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Preweanling naltrindole administration differentially affects clonidine induced antinociception and plasma adrenaline levels in male and female neonatal rats

[1]Israel Alberti, [1]Beatriz Fernández, [2]Luis Fernando Alguacil, [2]Antonio Aguilar, [2]Manuel Caamaño, [1]Eva M. Romero & *,[1]M. Paz Viveros

[1]Departamento de Biología Animal II, Facultad de Biología, Universidad Complutense, 28040 Madrid, Spain and [2]Departamento de Ciencias Biomédicas, Lab. Farmacología, Universidad San Pablo CEU, Boadilla, Madrid, Spain

1 The influence of a chronic treatment with the δ-selective opioid antagonist naltrindole (1 mg kg^{-1}) during the preweanling period (daily injections from birth to postnatal day 19), on the antinociceptive and sympatholytic effects of the α_2-adrenergic agonist clonidine in male and female rats of 20 and 25 days of age was investigated.

2 Nociception was assessed using the tail immersion test (water at 50°C) and plasma levels of adrenaline were measured by high-performance liquid chromatography.

3 The dose of clonidine (1.5 mg kg^{-1}) and the time point at which nociceptive responses were recorded (30 min after the administration of the drug) were chosen on the basis of dose-response (0.5, 1, 1.5 and 2 mg kg^{-1}) and time-response (5, 10, 15, 30 and 60 min) curves which were previously carried out in naive control neonatal rats.

4 In females, the functional blockade of the δ-receptor by neonatal naltrindole treatment did not modify the sympatholytic effect of clonidine but prevented clonidine induced antinociception. Conversely, in males naltrindole treatment allowed the appearance of clonidine antinociception and the sympatholytic effect of clonidine.

5 The results indicate that the δ-receptor is involved in the modulation of antinociceptive and sympatholytic responses to clonidine in neonatal rats and suggest the existence of sex differences in the interactions between δ-opioid and α_2-adrenergic receptors.

Keywords: Neonatal rats; δ-opioid receptor blockade; naltrindole; clonidine-induced-antinociception; plasma adrenaline levels

Abbreviations: A, adrenaline; ANOVA, analysis of variance; CLON, clonidine; DHBA, dihydroxibenzilamine; EDTA K_3, ethylenediaminetetraacetic acid. Tripotassium salt; EDTA Na_2, ethylenediaminetetraacetic acid. Disodium salt; HPLC, high-performance liquid chromatography; ICI-174864, N,N-diallyl-Tyr-Aib-Aib-Phe-Leu; KH_2PO_4, potassium dihydrogen phosphate; NC, naive controls; NTI, naltrindole; RBI, Research Biochemicals International; SD, standard deviation; SIA, stress-induced-antinociception; SS, saline

Introduction

Opioid and α_2-adrenergic mechanisms appear to interact with each other in the modulation of nociception (Bernard *et al*., 1994; Sierralta *et al*., 1996). The analgesic effects of noradrenergic and opioid agents, including the α_2-agonist clonidine and morphine, appear to be additive or synergistic in different animal models depending on the level of the pharmacological action (spinal or supraspinal), the dose and route of administration and the nociceptive test used (Wilcox *et al*., 1987; Drasner & Fields, 1988; Monasky *et al*., 1990; Ossipov *et al*., 1990a,b; Bernard *et al*., 1994). Some results indicate that the α_2-antagonist yohimbine reduces morphine-induced analgesia (Iglesias *et al*., 1992) and decreases behavioural withdrawal in morphine treated rats upon naloxone challenge (Taylor *et al*., 1991; Iglesias *et al*., 1992). Other authors have reported that yohimbine enhances morphine analgesia in humans (Gear *et al*., 1995). It has been shown that cross-tolerance develops between morphine and the α_2-adrenergic agonists such as dexmedetomidine (Kalso *et al*., 1993) and clonidine (Post *et al*., 1988), whereas other data indicates absence of cross-tolerance between δ-opioid and α_2-adrenergic receptors (Kalso *et al*., 1993). Previous data suggests that μ- rather than δ-opioid receptors are involved in the synergism of spinal opioid and α_2-adrenergic antinociception (Sullivan *et al*., 1992). Other results indicate that combined administration of δ-agonists and clonidine produced significant synergistic suppression of noxiously evoked neuronal activity in the spinal dorsal horn, whereas the selective μ-agonist DAGO did not show any synergistic action with clonidine (Omote *et al*., 1991). It has been also proposed that α_2-adrenoceptor-mediated antinociception may occur independently of opioid receptor mechanisms (Ossipov *et al*., 1989). Taken together these findings suggest that the interactions between noradrenergic and opioid analgesic mechanisms are complex and controversial. Most of the studies focused on this issue have been performed in adult rodents and there is scarce information about these functional relations in neonatal rats. There is evidence for the involvement of endogenous opioids in the hypotension elicited by α_2-adrenoceptor agonists (see for review Rubio *et al*., 1992) and different opioid receptor subtypes appear to be involved in the analgesic as compared to the cardiovascular effects of clonidine (Mastrianni *et al*., 1989). In the present study we studied possible correlations between the effects of functional blockade of δ-receptors on antinociceptive and adrenaline responses to clonidine in neonatal rats. For this purpose we

