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# Perinatal Exposure to Morphine Disrupts Brain Norepinephrine, Ovarian Cyclicity, and Sexual Receptivity in Rats

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SIDDIQUI, A., S. HAQ AND B. H. SHAH. *Perinatal exposure to morphine disrupts brain norepinephrine, ovarian cyclicity, and sexual receptivity in rats.* PHARMACOL BIOCHEM BEHAV **58**(1) 243–248, 1997.—The effect of perinatal exposure to morphine on the development of catecholaminergic and reproductive function in female rats was investigated. Adult rats received morphine intraperitoneally daily for 40 days. The dose of morphine was progressively increased at 10-day intervals from 5, 7.5, 10 to 15 mg/kg body weight until day 40. The rats were mated between days 38 and 45. Administration of morphine at dose rates of 20 and 30 mg/kg continued during pregnancy. The dose was increased to 40 mg/kg for 10 days postpartum. Results showed that morphine disrupted ovarian cyclicity in 52% of the females. Amongst the remaining females, 43% became pregnant when mated. In the female offspring born to such dams, sexual maturation was delayed and body weight was reduced until weaning. At adulthood, lordosis behavior was inhibited when the female offspring were tested against stimulus males. Plasma estradiol and ovarian estradiol and progesterone levels were reduced. Norepinephrine concentration in the hypothalamus was reduced, whereas it remained unchanged in the amygdala. Dopamine concentrations in both hypothalamus and amygdala were not influenced by perinatal morphine exposure. These results suggest that chronic morphine treatment during perinatal life selectively influences the development of noradrenergic mechanisms in the rat brain and this may in turn be responsible for reduced reproductive activity. © 1997 Elsevier Science Inc.

Perinatal Morphine Pregnancy Behavior Reproduction Catecholamines

IN HUMANS, morphine exposure during pregnancy results in general retardation of growth and development. A large proportion of infants born to morphine-addicted mothers are underweight and display disturbances in the development of the central nervous system (19,20,50). Common among these are difficulties in areas of self- and social adjustment, including uncontrollable temper, impulsiveness, poor self-confidence, and aggressiveness (8,43). A number of studies have shown that administration of drugs of abuse during the critical period of brain sexual differentiation results in abnormal postpubertal reproductive performance in humans and animals (11,42). Evidence suggests that in rats, such changes are accompanied by derangements in the brain sexual differentiation process that occurs perinatally (14). Thus, normal development of the neuroendocrine components involved with gonadal function and reproductive behavior of the animal appears to be highly sensitive to a critical steroid hormonal milieu during pregnancy (12,38,44). Previous studies have shown that ontogenesis of steroid receptors coincides with that of opiate receptors in the rat brain during fetal life (10,49). This suggests that perinatal opiate exposure may influence the organization of the brain sexual differentiation process (46).

The effects of morphine are produced as a result of its interaction with opioid μ, κ, and σ receptors (7,9,19,20). Morphine is considered to be a relatively selective agonist for μ-opioid receptors. All three opioid receptor types are coupled with the inhibitory guanine nucleotide binding proteins (Gi-proteins). Activation of Gi-protein inhibits adenylyl cyclase and thus causes a decrease in intracellular cyclic AMP (cAMP) levels (31). The modulation of the cAMP cascade is believed to be responsible for diverse actions of morphine (9,18). Recent studies have shown that chronic morphine

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treatment alters the expression of G-proteins (Gsα, Giα) (6,34) and opioid receptors (32). It regulates the activity of phospholipase C (41) and ion channels (13). Morphine also modulates the development and transmission of neurotransmitter systems, including norepinephrine (40) and dopamine (45), in various regions of the brain. Endogenous opiate peptides are known to be involved in regulation of the hypothalamo–pituitary–ovarian axis by suppressing noradrenergic input to the hypothalamus (22). We have recently shown that morphine exposure during gestation exerts deleterious effects on the outcome of pregnancy as a consequence of disruption of catecholaminergic activity in male offspring. This was primarily through luteinizing hormone (LH)-dependent reduced testicular steroidogenesis and spermatogenesis in male rats postpubertally (39). In continuation of our previous work, the present study was conducted to examine the effects of perinatal morphine exposure on postpubertal gonadal functions in relation to catecholaminergic changes in female rat offspring.

