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Naloxone prevents cell-mediated immune alterations in adult mice following repeated mild stress in the neonatal period

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1 Mild stress plus mild pain (solvent injection) applied daily to neonatal mice induces hormonal, behavioural and metabolic changes perduring in the adult life.

2 We investigated whether daily mild stress to neonatal mice induces also long-term defined changes of immune response, and whether immune changes are prevented through repeated administration of the opioid antagonist naloxone.

3 Mild stress plus solvent injection administered from birth to the 21st postnatal day causes not only behavioural and metabolic changes, but also long-term (up to 110 days of life) splenocytes modifications, consisting in: increased release of the Th-1 type cytokines interleukin-2 (IL-2) (from an average of 346 to 788 pg ml^{-1}), interferon-γ (from 1770 to 3942) and tumour necrosis factor-α (from 760 to 1241); decreased release of the Th-2 type cytokines IL-4 (from 49.1 to 28.4) and IL-10 (from 1508 to 877). Moreover, enhanced natural killer-cell activity; enhanced proliferative splenocytes properties in resting conditions and following phytohemoagglutinin and concanavalin-A stimulation are observed. Immunological, behavioural and metabolic changes are prevented by the opioid antagonist (−)naloxone (1 mg kg^{-1} per day s.c., administered instead of solvent) but not by the biologically inactive enantiomorph (+)naloxone.

4 In conclusion, endogenous opioid systems sensitive to naloxone are involved in long-lasting enhancement of the Th-1 type cytokines and cell-mediated immunological response caused by repeated mild stress administered postnatally.

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Abbreviations: ACTH, adrenocorticotropic hormone; C, Control group; Con-A, concanavalin-A, c.p.m., counts per minute; CRH, Corticotropin releasing hormone; HPA, hypothalamic-pituitary-adrenal; IFN-γ, interferon-γ; IL-1α, interleukin-1α; IL-1β, interleukin-1β; IL-10, interleukin-10; IL-2, interleukin-2; IL-4, interleukin-4; IL-6, interleukin-6; *ir*, Immuno-reactive; IU, International Units; LU_{33}, lytic units 10^{-7} effector cells; NA, Naloxone-treated group

Introduction

Repeated neonatal mild stress, such as manipulation of pups and/or a brief isolation from the dam performed daily for a few weeks, induces hormonal, behavioural and metabolic alterations which may perdure up to adult and senescent ages. Several endocrine systems contribute to these phenomena and, among them, the hypothalamic-pituitary-adrenal (HPA) axis is the most extensively studied. This is viewed as a complex system with different control pathways and crosstalks with neurotransmitters implicated in the regulation of several vegetative functions (Anand, 2000; Liu *et al*., 1997; Lopez *et al*., 1999).

The role of various neuromediators has been investigated in studies performed in newborn animals. However, a crucial problem at all such studies is that drugs are usually administered to pups through injection, and this introduces a new stressful condition, i.e., the repeated injection discomfort. When this model is used, two series of stressful events apparently interact: the isolation/manipulation stress plus the mild pain stress caused by drug administration. Some research groups (including ours) have published experimental designs in which pups were either treated with the active compound under investigation, or with the vehicle alone as control (d'Amore *et al*., 1990). Further investigation showed that the summation of saline injection to the 'classical' mild stress model (d'Amore *et al*., 1991) induced long-term changes which were in part analogous to those produced by the manipulation/isolation stress only (Meaney *et al*., 1991). Therefore, we have suggested that the stress induced by vehicle injection added to the 'classical' stress could be a suitable model to study neurotransmitter mechanisms triggered by postnatal repeated mild stress accompanied by mild pain (injection) stimuli. This model allowed the possibility of using drugs within the experimental process. We have observed that, besides HPA hormones, endogenous opioid systems play an important role on behavioural effects observed in adult rodents exposed to this procedure during the neonatal period (d'Amore *et al*., 1993;

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Pieretti *et al*., 1991). HPA hormones and endogenous opioid systems trigger several mechanisms involved in stress and immune responses, acting through central and peripheral pathways (Akil & Morano, 1995; Reisine & Pasternak, 1995), both in development and adulthood.

The responses to environmental injury and stress in adult organisms are of limited duration. On the other hand, the early neonatal period is a time of great plasticity during which specific ontogenetic processes are vulnerable to perturbation by external insults (Lauder & Krebs, 1986; Zagon & McLaughlin, 1993). Stressful manipulations and environmental injury, applied during this period, may induce delay or change of specific functions, which may last for a long time or forever (De Giorgis *et al*., 1996; Ruda *et al*., 2000; Vallee *et al*., 1999). In this context, data from our and other laboratories reported that, in neonatal mice, chronic exposure to gentle handling plus a mild nociceptive stress (i.e., removing pups daily from the home cage and grouping them in a container with fresh bedding material; then weighing and subcutaneously injecting them with water, and after a few minutes returning the pups to the home cage with the mother) produced behavioural and metabolic alterations perduring up to the adult life. Particularly, increase in pain threshold sensitivity – or pain threshold decrease, depending on experimental procedures – increased sensitivity to opioids, increased body weight increment curve, and alteration of glucose and lipids metabolism were observed. Most of these phenomena were prevented or reversed by the opioid antagonist naloxone (d'Amore *et al*., 1996; 2000; Pieretti *et al*., 1991), or by the δ-opioid antagonist naltrindole (Fernàndez *et al*., 1999). Reduction in pain sensitivity was reported also by other investigators who studied mice behaviour using the isolation/manipulation model (D'Amato *et al*., 1999), thus enforcing the possibility of data-transferring from the one model to the other.

