

**REPEATED MILD POSTNATAL STRESS IN MICE:
PERSISTENCE OF EFFECTS IN ADULT ANIMALS**

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Abstract

In the present paper, we used a new model of mild stress (maternal deprivation and subcutaneous administration of solvent) to study stress evolution from a pharmacological point of view considering whether daily mild stress to neonatal mice induce a persistence effects in adult animals such as weight, endocrine and immunological changes. The results of our experiments indicate that mild stress plus solvent injection administered from the birth to the 21st postnatal day causes a significative increase of body weight, an alteration of the endocrine (increase of ACTH) and immune system (increase of cytokines) thus indicating that repeated mild postnatal stress in mice may induce significative effects in adult animals.

Introduction

Stress is any external or internal factor capable of upsetting the homeostatic balance of an organism.

Sources of stress can be sensorial, like pain, changes in temperature, and noise, but also psychological, like fear, frustration, and anxiety (Ananad 2000, Liu et al., Lopez et al., 1999).

An organism's response to stress implies the activation of a series of defence mechanisms and, through this activation, the release of different types of hormones and neuromediators (d'Amore et al., 1993, Pieretti et al., 1991). Of the different control systems studied, that of the hypothalamic-pituitary-adrenal axis (HPA) hormones has been found to be the one most involved. Corticotropin-releasing hormone (CRH), released from the hypothalamus, induces the pituitary gland to produce proopiomelanocortin, a polypeptide precursor of a series of peptides including, on the one hand corticotropin (ACTH), which in turn stimulates the secretion, from the adrenal cortex, of steroid hormones, and on the other hand, the endogenous opioid peptide, β -endorphin. ACTH and β -endorphin subsequently act on specific receptors (Akil and Morano, 1995; Reisine and Pasternak, 1995).

In rodents, there is evidence that most receptors for these systems – the HPA hormone and endogenous opioid systems – originate and develop during the first weeks of life. The fact that even slight variations in the internal or surrounding environment in this period can alter the development of these important neuroendocrine structures explains why, in rodents, the perinatal period is referred to as a “critical period of development” (Lauder and Krebs, 1986, Zagon and McLaughlin, 1993).

Even mild stress occurring during this critical period can result in alterations that persist in the adult animal. The nature of these alterations is many-fold and involves responses of different kinds: behavioural, metabolic, pharmacological, immune and endocrine (De Giorgis et al., 1996, Ruda et al., 2000, Vallee et al., 1999).

The existence of a common precursor for ACTH and β -endorphin points to possible cross-talk between the steroid hormone system and the system of endogenous opioid peptides, both of which are involved both in the stress response and in other nervous functions: brain excitability, pain, immune response, etc. (Ortolani et al., 1990).

Rodents that are exposed daily, from birth until weaning, to brief periods of stimulation, or handling, show, as adult animals, a reduced responsiveness of the HPA axis to stress and an increased number of glucocorticoid receptors in the hippocampus; conversely, more severe stress, such as prolonged maternal deprivation, physical trauma or the administration of an endotoxin, increase the responsiveness of the HPA axis to stress (Meaney et al., 1988; Plotsky et al., 1993). Neonatally handled rats have been found to show, as adults, increased concentrations of opioid peptides such as dynorphin-A and -B in certain brain regions (hypothalamus, pituitary and hippocampus) (Ploj et al., 1999).

In the light of literature data regarding the effects of mild stress in the neonatal period, a series of studies were performed in our laboratory in an attempt to clarify the pathogenesis of these alterations. We used a new model of mild stress that, in addition to maternal deprivation, also involves the subcutaneous administration of solvent, the aim being to introduce a mild pain stimulus as a new variable, and to study stress evolution from a pharmacological point of view. Indeed, several groups have, in the past, published experimental models in which newborn animals were treated with an active principle or with vehicle (this group served as the control). However, subsequent studies indicated that the stress generated by solvent injection, when summed with the “classic” isolation stress, induced by itself long-term alterations that in part reflected those induced by handling/isolation alone, and thus that for pharmacological studies another control group (subjected neither to injection nor to handling) would have to be introduced.

The experimental model we adopted involved not only maternal deprivation for 10 minutes each day (during which the pups were placed in a cage with clean sawdust and weighed), but also the

subcutaneous administration of distilled water, 1ml/kg. This treatment was carried out for 21 days, after which the rats were weaned and divided into groups (3 per cage).