## METHODS

### *Animals*

Experiments were carried out on 4- or 5-day regularly cycling female Wistar rats weighing 180–220 g. The females were tested further for at least four consecutive cycles; cyclic regularity was determined by examining cell types in daily vaginal smears.

### *Materials*

Morphine (supplied in sulfate form) was obtained from Punjab Drug House (Lahore, Pakistan) and was dissolved in physiological saline solution. Estradiol benzoate and progesterone were purchased from Sigma, Inc. (Dorset, UK) and were dissolved in corn oil. All other chemicals and solvents used for chromatographic analysis were of analytical grade and were obtained from BDH (Poole, UK).

### *Treatment Protocol*

The treatments and experimental design were similar to those previously reported (39). Animals were randomly selected and divided into a morphine sulfate-treated experimental group ($n = 16$) and a saline-treated control group ($n = 6$). They were housed three to five per cage with free access to water and food, at a temperature of 25 ± 2°C and with lights on from 0600 to 1800 h. The experimental group received daily intraperitoneal (IP) treatment with increasing doses of morphine (5–15 mg/kg body weight) in 1 ml volumes of 0.9% saline, for a duration of 40 days. A dose of 5 mg/kg body weight was injected for the first 10 days of treatment. The dose regimen was progressively increased to 7.5, 10 and 15 mg/kg body weight every 10 days until day 40. To minimise stressful stimuli of IP administration, especially in the event of prolonged exposure, morphine treatment remained restricted to only once per 24 h. However, except for signs of some irritability, no other visible signs of craving and withdrawal were noted.

Vaginal smears were examined every day for cell types from the 25th day of treatment through the onset of pregnancy. On the 38th day of treatment, a mature male rat was placed in each cage of females for mating. Presence of spermatozoa in vaginal smears indicated day 1 of pregnancy. Morphine treatment of female rats continued daily throughout pregnancy and lactation. Perinatal exposure to morphine was chosen to cause neurobehavioral and neurochemical alterations in the offspring. Rats that became pregnant ($n = 10$) continued to receive morphine at 20 mg/kg per day for the first 10 days of pregnancy. The dose was further increased to 30 mg/kg per day for the latter half of pregnancy. Irrespective of whether parturition occurred on the 22nd day or was delayed, the dose was increased to 40 mg/kg for yet another 10 days, assuming that offspring that were exposed to morphine in utero would receive this exposure via suckling of milk (16). Parallel to the treatment regimen of the experimental animals, control female rats ($n = 6$) received injections of 0.9% saline.

Animals were observed for length of gestation, average litter size, body weight of each pup, and incidence of stillbirths. Some of these data have been reported elsewhere (39). At 25 days of age, the offspring were weaned from their mothers and, on day 35, male and female littermates were segregated. Because the morphine treatment of the dams had already been discontinued, other than moderate signs of irritability for up to 2 weeks, no other gross symptoms of withdrawal were discernible. Three to five animals of the same sex were housed in each cage and were maintained on a 12 L:12 D cycle (lights on at 0600 h) with food and water available ad lib. Occurrence of vaginal opening (VO), a reliable bioassay for the onset of sexual maturation, was recorded every day. Vaginal smears were taken for a period of 3 weeks to determine regularity of estrus cycles. To further assess ovarian function prior to ovariectomy, animals were bled from the tail vein for hormonal analysis.

### *Behavioral Testing*

At 75–80 days of age, both experimental and control animals were ovariectomized under ether anaesthesia. Ovaries were weighed, processed, and stored frozen for analysis of steroid hormone concentrations. After 2 weeks of recovery, females were tested for three consecutive weeks for reproductive behavior in response to estradiol benzoate (10μg) treatment for 2 days followed by progesterone (500 μg) on the third day. Tests with stimulus males began 5 h after the progesterone injection. The stimulus male was allowed to adapt to the testing arena for at least 5 min prior to the introduction of an experimental female. Males were permitted to mount female rats for 5 min, and the number of lordosis responses as well as the quality of each lordosis response was recorded. A lordosis quotient (LQ: number of lordosis responses/number of mounts × 100) was derived as a measure of estrus responsiveness. In addition, solicitation behavior, including darting, hopping, and ear-wiggling, was recorded whenever it occurred during the 5-min mount test.

