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Neonatally Administered Naltrexone Affects Several Behavioral Responses in Adult Rats of Both Genders

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DE CABO DE LA VEGA, C., A. PUJOL AND M. P. VIVEROS. *Neonatally administered naltrexone affects several behavioral responses in adult rats of both genders.* PHARMACOL BIOCHEM BEHAV **50**(2) 277-286, 1995.—The effects of a daily injection of the opioid antagonist naltrexone (NALTX, 1 mg/kg SC), from birth to weaning day, on several behavioral parameters were investigated in adult rats of both genders. This work uses three behavioral tests. The use of the open-field test in addition to the holeboard and plus-maze tests allows the measurement of complementary parameters, and provided further information on diverse components of behavior. The effect of repeated testing (habituation) over 3 consecutive days of open-field testing was also assessed. In accordance with previous reports, female rats showed higher locomotor activity and exploration and a lower level of emotionality than their male counterparts. The response of the animals to repeated testing seemed to be both sex and treatment dependent. In both males and females, NALTX treatment during the preweanling period did not affect locomotor activity, but increased the animals' levels of anxiety and emotionality and decreased exploratory behavior. Our results indicate that neonatal opioids may play an important role in the development of behavioral responses in the adult.

Naltrexone Neonatal opioid blockade Opioid plasticity Holeboard Open field Plus-maze
Repeated testing Anxiety Emotionality Exploration Locomotion Adult behavioral responses

SEVERAL experimental data indicate the existence of critical periods during the postnatal development of the endogenous opioid system (EOS) in the rat, during which the system would be particularly sensitive to both environmental conditions and pharmacologic treatment with opioid agonists or antagonists (3,5,14,15,44,59,64,73). There are also a number of works that show the involvement of the opioid system in the control of diverse aspects of behavior. In this regard, it has been found that opioid peptides are released as part of the adaptive reaction to particular stressful situations (19,40,63). Besides, it has been suggested that opioid substances may facilitate the exploratory behavior in novel or aversive environments (67), whereas opioid antagonists such as naloxone or naltrexone would depress locomotor activity and exploration (6).

According to these data, it is interesting to study the consequences of the unique plasticity of the EOS, during critical postnatal periods, on the establishment of adult adaptive responses to stressful environmental situations. Most of the works appearing in the literature in which opioid agonists or antagonist were used during the early postnatal development deal with nociception or somatic growth and neurobiologic maturation (5,34,36,41,48,73-80). However, there is less evidences dealing with the effects of substances acting on the opioid system during the postnatal development on behavioral responses such as activity, exploration, anxiety, or emotionality in adulthood (35,39,50,71,81), and the available results are still fragmentary.

In the present work, we studied the effects of intermittent functional opioid blockade (daily naltrexone administration) during the preweanling period on a range of behavioral parameters in adulthood. The paradigms employed involve novelty and uncertainty (holeboard) (43), aversion (29,30), and approach and avoidance conflict (plus-maze) (56). The convenience of an integrated study of behavior employing a battery

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of tests of this type has been recently suggested (38,43). However, these three tests have hardly been employed together previously: there is only one work found in the literature using a similar design (20).

METHOD

Animals, Experimental Conditions, and Dosing

We used 65 male (400–470 g) and female (240–300 g) Wistar albino rats (16 to 17 animals per treatment × sex cohort). Subjects were the offspring of four male and eight female rats purchased from IFFA-CREDO (Les Oncins, France) and mated (one male × two females) in our laboratory 2 weeks after their arrival. The pregnant dams were randomly assigned to one of two experimental groups (control or naltrexone treated). All animals were maintained at constant temperature (20 ± 1°C) and in a reverse 12-h dark–light cycle (lights on at 2000 h, with free access to food (commercial diet for rodents A04; Panlab, Barcelona, Spain) and water. The reversed light cycle allows for both testing of the animals during the dark phase in which they display their greater activity and applying the treatment during the phase in which both opioid peptides and receptors are seen to reach their circadian peaks in the brain (25,65). Litters were sex-balanced and culled to 10 ± 1 pups per dam. Neonates remained with the dam at all times except during drug administration, to minimize stress resulting from maternal deprivation, and were weaned at 22 days of age (regarding the day of birth as day 0). All experimental procedures were carried out between 0930 and 1430 h under similar temperature and illumination conditions as those in the animal facilities, except for the open field test (see subsequent description). The behavioral testing was performed in adulthood (80 days of age), and animals were equilibrated in a quiet, windowless laboratory at least 30 min before the test.

