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Prenatal Exposure to Morphine in Mice: Enhanced Responsiveness to Morphine and Stress

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CASTELLANO, C. AND M. AMMASSARI-TEULE. *Prenatal exposure to morphine in mice: Enhanced responsiveness to morphine and stress.* PHARMACOL BIOCHEM BEHAV 21(1) 103–108, 1984.—Some behavioral effects of prenatal morphine administration were studied in CD1 mice. Two sets of experiments were carried out. In a first set, which was performed during development: (a) measures of postnatal reflexes revealed only a light deficit in tests involving motor control, (b) activity measures showed a significant reduction of spontaneous activity which was evident only in the course of the first postnatal days. In a second set of experiments, in which adult mice were tested for activity, analgesia and passive avoidance learning: (a) no difference was observed, in baseline conditions, between the performances of the mice prenatally exposed to saline and those preexposed to morphine, (b) as compared with controls, enhanced responsiveness to morphine administration (for the activity and passive avoidance measures), and to morphine and stress (for the analgesic measures) were found.

Morphine Stress Prenatal administration Mice

A number of investigations have been carried out in laboratory animals in order to investigate the effects of prenatal opiates administration on offspring behavior during ontogeny and in the adulthood [19]. These researches have demonstrated that both physical and behavioral development can be altered in pups born by mothers exposed to opiates (morphine or methadone) during either gestation or lactation. It has been shown, in addition, that the abnormalities which can early be detected by analyzing neurochemical, physiological, and spontaneous or learned behavioral patterns can also persist at the adult age [14,18]. These data provide strong evidence of the involvement of the enkephalinergic systems in the early CNS organization [17]. However, the contrasting results existing in the literature, mainly concerning the strength and the duration of the effects consecutive to prenatal exposure to opiates, indicate that this involvement has to be better specified. It has been suggested that many of the observed discrepancies can be explained on the ground of differences in the injection schedule, in the total daily doses of opiates administered, and in the species and the sex of the animals tested [9]; it has thus been found useful to obtain a set of comparable behavioral measures established on the basis of a single schedule of drug administration and in the same animal species. For this purpose, in the present research, the behavioral parameters mainly analyzed in the experiments until now carried out—activity, analgesia and passive avoidance learning—have been studied, in adult male and female CD1 mice which had prenatally been injected with saline or morphine. In addition, measures of postnatal reflexes and motor activity during development were carried out. Finally, since it is known that a number of stressors produce an analgesic reaction, which has been related to the physiological and psychological factors that activate endogenous pain control and opiate system [12], additional experiments have been performed in which the degree of analgesia induced by immobilization stress in the adult offspring belonging to treated and control litters has been assessed.

METHOD

Subjects

Nulliparous adult females CD1 mice (Charles River, Como, Italy) were housed individually with food and water available ad lib. They were maintained on a 12 hr light-dark cycle (7 a.m.–7 p.m.). In order to avoid a high mortality rate in the offsprings, due to the association of morphine injections with the beginning of pregnancy, half of the females were subcutaneously injected initially, for a five day period, with increasing doses of morphine, according to a previously described method [14]: day 1: 10 mg/kg; days 2, 3 and 4: 20 mg/kg; day 5: 30 mg/kg. From day 6 to 18 no injection was given. At the sixth day of this drug-free period males were introduced (ratio: 2:1) and left for seven days. Pregnant females were then removed to individual cages. At this time females were injected with morphine for fourteen consecutive days. The initial dose of morphine (10 mg/kg) was increased

every 2nd day by 5 mg/kg until a maximum of 40 mg/kg. Parturition occurred within one week after the treatment termination. The remaining females served as controls, and received 0.9% sodium chloride on an injection schedule identical to that used to administer morphine. All injections were given between 9–10 a.m. After termination of treatments the cages were inspected daily for live births. The number of pups varied from 8 and 12, and the litters were culled to eight. Pups were weighed every other day, from day 1 to 21. After one month the litters were weaned. After weaning males and females belonging to the same litters were divided and housed in separate cages.