### *Tissue Processing and Catecholamine Measurement*

After the final behavioral test at 120 days of age, all female rats were sacrificed by decapitation between 1500 and 1600 h. The brains were removed immediately after sacrifice and kept on ice. The hypothalamus and amygdala were dissected out as described by Baron et al. (5). All tissue samples were weighed and homogenized in an appropriate volume of 0.1 M HCl containing 5 pmol/20 μl 3,4-dihydroxybenzylamine (Sigma, London, UK) as the internal standard. Homogenates were centrifuged at 500 × *g* for 5 min at 4°C. Supernatants were either stored at −70°C or processed directly for high-performance liquid chromatography (HPLC) analysis. Samples (20 μl) from the resulting supernatant were injected onto a reverse-phase HPLC column ($C_1$s μ Bondapak) coupled to an electochemical detector as described previously (38). Norepinephrine

TABLE 1

EFFECT OF PERINATAL MORPHINE SULFATE ON BODY WEIGHT GAIN OF PREPUBERTAL AND ADULT FEMALE RATS

| | Weight (g) at Days of Age | | | |
|---|---|---|---|---|
| Experimental Treatment | 0 | 25 | 60 | 120 |
| Saline | 4.1 ± 0.1 (20) | 42.1 ± 2.1 (19) | 72.8 ± 5.3 (19) | 160.0 ± 6.5 (19) |
| Morphine sulfate | 3.4 ± 0.2* (25) | 30.7 ± 2.0* (16) | 60.6 ± 1.6* (14) | 168.4 ± 7.9 (14) |

Values are mean ± SEM; number of offspring studied is given in parentheses.
*$p < 0.05$ vs. saline-exposed animals.

(NE) and dopamine (DA) concentrations were determined simultaneously.

*Radioimmunoassay*

Luteinizing hormone was assayed according to the method of Naftolin and Corker (30), as modified by Kendle et al. (23). The sensitivity of the assay was 2 ng/ml against the standard LER-$C_2$-1056 (potency 1.73 × NIH-LH SI). The intra- and interassay coefficients of variance were 9.9% and 7.8%, respectively. Plasma and ovarian estradiol ($E_2$) and progesterone (P) concentrations were determined by radioimmunoassay in duplicate using antisera from Guildhay Antisera Ltd. (Surrey, UK). The antiserum against $E_2$ was raised in sheep injected with a 17β-estradiol–3-carboxymethylether–ovalbumin conjugate. Mean intra- and interassay coefficients of variance were 5.7% and 8.2%, respectively. The P antiserum was raised in sheep (HP/S/53-IIA) injected with a progesterone II hemisuccinate–ovalbumin conjugate. Mean intra- and interassay coefficients of variance for all samples were 7.2% and 10.8%, respectively.

*Statistical Analysis*

Differences of all behavioral measures and catecholamine concentrations were subjected to analysis of variance followed by Duncan's multiple-range *t*-test, with $p < 0.05$ denoting a significant difference. Significance of differences in body weight and in concentrations of LH, $E_2$, and P in control and experimental groups was determined with Student's *t*-test.

## RESULTS

All control rats had normal 4–5 day estrus cycles. However, amongst the morphine-treated group, 52% of the females did not exhibit normal estrus cycles and were in constant metestrus–diestrus or occasionally proestrus phase. The remaining 48% of the morphine-treated females exhibited normal estrus cyclicity; however, only 43% became pregnant when mature male rats were placed with them. Pregnant rats treated with morphine experienced gestational length prolonged beyond the normal term of 22 days ($p < 0.01$) [these results have already been reported (39)]. Litter size was normal, but there were significantly more stillbirths in each litter, and live pups had decreased body weights.

The mean body weight gained by the female offspring born to morphine- or saline-treated animals is given in Table 1. Newborn female offspring born to morphine-treated rats showed a low body weight gain as compared with control rats. However, this difference did not persist postpubertally or in adulthood. Ovarian weights showed a significant reduction ($p < 0.05$) in the morphine-exposed group when compared with saline-exposed rats (Table 2).