Physical and psychological stressors can enhance or suppress the immune response, depending on the duration and severity (Lopez *et al*., 1999). In this study we investigated whether a mild stressful procedure plus a nociceptive stimulus applied to newborn mice, in addition to inducing long lasting behavioural/metabolic alteration, may also similarly affect the immune response with particular regard to the Th-1 – Th-2 types pattern balance, and whether immunological alterations can be due, at least in part to, a modulation of the endogenous opioid system.

We have previously found that mice stressed in newborn age showed higher body weight increment curve (d'Amore *et al*., 1996). Plasma leptin levels are directly related to body weight (Dallongeville *et al*., 1998), and in recent experiments Lord *et al*. (1998) showed that leptin was able to stimulate the release of pro-inflammatory cytokines *in vitro*, therefore we also evaluated plasma leptin levels in postnatally stressed mice.

Methods

Animals and breeding condition

The experiments were performed during the winter, because due to their circannual variations, the opioid receptor sensitivity is maximal in this period (Buckett, 1981; De Ceballos & 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 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 (± 1°C), with 50% (± 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 Instituto Superiore di Sanità. 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. Before treatments began, the experimental litters were randomly assigned to one of the following groups (each group consisting of 3 litters): (a) Control group (C): the pups were left undisturbed with their mother in the home cage, except for cage cleaning twice a week; (b) 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 (s.c.) injected with distilled water, 1 ml kg^{-1} using a microsyringe with a 27-gauge needle; after 10 min, the pups were returned to the home cage with the mother; (c) Naloxone-treated group (NA): the pups of each litter were manipulated as those in the W group except for

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, NA+, while animals of the C groups are left undisturbed, except for cage cleaning twice a week
Day 21	Treatment ends. Homogenous 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

injection of (−)naloxone hydrochloride, 1 mg kg^{-1} dose and 1 ml kg^{-1} volume, instead of water. This dose was the lowest effective dose of naloxone in newborn male mice in our experimental conditions (d'Amore *et al*., 1996 and unpublished data); (d) (+)naloxone-treated group (NA+): the pups were manipulated as those in the NA group except for injection of (+)naloxone, 1 mg kg^{-1} dose and 1 ml kg^{-1} volume, instead of naloxone. (+)naloxone is the (+)enantiomorph of naloxone and is considered biologically inactive (this compound displays about 10 000 times lower affinity for opioid receptors (Jimenez *et al*., 1990).

To avoid manipulation of control mice, 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. Another condition which is sometimes indicated in these studies, i.e., rearing the pups in mixed sex litters, was discarded in order to enhance possible differences among experimental groups. We are aware that developmental research is unique in many ways, and the issue of what constitutes a 'control' group has been a constant source of debate.

From weaning (21 days of age) the individuals in each treatment group were rehoused in post-weaning cages of three animals each. On the 35th postnatal day animals were weighed, and underwent thermal nociceptive test (tail-flick). At 70 and 110 days of age animals were weighed, and at 110 days they were sacrificed through rapid decapitation. Blood was collected and spleen was excised. The brain was rapidly extracted, and pituitary, hypothalamus and mesencephalon were isolated and put at −80°C for further analyses (not to be described).

Tail flick test

At 35 days of age, animals were tested to determine nociceptive threshold in the tail-flick test (d'Amore *et al*., 1992). 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 of about 1–2 s in the intact control animal. A cut-off time of 10 s was chosen.

Sample collection

Blood was collected using tubes prefilled 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 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 1×10^7 ml^{-1} in RPMI 1640, supplemented with 10% heat-inactivated foetal calf serum, L-glutamine and penicillin/streptomicin (Pacifici *et al*., 1992).

Response to mitogens

Aliquots to 0.1 ml of spleen cells obtained as above were put into microtitre plate-wells and stimulated with phytohemoagglutinin (PHA) 1 μg ml^{-1}, and with concanavalin-A (Con-A), 0.5 μg ml^{-1}. All cultures, in triplicate, were incubated at 37°C in CO_2, 5% humidity, for 24 h, pulsed with 1 μCi of [^{3}H]-methyl-thymidine and incubated for a further 18 h. Cells were harvested into a filter paper Skatron 7031, using a Skatron automatic cell harvester. Incorporation of [^{3}H]-methyl-thymidine was determined by counting in 3 ml of Lipoluma scintillation fluid using microbeta counter. Counts were expressed as counter per minute (c.p.m.).

Natural killer (NK)-cell activity assay

^{51}Cr-labelled YAC-1 cells were used as targets. Attacker cells were tested in triplicate at three different ratios against 10^4 targets, in a 4 h ^{51}Cr-release assay. The background c.p.m. were determined by incubating a sample of target cells in RPMI 1640 medium and the maximum c.p.m. were obtained by incubating target cells in saponin. Cytotoxic activities were expressed as lytic units LU_{33} 10^{-7} effector cells.

Cytokine assay

A first set of splenocytes was adjusted to a final concentration of 10^6 cells ml^{-1} in culture medium, and stimulated at 37°C in 5% CO_2 incubator 36 h with PHA (1 μg ml^{-1}) 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, U.S.A.). The tests were performed according to the supplier's instructions.

