European Journal of Neuroscience, Vol. 20, pp. 1655–1664, 2004

Beneficial effects of enriched environment on adolescent rats from stressed pregnancies

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Keywords: adolescence, cyclophosphamide, environmental enrichment, immunosuppression, prenatal stress, social behaviour

Abstract

The capacity of an early environmental intervention to normalize the behavioural and immunological dysfunctions produced by a stressed pregnancy was investigated. Pregnant Sprague-Dawley rats underwent three 45-min sessions per day of prenatal restraint stress (PS) on gestation days 11–21, and their offspring were assigned to either an enriched-environment or standard living cages throughout adolescence [postnatal days (pnd) 22–43]. Juvenile rats from stressed pregnancies had a prominent depression of affiliative/playful behaviour and of basal circulating CD4 T lymphocytes, CD8 T lymphocytes and T4/T8 ratio. They also showed increased emotionality and spleen and brain frontal cortex levels of pro-inflammatory interleoukin-1β (IL-1β) cytokine. A more marked response to cyclophosphamide (CPA: two 2 mg/kg IP injections) induced immunosuppression was also found in prenatal stressed rats. Enriched housing increased the amount of time adolescent PS rats spent in positive species-typical behaviours (i.e. play behaviour), reduced emotionality and reverted most of immunological alterations. In addition to its effects in PS rats, enriched housing increased anti-inflammatory IL-2 and reduced pro-inflammatory IL-1β production by activated splenocytes, also producing a marked alleviation of CPA-induced immune depression. In the brain, enriched housing increased IL-1β values in hypothalamus, while slightly normalizing these values in the frontal cortex from PS rats. This is a first indication that an environmental intervention, such as enriched housing, during adolescence can beneficially affect basal immune parameters and rats response to both early stress and drug-induced immunosuppression.

Introduction

Exposure to stress early in life can predispose individuals to the development of affective and physiological disorders that persist through adulthood, suggesting that such conditions may be, in part, prenatally programmed (Meijer, 1985; Heim & Nemeroff, 1999; Maccari *et al.*, 2003). Maternal stress during gestation produces reduction of social behaviour (Ward & Weisz, 1984; Dunn & Berridge, 1990; Takahashi *et al.*, 1992), and enhanced emotional reactivity and anxiety in novel situations (Joffe, 1978; Vallee *et al.*, 1997, 1999), as well as dysfunction of the hypothalamus-pituitary axis (HPA) (Fride *et al.*, 1986; Maccari *et al.*, 1995, 2003; Vallee *et al.*, 1997; Koehl *et al.*, 1999; Morley-Fletcher *et al.*, 2003). The brain can modulate or regulate immunologic processes, and intense stress during gestation has been shown to negatively affect immune functions in the offspring (Sobrian *et al.*, 1992; Kay *et al.*, 1998; Bauer *et al.*, 2001). At the time of puberty, rats from stressed pregnancies presented slightly reduced serum IgG levels, a marginal decrease in Natural Killer activity (Sobrian *et al.*, 1992; Tuchscherer *et al.*, 2002) and a suppressive effect in response to the B-cell mitogens lipopolysaccharid (Coe *et al.*, 2002; Tuchscherer *et al.*, 2002). Many of these effects, involving both the HPA axis and immunity, appear to reflect alterations in the regulatory feedback on pro-inflammatory cytokines, via the glucocorticoid receptor (Scheinman *et al.*, 1995).

Several studies have also investigated the effects (generally beneficial) of environmental enrichment as well as in animal models of developmental dysfunction (Caston *et al.*, 1999) in rats offspring exposed to different insults, such as alcohol (Hannigan *et al.*, 1993), undernutrition (Carughi *et al.*, 1989), neonatal anoxia (Iuvone *et al.*, 1996) or prenatal (Rea *et al.*, 2002; Morley-Fletcher *et al.*, 2003) or early postnatal stress (Francis *et al.*, 2002). Furthermore, neurogenesis by enrichment is promoted in adult rat hippocampus (Kempermann *et al.*, 1997; van Praag *et al.*, 1999; Brown *et al.*, 2003). These studies lend support for the ability of enrichment to promote plasticity and to protect against CNS insult. Environmental enrichment also influences the frequency and diversity of positive natural behaviour, decreasing the occurrence of abnormal behaviour (Olsson & Dahlborn, 2002). Enriched rats usually show a superior ability to adapt or cope, when a situation is highly conflicting/stressful and has to be solved by using complex strategies (Escorihuela *et al.*, 1994; Klein *et al.*, 1994).

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Received 20 January 2004, revised 15 June 2004, accepted 17 June 2004

doi:10.1111/j.1460-9568.2004.03597.x

Enriched housing was expected to have a positive effect on the immune system and can be hypothesized to act directly to enhance, or indirectly to buffer, the immune system upon exposure to an acute environmental or pharmacological challenge (Coe *et al.*, 1989, 1996; Kingston & Hoffman-Goetz, 1996). Recent reports show the beneficial effects of chronic exercise training on basal immune function in humans (Mackinnon, 2000). An increased CD4 and CD8 response to physical exercise in children under chemotherapy treatment for cancer (Shore & Shepard, 1999) as well as the positive effects of moderate exercise on CD4 and CD8 immune senescence values in men (Yan *et al.*, 2001) were found.

In particular, we wondered whether early environmental intervention could modulate the carry-over consequences of stressed pregnancies on immune function. Thus, we provided enriched rats with increased physical stimulation of exploratory behaviour, using a re-arrangeable set of platforms, tunnels and toys, larger housing and more opportunities for voluntary physical exercise (running wheel) and learning than standard laboratory living conditions. We designed our study to investigate the contribution of enriched housing during adolescence, an ontogenetic phase characterized by elevated basal levels of behavioural activation and a high propensity for the expression of affiliative and novelty-seeking behaviours as well as for a peculiar psychopharmacological responsivity profile (for reviews see Laviola *et al.*, 1999, 2003; Spear, 2000). The response to enriched housing and increased stimulation has been reported to be particularly marked in adolescent animals (Chapillon *et al.*, 2002; Roy & Chapillon, 2002).