*Author for correspondence;
E-mail: PAZVIVER@eucmax.sim.ucm.es

investigated the effects of a neonatal chronic treatment with naltrindole, a highly potent non-peptide and selective δ-antagonist (Portoghese *et al*., 1988) and measured antinociception and plasma adrenaline levels in response to acute administration of clonidine in pre- and postweanling rats of 20 and 25 days of age respectively. This study provides the first evidence for the involvement of the δ-opioid receptor in the modulation of antinociceptive and sympatholytic effects induced by an α_2-receptor agonist during early development, and the existence of sex differences in this interaction.

Methods

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 20.00 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 to 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 in order to identify each of them. From the day of birth to day 19 half of the animals within each litter (males and females) received a daily s.c. injection of naltrindole (NTI) (RBI) (1 mg kg^{-1}, 1 ml kg^{-1}) and the other half a s.c. injection with the same volume of 0.9 % saline solution.

The animals which were tested at day 25 were weaned at 22 days of age. We have previously used the dose chosen for the δ-antagonist for the study of functional interactions between opioid receptor subtypes in neonates (Antelo *et al*., 1998; Fernández *et al*., 1999c). Taken into account this neonatal treatment plus the different acute treatments detailed below, each experimental group contained individuals from a minimum of four different litters which were tested on at least two different days to minimize inter-litter and inter-day variability. All experimental procedures were carried out between 09.30 h and 14.30 h. On the day of testing the animals were equilibrated in a quiet laboratory at least 1 h before experimental procedures were begun. Behavioural tests were carried out under the same illumination conditions as those in the animal facilities (red light). All the experiments performed in this study 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 clonidine

At each age (20 and 25 days), for each sex, and within each of the above mentioned neonatally treated groups, two acute treatment groups were used. One of them received a single injection of clonidine (Sigma) (1.5 mg kg^{-1}, 0.1 ml 20 g^{-1}, s.c.) (CLON), and the other one was a control group receiving a single injection with the same volume of 0.9% saline solution s.c. (SS).

Nociception was assessed using the tail immersion test with water at 50°C (Kitchen *et al*., 1984). 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 cut-off was used. Response latencies were measured 15 min before administration of saline or clonidine and 30 min after treatment. Antinociception was quantified as previously described (Pujol *et al*., 1993; Antelo *et al*., 1998; Fernández *et al*., 1999c) using the following formula: Latency quotient = response latency after treatment/response latency before treatment. The dose of clonidine used in the study and the time point at which nociceptive responses were recorded were chosen from the analysis of the area under the curves and time course of antinociceptive responses obtained from dose-response (0.5, 1, 1.5 and 2 mg kg^{-1}) and time-response (5, 10, 15, 30 and 60 min) curves which were previously carried out in naive control (NC) 25-day-old rats which were not weighed or injected during the preweanling period.