Behavioral Measures

All behavioural measures were carried out between 9 and 12 a.m.

Postnatal Reflexes

From postnatal day 2 to postnatal day 20, all mice were tested every other day according to a previously described schedule [4]. The following developmental parameters were recorded: rooting, cliff aversion, hair growth, ears open, righting, forelimb placing, forelimb grasping, bar holding, vibrissae placing, eyes open, auditory startle response. The age at which a given response was present in the adult form indicated the score for each mouse.

Activity

Animex. The measures of spontaneous activity were performed, during development, on postnatal days 8, 16, 32, and 90. Each subject was put, for 5 min, in a cylinder of transparent Plexiglas (diameter: 8 cm, for 8–16 day old mice, and 15 cm, for 32 and 90 day old mice; height: 15 cm). The cylinder was placed upon a selective activity meter (Animex), set at a standard level, which allowed recording animals' motility. All animals were tested in this set of experiments.

Toggle-floor box. At 80 days of age two groups of 30 subjects, taken from the offspring of saline (control subjects: CS) or morphine (experimental subjects: ES) injected mothers, were tested for locomotor activity in Plexiglas toggle-floor boxes (24.5×9.0 cm) [11]. The number of crossings from one side to the other of the box was automatically recorded by means of a microswitch connected to the tilting floor of the box: this count constituted the score of the mouse. Each group of 30 subjects was divided into three subgroups (10 mice per group), which were injected, 20 min before testing, with saline, morphine 5 mg/kg and morphine 10 mg/kg respectively.

Analgesia

Tail-flick. Two groups of thirty animals, taken each from the offspring born by saline or morphine injected mothers were used. Each group was divided into three subgroups which were injected, respectively, with saline or morphine (0.25 mg/kg and 0.5 mg/kg) 20 min before testing. Two other groups of 30 saline or morphine pre-exposed animals were also divided into three subgroups (10 mice per group); two groups were immobilized for 30 and 60 min respectively, the remaining one was not immobilized (NI), and served as control group. Immobilization occurred immediately before testing. The tail flick latency was recorded automatically, and was defined as the time elapsed between the onset of a high intensity light beam focused on the tail of the animal and the withdrawal response. To avoid excessive injury a cut-off value of 30 sec was imposed on the response.

Immobilization stress was produced by placing the animals in a cylindric snug-fit restraining apparatus, such as that described by Amir, Amit and Brown [1].

Hot plate. One week after the tail flick experiments, a control of analgesia in absence of any treatment was performed. The 20 subjects (10 from CS and 10 from ES) which had previously been injected with the highest dose of morphine (0.5 mg/kg) were tested with the hot plate method [5] as modified by Goldstein and Sheehan [6]. The endpoint was the licking of the forepaws or hindpaws. Each mouse was removed as soon as it reacted or, if it failed to react, after 30 sec.

Passive avoidance. Two groups of 32 animals, taken each from the offspring born by saline or morphine injected mothers were used. All the subjects were tested according to a previously described method [3].

The animals were tested for passive avoidance learning during two days [3]. On the first day (training day) they underwent a single learning trial in which each mouse was placed on a lighted platform in front of which was a hole leading to a dark compartment with an electrified metallic grid floor. As soon as the mouse stepped through the hole off the platform and onto the metallic grid, it immediately received a footshock (0.7 mA, 50 Hz) for 1 sec. It was then removed to its home cage to await testing. All animals entered the dark box in less than 6 sec (see Table 2). Twenty-four hr later (test day) the animals were tested for retention. Test procedures were the same as training except that no shock was administered. On the test trial the animals not entering the dark compartment within 180 sec were assigned a step-through latency of 180 sec.