In keeping with the reports of alteration of immune function as a consequence of prenatal stress (Klein & Rager, 1995; Coe *et al.*, 2002), animals were also assessed for a number of peripheral and central immunological parameters. The function of the immune system was also challenged by administering the well-known immunosuppressant agent, cyclophosphamide (CPA) (Turk *et al.*, 1972; Luebke *et al.*, 1992). The animals' behaviour in response to acute drug challenge and the degree of recovery from the immunosuppression were investigated. Redistribution of circulating lymphocytes may be a very sensitive indicator of immunotoxic effects and reflect the capacity of a substance or a stressor to have adverse effects on the immune system (Bauer *et al.*, 2001).

The results show a certain degree of reversibility at the level of immune and behavioural mechanism and suggest that some measure of compensation occurred in rats from disturbed pregnancies as a result of enriched sensory experience and voluntary physical exercise.

Methods

Animals

Sprague-Dawley female rats (250 g) without prior breeding experience, were purchased from a commercial breeder (Charles River, Italy). Animals were housed in an air-conditioned room (temperature 21 ± 1 °C, relative humidity 60 ± 10%), with a regular 12 h light : 12 h dark cycle (lights-on at 8.00 p.m). Water and food (Enriched Standard Diet purchased from Mucedola, Settimo Milanese, Italy) were available ad libitum. During the week after arrival, animals were group-housed (4 per cage) to co-ordinate their oestrus cycle. Females were then placed with a sexually experienced male and daily inspected, using a vaginal smear, until the discovery of spermatozoids (designated as day of gestation 0), after which they were housed individually in Plexiglas cages (30 × 20 × 15 cm). Pregnant females (and their pups) were then randomly assigned to the prenatal stress (PS) group or the stress-free group.

Prenatal stress procedure

The stress procedure started on day 11 of pregnancy until delivery at 21 days (for procedure see Maccari *et al.*, 1995). Briefly, pregnant female rats were individually placed in plastic transparent cylinders (7 cm diameter, 19 cm long) and exposed to white light for 45 min. Animals were submitted daily to three stress sessions starting at 09 : 00, 12 : 00 and 17 : 00 h. The stress-free pregnant females were left undisturbed in their home cages. Brief restraint stress procedure is believed to be largely psychological in nature due to the feeling of confinement of the animal, and it has been shown to affect various endocrine parameters, including the autonomic nervous system and the HPA axis. Male and female offspring were weaned on day 22 after birth, and only male pups from litters containing a similar number of subjects from both sexes were used in the present study. In contrast to other studies (van Praag *et al.*, 1999), we kept the social component in the cage constant by housing two non-siblings, same sex, and the prenatal group rats, both in standard and in enriched living cages. Also, we chose to expose post-weaning-animals to enrichment because they actively explore their new environment, and thus actively pursue their sensory stimulation, whereas with preweaning enrichment, the young animals appear more passive in experiencing the stimuli (Kohl *et al.*, 2002).

Environmental enrichment

At weaning, a total of 96 male rats from PS and stress-free groups were housed in pairs of no-siblings from same-prenatal condition and assigned to one of the two rearing conditions. In the nonenriched condition animals were housed in standard (30 × 20 × 15 cm) cages with the only manipulation consisting in cleaning the cage, whereas for the enriched condition they were housed in larger and higher cages (40 × 25 × 30 cm). In order to prevent litter effects, no more than two male siblings within each original litter were assigned to each condition (Becker & Kowall, 1977; Chapman & Stern, 1979) to which they were maintained throughout the whole study. Enrichment was defined in terms of physical environment and not social housing (Renner & Rosenzweig, 1986), and consisted of adding to the cage: standard laboratory but differently coloured platforms (12 × 9x6 cm), rodent tunnels (12 × 5 cm), a carton house (16 × 12 × 8 cm), an igloo (12 cm diam. × 6 cm height), a ball (7 cm diam.), triangular metal toys (7 × 7x9 cm) and a running wheel (15 cm diam.). The objects were provided in three different sets, which were changed every 3 days. In this way animals had at the same time a shelter and an exposed environment. When the wheel was present, the rats were provided with either a platform or a container suitable for hiding. A water bottle was suspended above the ceiling and food pellets were provided on the floor. For both housing conditions the sawdust of the cage was changed once a week in association with the measurement of the animals' body weight.

Drug administration

On postnatal days 35 and 36, rats from all experimental groups underwent intraperitoneal (i.p.) administration either of the immunotoxic agent, cyclophosphamide (CPA, 2 mg/kg) or vehicle (saline solution) (Turk *et al.*, 1972; Luebke *et al.*, 1992).

Lymphocyte immunophenotyping

Animals were assessed for lymphocyte immunophenotyping before CPA administration to determine basal T-helper cell (CD4) and T cytotossic/suppressor cell (CD8) -levels. A second sample was taken when rats were killed, ~ 7 days later. Blood samples via the tail vein were collected in tubes filled with 100 μL of EDTA. A total of 100 μL of whole blood was stained using 20 μL of monoclonal antibody reagent. The LeucoGATE (CD4/CD14) fluorescent information, with forward and side scatter, was used to set an electronic gate around the lymphoid population. This gate included at least 95% lymphocytes and less than 5% nonlymphocytes (granulocytes, monocytes and debris). Dual-colour immunophenotyping was performed using the following CYTOPASS (TECNOGENETICS S.R.L.,Milan, Italy) matched rat monoclonal antibody reagents directly conjugated to phycoerythrin or fluorescent isothiocyanate (FICT): CD4/FICT (helper/inducer cells) and CD8/FICT (cytotoxic/suppressor cells).

Preparation of spleen cells

The rats were killed by decapitation on postnatal day (pnd) 43. Spleens were aseptically removed with scissors and forceps in cold phosphate-buffered saline (PBS, JRH Biosciences, Lenexa, KS, USA) 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 hypotonize lyses. Splenocytes were adjusted at final concentration of 1×10^6/well in 96-well microtitre plates (Costar, Data Packaging, Cambridge, MA,USA) in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum, L-glutamine and penicilin/streptomicin (Gibco Laboratories, Grand Island, NY, USA). A first set of splenocytes was stimulated at 37 °C in a 5% CO2 incubator for 36 h with 1 μg/mL phytohaemagglutinin (Sigma, St. Louis, MO, USA) for the induction of cytokines. A second set of splenocytes was cultured in triplicate for 36 h under the same conditions, yet without stimulation, to evaluate the spontaneous production of cytokines. After incubation, the culture supernatants were collected and stored at −80 °C to be analysed for quantifying cytokines.

