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**Maternal deprivation and early handling affect density of calcium binding protein-containing neurons in selected brain regions and emotional behavior in periadolescent rats.**

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# **MATERNAL DEPRIVATION AND EARLY HANDLING AFFECT DENSITY OF CALCIUM BINDING PROTEIN-CONTAINING NEURONS IN SELECTED BRAIN REGIONS AND EMOTIONAL BEHAVIOR IN PERIADOLESCENT RATS**

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**Abstract.** Adverse early life experiences can induce neurochemical changes that may underlie modifications in hypothalamic-pituitary-adrenal axis responsiveness, emotionality and cognition. Here, we investigated the expression of the calcium binding proteins (CBPs) calretinin, calbindin and parvalbumin, which identify subpopulations of GABAergic neurons and serve important functional roles by buffering intracellular calcium levels, following brief (early handling) and long (maternal deprivation) periods of maternal separation, as compared with non-handled controls. CBP-expressing neurons were analyzed in brain regions related to stress and anxiety. Emotionality was assessed in parallel using the social interaction test. Analyses were carried out at periadolescence, an important phase for the development of brain areas involved in stress responses. Our results indicate that density of CBP-immunoreactive neurons decreases in the paraventricular region of deprived rats but increases in the hippocampus and lateral amygdala of both early-handled and deprived rats when compared with controls. Emotionality is reduced in both early-handled and deprived animals. In conclusion, early handling and deprivation led to neurochemical and behavioral changes linked to stress-sensitive brain regions. These data suggest that the effects of early experiences on CBP containing neurons might contribute to the functional changes of neuronal circuits involved in emotional response.

**Key words:** maternal separation, hippocampus, paraventricular nucleus, amygdala, calcium binding proteins, anxiety.

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Abbreviations: BDNF, brain-derived neurotrophic factor; CB, calbindin; CBP, calcium binding protein; CR, calretinin; CRF, corticotropin releasing factor; DEP, maternal deprivation; EH, early handling; HPA, hypothalamic-pituitary-adrenal; NH, non-handling; PND, postnatal day; PV, parvalbumin; PVN, hypothalamic paraventricular nucleus; SRIF, growth hormone-releasing hormone inhibitory factor somatostatin.

Early environmental manipulation of the infant-mother relationship can produce short and long term effects on the offspring (Cirulli et al., 2003; Pryce and Feldon, 2003; Weaver et al., 2004). In the rat, the most common manipulation procedure is maternal separation which involves removing the infants from their mothers daily during the neonatal period. This experimental paradigm has been widely used to investigate neurobiological changes associated with the etiology and vulnerability to psychiatric diseases (Cirulli et al., 2003). Brief (3-15 min) daily separations from the mother in the first two postnatal weeks of life (early handling, EH) result in persistent effects on behavior in adults including reduced emotionality, lower corticosterone response to stressors and enhanced exploratory behavior (Levine, 1960; Denenberg and Smith, 1963). Thus, EH reduces stress- and fear-related responsiveness to environmental challenges in adulthood. On the other hand, prolonged maternal separations (3- 6 h, maternal deprivation, DEP) appear to be detrimental, increasing fear-related behavior and endocrine responses to stress (Plotsky and Meaney, 1993). However, a range of DEP schedules led to neurobiological and behavioral effects in the same direction as those induced by EH (Lehmann et al., 2002; Pryce and Feldon, 2003).

Long-term behavioral consequences of early life events are believed to be attributable to modifications of specific neural substrates. In particular, maternal separation can modify responsiveness of the hypothalamic-pituitary-adrenal (HPA) axis which is a fundamental regulator of stress response. The hypothalamic paraventricular nucleus (PVN) is a core component of the HPA axis. Upon stimulation by stress, neuroendocrine neurons in the PVN regulate glucocorticoid secretion via release of corticotropin releasing factor (CRF). HPA axis function is modulated by a complex feedback involving local hypothalamic inhibitory and excitatory systems as well as projections from stressor-sensitive brain regions such as the hippocampus and the amygdala (Herman and Cullinan, 1997; Herman et al., 2002a). These stressor-sensitive regions can potentially modulate CRF release through interaction with GABA-, glutamate- and peptide-containing neurons located in the preoptic area and the hypothalamus (Herman et al., 2002b). Previous studies have shown that EH and DEP influence expression levels of paraventricular CRF and HPA axis regulation, which may lead to consequences in stress response and behavior (Avishai-Eliner et al., 2001; Fenoglio et al., 2004; Husum and Mathe, 2002; Ladd et al., 2005).