After training, each group was divided into four subgroups of 8 subjects. Two groups were injected with saline and morphine (0.25 mg/kg) respectively immediately after training. The other two groups were injected with 0.5 mg/kg of morphine immediately, and 120 min after training respectively. Two additional groups of 16 subjects taken from animals previously tested in other control conditions (8 ES and 8 CS from the Animex experiment, and 8 ES and 8 CS from the toggle floor experiment) were used. These groups did not receive footshock on the training day. Immediately after training half of the subjects were injected with 0.5 mg/kg of morphine, and the others with saline.

Morphine HCl was dissolved in 0.9% NaCl and injected at the volume of 4 ml/kg. NaCl, 0.9% (4 ml/kg) was used for control injections.

Statistical Analysis

The results were statistically evaluated by one-way ANOVA, for the weights and the activity (Animex) measures, two-way ANOVA, for the activity (toggle-box) and the tail flick experiments, and two-way ANOVA for repeated measures, for the passive avoidance experiments. Since examination of the data did not reveal, in each situation, any difference between males and females performances, their scores were plotted together.

RESULTS

Weight Measures

The weights of the morphine preexposed mice were sig-

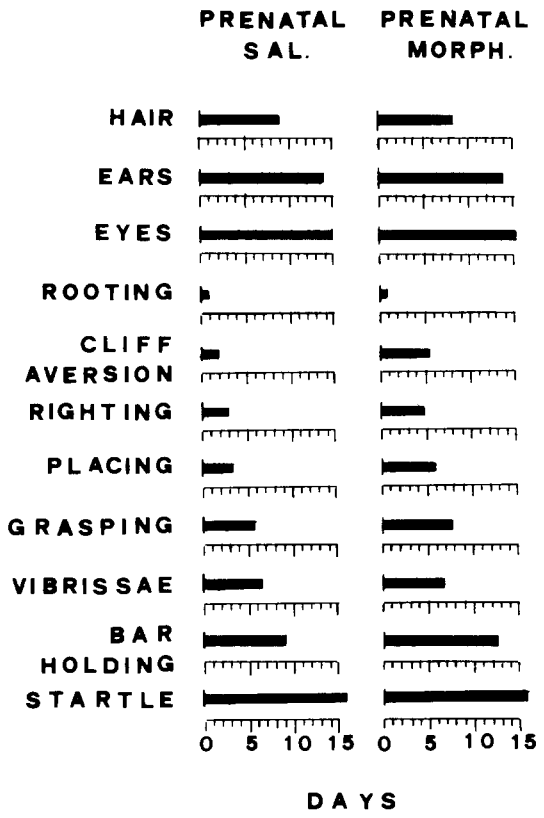

FIG. 1. Postnatal reflexes. Each line corresponds to the mean age in which a given response is present in its adult form.

TABLE 1
ACTIVITY MEASURES DURING DEVELOPMENT

Postnatal day	Prenatal Saline	Prenatal Morphine
8	138.4 ± 12.5	112.8 ± 13.3
16	278.8 ± 12.9	240.0 ± 16.0
32	253.7 ± 4.9	243.0 ± 8.9
90	391.6 ± 12.3	413.8 ± 20.9

Mean number of movements (±SEM) during development of different groups of CD1 mice prenatal exposed to saline or morphine.

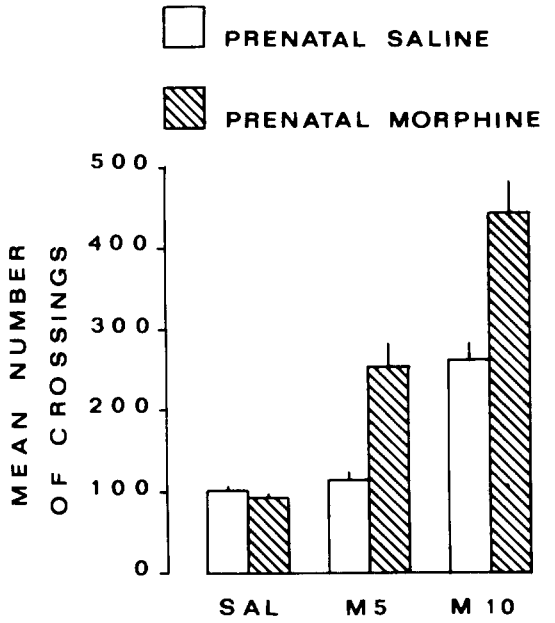

FIG. 2. Toggle-floor box activity in morphine and saline prenatally exposed mice injected with saline or two doses of morphine (5 and 10 mg/kg), in the adulthood.

