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Mild postnatal manipulation reduces proenkephalin mRNA in the striatum in developing mice and increases morphine conditioned place preference in adulthood

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Abstract

Stressful events during certain neonatal periods may increase the vulnerability of an individual to develop psychopathology and/or drug dependence later in life. Therefore, in the present study, we assessed activity levels, emotionality, sensitivity to the effects of morphine, as well as expression of proenkephalin and prodynorphin in several brain regions in 35 and 90-day-old male mice, subjected to postnatal manipulation consisting in brief exposures to clean bedding (CB). In comparison with controls, CB mice showed reduced emotionality expressed as percentage of time in open arms of the elevated plus maze both at 35 days of life and in adulthood. Increased nociceptive threshold was also present in both time points measured. Conversely, higher locomotor activity was recorded in 35 days of life but not in adulthood. Analysis of film autoradiograms revealed no changes in prodynorphin mRNA level, but statistically significant decrease in the level of proenkephalin mRNA in striatum in young CB mice in comparison with young controls; no difference was observed between adult CB and control animals. CB adult mice also showed hypersensitivity to the rewarding effect of morphine in comparison with controls in the place preference test. In conclusion, our results revealed that in the critical period of development the effects of manipulation were evident, not only on behavioral responses but also on the neurochemical markers considered in the present research. Postnatal manipulation could induce changes in the dynamic neuronal processes occurring during development with long-term behavioral effects.
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1. Introduction

In the last years, considerable attention has been given to the influence of early-life events on brain functioning and behavior in adult life. It has been suggested that stressful events during certain neonatal periods may increase the vulnerability of an individual to develop psychopathology and/or drug dependence later in life. Behavioral adaptations to environmental pressures occurred during critical neonatal periods.

The organism is protected against severe stressors, at least in part, by the immaturity of the Hypothalamo–Pituitary–Adrenal (HPA) axis functioning that characterizes the first weeks of pups' life, the so called "stress hyporesponsive period" (Levine, 1970 in the rat; D'Amato et al., 1992; Schmidt et al., 2003, in the mouse). However, even if pups do not show the HPA activation typical of adult animals, the effects of early stress can be detected till adulthood (Levine, 2001).

Several studies conducted by the Meaney's group support the idea that the amount of maternal care behavior received by the pups during the first postnatal week is correlated with later functional and anatomical parameters in the Central Nervous System (CNS) and in the HPA axis functioning (Liu et al., 1997; Caldji et al., 1998; Meaney 2001). The temporary absence of mother/nest odor (clean bedding procedure: CB) has been used as short maternal

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separation procedure in our previous studies. We have called it "clean bedding" to differentiate from the classical handling procedure. In fact, in our first study (D'Amato and Cabib, 1987) we demonstrated that it was not the handling procedure *per se* (manipulation deriving from removal from the home cage, transport to, isolation in a new box and return to the home cage) but the absence of mother/nest odor during the separation that was responsible for the long-term effects on emotionality (Cabib et al., 1993). Furthermore, we have evidenced that such experimental procedure increased the nociceptive threshold and morphine sensitivity in mice (D'Amato et al., 1999). The latter result suggests that changes in the opioid peptide system functioning may have occurred in this model of mother separation.

Similar effects of postnatal manipulations on nociception have been reported by Pieretti et al. (1991) in mice, and by others in rats (Smythe et al., 1994; Sternberg and Ridgway, 2003). In addition, Ploj and collaborators demonstrated that postnatal handling affected the opioid system in rats showing that, 7 weeks after the postnatal stress, the level of opioid peptides dynorphin B and Met-enkephalin-Arg^6-Phe^7 was changed in some brain structures like substantia nigra, hypothalamus and in the neurointermediate pituitary lobe (Ploj et al., 2003). More recently Vazquez et al. (2005) demonstrated that maternal deprivation (3 h of daily isolation from Day 1 to 14 of postnatal life) increased vulnerability to morphine dependence and disturb the enkephalinergic system in adult rats. By contrast, very short (1 min) early handling during the same developmental phase induced vulnerability to morphine consumption, but had no effects on the enkephalinergic system (Vazquez et al., 2006).