While many researchers have tended to focus primarily on HPA axis alterations induced by postnatal stress, other alterations provoked by this kind of treatment – raised pain threshold as measured using the tail-flick, hot-plate and formalin tests of nociception – indicate that at least the opioid system, too, could play an important role in the pathogenesis of alterations that persist into adulthood (Pieretti et al., 1991; D'Amato et al., 1999).

Therefore, applying pharmacodynamic methods, we evaluated the importance both of the endocrine component of the HPA axis (through the administration not of distilled water, but of an antisense oligonucleotide in order to block any endogenous ACTH-induced effects) (Spampinato et al., 1994) and of its opioid component (through the administration of naloxone in order to block the effects induced by the release of endogenous opioids).

Therefore, we have considered whether daily mild stress to neonatal mice induce a persistence effects in adult animals such as weight, endocrine and immune system.

Materials and Methods

Animals and breeding condition

The experiments were performed during the winter period, in order to take circannual variation of the opioid receptors at their maximal sensitivity (Buckett, 1980; De Ceballos and De Felipe, 1985). Laboratory-born CD-1 male mice were obtained from multiparous mothers, which were transferred on the 14th day of gestation to our laboratory by a commercial breeder (Charles River Italia, 22050 Calco, Italy). Upon their arrival, pregnant females were placed in individual nesting cages and, starting on the 19th day of pregnancy, examined twice daily (at 08.00 and 16.00 h) for the presence of pups. Within approximately 12 h from the detection of the pups in the cage, litters of homogeneous size (13 ± 1 subjects) were put together, and randomly culled to five male plus two female pups with homogeneous weight, so that all pups were randomly cross-fostered, and had approximately the same weight at the beginning of the experiment. The housing room temperature was maintained at 21°C ($\pm 1^\circ\text{C}$), with 50% ($\pm 5\%$) relative humidity and with a 12 h light-dark cycle. Animals were given a standard diet (Mucedola S.r.l. 20019 Settimo Milanese, Italy) and tap water. Animal care and use followed the rules of the Council of European Communities. The experimental procedures were approved by the Bioethical Committee of the Istituto Superiore di Sanita'. Animals were regularly examined by a veterinary surgeon and no health problems were ever observed.

Neonatal treatment and postweaning protocol

Procedures are summarized in Table 1

After fostering, the experimental litters were randomly assigned to one of the following groups:

- 1) Control group (C): the pups were left undisturbed with their mother in the home cage, except for cage cleaning twice a week
- 2) Water-treated group (W): the pups of each litter were removed daily from the home cage and grouped in a container with fresh bedding material; each pup was weighed and subcutaneously (sc) injected with distilled water, 1 ml/kg using a microsyringe with a 27-gauge needle; after 10 min the pups were returned to the home cage with the mother.
- 3) Naloxone-treated group (NA): the pups of each litter were manipulated as those in the W group except for injection of naloxone hydrochloride, instead of water, 1 mg/kg dose and 1 ml/kg volume. This dose was the lowest effective dose of naloxone in newborn male mice in our experimental conditions (Pieretti et al, 1991).

4) Antisense-treated group (AS-0.1): the pups were manipulated as those in the NA group except for injection of oligodeoxynucleotide antisense, instead of naloxone, 0.1 nmol/g dose and 1 ml/kg volume. The antisense prevents biosynthesis of ACTH (Spampinato et al, 1994).

The protocol described (i.e., complete litters receiving the same treatment) was preferred to the alternative (i.e., each litter contributing to all treatments), which is more usual in developmental psychobiological studies, to avoid manipulation of Control mice. From weaning (21 days of age) the individuals in each treatment group were rehoused in post-weaning cages of the five male animals. From 91 to 95 days of age, on each consecutive day one animal from each group was weighed, and was sacrificed through rapid decapitation. Blood was collected and spleen was excised. The brain was rapidly extracted, and pituitary was isolated and put at -80°C.