Naltrexone hydrochloride (Sigma Chemical Co., St. Louis, MO) (1 mg/kg) or distilled water was administered subcutaneously once daily from birth to day 21 at a dose volume of 1 ml/kg, using a Becton Dickinson microlance needle (Huesca, Spain), and delivered from a stand-supported Hamilton syringe (Bonaduz, Switzerland). This dosage has been shown to cause a 4–6 h blockade of the opioid system (73), and has been previously employed in developmental studies to assess the effect of neonatal opioids on other brain variables (73,77,78).

Behavioral Testing

Behavioral testing started at day 80 after birth and was completed within 12 days. Animals performed each test individually. The order of testing was as follows: holeboard, open field, plus-maze. We allowed 3–4 days among the three tests for recovery of the animals from the previous testing conditions. All behavioral procedures took place in the same room.

Holeboard Test

The holeboard was a box (60 × 60 × 45 cm) with matte-painted metallic walls and a plastic-covered wooden floor bearing four equally spaced holes (3.8 cm in diameter) and divided into 36 squares (10 × 10 cm). This test was carried out for only 1 day for 5 min under red light. The duration of the test was based on previous work (23) that revealed within-session habituation after this time. Frequency and duration of head dipping (HD), frequency of rearing (number of times that the animal stood on its rear limbs), defecation (number of boluses), external ambulation (number of line crossings in the periphery, by the walls), and internal ambulation (number of line crossings in the central area) were assessed.

Open Field Test

The open field arena consisted of a cylinder (75 cm diameter × 50 cm high) with a floor divided into 19 sections of a similar area by two concentric circles (17 cm and 45 cm diameter) and a series of radii, and was made of the same materials mentioned earlier. This test was performed for 3 consecutive days, each session lasting 3 min. The duration and number of the sessions were decided according to our previous observations and reports from other groups (4,8,30,33,62,66,68) to be representative of the activity of the animal and to reflect the most significant variations resulting from reexposition to the test. A white-light lamp of 100 W was placed 80 cm over the center of the arena during testing. The parameters measured were: external ambulation (number of squares adjacent to the wall entered with the four limbs), internal ambulation (number of squares in the central area entered with at least three limbs), frequency of rearing, frequency of facial grooming, immobility (duration of periods without activity, although movement of whiskers could be shown), and defecation.

Plus-Maze Test

The plus-maze consisted of two open arms (50 × 10 cm) and two enclosed arms of the same size with 40-cm-high walls arranged so that the arms of the same type were opposite each other. The junction of the four arms formed a central square area (10 × 10 cm). The apparatus was made of hard plastic material and elevated to a height of 62 cm. This test was carried out for only 1 day, for 5 min, under red light. The duration was established following the criteria of previous studies (56) based on the finding that the anxiogenic effect is maximal during that time but decreases toward the end of 10 min. (52). The measures recorded were frequency and duration of arm visits, separately for open and closed arms. An arm was considered to be visited when the animal entered it with at least the head and the two forelimbs. We also estimated the percentage of entries to the open arms to the total, percentage of time spent in the open arms and in the closed arms, and total number of entries and total time spent in both type of arms.

The open-field test was chosen to assess the effects of the treatment on repeated testing. The use of the other two tests for this purpose is discouraged by the rapid habituation observed for the holeboard (21) and the tolerance to pharmacologic effects in the plus maze when the test is repeated (24). In contrast, the open-field test has been carried out for several days as the habitual procedure since the early reports (33).

Each test was started by placing the animals in the area of the apparatus considered more behaviorally neutral [one of the corners (holeboard), an external square (open field), at the center of the apparatus facing one of the enclosed arms (plus-maze)] so that the animal was not artificially induced to perform a significant pattern. The three apparatus were thoroughly cleaned at the end of every test.

Statistical Analysis

All data were tested for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Bartlett's box test). Data from two variables of the open-field test (external ambulation and immobility) needed to be transformed into their corre-

sponding square roots to satisfy both conditions. Mean data from holeboard and plus-maze tests were analyzed by two-way analysis of variance (ANOVA) (factors: treatment and sex). Five animals accidentally fell from the plus-maze apparatus and were therefore excluded from the analysis. Hence, the degrees of freedom were different for this test. Regarding the open-field test, mean data were initially processed by three-way ANOVA (factors: treatment, sex, and repeated testing). From the behavioral point of view, the first day of the open-field testing was considered to be different from the rest, because of the concomitant neophobic effect from its being the first time that the animal was exposed to the test. To address this problem, the data obtained the first day of testing were complementarily processed in separate using two-way ANOVA (factors: treatment and sex). Duncan's multiple range test with a level of significance set at $p < 0.05$ was used for posthoc analysis. To evaluate more accurately the effect of the repetition of the test, the variables that showed significant variations as a result of repeated testing were further analyzed within each experimental group for differences from the scores shown on the first day of testing, by a series of paired Student t-tests to compare values from the same animal on different days (within-group comparisons).