The aim of the present study was to evaluate changes occurring in consequence of our mild postnatal stress procedure in several behaviors, generally accepted to be related to the endogenous opioid system modulation (Drolet et al., 2001). We measured locomotor activity levels, emotionality in the elevated plus maze and nociception in 35- and 90-day-old mice, subjected to postnatal clean bedding exposure. In addition, behavioral response to morphine in a place preference apparatus was evaluated in adult mice. Together with the behavioral analysis, a neurochemical evaluation of the biosynthetic activity of the opioid system has been performed, analyzing the levels of mRNAs coding for proenkephalin and prodynorphin in different brain structures.

2. Methods

2.1. Housing and subjects

Outbred albino NMRI mice served as subjects of this study. Nulliparous females aged 6 weeks and weighing 22–25 g (Harlan, Italy) were housed in social groups on a 12-h light–dark cycle 7 AM–7 PM with food and water available ad lib in a colony room at constant temperature 21 ± 1 °C. Seven days after their arrival, the females were housed in pairs in 33 × 15 × 13 cm Plexiglas cages. A male was introduced and left for 15 days. Pregnant females were then removed to individual cages, the floor of which was covered with sawdust. No other nesting material was supplied. Twice a day, around parturition time, cages were inspected for live pups. The day of birth was considered as Day 0. On Day 1 litters were culled to eight pups (four males and four females). Litters with less than eight pups were discarded from the sample. Data presented in this paper are based on a total of 60 lactating females with their offspring. During the first two postpartum weeks, home-cage bedding was changed only on the 10th day of the pup's life: mother and offspring were temporarily removed from their cage, soiled bedding was replaced with clean material, except for a very small amount that was scattered in the clean environment. Animals were then reintroduced into their cages.

These experiments were conducted in accordance with the Italian national laws and regulations concerning the use of animal for research, and NIH Guidelines on Animal Care.

2.2. Postnatal manipulation

Litters were randomly assigned to one of the two experimental conditions on Day 1: postnatal manipulation (Clean Bedding: CB, N=29) and unhandled (Control: C, N=31). Once a day, from Day 1 to 13, each whole CB litter was transferred to a new cage, the floor of which was covered with clean bedding and left for 15 min. During the entire 15 min of the procedure, the cage was placed on a hot plate set at a temperature of 35 °C, to prevent cooling of pups. During this procedure, the mother was left in its home cage. This experimental manipulation was randomly performed between 11 AM and 5 PM. Control litters were left undisturbed until weaning (except for cage cleaning as described).

2.3. Experimental groups

Mice were individually marked with diluted picric acid and weaned on Day 28 of life. Males were housed in groups of four with age-matched mice of the same experimental group, taken from different litters.

Behavioral tests and biochemical measurements were conducted in different 35- and 90-day-old groups of mice, with the exception of the biochemical measures that were conducted on the animals also tested for nociception, in order to confirm previous results (D'Amato et al., 1999).

2.4. Behavioral test

2.4.1. Elevated plus maze

The plus maze was an opaque Plexiglas apparatus with two open arms (27 × 5 cm) and two enclosed arms (27 × 5 × 15 cm). The arms extended from a central platform (5 × 5 cm) and the apparatus was mounted 38.5 cm above the floor. The subjects were individually tested in a single 5-min session. All mice were placed in the center facing an open arm to initiate the test session. Each test was videotaped and later, an experimenter recorded the behavioral items using a keyboard connected to an Apple computer. The number of entries (four paws in) and the time spent inside each type or arm were recorded. Three measures were considered for the analysis: total number of entries, percentage of entries into the open arms and percentage of time spent into the open arms.

A total of 12 CB and 12 C mice, belonging to different litters, were tested both at 35 and at 90 days of age. A two-way ANOVA (postnatal treatment and age) was used for statistical analysis.

2.4.2. Locomotor activity

Locomotor activity was measured in toggle-floor boxes, each one divided into two 20×10-cm compartments, connected by a 3×3-cm opening. The apparatus was computer controlled (Pavone et al., 1992). For each mouse the number of crossings from one compartment to the other was automatically recorded by means of a microswitch connected to the tilting floor of the box. Mice were subjected to a 60-min activity test.

A total of 12 CB and 12 C mice, belonging to different litters, were tested both at 35 and at 90 days of age. Data were analyzed by a three-way ANOVA for repeated measures (postnatal treatment, age, and 20-min blocks activity). Tukey HSD was used for post hoc analysis.

