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Neonatal Naltrindole and Handling Differently Affect Morphine Antinociception in Male and Female Rats

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FERNÁNDEZ, B., I. ALBERTI, I. KITCHEN AND M. P. VIVEROS. *Neonatal naltrindole and handling differently affect morphine antinociception in male and female rats.* PHARMACOL BIOCHEM BEHAV **64**(4)851–855, 1999.—The effects of a daily injection of the δ selective opioid antagonist naltrindole (1 mg/kg), from birth to postnatal day 19, on antinociceptive responses to morphine (2 mg/kg) in 20-day-old rats of both sexes were investigated. The effects of postnatal handling were studied by including two control groups—one group receiving daily injections of saline, and a naive unhandled group. Antinociception was assessed using the tail-immersion test and time–response curves (5, 10, 15, and 30 min) were carried out for all experimental groups. In all treatment groups females showed greater sensitivity to the noxious stimuli compared to males. No significant effect of naltrindole treatment on baseline latencies was found. Postnatal handling increased sensitivity to thermal pain in both sexes, and reduced the effect of morphine in males. No significant effect of chronic naltrindole administration on morphine antinociception was found in this sex. Naltrindole-treated females showed an increased antinociception when compared to unhandled animals of the same gender. The results indicate that preweanling handling stress and chronic naltrindole treatment differentially affected morphine antinociception in male and female neonatal rats.

Neonatal rats δ-Opioid receptor blockade Naltrindole Handling stress Morphine antinociception Sex differences

THERE is a lot of information about the existence of interactions and cooperativity between μ- and δ-receptors in adult rodents. These types of interactions, which affect antinociceptive responses (11,12,18,19), the development of physical dependence on morphine (22), and brain-stimulation reward (7), may have implications for the therapeutic use of opioids. Functional interactions between μ- and δ-receptors in the modulation of antinociception appear to be complex. Different δ-receptor agonists have been shown to positively or negatively modulate morphine antinociception. Thus [Leu^5] enkephalin and its analogs have been shown to increase, whereas [Met^5] enkephalin and its analogs decrease morphine antinociceptive potency in mice (11). The modulation of μ-mediated antinociception by δ-agonists also depends on the specific μ-receptor agonist employed (12,17,23). These results have led to the notion that different subtypes of opioid μ and δ (i.e., complexed and noncomplexed) receptors may exist, and that the μ-receptor subtype residing in the μ–δ-receptor complex may be selectively activated by some μ-agonists. Although some results suggest that μ_2 (11) and δ_2 (20,24) may be the receptor subtypes residing in the μ–δ-receptor complex, a definitive identification of the interacting opioid receptor subtypes has not been achieved. The available data on μ–δ-receptors interactions mostly refer to adult rodents, and there is much less information of these functional interactions in neonatal animals. We have recently reported that chronic naltrindole treatment during the preweanling period blocked the antinociceptive response to the μ-selective agonist alfentanil in male but not in female rats at postnatal day 20 (2,10). In the present study, we have investigated the effects of functional blockade of neonatal δ receptors by the same naltrindole treatment used previously (2,10), and measured antinociceptive responses to morphine in preweanling 20-day-old rats of both sexes. The comparison of the present results with our

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previous work on alfentanil responses in neonates (2,10) provides an indirect indication about a potential influence of the μ-agonist employed on the effects of neonatal naltrindole on μ-mediated antinociceptive responses. We have also addressed the effects of handling the animals during the preweanling period on pain sensitivity and morphine responses, and the influence of sex during early development in this respect.