RESULTS

Holeboard Test

The ANOVA did not reveal significant differences between control and treated groups regarding external ambulation. However, naltrexone-treated animals showed significantly lower levels of internal ambulation than the controls [$F(1, 63) = 10.99$, $p < 0.01$] (Table 1). The more representative parameters for this test—head dipping frequency and duration—were also affected by the treatment in a similar way. The ANOVA showed a general decrease of head-dipping frequency in the treated animals, when considering male and female groups together [$F(1, 63) = 6.10$, $p < 0.05$]. In accordance with this result, head-dipping duration was also lower in the naltrexone-treated groups [$F(1, 63) = 15.20$, $p < 0.0001$], a significant difference between the female groups for the Duncan's test. The naltrexone treatment did not significantly affect either rearing frequency or defecation (Table 1). Regarding sexual differences, in this test females showed significantly higher external and internal ambulation [$F(1, 63) = 8.44$, $p < 0.01$ and $F(1, 63) = 4.36$, $p < 0.05$, respectively], and higher rearing frequency [$F(1, 63) = 6.63$, $p < 0.01$], as well as longer head-dipping duration [$F(1, 63) = 4.65$, $p < 0.05$]. These differences only reached significance for the Duncan's test in the case of external ambulation and rearing frequency. The interaction of treatment × sex was not significant for any of the parameters studied; therefore, a different effect of the treatment according to sex cannot be concluded.

Open-Field Test

The three-way ANOVA revealed that both treatment and sex factors significantly affected the following variables studied: external ambulation [$F(1, 193) = 13.27$, $p < 0.0001$, and $F(1, 193) = 41.64$, $p < 0.0001$, respectively], internal ambulation [$F(1, 193) = 15.64$, $p < 0.0001$, and $F(1, 193) = 25.32$, $p < 0.0001$], rearing frequency [$F(1, 193) = 54.94$, $p < 0.0001$, and $F(1, 193) = 32.05$, $p < 0.0001$], defecation [$F(1, 193) = 7.08$, $p < 0.01$, and $F(1, 193) = 41.60$, $p < 0.0001$]. An effect of treatment alone was found for immobility [$F(1, 193) = 117.49$, $p < 0.0001$]. An interaction between treatment and sex also appeared for immobility [$F(1, 193) = 8.82$, $p < 0.01$] and grooming frequency [$F(1, 193) = 13.20$, $p < 0.0001$]. In summary, naltrexone treatment decreased both types of ambulation (Table 2) and rearing (Table 3), and increased immobility (Table 3) and defecation (Table 4).

A significant effect of repeated testing was also found for external ambulation [$F(2, 192) = 6.15$, $p < 0.01$], internal ambulation [$F(2, 192) = 14.51$, $p < 0.001$], and rearing frequency [$F(2, 192) = 4.56$, $p < 0.05$] (reducing the scores of the three parameters), as well as for immobility [$F(2, 192) = 7.23$, $p < 0.001$] (increasing the values). No significant interactions of repeated testing with the other factors were found.

Grooming and defecation were not significantly affected by repeated testing. Therefore, the total scores for the 3 days were analyzed for posthoc comparisons. Regarding defecation, Duncan's test was significant for the difference between control and treated females, but not between males, possibly because of a ceiling effect (Table 4). The effect on grooming was complex: It increased for the males and decreased for the females (Table 4). Besides, control females showed higher scores for external and internal ambulation and rearing, and lower levels of defecation and immobility than the males.

First Day of Testing

Neonatal naltrexone treatment significantly reduced internal ambulation [$F(1, 63) = 5.80$, $p < 0.05$] (Table 2) and rearing frequency [$F(1, 63) = 33.27$, $p < 0.05$] (Table 3), and

TABLE 1

EFFECTS OF PREWEANLING NALTREXONE TREATMENT ON HOLEBOARD ACTIVITY IN ADULTHOOD

	External Ambulation (Line Crossings)	Internal Ambulation (Line Crossings)	HD Frequency	HD Duration (s)	Rearing (No. of Rears)	Defecation (No. of Boluses)
Males						
CONTR	125.5 ± 10.2	55.8 ± 3.7	15.8 ± 1.1	38.7 ± 4.0	23.3 ± 2.1	2.1 ± 0.6
NALTX	126.9 ± 8.9	41.1 ± 3.1*	13.5 ± 1.2	26.8 ± 2.3	19.0 ± 1.7	3.4 ± 0.7
Females						
CONTR	162.8 ± 12.6†	65.4 ± 5.9	16.8 ± 1.1	54.9 ± 6.6†	30.7 ± 2.4†	2.6 ± 0.7
NALTX	151.8 ± 11.0†	50.3 ± 4.8*	13.3 ± 1.2	30.4 ± 4.7*	26.2 ± 2.9†	2.8 ± 0.8

Values represent the mean ± SEM of 16 to 17 animals per treatment × sex cohort.
*$p < 0.05$ vs. the corresponding control group (Duncan's multiple range test).
†$p < 0.05$ vs. the corresponding male group (Duncan's multiple range test).