2.4.3. Tail-flick test

Animals tested for in situ hybridization were previously tested for nociception to confirm the already reported changes in pain sensitivity (D'Amato et al., 1999). Pain threshold was evaluated by the tail-flick test. Immediately before the test, mice were gently blocked in a dark plastic tube and radiant heat focused on the animal's tail. If no tail flick occurred within 10 s, the test was terminated to prevent tail injury and the score for the animal was 10. The test was repeated three times with an interval of 15 min between successive measurements. Due to the repetitive nature of the test procedure adopted, each animal was assigned a tube and was always tested in its own tube in order to avoid confusing effects due to odor of conspecifics.

A total of 8 CB and 8 C mice, derived from different litters, were tested at 35 days of life, whereas 7 CB and 8 C mice were examined when at 90 days old. A two-way ANOVA was used for statistical analysis (postnatal treatment and age).

2.4.4. Place preference conditioning

The CPP experiment was performed in boxes formed by two gray Plexiglas chambers (15×15×20 cm) and a central alley (15×5×20 cm). Two sliding doors (4×4 cm) connected the alley to the chambers. In each chamber, two triangular parallelepipeds (5×5×20 cm) made of black Plexiglas and arranged to form different patterns (always covering the same surface of the chamber) were used as conditioned stimuli. This apparatus is characterized by a neutral set of cues devoid of rewarding or aversive properties which eliminates baseline chamber preferences (Cabib et al., 1996).

On Day 1 (pretest), mice were free to explore the entire apparatus for 10 min. During the following 8 days (conditioning phase), mice were confined daily for 30 min alternatively in one of the two chambers. For each animal of both experimental CB and C groups, during the conditioning phase, one of the patterns was consistently paired with saline injection and the other one with morphine injection. Pairings were balanced so that for half of each experimental group morphine was paired with one of the patterns and half of them with the other one. Testing was conducted on Day 10 similarly to the pretest procedure. Test sessions were videotaped and, later on, the time spent within each chamber of the apparatus was quantified by an experimenter unaware of the treatment conditions, using the Observer program (version 3.0, System for Macintosh, Noldus, Wageningen 1997). Difference in time spent by the animal in the drug-paired versus the saline-paired compartment was used as the CPP score for each animal.

A total of 34 CB and 32 C adult mice were tested for morphine (0, 10 and 20 mg/kg i.p.) place preference conditioning. Only adult animals were tested as the long procedure required for the conditioning procedure would have required an anticipation of the weaning time. A two-way ANOVA was used for statistical analysis (postnatal treatment and morphine) followed by the Tukey HSD post hoc. Attention has been paid in experimental group composition to avoid a litter effect.

2.5. Biochemical measurements

We examined the effect of CB procedure, in young (35 days old) and adult (90 days old) mice, on the level of proenkephalin and prodynorphin mRNAs in different brain structures rich in endogenous opioids: striatum, amygdala and nucleus accumbens. Animals tested for nociception were used for biochemical analysis (young: CB=8, C=8; adult: CB=7, C=8).

2.5.1. Measurement of proenkephalin and prodynorphin mRNA level by in situ hybridization

Mice from the C and CB groups were decapitated at 35 and 90 days of life, 5 to 7 h after the nociceptive test. Their brains were immediately removed and frozen on dry ice. They were then cut into 12-μm thick coronal sections on a cryostat microtome (Leica Microsystems, Nussloch, Germany), the sections were thaw-mounted on gelatin–chrom–alum-coated slides and processed for in situ hybridization according to the method of Young et al. (1986). Briefly, the sections were fixed with 4% paraformaldehyde, washed with PBS and acetylated by incubation with 0.25% acetic anhydrite (in 0.1 M triethanolamine and 0.9% sodium chloride). The sections were then dehydrated using increasing concentrations of ethanol (70–100%), treated with chloroform for 5 min, and rehydrated with decreasing concentrations of ethanol.

The sections were hybridized for approximately 15 h at 37 °C with proenkephalin and prodynorphin oligonucleotide probes complementary to residues 388–435 of the rat proenkephalin mRNA (Yoshikawa et al., 1984) and 862–909 of the rat prodynorphin gene (Civelli et al., 1985), respectively. The probes were labeled with ^{35}S-dATP by the 3′-tailing reaction using terminal transferase (Roche Diagnostics, Mannheim, Germany). The specificity of the proenkephalin and prodynorphin probes had been extensively documented elsewhere, and was confirmed by a Northern blot analysis and competition experiments (Young et al., 1986). The patterns of hybridization signal found in brain sections fully agreed with the well-known distribution of proenkephalin and prodynorphin mRNA; moreover, they provided support for the specificity of the probes under the present experimental conditions.