nificantly lower, as compared with those of the control subjects, only on the first (CS: 1.68 g, ES: 1.38 g, $p<0.01$) and on the second (CS: 2.15 g, ES: 1.89 g, $p<0.05$) postnatal day. The following measures did not reveal any significant difference between the two groups.

Postnatal Reflexes (Fig. 1)

As compared with the subjects born by saline injected mothers, the animals born by morphine injected mothers revealed a light deficit in the tests involving motor control only.

Activity

Animex (Table 1). The measures recorded during development indicate a significant reduction of spontaneous activity on the 8th, $F(1,96)=4.9$, $p<0.05$, and the 16th, $F(1,94)=8.65$, $p<0.01$, day after birth in ES, as compared with CS. After this period, the activity rates of the two groups were similar, $F(1,95)=0.28$, n.s.

Toggle-floor box (Fig. 2). No difference was evident between CS and ES tested at 80 days of age in absence of morphine treatment.

Activity stimulation induced by morphine (5 and 10 mg/kg) has then been analyzed in CS and ES. ANOVA (two-way) showed: (a) a significant treatment effect, $F(2,59)=113.5$, $p<0.01$, indicating that morphine enhanced activity in both CS and ES, (b) a significant pretreatment effect, $F(1,59)=55.2$, $p<0.01$, showing a different reactivity of the two groups to morphine administration, since only the injection of 10 mg/kg modified the activity rates of CS, and (c) a significant pretreatment × treatment effect, $F(2,59)=15.8$, $p<0.01$, showing that, for each dose of morphine, activity stimulation was higher in ES than in CS.

Analgesia (Fig. 3)

Tail flick. No difference was evident between CS and ES tested in the adult age in absence of any treatment. Morphine (0.25 and 0.5 mg/kg) or stress- (30 and 60 min) induced analgesia have then been analyzed in CS and ES. ANOVA (two-eay) showed: (a) a significant treatment effect, $F(2,59)=100.9$, $p<0.01$, showing that morphine enhanced the degree of analgesia in both CS and ES, (b) a significant pretreatment effect, $F(1,59)=80.0$, $p<0.01$, indicating that morphine-induced analgesia was higher in ES than in CS,