Table 1: TIMING OF EXPERIMENTAL PROCEDURE	
Day -7	Pregnant mothers are transferred to our Institute
Day 1	Day of birth. Pups are culled to 5 male animals, and cross-fostered
Day 2	Treatment starts for groups W, NA while animals of the C groups are left undisturbed, except for cage cleaning twice a week
Day 21	Treatment ends. Homogeneous litters of animals are randomly set in groups of three per cage, weighed and weaned. Then, all animals are left undisturbed
Day 35	Animals are weighed and tested at the tail-flick apparatus
Day 70	Animals are weighed
Day 110	Animals are weighed, and sacrificed through rapid decapitation. Blood, spleen, pituitary and the brain are collected

Determination of pituitary ACTH

The ACTH has been quantified with radioimmunological methods previously described (Spampinato et al, 1994) in the pituitary.

Mice were killed by rapid decapitation 3 h after the last treatment, the anterior pituitary was homogenized in 0.1 M acetic acid (90° C) and processed as described (Spampinato and Goldstein, 1983). Immunoreactive (ir)-ACTH levels were measured by radio-immunoassay (RIA) of the pituitary acetic acid extracts by double-antibody precipitation using human ACTH antiserum and human ACTH standard obtained from Dr. AF Parlow (Harbor-UCLA Medical Center) and

[¹²⁵I]iodotyrosyl2-ACTH-(1-39) (Amersham Biosciences, Milano). The antibody recognizes mouse ACTH-(1-39), and there is no cross-reactivity with other peptides derived from the proopiomelanocortin precursor. At the time of the decapitation, trunk blood was collected from each mouse into edetate calcium disodium tubes placed on ice, spun in a refrigerated centrifuge, and plasma was collected and stored at -80°C

Sample Collection

Blood was collected using tubes pre-filled with 12 IU sodium heparin. Plasma was separated by centrifugation (300 r.p.m. for 10 min) and stored at -80°C until analysis. For murine spleen cell preparation, spleens were aseptically removed spleen cell from sacrificed mice, put in cold phosphate-buffered saline (PBS) and gently homogenized with a loose Teflon pestle. After allowing the tissue debris to settle for 3-5 min at 4°C, the cells were collected and washed three times with cold PBS. Red blood cells were removed by hypotonic lysis. Splenocytes were adjusted at a final concentration of 1x10⁷ ml in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum, L-glutamine and penicillin/streptomycin (Pacifici et al., 1992).

Cytokine assay

A first set of splenocytes was adjusted to a final concentration of 106 cells ml-1 in culture medium and stimulated at 37°C in % CO₂ incubator 36h with PHA (1 µg/ml) for the induction of interleukin-1β (IL-1β), IL-2, IL-4, IL-10, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α). A second group of splenocytes was cultured simultaneously in triplicate without stimulation to determine the spontaneous production of cytokines. After incubation, the culture supernatants were collected and stored at -80°C for the quantitation of cytokines. For quantitative measurement of murine IL-1β, IL-2, IL-4, IL-10, IFN-γ and TNF-α in supernatants of splenocytes cultures, specific solid-phase ELISA assays, employing the multiple antibody sandwich principle, were used (Genzyme Inc., Cambridge, MA, USA). The tests were performed according to the supplier's instruction. Samples from control and treated mice and interleukin standards were assayed simultaneously, in triplicate, in 96-well microtitre plate pre-coated with monoclonal anti-ILs. The standard curves (assay sensitivity between brackets) were as follows IL-1β, 15 to 960 (assay sensitivity=10) pg/ml; IL-2 15 to 960 (assay sensitivity=15) pg/ml; IL-4 20 to 540 (assay sensitivity=5) pg/ml, IL-10, 30 to 1080 (assay sensitivity=15) pg/ml; IFN-γ, 20 to 1620 (assay sensitivity=5) pg/ml; and TNF-α, 35 to 2240 (assay sensitivity=15) pg/ml. Tissue culture supernatants were diluted in wash buffered and dilution factor was considered for the calculation of IL amount. The specificity of monoclonal anti-mouse-IL was tested in our laboratory. Negative reactions with other murine cytokines such as IL-1α, IL-6 and lymphocyte inhibitory factor were

found. Assay performance was tested using three concentrations of cytokines in culture medium throughout the procedure. Mean intra- and inter-assay coefficients of variations were always less than 5% (Pacifici et al., 1997).

Tail Flick Test

At 35 days of age, animals were tested to determine nociceptive threshold in the tail-flick test (Capasso et al., 1991). The radiant heat was focused on a spot 1.5 cm from the tip of the tail and the light beam intensity was adjusted to obtain a reaction time about 1-2s in the intact control animal. A cut-off time of 10s was chose (Table 2).