Cytokine assay

For quantitative measurement of rats IL-1β, IL-2 and IL-10 in supernatants of splenocytes cultures, specific solid phase Enzime-Linked Immunosorbent Assay (ELISA), employing the multiple antibody sandwich principle were used (Genzyme Cambridge, MA,USA). All tests were performed according to the manufactorer's instructions. Samples from control rats, treated rats and cytokine standards were assayed simultaneously in triplicate, in 96-well microtitre precoated with monoclonal anti-ILs. The standard curve for IL-1β assay ranged between 10 and 400 pg/mL; that for IL-2 assay between 31.2 and 1000 pg/mL; and that for IL-10 assay between 7.8 and 500 pg/mL. Tissue culture supernatants were diluted in buffered wash and dilution factor was considered for calculating the quantify of cytokines. The sensitivity of the assay was 10 pg/mL for IL-1β, 0.2 pg/mL for IL-2 and 5 pg/mL for IL-10. The specificity of monoclonal antirat-ILs was tested in our laboratory. No cross-reactivity was encountered between tested cytokines. Assay performance was tested using three different concentrations of cytokines in culture medium throughout the procedure. Mean intra- and interassay coefficients of variation were consistently below 5%.

Cytokine assays in brain areas

Immediately after the rats were killed by decapitation, the brain areas were rapidly dissected, frozen on dry ice and stored at −80 °C until use. Frontal cortex and hypothalamus were homogenized in 50 mM Tris buffer, pH 7.5 containing 10 μg/mL indomethacin, and a protease inhibitor 0.2 mM leupeptin. The samples were vigorously vortexed, incubated for 5 min on ice before and centrifuged at 14 000 r.p.m. for 30 min at 4 °C. Supernatants were collected and stored at −70 °C until required. The level of IL-1β was assayed by specific ELISA, following the manufacturer's instructions. The range of determination was 10–1000 pg/mL. Each sample was tested in duplicate (the ELISA kit for rat IL-1β was from Endogen Inc., Woburn, MA, USA).

Radioimmunoassay for corticosterone

To investigate the HPA axis basal function, rats were moved to an adjacent room and individually placed in a restraint transparent tube. Briefly, blood samples were collected quickly (< 1 min) via the tail vein at 9 : 30 h, to determine basal corticosterone levels. A second blood sample was obtained when rats were killed. Blood was collected in tubes filled with 100 μL of EDTA.

Plasma corticosterone levels were obtained using a RIA Kit (ICN Biomedicals) with a highly specific corticosterone antibody and a detection threshold of 0.1 μg/100 mL. The intra- and interassay coefficients of variation were 5 and 11%, respectively.

Statistics

Behavioural, hormonal and immunological data were analysed using parametric analysis of variance (ANOVA) with two prenatal groups, two postweaning conditions and two drug conditions as between-subject factors (Winer, 1971; Chiarotti *et al.*, 1987). Planned comparisons were used for *post hoc* comparisons.

Behavioural measures

Behavioural assessments were carried out after 2 weeks of housing in standard conditions or in environmentally enriched conditions. The time-line in Fig. 1 illustrates the temporal sequence of events.

Open field test

One day after drug-administration, animals were tested for locomotor/exploratory activity in an open field. The apparatus was composed of a black Plexiglas floor (30 × 30 cm) surrounded by a transparent PVC wall (30 cm in height). Photocell beams were located on each of two sides of the arena. The floor was divided into a central zone (6 × 6 cm) and a peripheral part. At the beginning of the test, a single rat was placed in the central part and was allowed to freely explore the arena for 15 min. Velocity (cm/s), resting time (s) and distance moved (cm) were recorded by a computer. At the end of this session a new object (a ball, 7 cm diameter) was introduced. The latency to first approach, and time spent close to, the object were obtained. The apparatus was cleaned with a 30% ethanol solution between subjects.

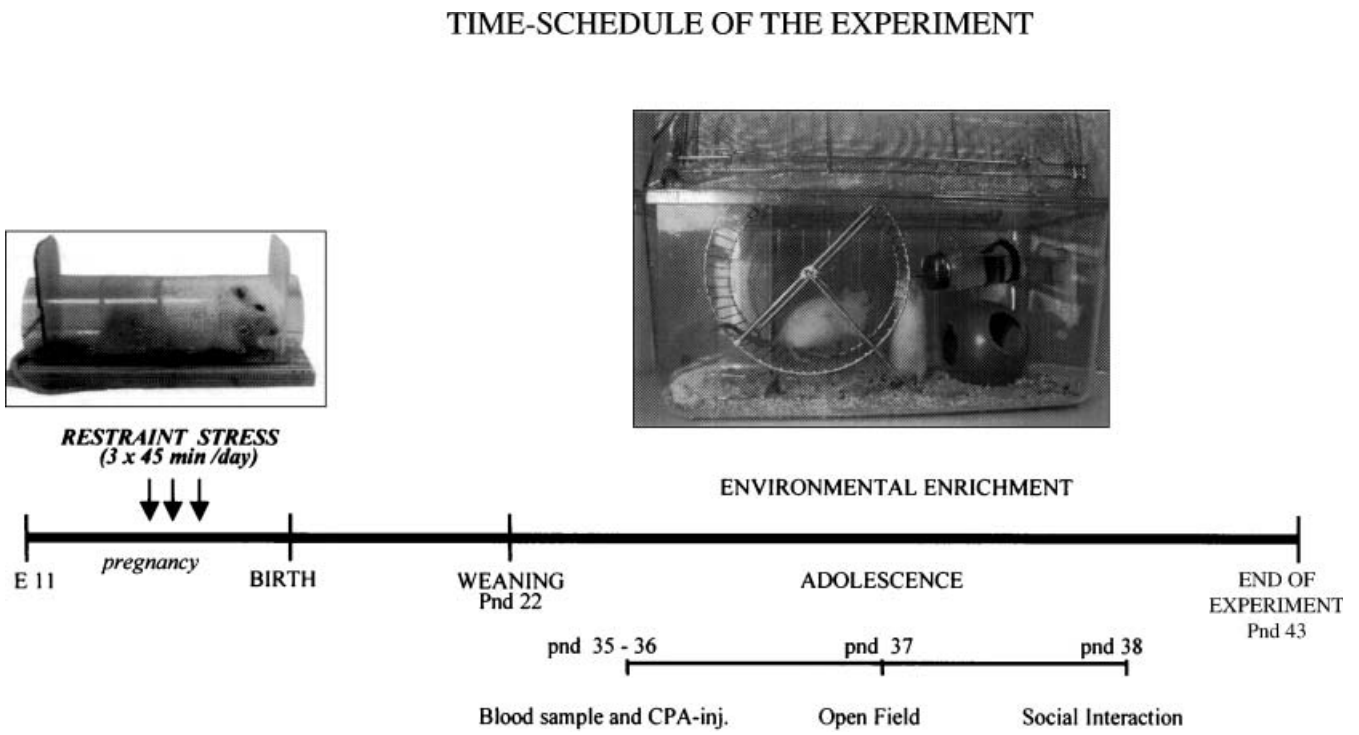

FIG. 1. Time-line for experimental protocol. It shows the age of animals (postnatal days) at time of testing. See Materials and methods for a description of the procedure of prenatal stress and environmental enrichment. CPA, cyclophosphamide (2 mg/kg); E, embryonic day; pnd, postnatal day.