The VO of the saline-exposed group occurred between 40 and 46 days of age, whereas in the morphine-exposed group VO ranged between 51 and 63 days of age. After initial irregularity in estrus cycles for 11–12 days, 88% of the females in the control group and nearly 74% of the morphine-exposed animals started showing normal cyclicity. Female offspring perinatally exposed to morphine exhibited a significant inhibition of adult lordosis behavior when compared with saline-exposed controls in all three tests performed at weekly intervals. The lordosis quotient was significantly lower in morphine-exposed animals than in controls (Fig. 1). Measures of solicitation (i.e., numbers of darts, hops, and ear-wiggles) showed a significant reduction in morphine-exposed animals (0.6 ± 0.2) compared with control animals (3.7 ± 0.5).

Our previous studies showed that perinatal morphine exposure causes a disruption in the sexual differentiation process and in gonadal function of male offspring (39); therefore, in this study we measured changes in plasma LH, $E_2$, and P

TABLE 2

BIOLOGICAL EFFECTS IN FEMALE RATS BORN TO MORPHINE- AND SALINE-TREATED DAMS

| | | | Estradiol | | Progesterone | |
|---|---|---|---|---|---|---|
| Experimental Treatment | Ovarian Weight (mg) | Plasma LH (ng/ml) | Plasma (ng/ml) | Ovarian (ng/g) | Plasma (ng/ml) | Ovarian (ng/g) |
| Saline | 56.0 ± 4.3 (18) | 1.1 ± 0.2 (18) | 19.6 ± 1.8 (18) | 20.8 ± 2.3 (18) | 13.1 ± 1.9 (18) | 22.7 ± 2.1 (18) |
| Morphine sulfate | 43.3 ± 4.1* (14) | 0.5 ± 0.1* (14) | 11.2 ± 3.0* (14) | 11.0 ± 2.3** (14) | 10.7 ± 2.7 (14) | 12.3 ± 2.0** (14) |

Values are mean ± SEM; number of offspring studied is given in parentheses.
*$p < 0.05$ and **$p < 0.001$ vs. saline-exposed animals.

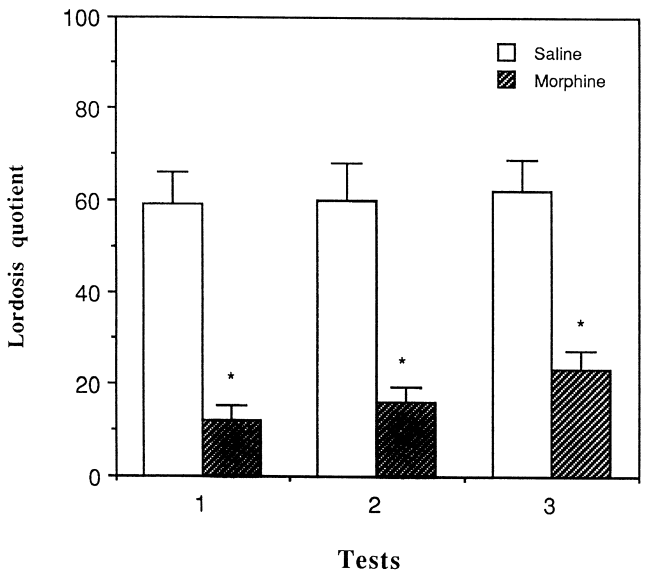


FIG. 1. Effects of perinatal morphine exposure on adult lordosis behavior. Animals were tested for estrus responsiveness in three consecutive weeks. Values are mean ± SEM ($n = 14$ in each group). *$p < 0.001$ vs. saline.

levels and in NE and DA concentrations in the hypothalamus and amygdala of ovariectomized female offspring born to morphine-treated dams. Morphine treament caused significant reduction in plasma LH ($p < 0.05$) and $E_2$ ($p < 0.05$) levels when compared with the control group. Similar declines were also observed in ovarian $E_2$ and P concentrations compared with control animals (Table 2). Plasma P concentrations in the morphine-exposed female rats, however, did not exhibit any change as compared with saline-treated animals (Table 2).