Plasma adrenaline levels

Fifteen min after the completion of the tail immersion test, the animals were killed by decapitation and blood samples were collected from the trunk and dispensed into tubes containing EDTA K_3. Blood samples were centrifuged (2500 r.p.m. for 15 min at 4°C) and plasma was stored at −80°C. Adrenaline levels were determined by high-performance liquid chromatography (HPLC). The chromatographic system included a pump (model 429), an automatic sampler injector (model 465) (Kontron Instruments) and the detector (Coulochem II) (ESA, Inc.). The mobile phase was a binary mixture acetonitrile : buffer (5 : 95 v v^{-1}). The buffer solution was composed by KH_2PO_4 25 mM, octanesulphonic acid 5.4 mM and EDTA Na_2 0.1 mM; pH was adjusted to 2.2 by adding o-phosphoric acid. The mobile phase was pumped isocratically at 1.5 ml min^{-1}. As stationary phase an analytical column (supelcosil LC-ABZ 5 μm 50 × 4.6 mm) (Supelco) was used, with a guard column (Nucleosil 120 C18 5 μm 30 × 4.6 mm) (Scharlau). Prior to the analysis, samples were treated using a plasma catecholamine extraction kit (ESA plasma catecholamine analysis kit, ESA, Inc., Chelmsford, MA, U.S.A.), which included a solid-phase extraction method. Standard solutions of adrenaline (A) and dihydroxibenzilamine (DHBA) (internal standard) were prepared daily and injected for the calibration curve. Retention times (min) [mean ± standard deviation (s.d.)] were: $A = 2.76 \pm 0.05$; $DHBA = 3.80 \pm 0.14$. Quantitation of A in plasma samples was performed using DHBA as internal standard. The mean recovery of A in samples was (mean ± s.d.) 29.85 ± 4.87 (%).

Statistical analysis

All data was analysed by ANOVA. The influence of handling and chronic naltrindole administration on baseline nociceptive latencies was evaluated by analysing the nociceptive thresholds of all the animals included in the study. The effects of neonatal naltrindole on clonidine-mediated antinociception were studied by comparing the latency quotients obtained from the animals treated neonatally with saline or naltrindole. Equivalent ANOVA were performed to analyse the effects of neonatal naltrindole treatment on clonidine-induced plasma adrenaline levels. The dose of clonidine used to evaluate the effects of neonatal naltrindole treatment was chosen from the analysis of the area under the curves corresponding to 25-day-old naive control animals, as previously indicated. Student-Newman-Keuls test with a level of significance set at $P < 0.05$ was used for *post hoc* comparisons.

Results

Dose-response curves

The analysis of the dose-response curves over 60 min showed that for both, males and females, the dose of 1.5 mg kg^{-1} produced the most marked antinociceptive effect, with significant differences being found between this dose and the other doses tested in the study ($P<0.05$). Peak antinociception was found 30 min after the administration of the drug (Figure 1).

Baseline nociceptive latencies

The analysis of the baseline responses in the tail immersion test showed that 25-day-old naive control females showed significantly reduced nociceptive thresholds compared to males ($P<0.05$) [NC males: 2.03 ± 0.08 ($n=52$), NC females: 1.64 ± 0.07 ($n=54$)]. Neonatal handling, i.e., daily handling and injecting, significantly increased pain sensitivity in both sexes ($P<0.05$) (comparisons between NC and SS groups at day 25), whereas no significant effect of neonatal naltrindole treatment on the baseline nociceptive latencies was found at either age. Postnatal day 25, SS males: 0.98 ± 0.07 ($n=21$), SS females: 1.13 ± 0.10 ($n=19$), NTI males: 1.10 ± 0.10 ($n=20$), NTI females: 1.31 ± 0.12 ($n=17$). Postnatal day 20, SS males: 0.87 ± 0.06 ($n=19$), SS females: 1.01 ± 0.09 ($n=19$), NTI males: 0.74 ± 0.05 ($n=22$), NTI females: 1.01 ± 0.09 ($n=14$).

Effects of naltrindole treatment on antinociceptive responses to clonidine

The antinociceptive effect of clonidine was evaluated by the statistical analysis of the latency quotients, as previously indicated. The groups included in the analysis were chronically treated with saline or naltrindole and subsequently studied for the acute effect of clonidine. The analysis of the data obtained from 20-day-old males showed that

