 hr light-10 hr dark cycle (lights on at 0500 hours). Sexually experienced male rats were introduced to each cage containing two females; once mating had occurred the female rats were transferred to individual cages. The presence of

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sperm in the vaginal smear was recorded as day zero of pregnancy.

The pregnant female rats were randomly assigned to one of two groups: (1) a morphine sulfate (MS) treated experimental group; or (2) a saline (S) treated control group. The experimental group of rats was injected twice on day five post-conception, once in the morning (0730 hr) and once in the afternoon (1630 hr) with 5 mg/kg MS subcutaneously (SC). On day six the animals were injected with 5 mg/kg MS in the morning and 10 mg/kg in the afternoon. On day seven and thereafter through day twelve the animals received two daily injections of 10 mg/kg MS. The control group of female rats received two daily injections of 0.8% NaCl on days five to twelve. These injection times were chosen to coincide with the first trimester of human pregnancy during which fetal development is most sensitive to insult by teratogens. The offspring of each group were left undisturbed until weaning. Only female offspring were used for further experimentation. They were housed individually in standard rat cages at 21 days of age and maintained on a 12-12 reversed light-dark cycle with lights off at 0930 hr. Purina rat chow and water were available ad lib.

The animals were checked daily for vaginal opening; then vaginal smears were taken for a period of three weeks to determine estrous cyclicity. Beginning on day 25, animals were also weighed once a week throughout the experiment. At 60 days of age they were ovariectomized under ether anesthesia and were given a priming injection of 2 μg estradiol benzoate (EB) three days later. Animals in each group were randomly assigned to receive one of three EB doses (2, 8, or 25 μg) for behavioral testing. Testing for female sexual behavior began ten days following ovariectomy.

Behavioral Testing

The animals were tested with sexually experienced males in glass-walled observation tanks (measuring 51×25×30 cm) with wood shavings on the floor. They were tested once a week during a three week testing period. The female rats received an SC injection of EB (2 μg, 8 μg, or 25 μg) and 500 μg progesterone, 54 and 5 hours, respectively, prior to testing. Male rats were allowed to adapt to the test chamber for at least ten minutes prior to the introduction of the female. Males were permitted to mount female rats ten times and the number of lordosis responses was recorded. A lordosis quotient (LQ, number of lordosis/number of mounts × 100) was derived and served as a measure of estrous responsiveness. Solicitation behavior, including darting, hopping, and ear wiggling, was scored whenever it occurred throughout the ten-mount test. In addition, the quality of lordosis was observed and scored on a scale of zero to three, where three was a maximum lordosis and zero was no lordosis.

For each measure, means were calculated for each subject based on her performance in three weekly tests of feminine sexual behavior. All behavioral measures were subjected to analysis of variance, and Duncan's multiple range tests were used for post-hoc pairwise comparisons.

Cytosol Estrogen Receptor Assays

At least two weeks after their final behavior tests, animals were sacrificed by decapitation and the brains quickly removed and placed on ice. The entire hypothalamus-preoptic area (HPOA) was dissected as described previously [4]. All procedures were performed at 0–4°C unless otherwise noted. The tissue was homogenized (4 hypothalami/ml) in TEDG (10 mM Tris HCl, 1.5 mM Na_2-EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol, pH=7.4 at 4°C) and centrifuged for 10 min at 850×g. The supernatant was recentrifuged for 1 hr at 105,000×g; the resultant cytosol was removed and used immediately.