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^{-1}; IL-2, 15 to 960 (15) pg ml^{-1}; IL-4, 20 to 540 (5) pg ml^{-1}; IL-10, 30 to 1080 (15) pg ml^{-1}; IFN-γ, 20 to 1620 (5) pg ml^{-1}; and TNF-α, 35 to 2240 (15) pg ml^{-1}. Tissue culture supernatants were diluted in wash buffered and the 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 variation were always less than 5% (Pacifici *et al*., 1997).

Leptin assay

Leptin concentration in plasma was determined by Linco's mouse leptin radioimmunoassay (RIA) kit (Alifax, Linco Research, MO, U.S.A.). The tests were performed according to the supplier's instructions. Samples from control and treated mice and leptin standards were assayed simultaneously, in triplicate. The sensitivity of the assay was

0.2 ng ml^{-1}. The limit of linearity was 20 ng ml^{-1}. Assay performance was tested using three concentrations of leptin (1 low and 2 high) throughout the procedure. Mean intra- and inter-assay coefficients of variation were always less than 10%.

Drugs

(−)naloxone HCI was purchased from Sigma Aldrich S.r.l.; (+)naloxone HCL was a generous gift of U.S.-NIDA. Doses are expressed as the weight of the base.

Statistical methods

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

Results

Behavioural and body weight data

As expected, the tail-flick test revealed that, at 35 days of age, there was a consistent difference among groups (ANOVA: d.f. = 3;56; F = 11.6; $P<0.001$). Mice of W group had a higher threshold reaction time to nociceptive stimulus than the C group, and this phenomenon was prevented by naloxone, 1 mg kg^{-1}, but not by the inactive enantiomer (+)naloxone (Table 2). Data relative to W and NA+ groups were consistently higher than C and NA groups (d.f. = 1, 28; P at least <0.01).

Table 3 shows the evolution of body weight in the four groups. W-group mice showed a consistently higher body weight than the C group; this effect was prevented by naloxone, while (+)naloxone was inactive. The difference was significant among groups at 70 days of age (d.f. = 3,56; F = 3.85; $P<0.05$). The difference was even more evident at 110 days of age (d.f. = 3,56; F = 9.0; $P<0.001$). At this age, the Bonferroni–Dunn *post-hoc* test showed significantly higher weight in W or NA+ groups as compared to C or NA groups (P at least <0.05).

Immunological data

There was a significant difference in cytokines of Th1-type released by stimulated splenocytes in all groups of mice, i.e., IL-2 (d.f. 3;16, F = 145.0; $P<0.0001$), IFN-γ (d.f. 3;16, F = 769.3; $P<0.0001$) and TNF-α (d.f. 3;16, F = 93.4; $P<0.0001$). The significance of C or NA groups *versus* W or NA+ was at least <0.002. Thus, these effects were prevented by naloxone, but not by (+)naloxone treatment (Figure 1A).

A consistently diminished release of IL-4 (d.f. 3;16, F = 89.5; $P<0.0001$) and IL-10 (d.f. 3;16, F = 44.5; $P<0.001$) was found in W group (Figure 1B). Again, the effect was prevented by naloxone, but not by (+)naloxone treatment. No consistent difference was recorded for IL-1β in the four groups.

We also studied NK cell activity, a parameter of non-specific natural immunity. Within the analysis of variance (d.f. 3;16, F = 40.9; $P<0.0001$), this activity was significantly enhanced in W group in comparison with C group. The increase was prevented by naloxone, but not by (+)naloxone treatment (Figure 2) (difference between C or NA *versus* W or NA+ was significant at least at $P<0.001$).

Finally, we investigated the splenocytes lymphoproliferative response. ANOVA was significantly different in resting conditions (d.f. 3;16, F = 18.7; $P<0.001$) and following mitogen stimulation with PHA (d.f. 3;16, F = 20.6; $P<0.001$) and with ConA (d.f. 3;16, F = 19.2; $P<0.001$). For all three conditions, a consistent increase was observed in the W group, and the effect was prevented by naloxone, but not by (+)naloxone treatment (Table 4). Difference between C or NA group *versus* W or NA+ group was significant at least at $P<0.01$.

Serum leptin mean levels were consistently different among groups (d.f. 3;16, F = 6.04, $P<0.01$); *post-hoc* analysis confirmed that leptin levels in the C group was consistently below other groups: P at least <0.02) (Table 5). In this case therefore naloxone treatment did not prevent the enhancement of leptin levels found in the W group.

Table 2 Pain sensitivity at 35 days of age

	Tail flick threshold			
Group	*C*	*W*	*NA*	*NA+*
Reaction time (s)	1.27	2.14	1.10	1.98
s.e.mean	0.14	0.20	0.12	0.13

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^{-1}; NA+ is for (+)naloxone injection, 1 mg kg^{-1} once a day s.c., for 21 days. At the analysis of variance F(3,56) = 11.6; $P<0.0001$); C and NA groups are consistently different from W and NA+ groups (P at least <0.01).

Discussion

Neonatal stress and immune effects

Up to now, the effects induced by neonatal handling or mild stress exposure on the immune system of the adult animal have been investigated only in a limited number of studies. Persinger & Falter (1992) found immuno-facilitating effects in 100 days old rats, which after infantile stimulation had received albumin injection at 80 days, while von Hoersten *et al*. (1993) found immunosuppressive effects in adult rats, which had undergone maternal deprivation daily from birth up to 28 days of age. Furthermore, an exacerbation of experimental encephalomyelitis in 60 days old rats which had undergone handling during the neonatal period was shown (Manni *et al*., 1998).