After hybridization, the slices were washed twice for 20 min with 1×SSC/50% formamide at 40 °C, and twice for 50 min with 1×SSC at room temperature. Then, the slices were dried

Table 1
Emotionality (plus maze test), nociception (tail-flick test) and locomotor activity of 35-and 90-day-old postnatally manipulated (CB) and control (C) male mice

PLUS MAZE	CB	C	CB	C	F_{treat} (1/29)	F_{age} (1/29)
	35-day-old mice		90-day-old mice			
% Time open	50.27 (4.16)	39.55 (2.64)	60.18 (3.60)	45.67 (4.84)	10.81**	4.36*
% Entries open	46.17 (3.77)	42.63 (2.20)	49.79 (3.10)	44.68 (4.32)	1.64	0.7
Total entries	19.25 (1.72)	19.68 (0.78)	21.75 (2.23)	23.25 (2.14)	0.29	2.9

TAIL-FLICK	CB	C	CB	C	F_{treat} (1/25)	F_{age} (1/25)
	35-day-old mice		90-day-old mice			
Latency (s)	5.13 (0.32)	3.65 (0.24)	4.36 (0.25)	3.31 (0.41)	15.49***	2.95

LOCOMOTOR ACTIVITY	CB	C	CB	C	F_{treat} (1/44)	F_{age} (1/44)	F_{time} (2/88)
	35-day-old mice		90-day-old mice				
0–20 min	73.08§ (3.83)	51.50 (2.88)	61.33 (5.15)	60.42 (6.81)	2.25	1.39	2.31
20–40 min	40.42 (5.79)	28.42 (3.63)	30.67 (4.61)	28.83 (4.35)			
40–60 min	31.33 (5.82)	28.67 (3.12)	20.42 (4.41)	22.42 (3.13)			

Data are means (S.E.) *$p<0.05$; **$p<0.01$; ***$p<0.001$. §versus same age C (Tukey HSD test $p<0.01$).

and exposed to Hyperfilm βmax films (Amersham) at room temperature for 14 days (proenkephalin), or for 21 days (prodynorphin). The resulting autoradiograms were analyzed using the MCID Elite system (Imaging Research, St. Catharines, Ontario, Canada). Mean optical density was measured in selected brain regions, which included: dorsal striatum, nucleus accumbens, and the central nucleus of amygdala. For each brain structure, data were collected from at least four sections per animal, bilaterally and the individual mean value was used in the statistics (two-way ANOVA: postnatal treatment × age). Background signal was measured over the corpus callosum and was subtracted from the hybridization signal in the regions of interest. Tukey HSD was used for post hoc analysis.

3. Results

3.1. Behavioral tests

Table 1 reports data for behavioral tests conducted in young and adult mice (35 and 90 days old). The effects of postnatal manipulation on emotionality (plus maze test) and nociception (tail-flick test) were already evident in juvenile animals and persisted till adult age. CB mice spent a greater percentage of time in the open arms, compared to C mice, both at 35 and 90 days of age. Moreover, the significant age effect on this parameter suggests that emotionality decreased with age. No significant interaction between postnatal treatment and age emerged. Juvenile as well as adult CB mice showed higher nociceptive thresholds than controls and no age and interaction effects were observed. As for locomotion, no significant main effects (treatment, age and time interval) resulted from the ANOVA. The significant postnatal treatment × time interval effect ($F(2/88)=3.61$, $p<0.05$), followed by the appropriate post hoc analysis Tukey HSD), indicated that juvenile CB animals showed higher locomotion during the first time interval of the activity test, in comparison with their age mate controls ($p<0.01$).

Place preference conditioning with morphine is shown in Fig. 1. The ANOVA indicated significant main effects of morphine ($F(2/60)=3.50$, $p<0.05$) and of postnatal CB treatment ($F(1/60)=4.51$, $p<0.05$). The highest dose of morphine (20 mg/kg) induced an increase in time spent in the paired compartment independently from the postnatal manipulation ($p<0.05$). However, CB mice spent more time than controls in the paired compartment also with the lower dose of morphine ($p<0.05$), suggesting a hypersensitivity to the rewarding effects of the drug in animals manipulated during development.