METHOD

Animals and Neonatal Treatments

Experiments were performed on Wistar albino rats of both sexes from the animal house of the Universidad Complutense of Madrid, which is served by Harlan Interfauna Ibérica S.A. (Barcelona, Spain). The animals were maintained at a constant temperature of 21°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/A03; Panlab, Barcelona, Spain) and water. Male rats were mated with females (one male × two females), and spermpositive females were then rehoused in individual cages for the duration of pregnancy. On the day of birth (postnatal day 0), litters were sex balanced and culled to 10 ± 1 pups per dam. The pups were marked subcutaneously with ink for identification. From the day of birth to day 19 half of the animals within each liter (males and females) received a daily SC injection of naltrindole (RBI) (1 mg/kg, 1 ml/kg), and the other half an SC injection with the same volume of 0.9% saline solution. The dose of naltrindole used in this study selectively antagonizes δ-receptors without affecting μ-receptors (5), and has been previously used in our recent work (2,10) on alfentanil responses in neonates. The effects of handling the animals were studied by including additional naive control groups that were not weighed or injected.

All the experiments are in compliance with the Royal Decree 223/1988 of 14 March (BOE 18) and the Ministerial Order of 13 October 1989 (BOE 18) about protection of experimental animals, as well as with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Antinociceptive Responses to Morphine

For each sex, and within each of the above-mentioned naive or neonatally treated groups, two acute treatment groups were used. One of them received a single injection of morphine sulfate (a generous gift of Dr. Ambrosio) (2 mg/kg, 0.1 ml/20 g, IP), and the other was a control group receiving a single injection with an equivalent volume of 0.9% saline solution IP. In pilot studies, we found that the dose of morphine used in this work produced submaximal responses without inducing any remarkable sedative effect, which might have interfered with the performance of the tail-immersion test. Each experimental group contained 8–12 individuals, from a minimum of four different litters, which were tested on at least two different days to minimize interlitter and interday variability. Nociception was assessed using the warm water (50°C) tail-immersion test (15). Nociceptive responses (tail-immersion latencies) were measured as the time elapsed prior to removal of the tail from the water surface, and a maximum 10-s cutoff was used. Response latencies were measured 15 min before administration of saline or morphine and 5, 10, 15, and 30 min after treatment. In a parallel experiment we studied if an acute injection of naltrindole (1 mg/kg, 0.1 ml/20 g IP), administered 10 min before the acute administration of morphine, could antagonize the effect of the μ-agonist. Antinociception was quantified as previously described (2,10,21) using the following formula: Latency quotient = response latency after treatment/response latency before treatment. All experimental procedures were carried out between 0930 and 1430 h. On the day of testing the animals were equilibrated in a quiet laboratory at least 1 h before experimental procedures were begun. Behavioral tests were carried out under the same illumination conditions as those in the animal facilities (red light).

Statistical Analysis

The effect of sex and neonatal treatments (postnatal handling and chronic naltrindole administration) on baseline nociceptive responses, and the influence of sex on morphine antinociception in naive animals were evaluated by two-way ANOVA. The effects of neonatal treatments on morphine antinociception were studied by comparing the areas under the curve calculated from the latency quotients obtained over the 30-min period using two-way ANOVA [neonatal treatment, acute treatment (saline, morphine)]. Additional one-way ANOVA was performed where appropriate. Student–Newman–Keuls test with a level of significance set at $p < 0.05$ was used for post hoc comparisons.

RESULTS

Baseline Latencies

The analysis of the baseline responses to thermal stimuli revealed a significant effect of postnatal handling, $F(1, 88) = 34.87$, $p < 0.001$, and post hoc comparisons indicated that naive control animals of both sexes showed significantly higher baseline latencies compared to handled animals. In all treatment groups females showed greater sensitivity to the noxious stimuli compared to males, $F(1, 88) = 122.45$, $p < 0.001$; $F(1, 106) = 202.94$, $p < 0.001$ (Table 1). No significant effect of the chronic naltrindole treatment, $F(1, 106) = 3.34$, $p = 0.07$, or interaction between sex and naltrindole treatment, $F(1, 106) = 0.01$, $p = 0.9$, were found.