Pharmacological mechanisms involved in late behavioural and hormonal alterations triggered by early chronic stressful events were studied: drugs active on serotonin, GABA-benzodiazepine and noradrenaline receptors were investigated (Caldji *et al*., 2000; Escorihuela *et al*., 1995; Liu *et al*., 2000; Smythe *et al*., 1994). However, pharmacological treatments of pups were not fully effective in preventing alterations induced by repeated neonatal mild stress. Likewise, hormonal manipulation (e.g., dexamethasone treatment) was ineffective, while variation in maternal care was suggested as an

important factor in reducing the response to stress in the offspring (Liu *et al*., 1997; van Oers *et al*., 1999). The possibility to use neuroactive drugs to modulate long-term immune alterations induced by postnatal stress still needs to be investigated.

The present experiments show 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 and of Th-1 type (pro-inflammatory) cytokine release, accompanied by behavioural/metabolic responses. In the present investigation we tested the hypothesis that immune alterations induced by a neonatal stressful procedure could be prevented by naloxone. In our hands, (−)naloxone but not (+)naloxone prevented immunological and behavioural/metabolic changes. Therefore, we show the involvement of endogenous opioid systems: (i) in the pathogenesis of several long-term alterations induced by neonatal mild stressful procedures, and (ii) in the possibility of preventing them.

The HPA-opioid interaction hypothesis

A complex interaction can be hypothesized between the HPA and the opioid systems on immune function, as it was previously shown to happen for other functions, such as nociception and cerebral excitability (Ortolani *et al*., 1990; Pieretti *et al*., 1994). Acute and chronic treatment with μ and μ-δ opioid agonist drugs have an inhibitory role in the immune system of the adult organism, probably through modification of the Th-1 and Th-2 types response (Panerai & Sacerdote, 1997). Moreover, acute and repeated treatment with naloxone (5 mg kg^{-1}) in the adult mouse can induce a shift from Th-2 to Th-1 type cytokine pattern, and this effect was attributed to the removal of opioid peptides effect, mainly the endogenous β-endorphin produced by immunocytes (Sacerdote *et al*., 2000). Conversely, both supraphysiological and stress levels of dexamethasone decrease production of Th-1 type cytokines, and increase or maintain production of Th-2 type cytokines, although other factors in addition to glucocorticoids can be relevant for this phenomenon (Agarwal & Marshall, 2001; Moynihan *et al*., 1998).

Table 3 Body weight increment curve

	Body weight of experimental groups			
PN day	*C*	*W*	*NA*	*NA+*
35	32.9 (0.3)	33.9 (0.4)	33.4 (0.6)	34.3 (0.5)
70	42.3 (0.9)	45.3 (0.8)	42.5 (1.1)	45.4 (0.6)
110	46.4 (0.8)	48.9 (0.8)	44.7 (0.7)	49.0 (0.4)

Body weight in g, mean and (standard error). Groups as in Table 2. Analysis of variance showed significant difference among the groups at postnatal day 70 (F 3;56 = 3.85; $P<0.05$) and day 110 of age (F 3;56 = 9.0; $P<0.001$).

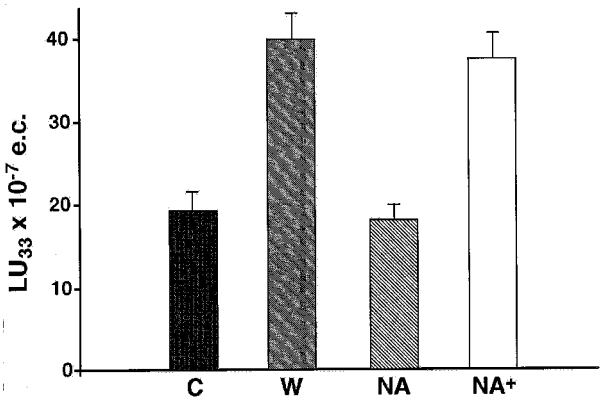

Figure 2 Effects induced by mild repeated post-natal stress on natural killer cell activity of splenocytes of CD-1 male mice. Cytotoxic activities are expressed as lytic units LU_{33} 10^{-7} effector cells. Mice underwent treatment up to day 21 of life, and analyses at day 110.