Cytosol estrogen receptor levels were quantified using a modification of the procedure of Chamness *et al.* [1]. Aliquots of cytosol were incubated with varying concentrations of ^{3}H-estradiol (2, 4, 6, 7-^{3}H-E_2, New England Nuclear Corp.) in the presence or absence of 100-fold excess unlabeled diethylstilbestrol to assess nonspecific binding. After a 4 hr incubation at 0–4°C, 300 μl of hydroxylapatite (HAP, Boehringer-Mannheim) in suspension with TE (10 mM Tris HCl, 1.5 mM Na_2-EDTA, pH=7.4) were added to each tube and vortexed every 5 min for 30 min. The tubes were centrifuged at 850×g for 2 min and the supernatant discarded. The HAP pellet was washed 3 times with 2 ml of 1% Tween-80 in TE and recentrifuged for 2 min at 850×g. The ^{3}H-E_2 in the final pellet was extracted at room temperature with 2×0.5 ml absolute ethanol; the ethanol was removed from the pellet following a 2 min centrifugation at 2,000×g. Toluene-based scintillation fluid (5.0 ml; 0.5% PPO + 0.005% POPOP) was added to the combined ethanol extracts and radioactivity counted in a Beckman LS-100C liquid scintillation counter with automatic external standard. Protein content of the cytosols was estimated using the method of Lowry *et al.* [11].

RESULTS

The vaginal openings of the control group occurred between 34–36 days whereas in the morphine group, the vaginal openings ranged from 30 to 44 days of age. The difference in this measurement appears to be due to a litter effect. Two out of six MS litters had vaginal openings as early as 30 days and two litters had vaginal openings as late as 44 days.

The weight of the experimental animals at all ages was significantly higher, $F(1,31)=15.33$, $p<0.01$, than that of the control animals. For instance, at age 25 days, the mean weight for the saline group was 125.8 g compared to 132.7 g for the morphine group. This difference was still present at age 80 days; 258.1 g was the mean weight for the control group compared to 287.1 g for MS animals.

The saline treated animals generally scored higher than the morphine treated animals on all behavioral measures in the three EB conditions (Table 1). Prenatal exposure to MS significantly decreased the frequency of lordosis responses that the animals exhibited, $F(1,31)=7.52$, $p<0.02$, and the quality of each lordosis was significantly lower than in the control group of animals, $F(1,31)=14.95$, $p<0.01$. Darting, $F(1,31)=18.09$, $p<0.01$, and ear wiggling, $F(1,31)=8.07$, $p<0.01$, also were decreased significantly. There was no significant interaction between prenatal treatment and EB test dosage.

Under the assay conditions described, HPOA cytosols from ovariectomized adult female rats contain a limited number ($B_{max}=80.5$ fmole/mg protein) of high affinity ($K_d=0.44$ nM) estrogen binding sites. Furthermore, the binding is inhibited by excess unlabeled E_2, diethylstilbestrol, and tamoxifen but not by testosterone, dihydrotestosterone, R5020, or corticosterone (Etgen, unpublished observations). Thus the E_2 binding species in HPOA cytosols measured using the HAP assay has all the properties (saturability, specificity, high affinity) of classical steroid receptors.

TABLE 1

PERFORMANCE MEASURES OF FEMININE SEXUAL BEHAVIOR OF FEMALE RATS

EB Dosage	Experimental Treatment*	N	Parameters LQ	QL	DT	EW
2 μg	Morphine	6	2 ± 1	0	0	0
	Saline	6	33 ± 13	1.0 ± 0.3	3.0 ± 1.4	3.5 ± 1.6
8 μg	Morphine	5	25 ± 9.0	0.3 ± 0.1	1.0 ± 1.0	2.0 ± 0.7
	Saline	5	41 ± 9.0	0.7 ± 0.2	2.4 ± 0.7	4.4 ± 1.0
25 μg	Morphine	5	77 ± 6.0	1.3 ± 0.1	4 ± 1.5	9 ± 4.0
	Saline	5	86 ± 8.0	2.0 ± 0.2	16 ± 3.0	17 ± 2.0
Conditions	Morphine	16	33 ± 8.0	0.5 ± 0.1	1.4 ± 0.6	3.4 ± 1.6
Combined	Saline	16	51 ± 8.0	1.2 ± 0.2	7.0 ± 2.0	8.0 ± 2.0

Values are group means ± SEM.
(LQ=lordosis quotient; QL=quality of lordosis; DT=dart; EW=ear wiggle.)
*Significant main effects (p's<0.02) of treatment and dosage of EB were found for all measures.