TABLE 1

EFFECTS OF NEONATAL HANDLING AND CHRONIC NALTRINDOLE TREATMENT ON BASELINE LATENCIES (s) IN THE TAIL-IMMERSION TEST

	20-Day-Old Rats	
	Males	Females
NC	2.28 ± 0.11	1.46 ± 0.07*
SS	1.86 ± 0.07†	0.98 ± 0.07*†
NTI	1.74 ± 0.06	0.87 ± 0.05*

Values represent mean ± SEM of 15–30 animals. Animals were treated neonatally with saline (SS) or naltrindole (NTI, 1 mg/kg) SC, from birth to day 19. The effect of handling was studied by including additional naive control groups (NC) (not weighed or injected during days 0–19). ANOVA: significant effects of sex and handling. Student–Newman–Keuls: *$p < 0.05$ vs. male groups; †$p < 0.05$ vs. naive controls.

Antinociceptive Responses to Morphine

The analysis of the latency quotients (15 vs. 30 min) in naive control animals injected with morphine rendered a significant interaction between sex and time, $F(1, 32) = 5.34$, $p < 0.05$, and post hoc comparisons showed that the effect of morphine significantly increased between 15 and 30 min in males, but not in females. Accordingly, the latency quotients obtained 30 min after the administration of the drug were significantly higher in males than in females ($p < 0.05$) (Fig. 1, upper panels). No significant interaction between sex and time was found in the corresponding control groups injected acutely with saline, $F(1, 30) = 0.78$, $p = 0.4$. The analysis of the areas under the curve rendered a significant effect of postnatal handling in males, $F(1, 31) = 5.34$, $p < 0.05$, with handled animals showing the lowest values. A visual inspection of the data indicates that there is an interaction between handling and acute treatment that did not reach statistical significance ($p = 0.17$). In fact, a multiple comparison of the means by one-way ANOVA revealed significant differences between the animals injected with morphine ($p < 0.05$), but not between the controls injected acutely with saline (Fig. 1, lower panels). These results indicate that handling reduced the antinociceptive effect of morphine in males. No significant effect of chronic naltrindole administration on morphine antinociception was found in these animals. Postnatal handling or chronic naltrindole alone did not induce significant effects on morphine antinociception in females. However, comparisons between naive controls and naltrindole-treated groups revealed that the areas under the curve of naltrindole-treated females were significantly greater than those of the corresponding unhandled animals, $F(1, 36) = 7.04$, $p = 0.01$. Multiple comparison of the means showed significant differences between the animals injected with morphine ($p < 0.05$), but not between the controls injected acutely with saline (Fig. 1, lower panels).