TABLE 2

EFFECTS OF PREWEANLING NALTREXONE TREATMENT ON OPEN FIELD ACTIVITY IN ADULTHOOD. PARAMETERS SIGNIFICANTLY AFFECTED BY REPEATED TESTING (I)

	External Ambulation (Squares Entered)			Internal Ambulation (Squares Entered)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Males						
CONTR	45.4 ± 3.1	44.6 ± 4.7	33.3 ± 4.3*	6.4 ± 1.5	3.4 ± 1.2*	1.5 ± 0.5†
NALTX	42.6 ± 4.0	28.9 ± 4.4‡,§	29.0 ± 4.2¶	4.1 ± 0.7	0.4 ± 0.2§	1.6 ± 1.0†
Females						
CONTR	62.4 ± 3.9#	56.0 ± 5.1	60.5 ± 3.5#	13.4 ± 2.6#	8.5 ± 2.5*	8.4 ± 2.3#
NALTX	56.6 ± 4.9#	47.7 ± 6.2*#	40.8 ± 4.9‡¶	5.9 ± 1.0‡	3.7 ± 1.2#	3.9 ± 2.0‡

Values represent the mean ± SEM of 16 to 17 animals per treatment × sex cohort.
*$p < 0.05$ vs. scores shown on day 1 (paired Student t-test, repeated testing effects of within-group comparisons).
†$p < 0.01$ vs. scores shown on day 1.
‡$p < 0.05$ vs. the corresponding control group (Duncan's multiple range test).
§$p < 0.0001$ vs. scores shown on day 1.
¶$p < 0.001$ vs. scores shown on day 1.
#$p < 0.005$ vs. the corresponding male group (Duncan's multiple range test).

TABLE 3

EFFECTS OF PREWEANLING NALTREXONE TREATMENT ON OPEN FIELD ACTIVITY IN ADULTHOOD. PARAMETERS SIGNIFICANTLY AFFECTED BY REPEATED TESTING (II)

	Rearing (No. Rears)			Immobility (s)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Males						
CONTR	12.9 ± 1.5	9.8 ± 1.7*	7.3 ± 1.1†	3.2 ± 0.9	12.3 ± 4.5	19.0 ± 7.2*
NALTX	5.5 ± 0.7‡	4.9 ± 1.0‡	6.1 ± 1.1	14.6 ± 3.7‡	36.9 ± 6.3†‡	33.5 ± 7.0‡§
Females						
CONTR	19.1 ± 1.8¶	15.4 ± 1.9¶	14.1 ± 1.0§¶	3.8 ± 2.2	6.2 ± 3.2	1.3 ± 0.8¶
NALTX	9.4 ± 1.6‡	8.6 ± 1.6‡	7.7 ± 1.3‡	21.9 ± 3.9‡	44.0 ± 8.8*‡	35.6 ± 5.5*‡

Values represent the mean ± SEM of 16 to 17 animals per treatment × sex cohort.
*$p < 0.05$ vs. scores shown on day 1 (paired Student t-test, repeated testing effects of within-group comparisons).
†$p < 0.0001$ vs. scores shown on day 1.
‡$p < 0.05$ vs. the corresponding control group (Duncan's multiple range test).
§$p < 0.01$ vs. scores shown on day 1.
¶$p < 0.05$ vs. the corresponding male group (Duncan's multiple range test).

TABLE 4

EFFECTS OF PREWEANLING NALTREXONE TREATMENT ON OPEN FIELD ACTIVITY IN ADULTHOOD. PARAMETERS NOT SIGNIFICANTLY AFFECTED BY REPEATED TESTING

	Defecation (No. Boluses)				Grooming (Frequency)			
	Day 1	Day 2	Day 3	Total	Day 1	Day 2	Day 3	Total
Males								
CONTR	3.5 ± 0.5	3.2 ± 0.6	3.0 ± 0.6	9.7 ± 1.2	0.8 ± 0.3	0.9 ± 0.3	2.0 ± 0.6	3.7 ± 1.0
NALTX	3.2 ± 0.4	3.5 ± 0.4	3.9 ± 0.5	10.6 ± 0.9	2.3 ± 0.5	2.1 ± 0.6	2.6 ± 0.5	6.9 ± 1.0*
Females								
CONTR	1.8 ± 0.5	0.6 ± 0.3	0.8 ± 0.3	3.1 ± 0.9†	2.4 ± 0.4	1.0 ± 0.4	1.8 ± 0.4	5.3 ± 0.5
NALTX	2.4 ± 0.4	1.9 ± 0.5	2.3 ± 0.6	6.2 ± 1.0*†	1.3 ± 0.4	0.9 ± 0.3	0.9 ± 0.3	2.9 ± 1.0†