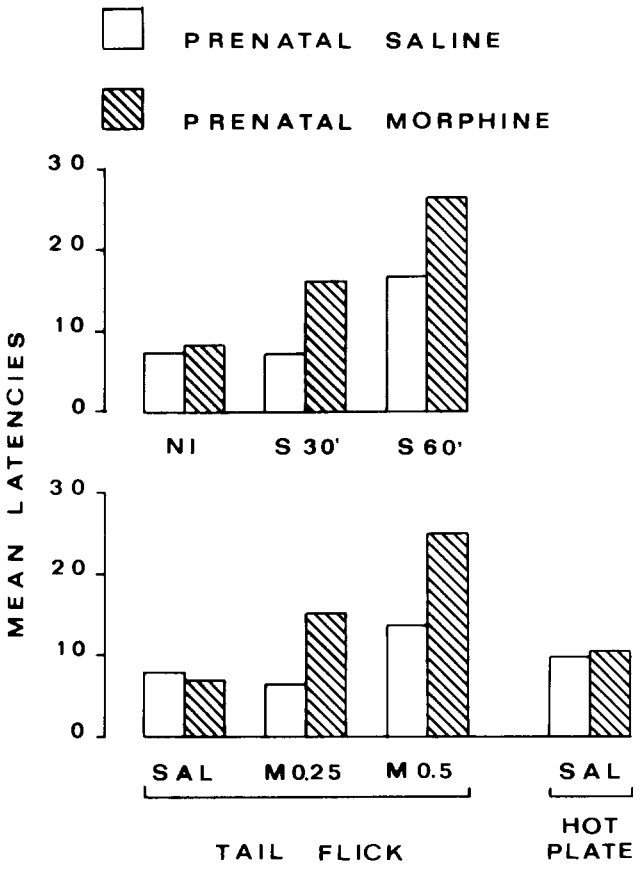

FIG. 3. Mean tail flick latencies of morphine and saline prenatally exposed mice subjected to different experimental treatments in the adulthood. On the top, analgesia was assessed after two periods of immobilization stress (30 or 60 min) or in nonimmobilized mice (NI). On the bottom, analgesia was assessed after the injection of saline or of two doses of morphine (0.25 or 0.5 mg/kg). Successively, the two groups (prenatal saline and prenatal morphine) previously injected with 0.5 mg/kg of morphine were tested, in absence of treatment, with the hot plate method, in order to control eventual effects of previous morphine injections on the baseline levels of analgesia.

and (c) a significant pretreatment × treatment effect, $F(2,59)=22.1$, $p<0.01$, showing that, for each dose of morphine, the degree of analgesia was higher in ES than in CS. The data obtained with stress were analyzed in the same way, and similar effects were observed. Stress induced analgesia in both CS and ES (significant treatment effect: $F(2,59)=175.6$, $p<0.01$). In addition, stress-induced analgesia was higher in ES than in CS (significant pretreatment effect: $F(1,59)=96.2$, $p<0.01$). Finally, ES showed the highest degree of stress-induced analgesia (significant pretreatment × treatment effect: $F(2,59)=22.8$, $p<0.01$).

Hot plate. No difference was observed between CS and ES, $F(1,18)=0.22$, n.s. This indicates that previous morphine treatments did not affect the baseline level of analgesia in the ES.

Passive avoidance (Table 2). The data were statistically evaluated by a two-way ANOVA (pretreatment × treatment) for repeated measures, in which the differences between the mean step-through latencies of the mice receiving the injections of saline or morphine immediately after training were compared in the two stages (training-test) of the experiment. The results showed a significant treatment effect, $F(2,95)=72.9$, $p<0.01$, since retention impairments were evident in both morphine and saline pre-exposed subjects injected with 0.5 mg/kg of morphine. Moreover, significant pretreatment, $F(1,95)=37.1$, $p<0.01$, and pretreatment × treatment, $F(2,95)=14.8$, $p<0.01$, effects were observed, since, in the morphine-pre-exposed subjects, this impairment was more pronounced, and was also evident after the injection of lower doses of morphine (0.25 mg/kg). The performances of the mice injected with morphine (0.5 mg/kg) 120 min after training were not different from those of the saline injected group. This result shows that the effects observed following the immediately posttrial administration of morphine were time dependent posttrial effects on memory consolidation. Finally the mean step-through latencies on the test day of the CS and the ES which had not received footshock on the training day, but only posttrial injections of saline or morphine, did not differ from each other. This result shows that the effects observed were not linked to factors other than learning (i.e., they were not due to nonspecific proactive pharmacological actions of the drug lasting more than 24 hr).