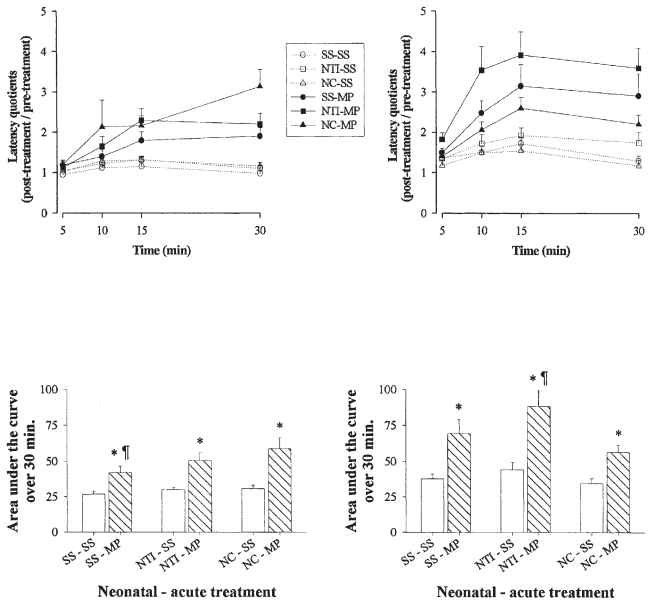

FIG. 1. Effects of postnatal handling and chronic naltrindole administration on antinociceptive responses to morphine (MP) in the tail-immersion test in 20-day-old male (left) and female (right) rats. Time course of antinociceptive response to MP (upper panels) and area under the corresponding curves over 30 min (lower panels). Values represent mean ±SEM of 8–12 animals. The animals were treated neonatally with saline (SS) or naltrindole (NTI) (1 mg/kg SC), from birth to day 19, and subsequently studied for the acute effects of MP (2 mg/kg, IP). The effects of handling were studied by including additional naive control groups (NC) (not weighed or injected during days 0-19). Response latencies were measured 15 min before acute administration of SS or MP (pretreatment latencies), and 5, 10, 15, and 30 min after treatment (posttreatment latencies). ANOVA: significant effects of acute and neonatal treatments. Student–Newman–Keuls: *$p < 0.05$ vs. the groups injected acutely with SS; ¶$p < 0.05$ vs. naive control groups.

The possible antagonism of morphine antinociception by pretreatment with a single naltrindole injection was studied by analyzing the latency quotients obtained 15 min after the administration of the opioid (peak antinociception). The results indicate that acute naltrindole administration did not antagonize or reduce the effect of morphine in control animals injected neonatally with saline [males, acute saline (SS): 1.15 ± 0.10, acute morphine (MP): 1.79 ± 0.22, acute naltrindole (NTI) plus MP ($n = 7$): 2.61 ± 0.45. Females, acute SS: 1.72 ± 0.16; acute MP: 3.14 ± 0.53, acute naltrindole NTI plus MP ($n = 7$): 3.95 ± 0.38]. However, in females treated neonatally with naltrindole, the antinociceptive effect of morphine was slightly decreased by pretreatment with acute naltrindole [acute SS: 1.92 ± 0.19, acute MP: 3.91 ± 0.57, acute NTI plus MP: 3.40 ± 0.42 ($n = 7$)], with no significant differences being found between the control group injected acutely with saline and the group injected with acute NTI +MP.

DISCUSSION

There is evidence for the existence of sex differences in nociception and antinociceptive responses to morphine in adult rodents (3,4). The present results indicate that in all treatment groups females showed greater sensitivity to noxious thermal stimulation compared to males. These data are in accord with previous results indicating that shock thresholds of adult female rats are significantly lower than those of male rats (3), and extends these findings to neonatal rats. Adult female rats and mice display significantly less morphine antinociception than males (3,4) and these gender differences appear to reflect inherent differences in the sensitivity of the brain to morphine (4). In this work, the antinociceptive effect of morphine appeared to be of a longer duration in naive control males than in females at day 20. In accordance with our previous studies (2,8,10), chronic naltrindole administration did not modify the baseline nociceptive responses. This data suggests that the δ-receptor does not play a tonic role in setting pain sensitivity, and is in good agreement with the results of knockout studies where deletion of the δ-receptor gene does not alter nociceptive threshold (25). Postnatal handling stress increased sensitivity to thermal pain in both males and females. It has been previously found that neonatal handling procedures (days 2–19) increased nociceptive thresholds in the tail-flick test in 30–day-old mice (6). These different results may well be attributable to species-related differences, because our results are in good agreement with previous data indicating that chronic stress usually depletes the central opioid pathways in rats (16), and this might account for the reduced nociceptive thresholds of handled animals.

The present results show that postnatal handling significantly reduced the effect of morphine in 20-day-old males. It has been reported that postnatal handling reduces the antinociceptive effect of β-endorphin (6) in male mice. Acute handling stressful procedures during early postnatal stages in-

duces the release of pituitary β-endorphin in rat pups (13). It has been proposed that chronic handling procedures might produce an exaggerated release of endogenous β-endorphin, which might account for the reduced responsiveness to this peptide (6). The reduced response to morphine in handled males might be attributable to a phenomenon of crosstolerance between exogenous morphine and endogenous β-endorphin. Little is known about sex-related differences in the effects of handling upon antinociceptive responses to opioids during the neonatal period. The present results indicate that the influence of handling stress on morphine antinociception was more marked in males than in females. We have recently reported that this handling procedure prevents the stress-induced rise in corticosterone in males, but does not modify the corticosterone response to stress in females (9). Taken together, this data points to the existence of gender differences in the effects of handling on both opioid-mediated antinociceptive responses and physiological responses to stress in neonatal rats. In adult rats it has been also shown that males are more sensitive than females to the consequences of stress on nociception and β-endorphin content in the periaqueductal gray matter (1).