3.2. Proenkephalin and prodynorphin mRNA level

Analysis of film autoradiograms of brain slices revealed changes in proenkephalin mRNA level in striatum in young animals exposed to postnatal manipulation during development. No other significant changes in proenkephalin and prodynorphin mRNA level were observed (Fig. 2).

3.2.1. Proenkephalin in the striatum

The two-way ANOVA indicated no significant treatment effect ($F(1/47)=2.16$, $p=0.14$) but a significant age ($F(1/47)=6.18$, $p<0.05$) and age × treatment effect ($F(1/47)=4.28$, $p<0.05$). Statistically significant decrease in the level of proenkephalin mRNA was observed in young CB mice in comparison with young controls ($p<0.01$, Figs. 2 and 3), whereas no difference was observed between control and CB adult animals. In the control group, the level of proenkephalin mRNA in striatum was higher in young in comparison with adult animals ($p<0.05$, Fig. 2). Conversely, no difference was observed between young and adult CB mice.

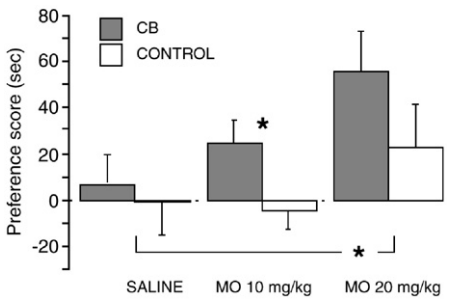

Fig. 1. Effect of i.p. administration of morphine (10 and 20 mg/kg) or saline on place preference in adult CB (postnatally manipulated, $n=34$) and control mice ($n=32$). The results are expressed as the differences in time spent on the test day in the paired versus the unpaired compartment. *$p<0.05$ (Tukey HSD test).

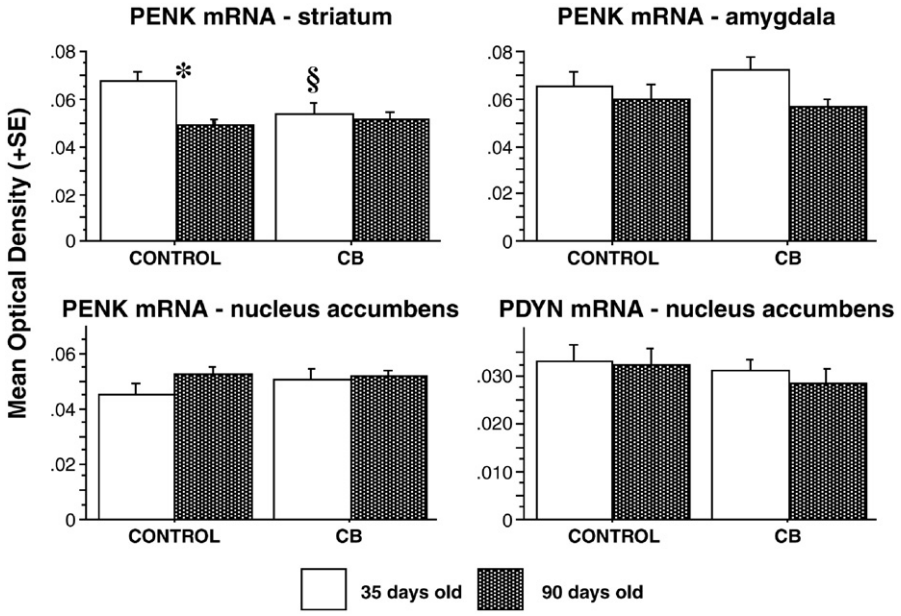

Fig. 2. Effect of postnatal manipulation (CB) on proenkephalin (PENK) mRNA level in striatum, amygdala and nucleus accumbens, and prodynorphin (PDYN) mRNA level in nucleus accumbens in 35 (CB: $n=8$; CONTROL: $n=8$) and 90 (CB: $n=7$; CONTROL: $n=8$)-day-old mice. The results are expressed as mean optical density ± SEM. Analysis of film autoradiograms of brain slices reveals higher proenkephalin mRNA levels in striatum of young control animals in comparison with that observed in young CB and adult control mice. No other structures show significant changes in the level of these prohormone mRNA. $^{*}p<0.01$ between young and adult CONTROL (Tukey HSD test). $^{\S}p<0.05$ versus same age CONTROL (Tukey HSD test).