The neurochemical basis for alterations in the lordosis response of the ovariectomized but steroid-primed morphine-exposed female rats was investigated immediately after the third behavioral test by measuring catecholamine concentrations in the hypothalamus and amygdala. Perinatal morphine exposure of the female offspring produced a mean decrease of 29% in the hypothalamic NE concentration ($p < 0.05$) as seen on day 120 postpartum, whereas the NE concentration did not change significantly in the amygdala. Concentrations of DA in the hypothalamus and amygdala were not influenced by the morphine treatment (Table 3).

## DISCUSSION

The present study examined the neuroendocrine and behavioral changes in female offspring of rats given morphine during pregnancy and neonatal life. We have shown that perinatal morphine exposure profoundly affected the onset of sexual maturation, as indicated by delay in VO, and impaired the ability of the ovariectomized but steroid-primed female rats to exhibit estrus behavior in response to the presence of stimulus male rats. Throughout the treatment period, an incremental dose regimen of morphine was given to the adult female rats to cover the effects of tolerance and physical dependence on the morphine treatment (9).

The body weight of the female pups born to the morphine-exposed mothers was less than the control pups, which is similar to our previous observations in male pups (39). This trend persisted until the animals were 60 days of age. Reduced birth weight of the pups and slowness in weight gain prepubertally can be attributed to the decreased food consumption (objective measurements were not made) associated with the reduced physical activity by the mother during the treatment period (39), although the possibility of an effect on the feeding behavior or food consumption cannot be excluded (1). Earlier studies in rats show no change of morphine-induced facilitation of feeding (45). Our findings on weight gain are also in conflict with earlier studies by Vathy and Katay (46), who reported significantly higher weight gain in morphine-exposed animals than in controls, which may be due to the lower dosage regimen employed in that study. While looking for the mechanism, it has recently been shown that blockade of dopaminergic transmission leads to enhanced feeding response in rats (15,45). Our studies show no change in the level of DA in chronically morphine-treated rats compared with controls. Such an effect is documented even after acute morphine administration at a dosage of 30 mg/kg, where it failed to induce any change in the hypothalamic DA concentrations (26).

In the present study, induction of opiate tolerance and dependence was assured by its incremental administration over the whole treatment period. The highest dose of morphine used in this study was 40 mg/kg body weight per day, assuming a bioavailability of 20% (25). This exposure given during pregnancy was found sufficient to affect the reproductive function in the female offspring, parallel to the observations in males in our previous studies (39). The reliable bioassay of VO demonstrated that the induction of morphine tolerance/dependence delays but does not prevent sexual maturation and estrus cyclicity, which was irregular initially but eventually became regular. Such an effect of morphine or its μ-receptor agonist fentanyl citrate has been documented pre-

TABLE 3

EFFECT OF PERINATAL MORPHINE ON CATECHOLAMINE CONCENTRATIONS IN THE HYPOTHALAMUS AND AMYGDALA OF FEMALE RATS

| | Norephinephrine | | Dopamine | |
|---|---|---|---|---|
| Experimental Treatment | Hypothalamus | Amygdala | Hypothalamus | Amygdala |
| Saline | 36.0 ± 4.0 (18) | 12.6 ± 2.0 (18) | 8.7 ± 0.9 (18) | 7.5 ± 0.8 (18) |
| Morphine sulfate | 25.4 ± 2.5* (14) | 11.7 ± 1.7 (14) | 8.4 ± 1.1 (14) | 9.0 ± 0.9 (14) |

Catecholamine concentrations are given in pg/mg protein. Values are mean ± SEM; number of offspring studied is given in parentheses.

*$p < 0.05$ vs. saline-exposed animals.

viously (25). In that study it was shown that, as with morphine, the fentanyl-exposed rats were able to ovulate or at least to reach proestrus even though VO was delayed.