Functional interactions between μ- and δ-opioid receptors subtypes in the mediation of antinociception has been mostly studied in adult rodents (8,11,12,14,18,19). We have recently reported that chronic naltrindole administration during the preweanling period blocked the antinociceptive response to the μ-selective agonist alfentanil in male but not in female rats at postnatal day 20 (2,10). The present results indicate that the same naltrindole treatment does not significantly affect the responses to morphine in males, which is in accord with other previous study indicating that chronic naltrindole administration does not modify the inhibitory effect of morphine on vocalization responses to noxious electrical stimulation in adult male rats (8). In contrast, however, chronic naltrindole treatment appears to positively modulate morphine responses in females, possibly potentiating the influence of handling, because this procedure tended to increase morphine antinociception in this sex. The comparison of the present work (using morphine) with our already mentioned articles on alfentanil responses (2,10) provides a good indication about both, sex- and specific agonist-related differences in the effects of preweanling chronic naltrindole administration. It has been previously shown that modulation of μ-mediated antinociception by the δ agonist DPDPE is only observed for morphine and normorphine, but not for other μ-receptor agonists including sufentanil and the peptides DAMGO and β-endorphin in adult male mice (12), although using a different approach synergy with DPDPE has been shown to be greater for DAMGO than for morphine in adult male rats (17,23). These results have led to the proposal that different μ-receptor subtypes that might correspond to $\mu_{complexed}$ and $\mu_{noncomplexed}$ may exist, which could be activated by different μ-opioid agonists and correspondingly differentially modulated by endogenous or exogenous δ-agonists (12). According to this interpretation, the different effects of chronic naltrindole treatment on the antinociceptive responses to alfentanil (2,10) and morphine might support the involvement of different μ-receptor subtypes. Alternatively, these results might reflect differences in the efficacy of the two μ-agonists, and could be related to altered coupling or receptor occupancy phenomena. This latter explanation appears to be supported by recent data obtained from μ-opioid receptor deficient mice, because these studies have not revealed the existence of μ-receptor subtypes encoded by different genes, and it has been suggested that pharmacological heterogeneity might arise from variable responsiveness of the μ-receptor, depending on the interacting ligand or intracellular effectors (14). It has been proposed that the positive and negative modulatory actions of δ-agonists on morphine antinociception may be mediated through opioid δ-receptors, as the modulation, but not the direct μ-mediated effect, can be antagonized by opioid δ antagonists (12,20). The present results indicate that the direct effect of morphine was not reduced by pretreatment with a single injection of naltrindole in either sex, which supports previous data indicating that the dose of naltrindole used in this study selectively antagonises δ-receptors without affecting μ-receptors (5). However, acute naltrindole induced a modest reduction in the positive modulatory action of chronic naltrindole treatment on morphine responses in 20-day-old females. Taken together, these results suggest that the positive modulation of morphine antinociception induced by chronic naltrindole administration was, in fact, mediated by the δ-receptor. To address the molecular basis of these functional responses, we are currently evaluating by autoradiographic mapping how the chronic administration of naltrindole influences the expression of brain and spinal μ- and δ-opioid receptor in neonatal rats of both sexes.

In conclusion, the present results indicate that handling stress and chronic naltrindole administration during the preweanling period affected antinociceptive responses to morphine in neonatal rats, and point to the important influence of sex on these effects.

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