3.2.2. Proenkephalin in the amygdala

Within the amygdala, high levels of proenkephalin mRNA were detected only in the central amygdaloid nucleus (Fig. 2). Neither treatment nor treatment × age interaction effects on proenkephalin mRNA amount in the central nucleus reached the statistical significance ($F(1/39)=0.18$, ns; $F(1/39)=1.15$, ns, respectively). However, proenkephalin mRNA levels were higher in young in comparison with adult animals, this difference approaching statistical significance ($F(1/39)=3.90$, $p=0.055$).

3.2.3. Proenkephalin in the nucleus accumbens

No significant differences in proenkephalin mRNA levels were observed in nucleus accumbens between control and postnatally manipulated mice, independently of the age of animals (postnatal treatment: $F(1/36)=0.43$, ns; age: $F(1/36)=1.44$, ns; treatment × age ($F(1/36)=0.73$, ns).

3.2.4. Prodynorphin in the nucleus accumbens

The prodynorphin gene is expressed at particularly high levels in the nucleus accumbens; however, no significant differences in prodynorphin mRNA levels were observed in this brain region between C and CB mice independently of the age of animals (postnatal treatment: $F(1/28)=0.92$, ns; age: $F(1/28)=0.25$, ns; treatment × age ($F(1/28)=0.10$, ns).

4. Discussion

There has been a great interest in the recent years in investigating the long-term effects of postnatal manipulation in

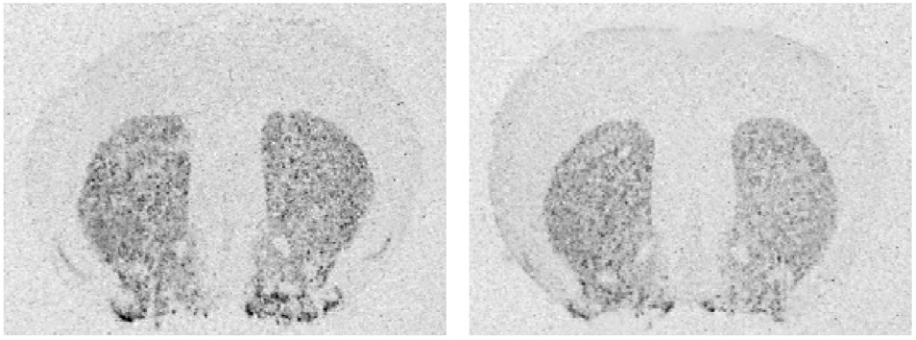

Fig. 3. Representative in situ hybridization autoradiograms showing proenkephalin mRNA signal in the striatum of 35-day-old control (left) and CB (right) mice. Note the lower optical density of the hybridization signal in the CB mouse brain.

the CNS and in hormonal functioning in animal models of early postnatal stress. Almost all studies have been conducted in rats, with few studies analyzing these effects in mice. In this paper we further clarify and extend our previous reports on postnatal manipulation and long-term behavioral effects (Cabib et al., 1993; D'Amato et al., 1999). Animals exposed to clean bedding 15 min daily, during the first weeks of life show higher nociceptive threshold in the tail-flick test and reduced emotionality in the plus maze test in adulthood. Data reported here demonstrate that these differences are already present at 35 days of life (3 weeks after the end of the CB procedure) and are permanent, suggesting stable modifications in the neural systems involved. Conversely, the increase in locomotor activity observed in CB young animals during the first part of the test, when the animals are more reactive to the novelty of the situation, disappears in adulthood. Moreover, CB adult mice seem to be more vulnerable to the rewarding effects of morphine as they showed place preference at a lower dosage, in comparison with their controls. Although the place preference apparatus used in this study was not able to significantly demonstrate a preference for the morphine-paired compartment in control groups, it provides evidence for the effect of postnatal manipulation. Unfortunately we could not test 35-day-old animals as the long conditioning procedure would have implied changes in weaning time. The hypersensitivity reported here is in contrast with the hyposensitivity we reported for the analgesic effect of morphine in the tail-flick test (D'Amato et al., 1999), which can be explained by the involvement of different neural pathways.