DISCUSSION

A number of points emerge from the results of the present study: (a) for each behavioral measure, no difference was evident between the baseline performance of adult mice prenatally exposed to saline and those preexposed to morphine (b) as compared to the baselines, morphine administration induced behavioral modifications in both offspring of control and treated litters and (c) these modifications were more pronounced in the mice prenatally exposed to morphine. In addition, during ontogeny, significant differences between the baseline levels of the saline and morphine prenatally exposed subjects were only found when activity measures were performed. It must be noted, on this point, that Sobrian [14] observed an early hyperactivity in rats prenatally exposed to morphine while an hypoactivity was recorded in the present experiment. Differences in procedure possibly related to the length of the experimental session might account for this discrepancy. In particular, the hypoactivity observed in our conditions might depend on the short test time used for recordings, which could mainly reveal in our subjects the emotional reaction to a novel environment, while the longer test sessions of Sobrian's experiments might rather evidentiate the CNS arousal widely described in subjects prenatally treated with opiates [7,14]. Moreover, the slight motor neglect observed during the first postnatal week in the morphine preexposed subjects could represent an additional factor responsible for the early hypoactivity, while its rapid recovery appears consistent with the similar rates of activity recorded in Sobrian and our pre-exposed subjects tested in adulthood.

In this research, the only evident effect observed in adult mice born by mothers injected with morphine during pregnancy was an enhanced responsiveness to morphine re-exposure, whatever the behavioral test considered, and to stress (for the analgesic measures). As concerns tail-flick experiments, it must be pointed out that our results agree with those of Kirby *et al.* [9] whose reported increased latencies in morphine pre-exposed rats without alteration of the baseline level. Conversely, other researches [8,10] de-

TABLE 2

MEAN STEP-THROUGH LATENCIES (IN SEC) (±SEM) ON THE TRAINING (A) AND THE TEST (B) DAY OF DIFFERENT GROUPS OF SALINE AND MORPHINE INJECTED CD1 MICE PRENATALLY EXPOSED TO SALINE OR MORPHINE

Prenatal treatment	Treatment	mg/kg	a	b
Saline	Saline		3.5 ± 0.5	85.2 ± 5.4
	Morphine	0.25	3.7 ± 0.4	93.1 ± 4.5
	Morphine	0.50	2.7 ± 0.2	40.6 ± 3.1
	Morphine	0.50[a]	3.6 ± 0.6	83.3 ± 7.0
	Saline		4.5 ± 0.6[b]	5.0 ± 0.7
	Morphine	0.5	4.6 ± 0.6[b]	5.3 ± 0.8
Morphine	Saline		4.2 ± 0.7	88.7 ± 8.3
	Morphine	0.25	3.2 ± 0.4	40.5 ± 4.5
	Morphine	0.50	3.8 ± 0.5	9.6 ± 2.4
	Morphine	0.50[a]	3.7 ± 0.6	85.5 ± 5.8
	Saline		5.6 ± 0.6[b]	5.8 ± 0.7
	Morphine	0.5	4.7 ± 0.6[b]	5.7 ± 0.6

a: Injected 120 min since the beginning of training.
b: No shock groups.

scribed a resultant tolerance to the analgesic effect of morphine in pre-exposed rats. This discrepancy indicates that a large attention must be paid on the schedules of morphine administration and more particularly on the doses injected to pregnant mice, which might alternatively determine tolerance or acute responsiveness in the adult offspring re-exposed to morphine. In the present experiment, the total lack of tolerance consecutive to our pretreatment is confirmed by the fact that the same morphine pre-exposed subjects tested firstly for tail-flick analgesia following morphine injection and secondly for hot plate analgesia in absence of morphine injections show in the latter case baseline latencies identical to those of the saline pre-treated subjects.

Finally, the results obtained in testing passive avoidance also revealed no difference between baseline performances of the subjects born by treated and control mothers, while an impairment was observed, in pre-exposed mice, after morphine administration. As concerns this point, it has previously been reported that rats prenatally exposed to morphine exhibit faster or slower acquisition of a conditioned avoidance response depending on the dose of drug administered. This early morphine interference with subsequently learned behavior has been interpreted as the consequence of a persistent alteration of cerebral protein synthesis due to opiates treatment [2,15].

Further experiments are now in program aimed to compare the effects of various doses and schedules of prenatal morphine and methadone treatment, in order to provide a behavioral frame for investigating the alterations of receptor binding and neurotransmitters systems associated with prenatal opiate administration.

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