Values represent the mean ± SEM of 16 to 17 animals per treatment × sex cohort.
*$p < 0.05$ vs. the corresponding control group (Duncan's multiple range test).
†$p < 0.05$ vs. the corresponding male group (Duncan's multiple range test).

increased immobility [$F(1, 63) = 36.01$, $p < 0.05$] (Table 3) on the first day of testing. In the case of internal ambulation, the difference between the male groups was not significant according to Duncan's test. This could be because the levels of control males for this parameter were already low (Table 2). There was no significant effect of treatment on external ambulation (Table 2). Regarding sexual differences, females showed higher external [$F(1, 63) = 15.02$, $p < 0.0001$] and internal [$F(1, 63) = 7.41$, $p < 0.01$] ambulation and rearing frequency [$F(1, 63) = 11.67$, $p < 0.001$] than the males. The interaction of treatment × sex was not significant in any case, but it should be noted that sexual differences for both internal ambulation and rearing frequency were only significant between the control groups.

Repeated Testing

The analysis of external ambulation data showed that, whereas control males only showed a significant decrease by the third day of testing ($p < 0.05$) compared with first-day values, the values for treated males decreased for this parameter from the second day ($p < 0.0001$) and were still lower on day 3 of testing (Table 2). A similar situation was found for the treated females, although the level of significance for the second day was lower (Table 2). However, control females kept similar values of external ambulation during the 3 days of testing. There were also significant decreases resulting from repeated testing for internal ambulation in both male groups (Table 2). The most marked reduction vs. day 1 in this parameter was found for the naltrexone-treated males at day 2 (90% decrease, $p < 0.0001$). Regarding the females, controls showed a significant decrease on the second day ($p < 0.05$), whereas naltrexone-treated females did not experience reductions vs. first-day scores. This is most probably a consequence of the low values of internal ambulation already shown by this group on day 1, as a result of the effect of naltrexone treatment. A similar situation appeared for rearing frequency: Male and female treated animals scored very low from the first day, and therefore, no further significant reduction vs. those values could be detected on the following days (Table 3). With respect to controls, the rearing frequencies of both males and females decreased with the day of testing; this effect was more marked in the control males. Concerning immobility, control males and naltrexone-treated animals dramatically increased during the 3 days of testing. Again, the stronger effect was found for the treated males, which also showed higher levels of significance from day 2 ($p < 0.0001$) (Table 3). Control males showed a similar tendency, although the increment of immobility was only significant by the third day of testing ($p < 0.05$). The immobility values for control females did not vary significantly among the three sessions of testing.

Plus-Maze Test

The ANOVA indicated that naltrexone-treated animals showed significantly lower scores for both frequency [$F(1, 58) = 17.14$, $p < 0.0001$] and duration [$F(1, 58) = 10.83$, $p < 0.01$] of open-arms visits (Table 5). There was no significant effect of the treatment on the frequency of visits to the closed arms. However, the duration of the visits to the closed arms was longer for naltrexone-treated animals [$F(1, 58) = 15.44$, $p < 0.0001$]. In accordance with these results, treated animals showed a lower percentage of entries to the open arms per total [$F(1, 58) = 14.22$, $p < 0.0001$] and percentage of time spent in the open arms [$F(1, 58) = 13.25$, $p < 0.001$], as well as a higher percentage of time spent in the closed arms [$F(1, 58) = 13.25$, $p < 0.001$] than the controls (Table 6). The total number of entries and total time spent in the arms were not significantly affected by the treatment. The ANOVA only revealed a significant effect of sex for the total number of entries; the females showed a greater number than the males [$F(1, 58) = 4.07$, $p < 0.05$]. In both cases, Duncan's test only found significance for the comparisons between treated animals. However, the interaction of treatment × sex was not significant for any of the parameters studied.