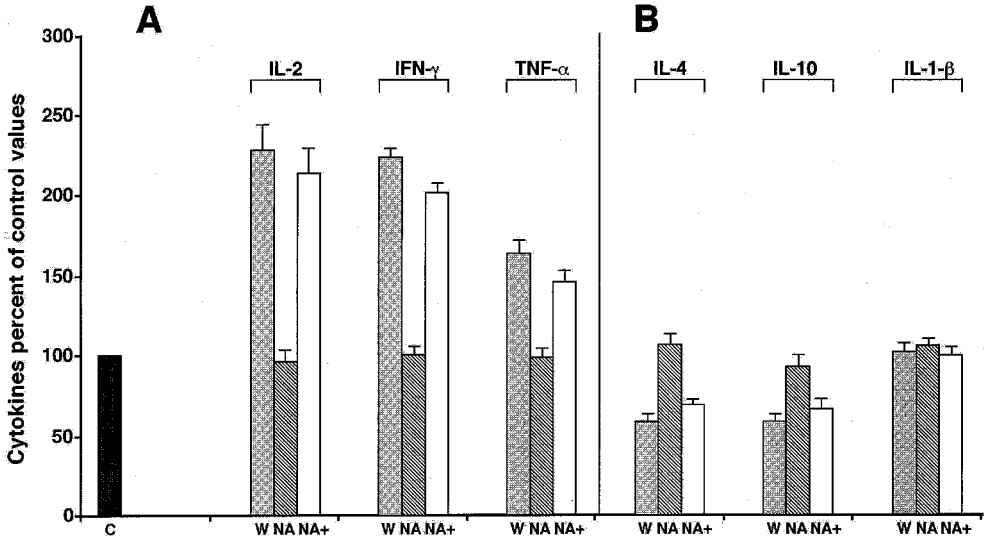

Figure 1 Effect of mild repeated post-natal stress on cytokine production by PHA-stimulated murine splenocytes, in the four groups of animals. Values are expressed in per cent of controls. (A) Th1-type cytokines. Mean control values in pg ml^{-1} are: IL-2: 346 (s.e.mean 31); IFN-γ: 1770 (75); TNF-α: 760 (53); (B) Th2-type cytokines. Mean values (s.e.mean) are: IL-4: 49 (3); IL-10: 1508 (135); IL-1β: 376 (28). Differences of C and NA groups *versus* W and NA+ are always highly significant, with the exception of IL-1β levels, which showed quite similar values in all groups. Mice underwent treatment up to post-natal-day 21, and analyses at day 110.

Table 4 Proliferative activity of mice splenocytes in resting conditions and after stimulation with phytohemoagglutinin (PHA) and concanavalin-A (ConA)

	Splenocyte proliferative activity			
Condition	*C*	*W*	*NA*	*NA+*
Resting	7.3 (0.6)	15.2 (1.3)	7.2 (0.7)	14.7 (1.3)
PHA	152.8 (12.5)	312.2 (27.4)	154.6 (14.2)	314.1 (23.1)
ConA	52.3 (4.5)	106.8 (9.7)	53.2 (4.4)	109.0 (8.8)

Proliferative response is expressed in c.p.m. $\times 10^{-3}$. Differences between control or naloxone groups *versus* water or (+)naloxone groups are significant at $P<0.001$. Mice underwent treatment up to day 21 of life, and analysis at day 110.

Table 5 Serum leptin mean level in the four experimental groups (ng ml^{-1})

	C	*W*	*NA*	*NA+*
Mean	4.5	6.9	6.3	6.4
s.e.mean	0.4	0.5	0.4	0.4

Significant difference between control values and any other groups ($P<0.001$). Mice underwent treatment up to day 21 of life, and analysis at day 110.

The involvement of endogenous opioids in the control of basal activity of the HPA axis is not clear: the effect of naloxone in the activation of the HPA axis in the adult rodent was suggested to be dependent on the dose of naloxone and the type of stressor (Pechnick, 1993); the status of the host's immune system can be relevant in order to direct the opioid control of the immune responses. Our data suggest that chronic mild stress to neonate rodents induces long lasting alteration of endogenous central and/or peripheral opioid (and perhaps HPA hormones) regulation, with consequent increased production of Th-1 type cytokines, and this increase is prevented by repeated low doses of naloxone. These effects are apparently opposite to the ones suggested by the previously cited investigators (Panerai & Sacerdote, 1997; Sacerdote *et al*., 2000; Agarwal & Marshall, 2001; Moynihan *et al*., 1998). In our opinion, the reason lies not only on the different experimental immunological conditions of animals, but also in the age of subjects, i.e., neonate in our experiments *versus* adult in the previously cited investigators.

Up to now, it is not known whether endogenous opioid systems are also involved in the changes induced by mild stressors which do not imply painful stimuli, such as gentle handling and/or repeated short-lasting maternal separation, although increased pain threshold was also demonstrated using these latter procedures (D'Amato *et al*., 1999). Interestingly, when adopting these stress procedures, an involvement of the HPA axis – which is strictly connected to the opioid system – was observed (Anand, 2000; Durand *et al*., 1998; Levine, 1957). Moreover, unpublished data of our laboratories (Loizzo & Spampinato, manuscript in preparation) confirm data from these previous findings: we in fact observed a consistent enhancement of pituitary *ir*ACTH and *ir*β-endorphin levels in W-treated 22 days old mice, 24 h after the last W injection, while this effect was completely prevented in Na-treated mice. These data further suggest that the HPA system and the endogenous opioid system are strictly related in triggering the previously described phenomena.

One possible interpretation of our data is that not all opioid receptors, or endogenous opioids are affected by postnatal treatment and interact with HPA hormones in the same direction and with the same intensity. Glucocorticoids interact differently with different opioid receptors: dexamethasone enhances the analgesic effect of κ opioid-agonists, while it prevents analgesia induced by selective μ and δ opioid drugs in mice (Pieretti *et al*., 1994). In this frame, Ploj *et al*. (1999; 2001) found persistent neurochemical changes in the opioid peptide and nociceptin systems (dynorphins, Met-enkephalin Arg(6)Phe(7) and nociceptin) in the brain of 3 months old rats, which between postnatal day 1 and 21 underwent brief daily handling procedures. Dynorphin-A 1-17, which is considered an endogenous ligand for κ opioid receptors (Smith & Lee, 1988; Spampinato & Candeletti, 1985), enhances IL-2 production and Con-A-stimulated proliferation of rat splenocytes in a dose-dependent fashion, and these effects are blocked by naloxone and by the selective κ opioid antagonist norbinaltorphimine (Ni *et al*., 1999). These effects were quite similar to the ones observed in our experiments (see Figure 2 and Table 4). Selective modulation of opioid receptor sensitivity (perhaps κ opioid) may therefore be hypothesized as an important mechanism to explain our present results. However, further studies need to be performed using selective opioid agonists and antagonists, since there are contrasting data in the literature on the effects induced by κ opioid stimulating drugs on the immune system of the adult organism (Belkowski *et al*., 1995).