Social interaction test

In order to increase levels of social behaviour, rats were individually housed for 24 h (Panksepp, 1981; Cirulli *et al.*, 1996; Terranova *et al.*, 1999). Then, each pair of rats from the same cage and housing condition was placed in the test arena (30 × 20 × 15 cm) for a single 20-min session. The order of testing was counterbalanced for all treatment groups. The whole session was video-recorded using a professional Sony videocassette recorder VO-58OOPS apparatus, and automatically subdivided into 5-min intervals. The first and last 5 min of each session were manually scored (Observer 20, Noldus), according to an 'all-occurrence' sampling method (Martin & Bateson, 1986) by an observer blind to the assignment of animals to the different groups. Separate scores were obtained for each individual in a pair, but as the two values cannot be considered statistically independent, pair means were used for further analysis. The following social and nonsocial behavioural categories were recorded. (1) Social behaviours (investigative and affiliative elements): (i) social investigation (anogenital and body sniffing); (ii) allogrooming; (iii) play-soliciting (mutual circle – partners are mutually sniffing each other's anogenital region while making a circle with their reciprocal movements; crawl over – the subject crawls over the partner's body, crossing it transversely from one side to the other; on top posture – the subject puts its forepaws on the partner's back); (iv) social rest: the subject is lying flat or standing still while maintaining physical contact with the partner, which may be in turn either inactive or involved in activities which do not require movements around the cage (i.e social sniffing or maintenance activities). This social rest behaviour can elicit allogrooming by the partner. (2) Rough-and-tumble play: pouncing (the subject lunges toward the side or back of the partner with the forepaws extended) wrestling and pinning (one of the animals lying with its dorsal surface on the floor with the other animal standing over it) with the partner. (3) Non-social behaviours (exploration): sniffing the air, rearing, moving around.

Results

Immunological parameters in the blood

At late adolescence rats from disturbed pregnancies exhibited a significant decrease in circulating CD4 T lymphocytes (80%) and the T4/T8 ratio, compared with stress-free animals (Group $F_{1,22} = 20.63$ and 15.21, respectively, both $P < 0.01$). At the same time, no differences were found in the number of CD8 T lymphocytes. Further, the CD4 and T4/T8 ratio were markedly affected by cage living (Condition × Group, $F_{1,22} = 6.15$ and 7.76; $P < 0.01$). Under standard conditions (see panel B in Figs 2 and 3a), prenatally stressed rats were significantly impaired on the CD4 T-cells and T4/T8 ratio in comparison to prenatal controls ($P < 0.01$). Enriched housing completely reverted the abnormal profile.

In rats injected 1 week before with cyclophosphamide, the expected immunonuppression (see Method) consisted of a reduction of: CD4 T lymphocytes (90%), CD8 T lymphocytes (60%) and the T4/T8 ratio (60%) (Drug $F_{1,22} = 56.56$, 5.11 and 22.42, respectively, all $P < 0.05$ or less). Interestingly, the animals from stressed pregnancies were particularly vulnerable to the drug-precipitated immunosuppression in comparison to prenatal controls: only a tendency for CD4 T cells (Group × Drug $F_{1,22} = 3.46$, $P = 0.07$, Fig. 2a) or heavily marked for T4/T8 ratio ($F_{1,22} = 7.76$; $P < 0.01$, Fig. 3c). Figures 2 and 3 present differences and changes, whereas Table 1 shows actual mean data.

The degree of recovery from drug effects was also a function of the animals' housing conditions. The increment values (see Methods) of CD4 T lymphocytes and the T4/T8 ratio (Condition × Drug $F_{1,22} = 43.38$ and 34.22, respectively, both $P < 0.01$) (see Figs 2c and 3b), were still consistently reduced in rats that received CPA injections under standard conditions ($P < 0.01$). Whereas, the drug-precipitated immunodepression, in contrast was completely absent in enriched rats. Further, a Group × Condition × Drug interaction was found ($F_{1,22} = 4.9$; $P < 0.05$).