DISCUSSION

Holeboard Test

The holeboard test is a paradigm involving novelty and uncertainty (43), in which the site-directed exploratory head-dipping frequency and duration are considered to be distinct parameters (42,43,47,50,60). It is interesting that these two variables were reduced in the naltrexone-treated animals. Besides, in the present work, we separately evaluated external and internal ambulation for this test. Although external ambulation in the periphery of the apparatus, by the wall, can be considered an estimation of locomotion, ambulation in the central area of the arena, around the holes, may have greater exploratory value. Internal ambulation may also indicate lower emotionality, given the characteristic thigmotaxis of the rat (2). In this context, it is interesting that naltrexone treatment, which decreased exploratory head-dipping parameters, reduced internal ambulation. Neither external ambulation nor rearing frequency were significantly affected. Although rearing frequency has been considered an exploratory parameter for other tests (29,41,50,68), in the holeboard this behavior could be considered to be "general activity," as opposed to site-directed exploration represented by the head dips (23,60). In this sense, the results obtained would suggest that neonatal naltrexone caused a decrement in the more clearly exploratory parameters of this test—head-dipping frequency and duration and internal ambulation—whereas it did not seem to alter those mainly related to general locomotor activity—external ambulation and rearing frequency.

The higher levels of internal and external ambulation and rearing shown by the females indicate a greater level of locomotor and exploratory activity. These data agree with most of the previous behavioral studies, which showed a greater activity for the females (7,27,28,29,50,57,66,68,70), although these differences were not always found for control animals in the holeboard (2,60). The neonatal naltrexone treatment does not seem to interfere with the sexual differences found in this test.

Open-Field Test

The results found for the first day of testing, and those parameters not affected by repeated testing, seem to be in accord with those obtained for the holeboard test. In the first open-field session, we observed that although a parameter basically related to locomotion such as external ambulation was not affected by neonatal naltrexone treatment, there was a significant effect on those considered to be more indicative of the degree of the animal's emotionality (decreasing internal ambulation and increasing immobility) (27,29,68). It is noteworthy that naltrexone treatment only affected external ambulation after repeated testing (but not on the first day). In this test, unlike that observed for the holeboard, naltrexone diminished rearing frequency and increased defecation, a parameter taken to be an indication of the emotional reactivity of the

TABLE 5

EFFECTS OF PREWEANLING NALTREXONE TREATMENT ON PLUS-MAZE ACTIVITY IN ADULTHOOD. PARAMETERS MEASURED

	Open Arms		Closed Arms	
	Frequency	Duration (s)	Frequency	Duration (s)
Males				
CONTR	9.9 ± 0.8	97.5 ± 16.2	9.4 ± 0.7	139.0 ± 12.8
NALTX	5.6 ± 0.6*	56.7 ± 11.7*	9.8 ± 0.9	186.5 ± 10.4*
Females				
CONTR	9.3 ± 1.0	86.1 ± 14.6	10.9 ± 0.5	156.6 ± 12.2
NALTX	6.8 ± 1.0*	41.1 ± 9.1*	12.6 ± 1.1†	197.0 ± 9.3*

Values represent the mean ± SEM of 16 to 17 animals per treatment × sex cohort.
*$p < 0.05$ vs. the corresponding control group (Duncan's multiple range test).
†$p < 0.05$ vs. the corresponding male group (Duncan's multiple range test).

animal (7,8,11,27-33,68). These results suggest a different involvement of the opioid system in the regulation of these responses in the open field and in the holeboard. Facial grooming was also sensitive to preweanling treatment with naltrexone. The increase found for the treated males seems to be in accordance with other effects of the treatment, because grooming is considered to be a possible indication of stress reactivity or emotionality by some authors (6,29,33,61,68). This parameter has also been interpreted as a displacement activity or a way of releasing the tension caused by a stressful situation (2,17). The effect of the naltrexone treatment on the female rats was opposed to that found for the males, showing lower facial grooming scores than the controls. An equivalent phenomenon of opposite differences in grooming depending on the gender has been observed when comparing wild rats (more reactive to open-field test) with laboratory rats (less reactive) (57); and similar results to our treatment have been found in restrained male and female rats for this parameter (2). In view of these data, it could be that the conflict aroused by the stressful open-field conditions was diverted toward other emotional behaviors in the naltrexone-treated female rats. In fact, treated females showed higher increases for both immobility and defecation than treated males.

The sexual differences found in this test were similar to those observed for the holeboard test: The female rats showed higher external and internal ambulation, as well as greater rearing frequency, than the males. These results coincide with those reported by other authors for the open field (7,28,29, 57,66,68). Except for facial grooming, there were no significantly different effects of the treatment depending on the sex of the animals. However, it should be noted that naltrexone treatment abolished sexual differences between treated animals regarding immobility.