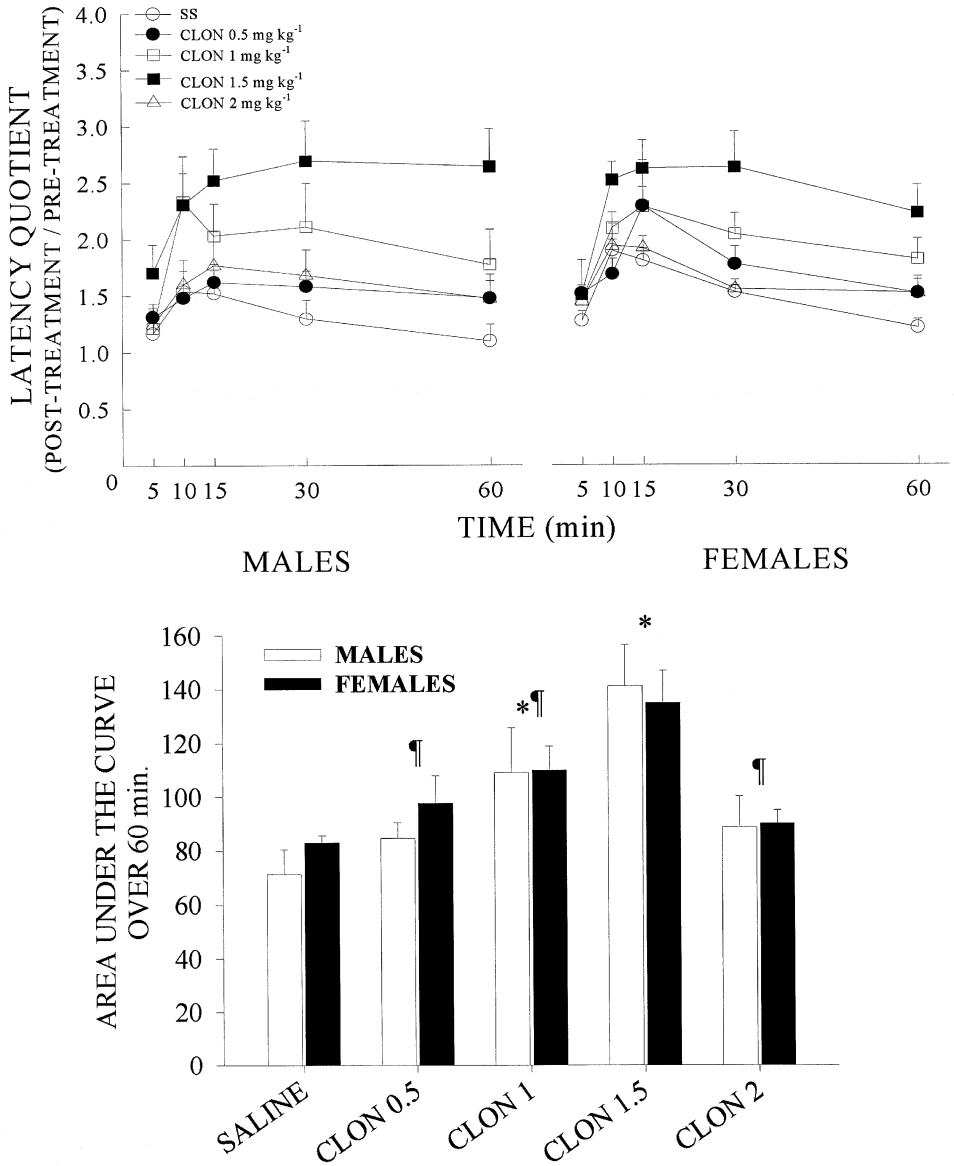

Figure 1 Time course of antinociceptive responses to clonidine (CLON) (0.5, 1, 1.5 and 2 mg kg^{-1}) and area under the corresponding curves over 60 min in 25-day-old naive control male and female rats, in the tail immersion test. Values represent mean$\pm$s.e.mean of 10–11 animals. Response latencies were measured 15 min before acute administration of saline (SS) or CLON (pre-treatment latencies) and 5, 10, 15, 30 and 60 min after treatment (post-treatment latencies). Student-Newman-Keuls: *$P<0.05$ vs the groups injected acutely with SS; ¶$P<0.05$ vs the groups injected acutely with CLON 1.5 mg kg^{-1}.

clonidine only produced a significant antinociceptive effect in the animals treated neonatally with naltrindole ($P<0.05$), whereas no significant effect was found in the control animals injected neonatally with saline. According to this result, the animals treated neonatally with naltrindole and acutely with clonidine showed significantly higher latency quotients than the corresponding control groups injected neonatally with saline and acutely with clonidine ($P<0.05$). In contrast, in 20-day-old females clonidine only induced antinociception in the control saline injected rats ($P<0.05$), and no significant effect was found in the animals treated with naltrindole (Figure 2a). The analysis of the data obtained at postnatal day 25 showed that, as at 20 days of age, clonidine induced a significant antinociceptive effect in the males treated neonatally with naltrindole and in the control females injected neonatally with saline ($P<0.05$), whereas no significant effect was found in either the control males injected neonatally with saline or in the females treated with naltrindole during the preweanling period (Figure 2b).