The reactions to stressful stimuli are generally expected to involve opioid systems activity (Drolet). This is the first study trying to explore if postnatal treatment has long-term effects on mRNA level for prohormones of the opioid peptides, proenkephalin and prodynorphin in mice. The biosynthesis rate of opioid peptide prohormones reflects the functional activity of the opioid systems. In this study we observed a significant decrease of proenkephalin in the striatum in young CB mice compared to young controls, as shown by the lower optical density of the hybridization signal in the CB mouse brain (see Fig. 3). Striatum proenkephalin mRNA levels in adulthood did not change between CB and control mice.

No differences were observed in proenkephalin mRNA levels in the amygdala between CB and control animals independently from the age, while within the manipulated mice, a tendency towards an age effect was observed with young CB showing higher level of proenkephalin than adult CB animals. Ploj and colleagues have investigated in rats the effects of different maternal separation procedures (15 min versus 360 min daily from day 1 to 21 of rat pups' life) on opioid peptides levels (Ploj et al., 1999, 2001, 2003). They found relatively little effects of postnatal treatment in adult rats and in few structures: 15 min daily of maternal separation resulted in higher Met-enkephalin-Arg-Phe and dynorphin B levels only in the hypothalamus. The effects of the more severe stress procedure were even less pronounced. More recently Vazquez et al. (2005) found hypersensitivity to the reinforcing properties of morphine and a decrease in preproenkephalin mRNA expression in the nucleus accumbens and the caudate-putamen of postnatally manipulated rats (3 h daily of isolation during the first 2 weeks of life). The presence of PENK effects only in the striatum and their disappearance in adult mice can be due to our procedure – mild postnatal manipulation – that could determine less stable changes than the invasive maternal deprivation procedure.

During the first postnatal weeks, i.e. in the period when the mouse pups are subjected to the CB procedure, the striatal enkephalinergic system is still immature. This immaturity involves not only the pattern and levels of the proenkephalin gene expression in the striatum, which reach the adult characteristics around the postnatal Day 15 in the rat (Song and Harlan, 1993), but also the lack of functional influences exerted by enkephalins in adult brain via the delta opioid receptors (De Vries et al., 1990). In contrast, the regulatory mechanisms within the nigrostriatal pathway mediated by the mu and kappa receptors are fully functional in 17-day-old rat embryos (De Vries et al., 1990; Winzer-Serhan et al., 2003). Thus, the development of the proenkephalin gene and the delta receptor expression, as well as their functional coupling, may be particularly vulnerable to modification by external influences in this early postnatal period. The lower level of the proenkephalin mRNA in the striatum of 35-day-old CB mice, in comparison to control animals, might reflect a disturbance in development of the striatal enkephalinergic system by the manipulation procedure, an effect that disappears at postnatal Day 90. Actually, the development of this system may be accelerated in CB mice, since the natural course of the proenkephalin gene expression seems to involve a drop in mRNA levels between Days P35 and P90, and CB mice have already reached the adult proenkephalin mRNA level by P35. In line with this reasoning, Ploj and coworkers came to the conclusion that the delta, but not mu or kappa, opioid receptor signaling is altered in postnatally-handled animals (Ploj et al., 2003; Ploj and Nylander, 2003). Interestingly no changes were observed in μ-opioid receptor density (Ploj et al., 2003) that is known to be important for mediation of natural rewards, and also for relation between mother and pups expressed as infant attachment behaviors (recently confirmed in the mu receptor knock-out mice by Moles et al., 2004). In line with this result, also Vazquez et al. (2005) did not find any effect in μ-opioid receptor density in the striatum and mesencephalon in maternally deprived rats.