Calcium binding proteins (CBPs) calbindin (CB), calretinin (CR) and parvalbumin (PV) are widely expressed throughout the nervous system where they are considered modulators of intracellular calcium concentration (Schwaller et al., 2002; Muller et al., 2005). Studies on deficient mice indicate these proteins can modulate the neuron's ability to sustain firing (Schiffmann et al., 1999; Gall et al., 2003) and regulate calcium pools critical for synaptic plasticity (Schurmans et al., 1997; Gurden et al., 1998; Schwaller et al., 2002). Moreover CBPs are particularly useful in identifying various subpopulations of GABAergic interneurons (Freund and Buzsaki 1996; DeFelipe, 1997). It has been shown that 6 h of maternal separation increases hippocampal content of CR and CB, while it reduces CR and CB expression in the hypothalamus immediately after the separation procedure, suggesting possible modified feedback mechanisms in HPA axis function during the stress hyporesponsive period (Lephart and Watson, 1999). The present study has been designed to

test the hypothesis that early environmental manipulations of the mother-infant relationship may induce lasting changes in the density of CBP containing neurons in selected brain regions involved in the regulation of stress response and emotional behavior. EH and DEP rats were compared with nonhandled (NH) rats in periadolescence, a peculiar developmental period of anatomical and neurochemical remodeling of different stressor-sensitive brain areas (Spear, 2000). Taking advantage of the high degree of affiliative and playful social interactions displayed in adolescence (Terranova et al., 1999; Spear, 2000), we assessed whether EH and DEP would affect emotionality in the social interaction test, a behavioral test which is sensitive to modifications in the stress-related brain regions studied (File and Seth, 2003). Our results indicate a relationship between early postnatal manipulations and the development of the neuronal populations analyzed, and suggest a link with the behavioral effects observed.

## **EXPERIMENTAL PROCEDURES**

### **Animals and maternal separation**

Pregnant female Sprague-Dawley rats (Charles River, Calco, Italy) were individually housed in 40×25×20 cm Plexiglas boxes, with a metal top and sawdust as bedding and were maintained at constant room temperature ( $21 \pm 1$  °C) and relative humidity ( $60 \pm 10\%$ ) with lights on from 08:00 to 20:00 h. Pellet food and tap water were freely available.

Birth was designated as postnatal day (PND) 0. On PND 1, pups were culled to six males and two females to ensure the presence of both sexes in the litters (Cirulli et al., 1997). Only the male offspring was used. Whole litters were randomly assigned to one of the following groups: NH control, DEP or EH.

NH: all pups were left undisturbed with their mother until weaning. Litter was not cleaned although some clean sawdust was added from the top of the cage.

DEP: all pups were separated from their dam for 3 h (09:30 -12:30 h) each day from PND 2 until PND 14. During the deprivation time, pups were removed from the home cage and housed as a group in a clean cage placed in an incubator maintained at a constant temperature ( $30 \pm 1$  °C). EH: all pups were separated from their dam for 15 min each day from PND 2 until PND 14. The handling procedure consisted of removing pups from their cage and housing them as a group in a clean cage. Pups were weaned at PND 21, group housed in cages of the same type as the home cage, and left undisturbed.

All experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and with the institutional guidelines on animal welfare (DL 116/92). These experiments were designed to minimize the number, and discomfort, of the animals used.

### **Tissue processing**

At PND 35, animals were deeply anesthetized with a ketamine/xylazine solution (100 mg/kg body weight: 33 mg/kg body weight) and transcardially perfused with saline solution (0.9% NaCl) followed by a solution of 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer, pH 7.4. Brains were then post-fixed overnight, cryoprotected, frozen at -80 °C, and cryostat sectioned (25 µm) in series.