Effects of naltrindole treatment on plasma adrenaline levels

The ANOVA performed on data obtained from adrenaline determinations showed that, at day 20, neonatal naltrindole treatment significantly increased plasma adrenaline concentration in males ($P<0.05$). In this sex, clonidine significantly reduced adrenaline levels in the group treated neonatally with naltrindole ($P<0.05$) but did not modify the levels of adrenaline in the control males injected neonatally with saline. In 20-day-old females, clonidine significantly decreased adrenaline levels in the control group injected neonatally with saline and in the group treated neonatally with naltrindole

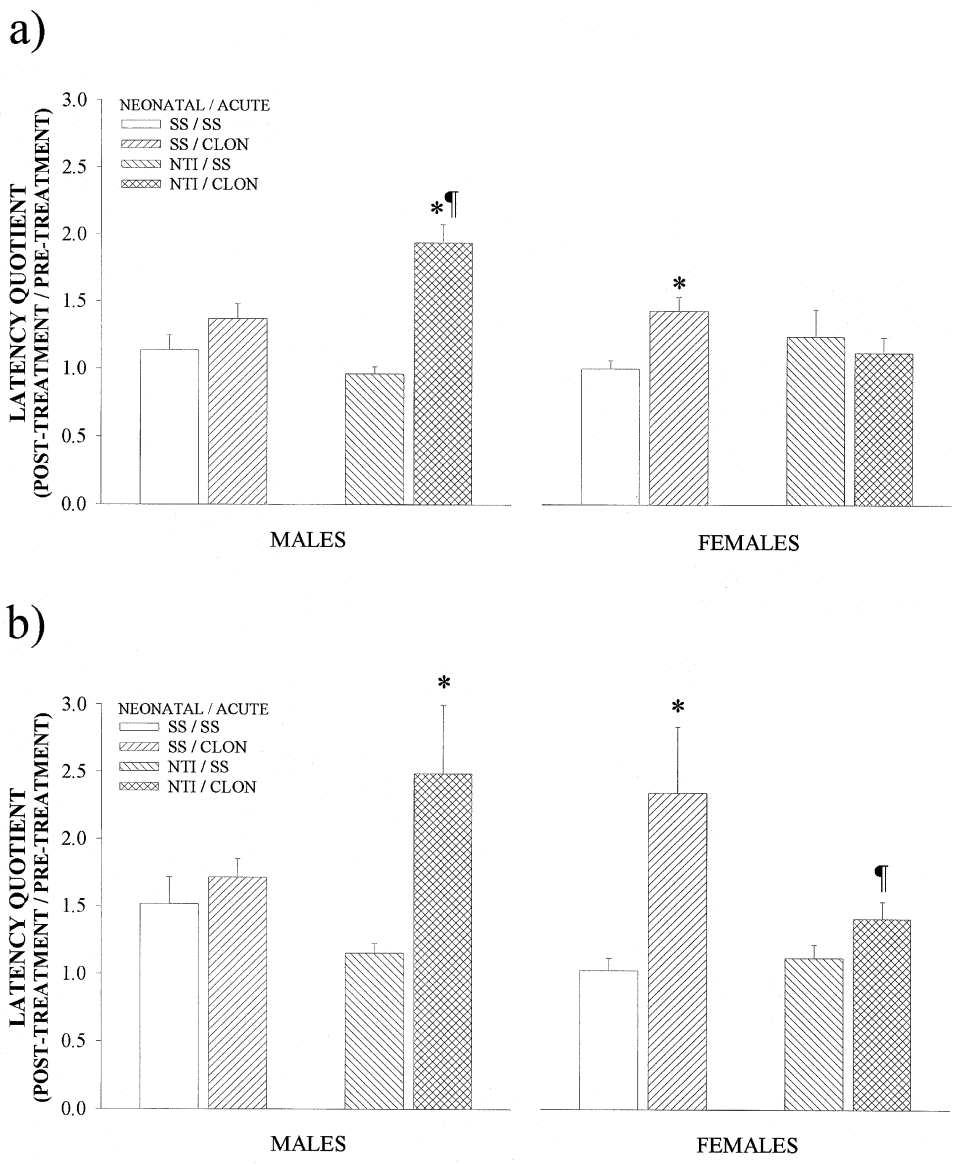

Figure 2 Effects of neonatal naltrindole treatment on antinociceptive responses to clonidine (CLON) in the tail immersion test in 20-day-old (a) and 25-day-old (b) rats. The animals were treated neonatally with saline (SS) or naltrindole (NTI) (1 mg kg^{-1}) (from birth to day 19) and subsequently studied for the acute effects of CLON (1.5 mg kg^{-1}). Histograms represent the mean $\pm$ s.e.mean of 7–12 animals. Response latencies were measured 15 min before acute administration of SS or CLON (pre-treatment latencies) and 30 min after treatment (post-treatment latencies). Student-Newman-Keuls: *$P<0.05$ vs the group of the same sex treated with the same neonatal treatment and injected acutely with SS; ¶$P<0.05$ vs the group of the same sex treated neonatally with SS and injected acutely with CLON.