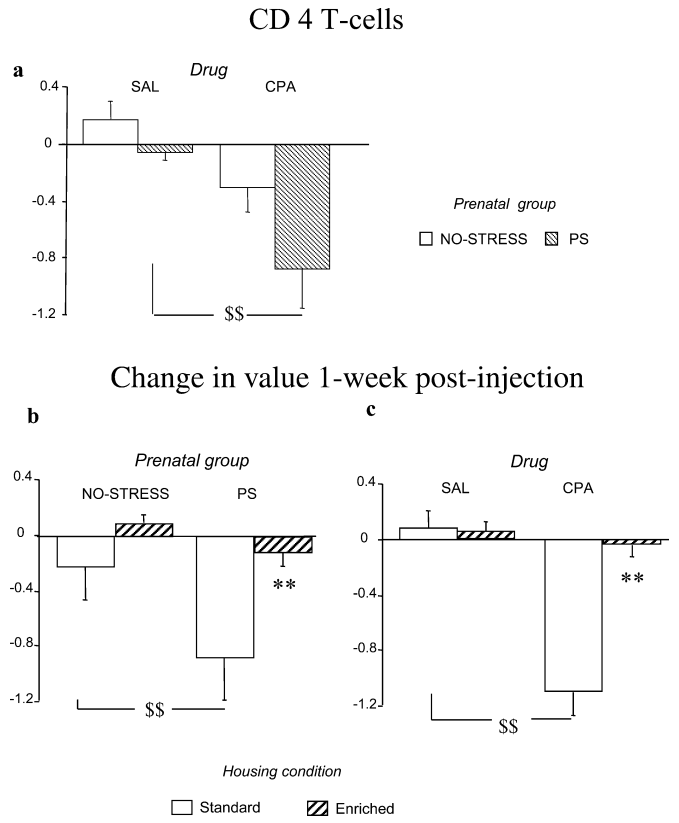

FIG. 2. Peripheral immunological assessment. Increment of CD4 T-cells in peripheral blood. (a) Significantly fewer cells percentage was found in cyclophosphamide (CPA)-injected prenatally stressed rats as compared with the control group ($^{\$\$}P < 0.01$). (b) Environmental enrichment significantly reversed CD4 T-cells percentage in PS rats. $^{**}P < 0.01$ (PS group, enriched vs standard rats) and $^{\$\$}P < 0.01$ (standard conditions, no-stress vs PS rats). (c) Environmental enrichment significantly reversed CD4 T-cells percentage in CPA-injected rats. $^{**}P < 0.01$ (CPA-treated group, enriched vs standard rats) and $^{\$\$}P < 0.01$ (standard conditions, SAL vs CPA rats).

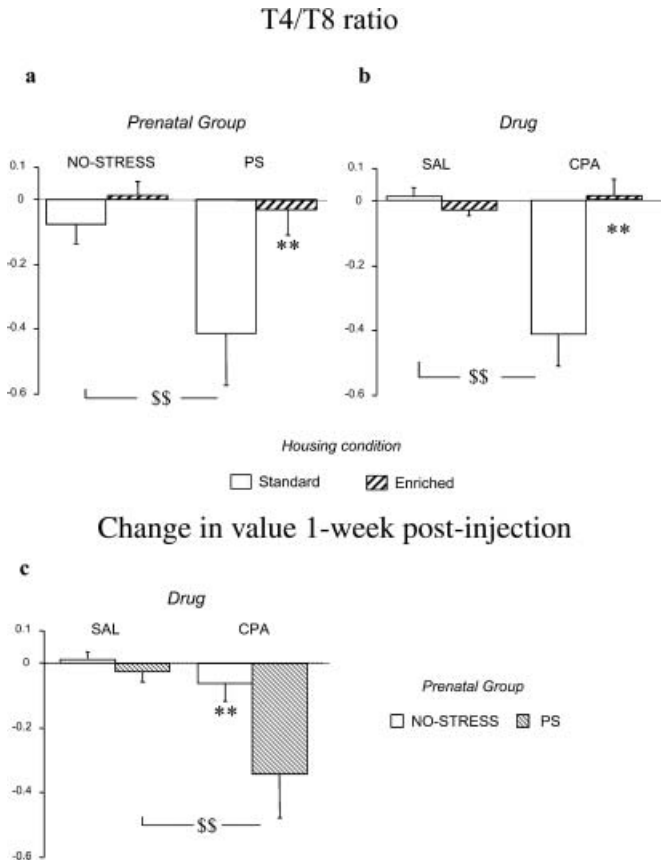

FIG. 3. Peripheral immunological assessment. Increment of T4/T8 ratio in peripheral blood. (a) Significantly fewer cells percentage was found in CPA-injected prenatally stressed rats as compared to the control group. $^{**}P < 0.01$ and $^{\$\$}P < 0.01$. (b) Environmental enrichment significantly reversed T4/T8 ratio in PS rats. $^{**}P < 0.01$ and $^{\$\$}P < 0.01$. (c) Environmental enrichment significantly reversed T4/T8 ratio in CPA-injected rats. $^{**}P < 0.01$ and $^{\$\$}P < 0.01$.

Cytokines in spleen cells

Rats from the disturbed pregnancies produced significantly higher (40%) concentrations of splenic pro-inflammatory cytokine IL-1β (Group $F_{1,32} = 24.29$; $P < 0.01$), in comparison to prenatal controls. Interestingly, animals from enriched housing presented approximately 60% lower levels than animals under standard conditions (Condition $F_{1,32} = 23.93$, $P < 0.01$). A Group × Condition interaction ($F_{1,32} = 17.18$, $P < 0.01$) indicated (see Fig. 4a) that the abnormal elevation of IL-1β production presented by PS rats under standard conditions was completely reverted by enriched housing ($P < 0.01$). No carry-over effects of CPA administration were found.

For anti-inflammatory IL-2 production, rats from enriched living exhibited ~20% higher levels than animals under standard conditions (Condition $F_{1,32} = 6.06$, $P < 0.01$). As a consequence of CPA administration 1 week before, a 50% reduction in IL-2 production was still evident (Drug $F_{1,32} = 85.06$; $P < 0.01$). Furthermore, such a profile was consistently more marked in rats under standard condition (see Fig. 4b) (Condition × Drug $F_{1,32} = 13.23$, $P < 0.01$). No carry-over effects of disturbed pregnancy were demonstrated.

For anti-inflammatory IL-10 production, enriched housing was associated with 50% lower levels than standard living conditions (Condition $F_{1,32} = 34.30$, $P < 0.01$). In general, amounts of this cytokine also appeared consistently elevated (60% more than saline-controls) in rats injected 1 week before with CPA (Drug $F_{1,32} = 106.48$, $P < 0.01$). As shown in Fig. 4c, in animals under standard conditions, the elevated amount reached 200%, whereas no carry-over effects were found in the enriched group.

Levels of IL-1β in brain areas

Separate ANOVA performed on data from prenatal controls indicated that only enriched housing was associated with a six-fold elevation of IL-1β production in the hypothalamus (Condition $F_{1,21} = 5.54$, $P < 0.05$). A Group × Condition interaction ($F_{1,37} = 4.72$, $P < 0.05$) indicated that rats from the disturbed pregnancies showed under standard conditions a 30% elevation in levels of IL-1β in frontal cortex (Fig. 4d). In contrast, enriched housing almost normalized the profile. Also, a Condition × Drug interaction ($F_{1,37} = 4.37$, $P < 0.05$) suggested that whereas in animals under standard conditions CPA administration tended to increase levels of IL-1β in the cortex, in rats in enriched living the drug failed to affect the profile.