In accordance with previous works (9,10,30,32,66), repeated testing did not significantly affect defecation or grooming. The effect of repeated testing, in control and treated animals alike, found for the other variables appeared as a general decrease of activity (ambulation and rearing) and an increase of immobility along the days of testing, a phenomenon previously observed by others (9,33,62,66). A lesser decrease in activity than that of the males was found for the female rats in our study, in accordance with previous reports (30,66). Generally speaking, naltrexone-treated animals showed a more important decrease in activity and increment in immobility than the controls. This was especially true for the treated males, which showed more marked reductions in external ambulation and a greater increase in immobility during the 3 days of testing; the opposite was true for the control females. It has been found that handled animals, considered to be less emotional (1,4,16,22,45,46), present smaller decreases in open-field activity after 3 days of testing (66), and that rats from a stress-reactive strain show a higher tendency to reduce

TABLE 6

EFFECTS OF PREWEANLING NALTREXONE TREATMENT ON PLUS-MAZE ACTIVITY IN ADULTHOOD. ESTIMATED VARIABLES

	% Open/ Total Entries	% Time in Open Arms	% Time in Closed Arms	Total Time in Both Types of Arms (s)	Total Entries Into Both Types of Arms
Males					
CONTR	51.4 ± 2.5	39.6 ± 5.5	60.4 ± 5.5	236.4 ± 12.4	19.4 ± 1.1
NALTX	35.6 ± 3.8*	22.3 ± 4.3*	77.7 ± 4.3*	243.2 ± 9.2	15.3 ± 1.1
Females					
CONTR	44.3 ± 3.3	34.6 ± 5.5	65.4 ± 5.6	242.7 ± 6.6	20.1 ± 1.1
NALTX	33.3 ± 4.3*	16.9 ± 3.7*	83.1 ± 3.7*	238.2 ± 7.0	19.4 ± 1.6†

Values represent the mean ± SEM of 16 to 17 animals per treatment × sex cohort.
*$p < 0.05$ vs. the corresponding control group (Duncan's multiple range test).
†$p < 0.05$ vs. the corresponding male group (Duncan's multiple range test).

external ambulation than rats from nonreactive strains (33). Furthermore, it has been observed that the decrement in open-field activity hardly appears when the rat is tested together with another individual (9), a situation that presumably mitigates the degree of stress experienced by the animal. In view of these data, it is possible that the reduction of activity with repeated testing found in this work for the naltrexone-treated groups could be indicative of a higher level of emotionality in the treated animals. However, a decrease in their exploratory drive cannot be excluded as a possible interpretation for these results.

Plus-Maze Test

Both behavioral and physiologic evidence has demonstrated that the animals' tendency to avoid the open arms is motivated by fear or anxiety (56). In this sense, the results obtained for this test would indicate that preweanling naltrexone treatment increased adult animals' level of anxiety. In fact, the treatment induced a decrease in both the frequency and duration of visits to the open arms, together with an increase in the duration of visits to the closed arms. However, neither the total number of entries nor the frequency of visits to the closed arms were significantly affected. These latter parameters are basically related to general activity (26,38,56, 70). Taken together, these data allow us to conclude that naltrexone has an effect on those parameters directly related to anxiety that cannot be explained by a general decrease in the activity of the animals.

In accordance with previous research (60), no sexual differences were found in the control animals for the variables mainly related to the level of anxiety, although sex differences appeared in the parameters to be indicative of general activity. In contrast, it is worth mentioning that other studies (2,38,70) have reported sexual differences in the levels of anxiety.

GENERAL COMMENT

Close functional relations appear to exist between the opioid system and those systems regulating behavioral responses. In particular, it has been proposed that endogenous opioids would be released as a reaction to different stressful situations (19,40,63), and may have a facilitating role in the exploration of a novel or adverse environment (67). In accordance with this view, it has been reported that opioid agonists increase exploration in novel environments, whereas antagonists such as naloxone or naltrexone reduce this behavior (6). In addition, the anxiolitic and antidepressant properties of low doses of opioid substances in human beings are well known, whereas naltrexone might have an opposite effect (18,37). The opioid system also seems to be involved in the modulation of locomotor activity. In this sense, recent studies using selective agonists for diverse opioid receptors administered in discrete brain areas have shown that opioid actions on locomotor- and anxiety-related patterns appear to depend on the receptor type and the brain region studied (12,49,51,53). These latter studies demonstrate the complexity of the actions of the opioid agonists, but nevertheless support the involvement of the opioid system in the regulation of this type of behavior.

On the other hand, it has been suggested that 1 day after the interruption of a given naltrexone treatment, diverse phenomena, such as receptor increased number and/or sensitivity, as well as enhanced opioid levels, may occur (58,62). Moreover, it has been shown that at the end of a treatment involving daily transient blockade of the opioid system (naltrexone 1 mg/kg), there is an enhanced opioid action on other functional variables, probably as a result of the autocompensatory capacity of the system (73,77,78).