In our experimental conditions the changes induced on the immune system by postnatal stressful procedures were evident at least for 3 months after treatment. We can forward some hypotheses: (i) long lasting change of production/bioavailability of endogenous opioid substances, as suggested by Ploj *et al*. (1999; 2001) and, (ii) enhanced bioavailability of endogenous active substances (for example, the ones produced by changed enkephalin-degrading peptidase activity), as suggested by data of Irazusta *et al*. (1999). However, other hypotheses could be involved, such as for example, increased synthesis or release of HPA hormones. Following a single saline injection and isolation during development, the central component of the HPA axis may be stress hyperresponsive, rather than hyporesponsive (Dent *et al*., 2000), thus indirectly enforcing the hypothesis of critical periods of development of opioid (McDowell & Kitchen, 1987) and possibly other receptors as being fundamental in the pathogenesis for these phenomena.

Lord *et al*. (1998) found that at physiological concentrations leptin increases some Th-1 (INF-γ and IL-2) and suppresses one Th-2 (IL-4) cytokine-type production *in vitro*. In our mice increase of Th-1 (IL-2, INF-γ and TNF-α) and decrease or no change of Th-2 type cytokines (IL-4, IL-10 and IL-1β) were observed, in parallel with the increase of plasma leptin level (Figure 1 and Table 5). However, naloxone prevented immune responses but did not prevent the increase of serum leptin levels. We cannot explain this data. A possible hypothesis is that leptin is not directly

sensitive to opioids, and is sensitive to stress hormones instead (De Vos *et al*., 1998).

Discussion of our data requires a certain self-criticism. Our experimental condition is based on the transfer of pregnant mice from the producer to our laboratories. The prenatal stress due to travel could induce some interference on the effects to be studied. In our opinion, this was minimized in our experimental conditions, by its extension to all experimental groups, but further data could define possible interaction between the two phenomena.

This study deals with a postnatal pharmacological manipulation of immune function, and may help to better address further investigation on these mechanisms in developing organisms. Inter-relationships between the endogenous opioid systems and the contribution of hormones (CRH, ACTH, corticosteroids) in this period of life on the immune system remain to be elucidated.

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References

AGARWAL, S.K. & MARSHALL, JR G.D. (2001). Dexamethasone promotes type 2 cytokine production primarily through inhibition of type 1 cytokines. *J. Interferon Cytokine Res.,* **21,** 147 – 155.

AKIL, H.A. & MORANO, M.I. (1995). Stress In *Psychopharmacology. The Fourth Generation of Progress*. Bloom, F.E., Kupfer, D.J. ed. pp. 773 – 786 NY: Raven Press.

ANAND, K.J. (2000). Effects of perinatal pain and stress. *Progr. Brain Res.,* **122,** 117 – 129.

BELKOWSKI, S.M., ALICEA, C., EISENSTEIN, T.K., ADLER, M.W. & ROGERS, T.J. (1995). Inhibition of interleukin-1 and tumor necrosis factor-alpha synthesis following treatment of macrophages with the kappa opioid agonist U50, 488H. *J. Pharmacol. Exp. Ther.,* **273,** 1491 – 1496.

BUCKETT, W.R. (1981). Circadian and seasonal rhythm in stimulation-produced analgesia. *Experientia,* **37,** 878 – 879.

CALDJI, C., FRANCIS, D., SHARMA, S., PLOTSKY, P.M. & MEANEY, M.J. (2000). The effects of early rearing environment on the development of GABAA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. *Neuropsychopharmacology,* **22,** 219 – 229.

D'AMATO, F.R., MAZZACANE, E., CAPONE, F. & PAVONE, F. (1999). Effects of postnatal manipulation on nociception and morphine sensitivity in adult mice. *Dev. Brain Res.,* **117,** 15 – 20.

D'AMORE, A., CAIOLA, S., MAROCCIA, E. & LOIZZO, A. (2000). Postnatal stress in mice: effects on body fat, plasma lipids, glucose and insulin. *Nutri. Neurosci.,* **3,** 207 – 214.

D'AMORE, A., CHIAROTTI, F. & RENZI, P. (1992). High-intensity nociceptive stimuli minimize behavioral effects induced by restraining stress during the tail-flick test. *J. Pharmacol. Toxicol. Methods,* **27,** 197 – 201.

D'AMORE, A., MARANO, G. & LOIZZO, A. (1993). Reduced antinociceptive response to beta-endorphin in adult mice after chronic neonatal handling. *Physiol. Behav.,* **53,** 1025 – 1027.

D'AMORE, A., MAZZUCHELLI, A., RENZI, P. & LOIZZO, A. (1996). Effect of naloxone on the long-term body weight gain induced by repeated postnatal stress in male mice. *Behav. Pharmacol.,* **7,** 430 – 436.

D'AMORE, A., PIERETTI, S., CHIAROTTI, F. & LOIZZO, A. (1991). Chronic treatment with MIF-1 prevents the painful stimuli threshold elevation induced by neonatal handling in mice. *Peptides,* **12,** 1291 – 1294.