Basal blood corticosterone

For the consequences of disturbed pregnancies, rats kept under standard conditions were significantly impaired in basal corticosterone

TABLE 1. T lymphocyte numbers and ratios

	T lymphocytes (per 100 μL of whole blood)*		
	CD4	CD8	T4/T8
No prenatal stress + standard environment			
Saline			
Pre-injection	47.30 ± 4.59	22.48 ± 0.83	2.20 ± 0.08
Post-injection	50.03 ± 1.02	22.13 ± 0.90	2.25 ± 0.06
Cyclophosphophamide			
Pre-injection	50.98 ± 1.10	22.95 ± 0.65	2.20 ± 0.00
Post-injection	43.50 ± 1.15	21.63 ± 0.45	2.00 ± 0.00
No prenatal stress + enriched environment			
Saline			
Pre-injection	49.73 ± 1.96	21.87 ± 1.36	2.27 ± 0.12
Post-injection	50.73 ± 0.38	22.70 ± 1.08	2.23 ± 0.06
Cyclophosphophamide			
Pre-injection	48.63 ± 1.97	20.58 ± 1.69	2.38 ± 0.22
Post-injection	49.37 ± 0.63	20.43 ± 1.10	2.43 ± 0.12
Prenatal stress + standard environment			
Saline			
Pre-injection	49.40 ± 0.56	21.27 ± 1.35	2.33 ± 0.15
Post-injection	47.93 ± 0.75	20.73 ± 0.12	2.33 ± 0.06
Cyclophosphophamide			
Pre-injection	49.55 ± 1.10	22.10 ± 0.55	2.23 ± 0.05
Post-injection	32.78 ± 2.18	21.38 ± 1.24	1.50 ± 0.18
Prenatal stress + enriched environment			
Saline			
Pre-injection	49.25 ± 2.47	21.00 ± 0.14	2.35 ± 0.07
Post-injection	48.95 ± 1.06	21.25 ± 0.49	2.30 ± 0.14
Cyclophosphophamide			
Pre-injection	47.90 ± 3.84	22.33 ± 1.37	2.13 ± 0.19
Post-injection	46.13 ± 2.33	21.67 ± 0.95	2.10 ± 0.14

Post-injection, 1-week post-injection values; Pre-injection, basal pre-injection values. *Data are shown as mean (± SD) T lymphocytes (per 100 μL of whole blood ($n = 4$ rats in each of the four groups of animals).

levels at adolescence. Enriched housing however, almost normalized the profile (Group × Condition $F_{1,32} = 4.92$, $P < 0.05$, see Fig. 5).

Body weight gain

Adolescent rats from disturbed pregnancies (means ± SD for the period pnd 21–41, 151.43 ± 29.70 g) were in general heavier than prenatal controls (136.66 ± 24.28 g) (Group × Day $F_{2,40} = 6.82$, $P < 0.002$). No influence of enriched housing was found. Further, as expected, rats injected with CPA (177.74 ± 37.22 g) were slightly retarded in body growth (Drug $F_{1,20} = 4.93$; $P < 0.05$), in comparison to saline-injected controls (189.15 ± 32.26 g).

Social interaction

Adolescent rats from enriched housing spent approximately 20% less time in nonsocial *rearing* and cage *explore* behaviours (Condition $F_{1,40} = 4.1$ and 15.4, respectively, $P < 0.05$ or less), but were more often involved in affiliative and play-soliciting behaviours *mutual-circle, follow, crawl-over and push-under* (Condition $F_{1,40} = 6.67$, 4.95, 9.24 and 13.79, respectively, all $P < 0.01$) as well as *rough-and-tumble play* (Condition $F_{1,40} = 3.28$, $P = 0.07$) than animals under standard conditions. Rats from disturbed pregnancies were significantly less involved in affiliative *mutual-circle*, *follow*, *crawl-over* activities and *rough-and-tumble play* behaviour than prenatal controls (Group $F_{1,40} = 48.6$, 20.25, 4.49 and 20.02, respectively, all $P < 0.05$ or less).

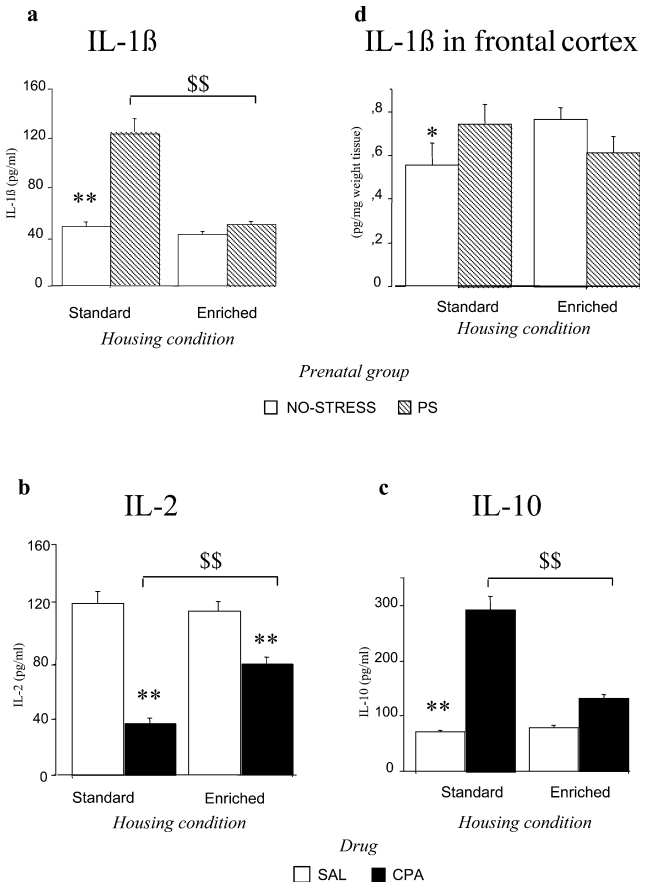

FIG. 4. Cytokines in spleen cells. (a) Levels of pro-inflammatory IL-1β. Environmental enrichment completely reversed the elevated values in PS rats. $^{**}P < 0.01$ and $^{\$\$}P < 0.01$. (b) Levels of anti-inflammatory IL-2. Environmental enrichment increased amounts of IL-2 reverting the inhibitory effects of CPA. $^{**}P < 0.01$ and $^{\$\$}P < 0.01$. (c) Amounts of pro-inflammatory IL-10. Environmental enrichment significantly reversed the effects of CPA-administration. $^{**}P < 0.01$ and $^{\$\$}P < 0.01$. (d) Amounts of IL-1β in frontal cortex. Prenatally stressed rats raised in standard conditions were associated with higher levels of IL-1β in comparison to those raised in enriched housing. $^{*}P < 0.05$ (PS vs no-stress rats).