Although the difference in the striatal proenkephalin gene expression between the CB and control mice does not persist till adulthood, lower level of proenkephalin mRNA, and particularly its peptide products, in the CB animals, may have produced permanent changes in other neurotransmitter systems by influencing their development. Indeed, numerous studies demonstrated that endogenous proenkephalin-derived peptides, especially Met-enkephalin, are potent inhibitors of neuronal growth and differentiation. During embryonic life and early postnatal period, they seem to inhibit DNA synthesis, nerve cell divisions, growth of dendrites and dendritic spine formation, and affect expression of the nerve growth factor (NGF) and its receptors (Zagon and McLaughlin, 1986; 1987; Hauser et al., 1987; Hammer et al., 1989; Perez-Navarro et al., 1993). Thus, even transient alterations in the inhibitory growth factor-like

effects of proenkephalin-derived peptides may influence the development of other neuronal populations. If this happens during periods critical for organization of neuronal connections, the consequences may be long-lasting, leading to changes in different behavioral characteristics of animals. In agreement with this suggestion, we observed long-lasting anxiety and nociceptive threshold changes in postnatally manipulated mice.

The results concerning locomotor activity in young animals could be, at least in part, a result of adaptive alterations of the developing striatal proenkephalin system. Proenkephalin is expressed in the striatum predominantly in the subpopulation of GABAergic projection neurons, which innervate the globus pallidus (Albin et al., 1989; Gerfen, 1992; Maneuf et al., 1994). Proenkephalin-derived peptides regulate neurotransmission in the striatopallidal pathway. On one hand, they are released from the striatal neuron terminals in the globus pallidus, where they reduce GABA release by acting at presynaptic opioid autoreceptors (Maneuf et al., 1994). On the other hand, proenkephalin-derived peptides released within the striatum seem to inhibit responsivity of striatopallidal neurons to activating stimuli (Steiner and Gerfen, 1999). Thus, reduced levels of striatal proenkephalin mRNA in CB 35-day-old mice suggest a deficiency in striatopallidal transmission and therefore increased motor activity, which indeed proved to be the case.

The other important consequence of lower level of proenkephalin mRNA in the striatum in 35-day-old CB mice is the possible influence of this change on development of dopamine (DA) system that may be reflected by changes in behavior of adult animals. In our previous study we have already demonstrated an increased behavioral response to a DA agonist (apomorphine) at the end of the postnatal treatment, confirming that this stressor modified DA functioning in 2-week-old mice (D'Amato and Cabib, 1987). This altered sensitivity of DA receptors was maintained until weaning occurred (postnatal Day 28) in the absence of further manipulation; 35- and 90-day-old CB mice did not differ any more from their controls in their behavioral response to apomorphine (D'Amato and Cabib, 1990, Cabib et al., 1993). However, the persistence of an altered DA functioning emerged if CB mice were exposed to chronic restraint stress in adult age.

The handling-produced decrease in anxiety reported in this and several other studies may be due, at least in part, to elevated levels of GABA A/benzodiazepine receptor subunits in fear-related brain regions, including the central and basolateral amygdaloid nuclei, locus coeruleus and the nucleus of the solitary tract (Caldji et al., 2000; Hsu et al., 2003). Similar results were obtained exposing animals during their first weeks of life to other postnatal stressing manipulation. The level of fearfulness in the adulthood has been reported to be correlated to the amount of care that pups receive from their mother (Caldji et al., 1998; 2003). The increase amount of care that handled pups receive from their mother was also correlated with the expression of some GABA A receptor subunits in the amygdala and locus coeruleus (Caldji et al., 1998; 2003). Moreover changes in second messengers or other intracellular mechanisms that affect the functional response have to be taken in account. In fact, it was evidenced that the reactivity of endogenous opioid system (measured as the extracellular level of Met-enkephalin in microdialysis study) could be reduced in stress-induced model of anhedonia (Bertrand et al., 1997).

From these data an important point emerges: during development the effects of handling are evident not only on behavioral responses, but also on the neurochemical markers considered in the present research. By contrast, no differences are detectable in the in situ hybridization studies when proenkephalin and prodynorphin were measured in adult mice. We suggested that mild postnatal stress induces a rearrangement in the dynamic neuronal processes occurring during development resulting in a disequilibrium in these processes, which is progressively balanced during the life.

In conclusion, it is important to stress that 1) this is the first study which investigates changes in precursor opioid levels occurring in postnatally manipulated mice, 2) this study is conducted in mice (majority of results following postnatal handling concerned rats) opening the possibility to use the knock-out mice for a deeper investigation of mechanisms involved and, 3) we have looked at the developmental changes and their stability by examining animals at different ages and we suggest that early changes in biosynthetic activity of proenkephalin system might be responsible for the long-lasting changes in response to environmental pressure in postnatally manipulated mice.

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