D'AMORE, A., PIERETTI, S., PALAZZESI, S., PEZZINI, G., CHIAROTTI, F., SCORZA, T. & LOIZZO, A. (1990). MIF-1 can accelerate neuromotor, EEG and behavioral development in mice. *Peptides,* **11,** 527 – 532.

DALLONGEVILLE, J., FRUCHART, J.-C. & AUWERX, J. (1998). Leptin, a pleiotropic hormone: physiology, pharmacology and strategies for discovery of leptin modulators. *J. Med. Chem.,* **41,** 5337 – 5352.

DE CEBALLOS, M.L. & DE FELIPE, C. (1985). Circannual variation in opioid receptor sensitivity in mouse vas deferens. *Eur. J. Pharmacol.,* **106,** 227 – 228.

DE GIORGIS, G.F., NONNIS, E., CROCIONI, F., GREGORI, P., LEUZZI, V., ROSINI, P. & LOIZZO, A. (1996). Evolution of daytime quiet sleep components in early treated phenylketonuric infants. *Brain Dev.,* **18,** 201 – 206.

DE VOS, P., LEFEBVRE, A.-M., SHRIVO, I., FRUCHART, J.-C. & AUWERX, J. (1998). Glucocorticoids induce the expression of the leptin gene through a non-classical mechanism of transcriptional activation. *Eur. J. Biochem.,* **253,** 619 – 626.

DENT, G.W., SMITH, M.A. & LEVINE, S. (2000). Rapid induction of corticotropin-releasing hormone gene transcription in the paraventricular nucleus of the developing rat. *Endocrinology,* **141,** 1593 – 1598.

DURAND, M., SARRIEAU, A., AGUERRE, S. & MORMEDE, P. (1998). Differential effects of neonatal handling on anxiety, corticosterone response to stress, and hipoocampal glucocorticoid and serotonin (5-HT)2A receptors in Lewis rats. *Psychoneuroendocrinology,* **23,** 323 – 335.

ESCORIHUELA, R.M., TOBENA, A., DRISCOLL, P. & FERNANDEZ-TERUEL, A. (1995). Effects of training, early handling, and perinatal flumazenil on shuttle box acquisition in Roman low-avoidance rats: toward overcoming a genetic deficit. *Neurosci. Biobehav. Rev.,* **19,** 353 – 367.

FERNÀNDEZ, B., ANTELO, MT, GUAZA, C., ALBERTI, I., PINILLOS, ML, & VIVEROS, MP. (1999). Naltrindole administration during the preweaning period and manipulation affect adrenocortical reactivity in young rats. *Dev. Brain Res.,* **112,** 135 – 137.

IRAZUSTA, J., TEJEDOR-REAL, P., VARONA, A., COSTELA, C., GILBERT-RAHOLA, J. & CASIS, L. (1999). Effect of neonatal handling on brain enkephalin-degrading peptidase activities. *Neurochem. Int.,* **35,** 357 – 361.

JIMENEZ, I., IGLESIAS, T. & FUENTES, J.A. (1990). Stereoselectivity and the subtype of the opioid receptor involved in stress-induced hypertension. *Eur. J. Pharmacol.,* **182,** 155 – 160.

LAUDER, J.M. & KREBS, H. (1986). Do neurotransmitters, neurohumors and hormones specify critical periods? In *Developmental Neuropsychobiology*. Greenough, W.T., Juraska, J.M. ed pp. 119 – 174 Orlando: Academic Press.

LEVINE, S. (1957). Infantile experience and resistance to psychological stress. *Science,* **135,** 405.

LIU, D., CALDJI, C., SHARMA, S., PLOTSKY, P.M. & MEANEY, M.J. (2000). Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinephrine release in the hypothalamic paraventricular nucleus. *J. Neuroendocrinol.,* **12,** 5 – 12.

LIU, D., DIORIO, J., TANNENBAUM, B., CALDJI, C., FRANCIS, D., FREEDMAN, A., SHARMA, S., PEARSON, D., PLOTSKY, P.M. & MEANEY, M.J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal response to stress. *Science,* **277,** 1659 – 1662.

LOPEZ, J.F., AKIL, H. & WATSON, S.J. (1999). Neural circuits mediating stress. *Biol. Psych.,* **46,** 1461 – 1471.

LORD, G.M., MATARESE, G., HOWARD, J.K., BAKER, R.J., BLOOM, S.R. & LECHLER, R.I. (1998). Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. *Nature,* **394,** 897–901.

MANNI, L., MICERA, A., PISTILLO, L. & ALOE, L. (1998). Neonatal handling in EAE-susceptible rats alters NGF levels and mast cell distribution in the brain. *Int. J. Dev. Neurosci.,* **16,** 1–8.

McDOWELL, J. & KITCHEN, I. (1987). Development of opioid systems: peptides, receptors and pharmacology. *Brain Res.,* **434,** 397–421.

MEANEY, M.J., MITCHELL, J.B., AITKEN, D.H., BHATNAGAR, S., BODNOFF, S.H., INY, L.J. & SARRIEAU, A. (1991). The effects of neonatal handling on the development of the adrenocortical response to stress: implications for neuropathology and cognitive deficits in later life. *Psychoneuroendocrinology,* **16,** 85–103.