All this behavioral and pharmacologic evidence, together with the unique plasticity shown by the opioid system in response to pharmacologic treatments during the early stages of postnatal development (3,5,15,44,59,64,73), raises the question of the possible effects of the neonatal transient opioid blockade and its consequences on the behavioral responses of the adult animal. The paradigms employed in our study were chosen according to their behavioral value attributed by the literature to assess different aspects of behavior: a) site-directed exploration (holeboard) (43); b) emotionality (open field) (29,30); and c) anxiety (plus-maze) (56). This experimental approach can be instrumental in assessing the possible role of neonatal opioids in establishing the patterns of behavior displayed by the adult animal in different environmental situations.

At present, the data available in this field are fragmentary and often contradictory, possibly because only partial aspects of behavior were studied. Some previous reports have investigated the effects of neonatal administration of opioid substances on behavioral development. Thus, it was reported that chronic morphine between days 3 and 26 causes retardation in the acquisition of some social behaviors, whereas the chronic naloxone produces the opposite effect (55). The same treatment employed in this study (one daily administration of 1 mg/kg naltrexone during the preweanling period) was seen to increase open-field grooming in 21-day-old animals (similar to the result found in our work), whereas a dose of 50 mg/kg caused a decrease in this parameter (74). Neither dose had a significant effect on other emotionality-related parameters (i.e., defecation, rearing) at 21 days of age. These developmental findings illustrate the importance of assessing the effects of these drugs in adulthood, because some of the neonatal behavioral alterations seem to be transient (55) or not fully evident at these early ages (74). Furthermore, our data, together with those of Zagon and McLaughlin (74), provide a good indication that the behavioral actions of the naltrexone treatment employed are mainly long-term effects. The literature shows that the administration of a constant dose of naltrexone during postnatal days 1–10 causes an increase of exploratory behavior when the animals reach adulthood (sniffing and investigating), accompanied by either an increment (females) or a decrement (males) in rearing (50). It has also been reported that naltrexone administration in the drinking water given to the dam during pregnancy and/or lactation seems to facilitate the extinction of the conditioned emotional response of the offspring in adulthood (35). However, naloxone administration during the gestation period has been reported to impair learning in the Biel water maze (69). Taken together, our results indicate that naltrexone treatment during the preweanling period produces a decrease in site-directed exploration accompanied by increased levels of anxiety and emotionality, without noticeable modifications in general activity, when the animals reach adulthood. The discrepancies with two of the previously mentioned studies (35,50) could be due, at least in part, to the different period of development during which naltrexone was administered and/or the different dose employed. On the other hand, other authors (71) did not find changes in square open-field activity after pre- and postnatal treatment with β-endorphin in adult rats; neonatal exposure to methadone was seen to produce an increase in the activity of adult animals, as measured by different behavioral tests including the open field (81). Neonatal administration of another opioid agonist (met-enkephalin) has been shown to

facilitate the performance in a complex maze in adulthood (39). It is noteworthy that in the two latter reports using an opioid agonist, the effect obtained (i.e., an improved response) was somehow opposite to the effect we found employing an antagonist such as naltrexone. The results from both our study and these reports (39,81) support each other and lead us to postulate that the manipulation of the opioid system during the preweanling period affects exploratory behavior, with stimulation of the system enhancing and the blockade impairing the performance in certain environments when the animals become adults. Some of our results are similar to those obtained by other authors using opioid antagonists in adult rats (6) and seem to be, to some extent, opposite to some of the effects of agonists also in adult animals (67). In all cases, the behavioral battery employed allowed us to obtain a clear and coherent picture of the behavioral changes induced by the treatment.

Other research using one single 1 mg/kg administration of naltrexone have reported decreased cerebellar neural cell division in rats of 6 days of age (80,78). Besides, naltrexone treatment similar to the one employed in the present work was seen to cause morphometric changes (cerebellum and hippocampus) (76,77) and altered dendritic growth and spine formation (cortex and hippocampus) (36) in 21-day-old rats. Furthermore, a recent report showed that our naltrexone administration protocol produces changes in the brain monoamine content (striatum, midbrain, and hypothalamus) in preweanling rats (13). It is therefore possible that this type of modification in the development of the brain led to the behavioral alterations in adult behavior found in the present work. Given the reports of a known growth-related opioid peptide (met-enkephalin) and a zeta (ζ) opioid receptor that appear to play a role in the regulation of brain development (72,80), the actions of the 1 mg/kg naltrexone administered during the preweanling period on this system may be related to the effects on adult behavior shown in our work.

In conclusion, the present results indicate that an intermittent functional opioid blockade during the critical stages of early postnatal development causes some impairment in the performance of the individuals in three different environmental situations. Thus, endogenous opioids would play an important role in the development and establishment of some behavioral mechanisms that may facilitate the exploration of certain environments. Finally, our findings may be interesting from the clinical point of view, particularly in relation to the use of naltrexone to treat neonatal apnea in children (54) or in programs of drug detoxification in humans, employing opioid antagonists with regard to their application to pregnant or lactating mothers (35).

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