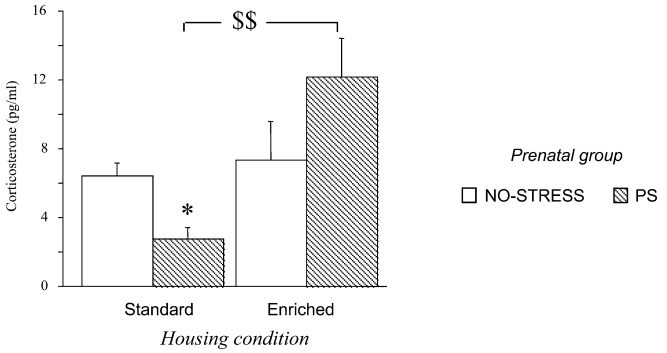

FIG. 5. Basal blood corticosterone levels. Under standard living conditions PS rats were significantly impaired in comparison to prenatal controls. (See Methods) $^{*}P < 0.05$ (no stress vs PS rats) and $^{\$\$}P < 0.01$.

With respect to drug effects, an increase of ∼80% *follow*, *crawl-over* and *mutual-circle* behaviours was found in CPA-injected rats (Drug $F_{1,40} = 20.25$, 6.81 and 32.26, respectively, all $P < 0.01$).

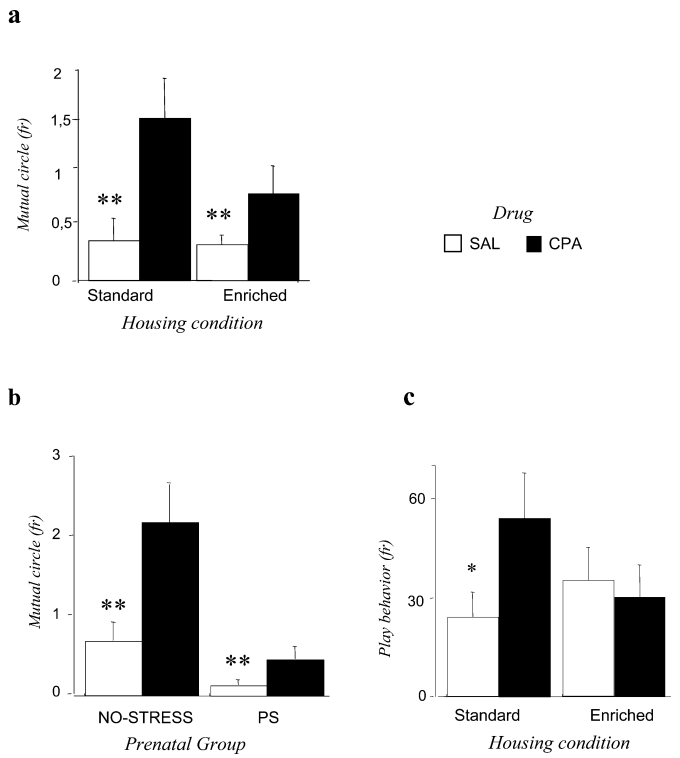

FIG. 6. Social interaction test (single 20-min session). (a) Frequency of soliciting mutual circle behaviour. The CPA-induced elevation of this behaviour was significantly less marked in enriched rats when compared to the standard living group. $^{**}P < 0.01$. (b) Frequency of mutual circle. PS rats were less responsive to pro-social drug effects than prenatal controls. $^{**}P < 0.01$. (c) Frequency of play behaviour. CPA-administration increased significantly levels of play behaviour in animals reared in standard environmental conditions. $^{*}P < 0.05$.

Further, drug effects appeared significantly less marked in enriched rats (Fig. 6a and c, *mutual-circle* and *rough-and-tumble play*: Condition × Drug $F_{1,40} = 5.4$ and 4.27, respectively, $P < 0.05$ or less). Also, rats from disturbed pregnancies were much less responsive to the pro-social drug effects than prenatal controls (Group × Drug $F_{1,40} = 13.06$, $P < 0.001$; Fig. 6b).

Open-field test

Enriched rats spent approximately 33% more time *lying-still* (Condition $F_{1,20} = 25.11$, $P < 0.001$) and were four times less *explorative* ($F_{1,20} = 5.57$, $P < 0.05$) than animals under standard conditions. When a novel object was presented, rats from disturbed pregnancies showed a six-fold higher latency than prenatal controls to first approach the object (Group $F_{1,20} = 7.82$, $P < 0.05$). No significant or reliable effects of CPA injection were found.

Discussion

Child intervention programs in humans can serve to offset the risk associated with compromised development as a function of early life adversity (Ramey & Ramey, 1998; DiPietro *et al.*, 2002). Animals studies reveal an apparent reversal of a number of adverse effects by means of nonpharmacological environmental manipulations (Wakshlak & Weinstock, 1990; Klein & Rager, 1995; Maccari *et al.*, 1995; Vallee *et al.*, 1997; Weinstock, 1997; Francis *et al.*, 2002; Schapiro, 2002; Tuchscherer *et al.*, 2002). The present study was designed to investigate the impact of behavioural intervention, such as experience of an enriched physical environment through adolescence, on the consequences of a stressed pregnancy (see also Morley-Fletcher *et al.*, 2003).

Repeated stress in pregnancy has been shown in the offspring here to decrease specific blood cell populations devoted to immune competence, especially the CD4 T lymphocytes and T4/T8 ratio, and to elevate concentrations of pro-inflammatory IL-1β both in the spleen and brain frontal cortex. Consistently, the disturbed offspring also exhibited a more marked response to the immunosuppressive drug CPA. The increased IL-1β production in the disturbed offspring concurs with the growing literature on adult humans indicating that, besides other intervening variables, including the disease process itself and inflammatory diseases, stress and psychopathology can be indexed by a hyper-secretion of pro-inflammatory cytokines (Sluzewsak *et al.*, 1996; see also Elenkov & Chrousos, 2002).