MOYNIHAN, J.A., CALLAHAN, T.A., KELLEY, S.P. & CAMPBELL, L.M. (1998). Adrenal hormone modulation of type 1 and type 2 cytokine production by spleen cells: dexamethasone and dehydroepiandrosterone suppress interleukin-2, interleukin-interferon-gamma production in vitro. *Cell Immunol.,* **184,** 58–64.

NI, X., LIN, B.C., SONG, C.Y. & WANG, C.H. (1999). Dynorphin A enhances mitogen-induced proliferative response and interleukin-2 production of rat splenocytes. *Neuropeoptides,* **33,** 137–143.

ORTOLANI, E., DI GIANNUARIO, A., NEROZZI, D., ZAPPONI, G.A. & LOIZZO, A. (1990). Some endorphin derivatives and hydrocortisone prevent EEG limbic seizures induced by corticotropin-releasing factor in rabbits. *Epilepsia,* **31,** 702–707.

PACIFICI, R., DI CARLO, S., BACOSI, A., PICHINI, S. & ZUCCARO, P. (1992). Immunomodulating properties of carbamazepine. *Int. J. Immunopharmacol.,* **14,** 605–611.

PACIFICI, R., DI CARLO, S., BACOSI, A., PICHINI, S. & ZUCCARO, P. (1997). Cytokine production in saquinovir treated mice. *Int. J. Immunopharmacol.,* **19,** 243–248.

PANERAI, A.E. & SACERDOTE, P. (1997). Beta-endorphin in the immune system: a role at last? *Immunol. Today,* **18,** 317–319.

PECHNICK, R.N. (1993). Effects of opioids on the hypothalamo-pituitary-adrenal axis. *Annu. Rev. Pharmacol. Toxicol.,* **33,** 353–382.

PERSINGER, M.A. & FALTER, H. (1992). Infantile stimulation produces mild enhancement in a primary humoral response of adult albino rats. *Psychol. Rep.,* **70,** 976–978.

PIERETTI, S., D'AMORE, A. & LOIZZO, A. (1991). Long-term changes induced by developmental handling on pain threshold: effects of morphine and naloxone. *Behav. Neurosci.,* **105,** 215–218.

PIERETTI, S., DI GIANNUARIO, A., DOMENICI, M.R., SAGRATELLA, S., CAPASSO, A., SORRENTINO, L. & LOIZZO, A. (1994). Dexamethasone-induced selective inhibition of the central mu opioid receptor: functional in vivo and in vitro evidence in rodents. *Br. J. Pharmacol.,* **113,** 1416–1422.

PLOJ, K., PHAM, T.M., BERGSTROM, L., MOHAMMED, A.H., HENRIKSSON, B.G. & NYLANDER, I. (1999). Neonatal handling in rats induces long-term effects on dynorphin peptides. *Neuropeptides,* **33,** 468–474.

PLOJ, K., ROMAN, E., BERGSTROM, L. & NYLANDER, I. (2001). Effects of neonatal handling on nociceptin/orphanin FQ and peptide levels in female levels. *Pharmacol. Biochem. Behav.,* **69,** 173–179.

REISINE, T. & PASTERNAK, G. (1995). Opioid analgesics and antagonists. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics.* Hardman, J.G., Limbird, L.E., Molinoff, P.B., Ruddon, R.W., Gilman, A.G. ed. pp. 521–556 New York, McGraw Hill.

RUDA, M.A., LING, O.D., HOHMANN, A.G., PENG, Y.B. & TAKIBANA, T. (2000). Altered nociceptive neuronal circuits after neonatal peripheral inflammation. *Science,* **289,** 628–631.

SACERDOTE, P., MANFREDI, B., GASPANI, L. & PANERAI, A.E. (2000). The opioid antagonist naloxone induces a shift from type 2 to type 1 cytokine pattern in BALB/cJ mice. *Blood,* **96,** 2031–2036.

SMITH, A.P. & LEE, N.M. (1988). Pharmacology of dynorphin. *Annu. Rev. Pharmacol. Toxicol.,* **28,** 123–140.

SMYTHE, J.W., ROWE, W.B. & MEANEY, M.J. (1994). Neonatal handling alters serotonin (5-HT) turnover and 5-HT2 receptor binding in selected brain regions: relationship to the handling effect on glucocorticoid receptor expression. *Brain Res. Dev. Brain Res.,* **80,** 183–189.

SPAMPINATO, S. & CANDELETTI, S. (1985). Characterization of dynorphin A-induced antinociception at spinal level. *Eur. J. Pharmacol.,* **110,** 21–30.

VALLEE, M., MACCARI, S., DELLU, F., SIMON, H., LE MOAL, M. & MAYO, W. (1999). Long term effects of prenatal stress and postnatal handling on age-related glucocortocoid secretion and cognitive performance: a longitudinal study in the rat. *Eur. J. Neurosci.,* **11,** 2906–2916.

VAN OERS, H.J.J., DE KLOET, E.R. & LEVINE, S. (1999). Persistent effects of maternal deprivation on HPA regulation can be reversed by feeding and stroking, but not by dexamethasone. *J. Neuroendocrinol.,* **11,** 581–588.

VON HOERSTEN, S., DIMITRIJEVIC, M., MARKOVIC, B.M. & JANKOVIC, B.D. (1993). Effect of early experience on behavior and immune response in the rat. *Physiol. Behav.,* **54,** 931–940.

ZAGON, I.S. & McLAUGHLIN, P.J. (ed). (1993). *Receptors in the Developing Nervous System.* Vol. II. Neurotransmitters pp. 1–256 London: Chapman & Hall.

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