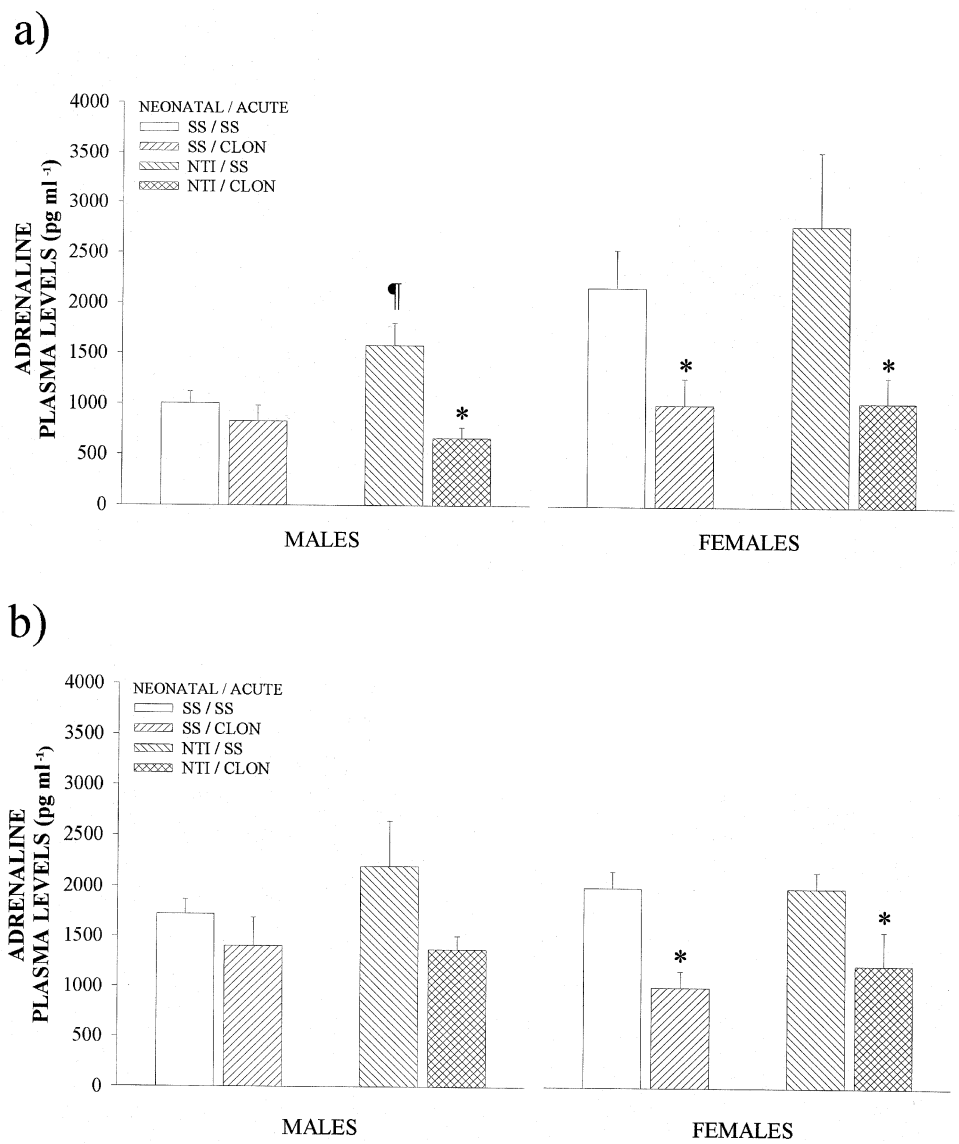

Figure 3 Effects of neonatal naltrindole treatment on plasma adrenaline levels (pg ml^{-1}) in 20-day-old (a) and 25-day-old (b) rats. The animals were treated neonatally with saline (SS) or naltrindole (NTI) (1 mg kg^{-1}) (from birth to day 19) and subsequently studied for the acute effects of clonidine (CLON) (1.5 mg kg^{-1}). Histograms represent the mean ± s.e.mean of 5–7 animals. Blood samples were obtained 15 min after the completion of the tail immersion test, i.e., 45 min after acute administration of SS or CLON. Adrenaline levels were measured by high-performance liquid chromatography (HPLC). Student-Newman-Keuls: $*P<0.05$ vs the group of the same sex treated with the same neonatal treatment and injected acutely with SS; $¶P<0.05$ vs the group of the same sex treated neonatally and acutely with SS.