Morphine-induced effects are primarily exhibited through μ-opioid receptor activation (9). The opioid μ-receptors interact with Gi proteins, leading to a decrease in cAMP levels. Activation of this signalling cascade can produce multiple effects, including stimulation of phospholipase C (41), opening or closing of $Ca^{2+}$ and $K^+$ channels (13), and regulation of Gsα or its mRNA (34). Also, recent studies show that chronic morphine administration causes increased expression of adenylyl cyclase mRNA in the locus coeruleus and the amygdala (28). Such an upregulation of the cAMP system (Gsα/adenylyl cyclase) might be a compensatory or protective mechanism to overcome the activation of the Gi pathway in response to morphine (6,28,33). The resultant enhanced phosphorylation of various substrate proteins by cAMP-dependent protein kinase A (PKA) may account for changes in various behavioral responses (9). It seems that the complexity of morphine-induced cellular responses is further enhanced by cross regulation between inhibitory (Gi) and stimulatory (Gs or Gq/11) signal transduction pathways (34–36). It is now well accepted that agonist-mediated persistent activation of G-protein coupled pathways leads to desensitization of the receptor and/or downregulation of the interacting G-protein (29,34,37). This has been demonstrated both in animal models and in transfected cells (4,29,34–36). It may be interesting to study the regulation of opioid μ-receptors along with G-proteins in perinatally morphine-treated animals to determine the basis of neurochemical, reproductive, and behavioral changes in the offspring.

Normal hypothalamic NE activity has been implicated as an important facilitator of female estrus behavior (48). Such effects of morphine on LH activity are mediated through tubero-infundibular DA secretion (17). We have shown that perinatal morphine exposure severely affected adult female sexual behavior. The frequency of lordosis responses was reduced, but execution was complete in spite of low scores for soliciting behaviors such as darting, hopping, and ear-wiggling. This suggests impairment of sensory processing of stimuli that regulate copulation without affecting the execution of estrus behavior in female rats. These results are in agreement with the findings of Vathy and Katay (46), who related such reduced lordosis responsiveness to opiate-mediated increases in the circulating levels of androgens during the critical period of brain sexual differentiation. Further studies are needed, however, to substantiate this proposition. One likely possibility is that morphine-induced changes in sexual behavior could arise from alterations in NE concentrations (25,47). Our findings indicate decreased NE levels in the hypothalamus of female offspring of the treated mothers, as shown in Table 3. In fact, prenatal morphine exposure can alter NE content and turnover in male and female rats differently in the sexually dimorphic area of the hypothalamus (47).

Morphine treatment caused an irregularity of estrus cyclicity and also reduced fertility. The dose regimen of morphine used in these studies was able to induce LH-dependent changes in estrus cyclicity. It is well documented that morphine inhibits LH release in adult rats of both sexes by affecting noradrenergic projection to the preoptic area of the hypothalamus (3,21,22). This projection is dependent on the levels of gonadal steroid hormones (26,27,51). Our studies indicate that plasma $E_2$ levels were reduced, whereas P concentrations remained unchanged. Ovarian weight in morphine-exposed rats was significantly low, indicating that the morphine treatment might have a direct effect on the ovary, thus affecting ovarian steroidogenic activity. Plasma LH levels in morphine-exposed animals were low, which is similar to previous reports (2,24). The low levels of LH might affect formation of the corpus luteum and follicular development, thus resulting in reduced plasma $E_2$ levels and lower levels of both $E_2$ and P within the ovary. The possibility that the toxic effects of morphine on reproductive functions are exerted upstream of the pathway, i.e., by altering noradrenergic activity at the hypothalamic level, cannot be excluded. Vathy et al. (47) have shown that prenatal exposure to morphine causes reduction in NE levels in females, in contrast to findings in males. Also, morphine treatment affects the development of noradrenergic system because it decreases tyrosine hydroxylase, NE uptake, and survival of NE neurons. Such an effect is thought to be mediated through modulation of the adenylyl cyclase cascade (40).

In summary, we have shown that chronic morphine treatment affects the female reproductive system, causing irregular estrus cycles and reduced fertility. In addition, the female offspring of these treated animals exhibited reduced estrus responsiveness to the presence of stimulus males. Although disturbance of noradrenergic development in the hypothalamus seems to be a possible cause for reduced steroid hormone levels and sexual activity, the possibility of a direct effect of morphine at the ovarian level cannot be ruled out. Further work is required to elucidate the sensitive time for morphine exposure, i.e., pregnancy or lactation, in relating the neurochemical and behavioral effects with the brain sexual differentiation process.

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