The ^3H-E_2 binding characteristics of brain cytosols prepared from morphine-treated and control females were subjected to Scatchard analysis. Both groups of rats showed a single class of high affinity (morphine: K_d=0.47 nM; saline: K_d=0.43 nM) estrogen receptors. Morphine-treated rats also had the same number of HPOA cytosol estrogen receptors (B_{max}=76.3 pmol) as the saline controls (81.2 pmol).

DISCUSSION

The results of the present experiment demonstrate that prenatal exposure to MS causes a measurable inhibition in adult feminine sexual behavior of female rats. Receptivity was reduced as indicated by decreases in both the frequency and quality of lordosis responses; darting and ear wiggling (proceptive behaviors) were also reduced when compared to saline-treated females.

The increased range of vaginal openings in the experimental group is somewhat inconsistent with the finding of precocial vaginal opening by Sonderegger and Zimmermann [18]. In the present study, the timing of vaginal opening varied between 30–44 days of age in the morphine-treated animals in contrast to the narrow time range in saline-treated animals (34–36 days of age). Two litters from the experimental group were comparable to the control group of animals. However, from the remaining four litters, two litters had precocial (day 30–32) and two litters had delayed (day 42–44) vaginal opening. Our finding that weights of the experimental animals were significantly higher than the weights of the control animals also stands in contrast to earlier findings on prenatal morphine exposure [3,17]. These differences might be explained by the fact that the earlier studies used higher doses of morphine in their treatments.

Our results indicate that the intensity of behavior rather than the absolute incidence of behavioral occurrence was affected by the MS; as EB dosage increased, so did the frequency and quality of the behaviors that morphine-treated animals exhibited. This may indicate that the action of the opiate system on the development of behavior is modulatory. Perhaps the sensitivity of the opiate and/or steroid receptors has been affected. Our data suggest that the behavioral changes cannot be explained by a change in either the number or affinity of HPOA cytosol estrogen receptors. However, we have not investigated the possibility that nuclear translocation and/or retention of estrogen receptors may be less efficient in morphine-treated animals.

Developmental alterations may have been produced in the maturing brain by morphine since it is readily transferred across placental barriers [9], and the developing organism has a higher sensitivity and greater vulnerability to narcotic actions during organogenesis. The influence of morphine on the development of female sexual behavior may indicate that the narcotic interferes with nerve cell maturation. These malformations may well affect the sensitivity of brain opiate and steroid receptors later in development.

There is another possible explanation for the MS effect on reproductive behavior. Perhaps the prenatal exposure to morphine interfered with the developing spinal cord rather than with the developing brain. Myers and Baum [14] have shown that the opiate receptor antagonists, naloxone or naltrexone, facilitate masculine sexual performance by acting on several neural sites, including the spinal cord. Therefore, we cannot rule out the possibility that the morphine resulted in developmental abnormalities in the spinal cord, perhaps resulting in altered sensitivity of the sensory nerves (e.g., pudendal) supplying the flanks and perineum. It is possible that estrogen exposure does not produce the same increase in sensory field size in morphine-treated females as is found in normal female rats [10]. Thus the morphine-treated females may not perceive the tactile stimulation from the male as sufficient for lordotic responding.

Because the pups were not cross-fostered, we cannot rule out the possibility that physiological and/or behavioral changes in the morphine dams may have influenced the development of the offspring. However, there is evidence in the literature [5] that body-weight differences between morphine-treated and control rats are not attributable to maternal effects. It must be noted also that morphine-induced alterations in liver metabolism of steroids could have made the animals less sensitive to estrogen.

Our results suggest that morphine sulfate exposure *in utero* can cause measurable reduction of adult female sexual

behavior. However, it is not known whether morphine sulfate disrupted hormonal events responsible for the activation of reproductive behavior or whether it has an independent non-hormonal mode of action. Given these effects of morphine, the possible consequences of opiate administration and opiate addiction on reproductive function await future investigation.

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