($P<0.05$) (Figure 3a). In 25-day-old males, the ANOVA only rendered a residually significant effect of the acute treatment ($P=0.06$). The data shows that, in spite of the lack of statistical significance, the trends were the same as at 20 days of age, i.e., the effect of clonidine was more marked in the animals treated neonatally with naltrindole. In 25-day-old females clonidine significantly reduced the levels of adrenaline in both neonatal treatment groups ($P<0.05$) (Figure 3b).

Discussion

The present results indicate that naive control females showed greater sensitivity to the noxious thermal stimulation compared to males. This data is in accord with previous studies which support the existence of sex differences in response to noxious stimuli with females displaying greater sensitivity (Bodnar *et al*., 1988; Fillingim & Maixner, 1995) and extends these findings to neonatal rats. Postnatal handling stress increased sensitivity to thermal pain in both males and females. These results are in good agreement with previous data indicating that chronic stress usually depletes the central opioid pathways in rats (Madden *et al*., 1977), which might account for the reduced nociceptive thresholds of handled animals. According to recent studies (Antelo *et al*., 1998; Fernández *et al*., 1999c), the results show that the functional blockade of the δ-receptors during the preweanling period by the administration of a chronic treatment with naltrindole did not modify the basal nociceptive responses of neonatal rats, suggesting that the δ-receptor does not play a tonic role in setting pain sensitivity. The data accords with the results of knockout studies where deletion of the δ-receptor gene does not alter nociceptive threshold (Zhu *et al*., 1997).

The analysis of dose response curves showed a biphasic effect of clonidine, with its antinociceptive effect increasing in a dose-dependent manner between 0.5 and 1.5 mg kg^{-1} and

declining at the highest dose tested (2 mg kg^{-1}). A biphasic behaviour of clonidine has been previously reported in both, adult *in vivo* observations (Mastrianni *et al*., 1989) and isolated neonatal rat spinal cord (Kendig *et al*., 1991). The decline in antinociception at high doses of clonidine has been proposed to reflect exacerbation of pain by activation of peripheral α_1-receptors (Mastrianni *et al*., 1989).