Table 2. Pain sensitivity at 35 days of age

TAIL FLICK THRESHOLD				
Group	C	W	NA	
Reaction time (s)	1.27	2.14	1.10	
sem	0.14	0.20	0.12	

Reaction time in s, mean and standard error, on the 35th day of life. C is for control group; W is for water injection, NA is for naloxone, 1 mg/kg/sc, for 21 d. At the analysis of variance $F(3,56) = 11.6$; $P < 0.0001$; C and NA groups are consistently different from W ($P < 0.01$).

Drugs

All drugs used in the experimental session were purchased from Sigma Chemical Co (St. Louis, USA) with the exception of the antisense compound (AS-ACTH) which was purchased from EUROBIO Laboratoires (Les Ulis Cedex, France) under instruction of one of us (S.S.). The 21 bases sequence, as phosphorothioate, MW 547.3 was: TCT GGC TCT TCT CGG AGG TCA Naloxone and AS-ACTH were used for subcutaneous injections in distilled water; preparations were stored at -30°C in small glass vials and used once after ice melting every day.

Statistical Analysis

All data were analysed by ANOVA followed by Bonferroni-Dunn post hoc comparison. Behavioral data were gathered from the 15 animals of each experimental group; immunological and chemical analyses were performed on five randomly selected sample per group.

Results and Discussion

Postnatal handling stress affects the animal's metabolism, particularly its lipid and glucose metabolism. Indeed, handled rodents observed several weeks after the end of treatment showed increases in bodyweight, in epididymal fat and in fat cell volume, as well as other metabolic alterations such as raised plasma leptin, insulin, glucose, cholesterol and triglyceride levels, while no significant changes were observed in their food consumption, or locomotor activity (d'Amore et al., 2000).

The data we obtained indicate not only that chronic naloxone treatment results in restoration of normal nociceptive test latencies (Table 2), but also that it can prevent some (not all) metabolic alterations in stressed animals. Indeed, animals treated with the opioid antagonist did not show a significant increase in bodyweight, while their leptin levels remained higher than those recorded in controls (Fig. 1).

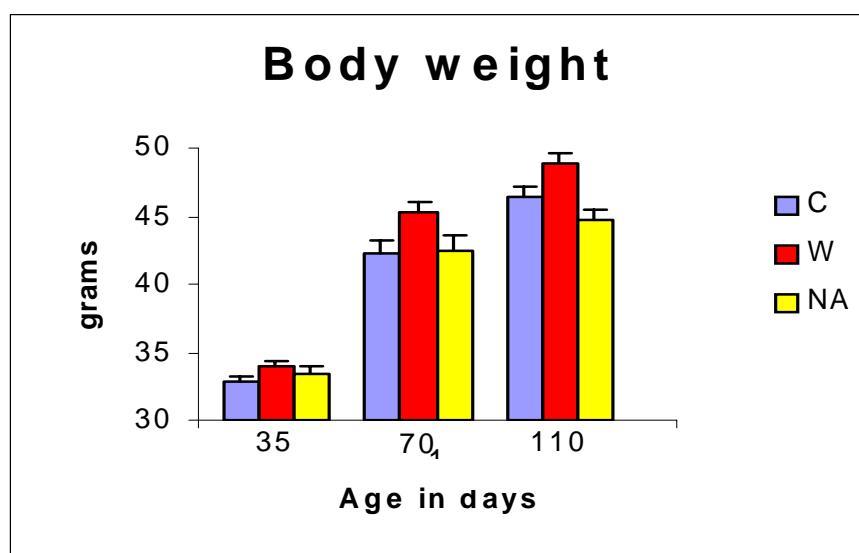


Figure 1. Weight in grams (mean and SE) recorded 35, 70 and 110 days after birth. Postnatal stress induces, as from the 70th day of life, a significant increase in the body weight growth curve, which persists through to the end of the observation period (110 days). Studies have shown that a weight increase of this kind is due above all to increased deposition of fat, due to increased fat cell volume (8). C is for Control animals; W is for water-injected (stressed) animals; NA is for (-)naloxone-treated animals

Some of the metabolic alterations observed can be attributed to the hormonal imbalances that mild neonatal stress induces. We therefore concentrated on verifying the influence of chronic antisense anti-ACTH and naloxone treatments on pituitary and plasma *ir*-ACTH levels. We found that mild but chronic postnatal stress induced a considerable increase (around 400%) in pituitary *ir*-ACTH 30 minutes after the last treatment (Fig. 2);