The data also indicate that enhancements to the environment, such as enriched living, positively impact young rats. In particular, the abnormal profile in CD4 T lymphocytes subset and T4/T8 ratio, as well as an unbalance toward pro-inflammatory cytokines, demonstrated in PS rats under standard conditions was completely reversed in animals from enriched living. Interestingly, the latter were also strongly protected by the additional immune depression produced by CPA challenge. It seems that at least for more responsive animals having suffered from severe stress during gestation, a qualitative nonpharmacological intervention such as enrichment of the physical environment was able to exert its influence. Enriched living, which included voluntary running-wheel exercise, has been reported to affect neurochemical markers such as acetylcholine and trophic factors, such as nerve growth factor and brain-derived neurotrophic factor (van Praag *et al.*, 2000). Earlier studies in humans documented that cytokine networks can be affected by both acute and chronic processes, including physical exercise (DeRijk *et al.*, 1997). Cytokines are known to be potent stimulators of corticosterone release, which has been suggested to provide an inhibitory feedback signal (Papanicolaou *et al.*, 1998) and also act directly on the corticosteroid receptors, strongly affecting density and affinity (see also Elenkov & Chrousos, 2002). We report here that under standard conditions, adolescent rats from disturbed pregnancies presented impaired basal levels of corticosterone (Kay *et al.*, 1998). It is possible that the current findings highlight an important developmental phenomenon, as an increased and prolonged corticosterone response to restraint stress is exhibited by PS rats at adulthood (Maccari *et al.*, 2003). A reduced response in the immature animal could later be manifested as a hyperactive response in adulthood. Precedents for such a bi-directional shift have already been reported (Heim & Nemeroff, 1999). Further, enriched living during adolescence was associated with an increased basal function of HPA. A similar profile, as a result of increased cage complexity (Barnard *et al.*, 1996) and increased voluntary physical activity in more complex cages (Olsson & Dahlborn, 2002), has been reported. Interestingly, for animals having suffered from severe stress during gestation there was apparently more room for the response to such extra stimulation during adolescence. Prenatal stress caused the offspring to be more reactive than the controls. They also responded differentially to the enrichment, perhaps because for them, it was more arousing. As a consequence, PS-induced alteration of a basal HPA index was almost normalized by enriched living.

Consistent with a very recent study on juvenile monkeys (Coe *et al.*, 2002), these findings indicate that maternal conditions during pregnancy can have a pervasive influence and continue to exert an

organizational influence on the cytokine network and emotional behaviour of rats up to late adolescence. This raises the possibility that the shift in physiological set points may persist into adulthood (Ader *et al.*, 1967; Vallee *et al.*, 1997; Weinstock, 1997). Diverse effects are implicated other than just corticosterone. The HPA axis does however, offer one hormonal pathway that could mediate many of these changes. A disturbed pregnancy can lead to the placental transfer of hormones that shape both prenatal and postnatal trajectory (Maccari *et al.*, 2003; Morley-Fletcher *et al.*, 2003). These observations span many laboratory and farm animal species, and have led to speculation that such effects may also occur in humans (Bakker *et al.*, 1995; Wadhwa, 1998). In accordance with the literature, adolescent rats from disturbed pregnancies were impaired in affiliative behaviour and exhibited a reduced amount of age-typical rough-and-tumble play (Ohkawa, 1987; Ward & Stehm, 1991). Meijer (1985) showed that children from stressed pregnancies were less sociable than their peers. Poor social interaction also characterizes psychiatric patients whose mothers experienced severe psychological stress during pregnancy or perinatal birth complications (Offord & Cross, 1969; Done *et al.*, 1994). In the absence of changes in general locomotion, PS rats exhibited increased emotionality, as measured by increased latency to first approach a novel object. This suggests that gestional stress may sensitize rats to the development of hyperanxiety (Weinstock, 2002), whereas an environmental intervention, such enriched living, had the opposite effect. Other studies have reported that modification of cage complexity affects levels of exploration and emotionality (Nevison *et al.*, 1997). In addition to its effects in PS rats, enriched rearing also determined an increase in the amount of positive species-typical behaviour (i.e. rough-and-tumble play) and reduced emotionality (Belzung & Le Pape, 1994; Chapillon *et al.*, 1999, 2002; Belzung, 2001).

As expected, as a consequence of CPA treatment, a slight reduction in body weight was demonstrated (Shalit *et al.*, 2002). Interestingly, this 'poorer' physical condition was not associated with reduction in general activity in the open field test. In contrast, CPA challenge determined a marked increase in the expression of affiliative and playful behaviours. With respect to mechanisms, a large body of evidence has implicated brain opioid systems and, in particular, μ-opioid receptors in the regulation of affiliative behaviour and play (Panksepp *et al.*, 1980; Terranova & Laviola, 2001) in rats and mice during adolescence. A positive interaction between immune-modifiyng agents, such as CPA, and functioning of the brain opioid system has been demonstrated (Dougherty *et al.*, 1986; Dafny & Reyes-Vazquez, 1987), which suggests that opioid-facilitated social activity may derive in part by CPA-induced immunomodulation (Peterson *et al.*, 1998), while social impairment observed in prenatally stressed offspring may be connected with PS-induced alteration of brain opioid function (Wang *et al.*, 2002).

These results show a certain degree of reversibility at the level of immune, hormonal and behavioural mechanisms, and suggest that some measure of compensation occurred in rats from disturbed pregnancies as a result of enriched sensory experience and voluntary physical exercise throughout adolescence. In the present study, experimental animals were all housed in pairs after weaning, and this allowed us to exclude possible confusion associated with social factors as part of the enrichment protocol (Kempermann *et al.*, 1997; van Praag *et al.*, 1999, 2000; Pietropaolo *et al.*, 2004). The nature of the proposed compensatory effect remains a matter of speculation, but the HPA emerges as a potentially interesting site for consideration. Environmental enrichment alters frontal cortex function, and the medial frontal cortex provides inhibitory regulation over HPA (Diorio *et al.*, 1993). Changes in the regulation of HPA axis and increased sleep quality have been reported as a function of regular voluntary exercise in mice (Droste *et al.*, 2003). These findings clearly suggest that neural systems that regulate behavioural and immunological responses can be influenced by events occurring at multiple stages in development. Later-developing systems with a more prolonged period of plasticity, can be stimulated to override altered trajectories within the system.

Acknowledgements

This research was supported as part of the Research Project 'Hypoxic-ischemic brain damage in the newborn: Epidemiological and experimental studies on diagnosis, therapies and rehabilitation' (0AN/F, Ministry of Health, Italy, to G.L.). We wish to thank Stefania Maccari for her kind advice on prenatal stress procedure and literature and Angelina Valanzano for her expert technical assistance.

Abbreviations

CPA, cyclophosphamide; ELISA, Enzyme-Linked Immunosorbent Assays; FICT, fluorescent isothiocyanate; HPA, hypothalamus–pituitary axis; IL, interleukin; PBS, phosphate-buffered saline; pnd, postnatal days.

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