The present results show that, in the control groups injected neonatally with saline, clonidine induced a significant antinociceptive effect in females but not in males. The absence of clonidine antinociception in the control males injected with saline during the preweanling period might be attributable to the handling stress i.e., weighing and injecting the animals during the preweanling period. We have recently found that the same handling procedure as the one used in this work reduced morphine antinociception in neonatal male rats and increased pain sensitivity in both sexes (Fernández *et al*., 1999a), which accords with the data obtained in the present work. Acute handling stressful procedures during early postnatal stages induce the release of pituitary β-endorphin in rat pups (Iny *et al*., 1987) and it has been proposed that chronic handling procedures might produce a repeated and exaggerated release of endogenous β-endorphin which might account for the reduced responsiveness to exogenous β-endorphin found in handled animals (D'Amore *et al*., 1993). The opioid component of clonidine-mediated antinociception may be produced by a β-endorphin-like opioid which is released in response to the interaction of clonidine with central α_2-adrenergic receptors (Mastrianni *et al*., 1989). From this point of view, the reduced response to clonidine in handled male rats might be attributable to a phenomenon of tolerance to endogenous β-endorphin. In addition, repeated testing might have contributed to unmask or potentiate clonidine antinociception in naive control unhandled animals. In fact, it has been shown that stress induced by immobilization during tail immersion test unmasked the antinociceptive properties of diverse α_2-adrenoceptor agonists, including clonidine (De Kock & Meert, 1997). Stressful handling procedures did not abolish clonidine antinociception in neonatal female rats. There is evidence of the existence of sex differences in nociception and antinociceptive responses to stress in adult rodents (Bodnar *et al*., 1988; Aloisi *et al*., 1994; Fillingim & Maixner, 1995). The present results point out the important influence of gender upon the effects of this kind of procedures on antinociceptive responses to clonidine during the early postnatal period. The present results also show that chronic naltrindole administration unmasked the antinociceptive effect of clonidine in male rats. This data appears to be in good agreement with previous reports indicating that chronic immobilization stress reduced the hypoactivity elicited by clonidine in adults male rats (Cancela *et al*., 1988; Keller *et al*., 1998), whereas a combined treatment with naltrexone (Cancela *et al*., 1988) or naloxone (Keller *et al*., 1998) and stress, completely antagonized the attenuating effect induced by chronic stress on clonidine induced sedation and hypoactivity. The attenuation of α_2-adrenoceptor reactivity and the corresponding subsensitivity to clonidine induced by chronic stressful events have been interpreted as adaptative changes of α_2-adrenoceptors that may be under a modulatory influence of an opioid mechanism (Cancela *et al*., 1988; Keller *et al*., 1998). In contrast to the results obtained in males, the chronic administration of the δ-opioid antagonist prevented the antinociceptive effect of clonidine in females. This lack of clonidine antinociception in naltrindole treated females parallels the decreased stress-induced-antinociception induced by the same naltrindole treatment in postweanling females which appeared to be a consequence of an impaired δ-receptor function (Antelo *et al*., 1998). If clonidine antinociception includes a δ-opioid component, a decreased δ-receptor function might account for the reduced antinociceptive effect of clonidine. There is scarce information about the interaction between noradrenergic and opioid analgesic systems in neonates, and the data obtained from adult animals have not yet revealed a clear picture about the exact nature of that functional interaction or the role played by specific opioid receptor subtypes (Ossipov *et al*., 1989; Omote *et al*., 1991; Roerig *et al*., 1992; Sullivan *et al*., 1992). The present results clearly indicate that δ-receptor exerts a differential modulation of clonidine antinociception in male and female neonatal rats. Previous studies indicate the existence of sex differences in opioid receptor density (Hammer, 1990; Limonta *et al*., 1991) and interactions between different opioid receptor types in the mediation of antinociception (Antelo *et al*., 1998; Fernández *et al*., 1999c) and a growing literature documents the important influence of sex on pain sensitivity and pain modulation (Bodnar *et al*., 1988; Mogil & Belknap, 1997; Fillingim & Maixner, 1995). The antinociceptive effect of clonidine and the influence of the naltrindole treatment on clonidine antinociception appeared to be more marked at day 25 than at 20 days of age. The δ-receptor shows a delayed development compared to the other opioid receptor subtypes, which extends to the fourth postnatal week, and the weaning process appears to be critical for the developmental expression of this opioid receptor (Kitchen & Pinker, 1990; Kitchen *et al*., 1995). It is likely that the more marked effects found in postweanling animals are due to the maturation of the δ-receptor after weaning.

Interestingly, the data obtained in our work with respect to adrenaline plasma levels in male rats parallels the results obtained in the antinociceptive test. Thus, the neonatal naltrindole treatment also allowed the sympatholytic effect of clonidine in males, an effect that was absent in the males injected neonatally with saline. In contrast, clonidine produced a sympatholytic effect in 20 and 25-day-old control females injected neonatally with saline, and this effect was not modified by the neonatal naltrindole treatment. The data suggests that the δ-receptor is involved in the modulation of clonidine induced sympatholytic effect in male but not female rats. The lack of effect of handling in females with respect to the sympatholytic response of clonidine parallels the absence of effect of handling on clonidine-mediated antinociception in this sex. These results suggest a greater vulnerability of neonatal male rats, compared to females, to the influence of handling on clonidine mediated responses, which is in accord with our recent studies on adrenocortical reactivity to stress (Fernández *et al*., 1999b) and morphine antinociception (Fernández *et al*., 1999a). No significant effect of the neonatal naltrindole treatment on the body weights of pre- or postweanling animals was found (data not shown). Therefore, the effect of the naltrindole treatment on clonidine induced antinociception and plasma adrenaline levels does not appear to be an indirect consequence of any effect of the δ-antagonist on the nutritional status or somatic growth of the animals.

In conclusion, the functional blockade of neonatal δ-receptor by a chronic naltrindole treatment did not modify the sympatholytic effect of clonidine but prevented clonidine induced antinociception in females. Conversely, in males naltrindole treatment allowed the appearance of clonidine antinociception and the sympatholytic effect of clonidine. The data provides the first evidence about a different involvement of the δ-opioid receptor in the modulation of antinociceptive and sympatholytic responses to clonidine in neonatal male and female rats.

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