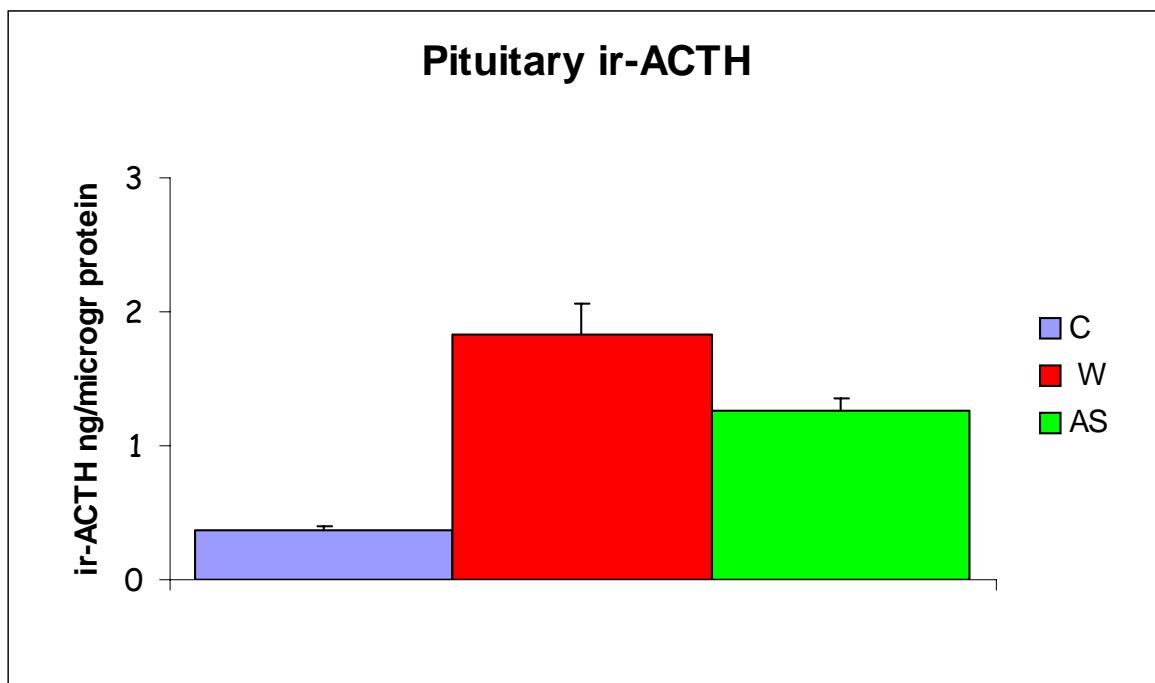


Figure 2 – Pituitary *ir*-ACTH levels 30 minutes after the last treatment, at the age of 21 days. This significant increase in *ir*-ACTH in stressed group (W) versus controls (C) is still present at 3 months of age. Antisense (AS) and naloxone treatment (not shown in the figure) produce a significant diminution in the ACTH levels. C is for Control animals; W is for water-injected (stressed) AS is for antisense-ACTH-treated animals

furthermore, this increase was still present both 24 hours and 3 months later. In plasma too, our findings indicate an alteration (significant increase) in concentrations of ACTH. In this case, too, on the third month of life chronic treatment with naloxone or antisense anti-ACTH (AS-ACTH) brought the ACTH concentrations back into line with those recorded in the control group. The efficacy of the action of both AS-ACTH and the opioid antagonist naloxone on the ACTH levels confirms the co-involvement of the two systems, i.e., of HPA hormones and of the opioid system, in the organism's response to repeated mild stress. These systems are also involved in controlling part of the immune response, in particular the cell-mediated and humoral responses. We therefore attempted to verify whether repeated mild stress can also influence the immune system.

Mice subjected to our model of postnatal stress showed, in adulthood (110 days old), selective cytokine synthesis alterations, in particular increased synthesis of pro-inflammatory, Th1-type, cytokines (IL-2, INF- γ and TNF- α), and a decrease in the levels of certain anti-inflammatory, Th2-type, cytokines (IL-4 and IL-10), while the IL-1 β cytokine showed no changes (Fig. 3).

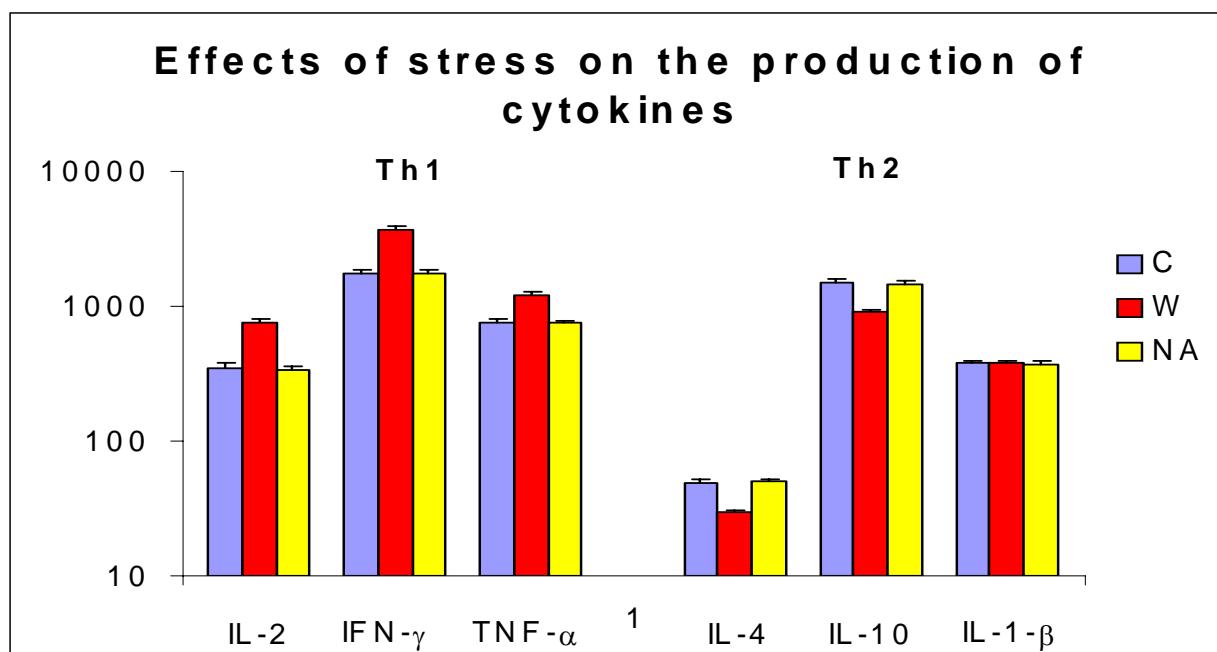


Figure 3 – Effects of mild postnatal stress on the production of cytokines by murine spleonocytes tested 110 days after birth. The values express pg/mL on a logarithmic scale. The increase in IL-2, IFN- γ and TNF- α levels is in the order of 200%. C is for Controls; W is for stressed; NA is for naloxone-treated animals

We also observed increased proliferation of T splenocytes, as well as significantly increased natural killer activity. These immune system changes can be added to the metabolic, behavioural and neurochemical alterations provoked by repeated mild postnatal stress in the rat that persist into adulthood. In this experimental model too, naloxone is able to bring the immune responses of the stressed animals back into line with the levels recorded in the control groups. Taken together, all these chronic perinatal stress-induced alterations suggest that there is a need for new models for investigating the possible involvement of HPA and opioid systems in the pathogenesis of certain metabolic and autoimmune disorders in man.

The present paper indicate that a mild stressor chronically applied to neonatal mice induces a selective and long term modulation of the immune response, consisting in enhancement of cell-mediated immunity of Th-1 type (pro-inflammatory) cytokines release, accompanied by behavioral and metabolic response: this effect were prevented by naloxone. Therefore, we can suggest the also the involvement of endogenous opioid systems in the pathogenesis of several long-term alterations induced by neonatal mild stressful procedures.

The relationship between the HPA and opioid systems on immune function was already previosly reported (Ortolani et al., 1990, Pieretti et al., 1994). In this respects, we cannot excluded several possibilities: 1) long lasting change of production/bioavailability of endogenous opioid substances, as suggested by Ploj et al., 1999, 2001 and 2) enhanced bioavailability of endogenous active substances (for example, the ones produced by changed enkephalin-degrading peptidase activity), as suggested by Irazusta et al., (1999).

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