## **Immunohistochemistry**

Immunohistochemical reactions were carried out either by using the biotin-avidin system or double immunofluorescence methods on sections incubated for 48 h at 4 °C with the following antibodies: rabbit anti-CR (1:10,000), mouse anti-CR (1:5000), mouse anti-CB (1:1500), rabbit anti-PV (1:15,000) (purchased from Swant, Bellinzona, Switzerland); rabbit anti-CB, rabbit anti-GABA (1:1000; Sigma, St. Louis, MO, USA), rabbit anti-CRF (1:500; Peninsula Laboratory, San Carlos, CA, USA); rabbit anti-SRIF (1:2000; generous gift of Dr. Benoit, University of Montreal, Canada). All the antibodies were diluted in a solution of 0.01 M PBS, pH 7.4, containing 0.5% Triton X-100. For the biotin-avidin system, sections were incubated with the appropriate biotinylated secondary antibodies for 1 h (1/250, Vector Laboratories, Inc., Burlingame, CA, USA) followed by the avidin- biotin-peroxidase complex (1:200; Vector) and developed in a solution of 0.015% 3,3'-diaminobenzidine, 0.0024% H<sub>2</sub>O<sub>2</sub> in 0.05 M Tris-HCl (pH 7.6). After adhesion on 3-aminopropyltriethoxysilane-coated slides (TESPA, Sigma, Milan, Italy), sections were dehydrated and coverslipped with Sintex (Nova Chimica, Cinisello Balsamo, Italy). For dual immunofluorescence, sections were incubated in a mixture of Cy3-conjugated secondary antibody (1:800; Sigma) and appropriated secondary biotinylated antibody (1:250; Vector) followed by avidin FITC (1:400; Vector). Sections were mounted, air dried, and coverslipped in polyvinyl alcohol with diazabicyclo-octane (DABCO; Sigma) as an anti-fading agent and analyzed with a laser scanning Olympus Fluoview confocal system (Olympus Italia, Milan, Italy).

## **Cell quantification**

To reduce to a minimum the likelihood of litter effect, an average of two subjects per litters was used to perform cell quantification. For the quantification of the CBP positive neurons in the hippocampus, three representative coronal sections of the rostral, medial and caudal regions respectively (approximately -3.8 mm, -4.8 mm, -5.8 mm Bregma level; NH n=6, EH n=5, DEP n=5) were chosen from each series. The hippocampus was divided into two regions: the dentate gyrus and the Ammon's horn. In the three sections selected for the analysis, labeled neurons were counted in the whole sectional area of each hippocampal layer, with the exception of CB-labeled cells in the granular and pyramidal layers, which were counted within a rectangular reference area (100-100 µm) placed onto the layer. In the pyramidal layer cells were counted moving the reference area from CA3 to CA1 (each step 1000 µm, 4 to 8 rectangular reference areas counted per section). Three representative sections of the paraventricular nucleus (approximately from -1.53 mm to -1.78 mm Bregma level; NH n=6, EH n=5, DEP n=5) and amygdala (approximately -1.78 mm to -2.45 mm Bregma level; NH n=10, EH n=9, DEP n=9) were used to determine the numbers of labeled neurons inside these regions.

In the hypothalamus, CBP positive neurons were counted within a triangular reference area (drawn among the dorsal and ventral tips of the third ventricle and the fornix), including the PVN and the immediately surrounding region.

Labeled neurons were counted in the whole sectional area of both basal and lateral nuclei of the amygdala. Sections were analyzed using Olympus IX 50 microscope, a CoolSNAP-Pro Color RS Photometrics video camera and the Image Pro-Plus software (Media Cybernetics L.P.). Surface areas of different regions analyzed were measured using the Image Pro-Plus software in three representative sections immunostained for CBPs. Adjacent sections stained with Cresyl Violet were used to unambiguously identify the experimental zones. Average densities of the neuronal populations in the studied sections were calculated by dividing the numbers of counted neurons by the surface area (mm<sup>2</sup>) of the specific region or layer analyzed.

For quantification of double-labeled cells an FV 200 Olympus Fluoview confocal laser-scanning microscope equipped with a 100- objective was used. Randomly selected cells were analyzed with fixed photo-multiplier settings, to minimize rating variations between sections and animals. Fifty cells per region of interest were examined for colabeling in each animal (n=3 animals for each group). Data are presented as the average percentage of colabeled cells.

### **Social interaction test**

After birth, 12 litters were randomly assigned to the three experimental groups: 4 to the NH, 4 to the DEP and 4 to the EH group. In order to prevent possible litter effects, only two males from each litter were tested in the social interaction test on PND 35. Software systems for collection and analysis of observational data were used for scoring duration and frequency of behaviors (The Observer 2.0 and Ethovision 1.7, Noldus 1991).

On PND 34 male rats were housed individually with some of their own sawdust in a cage identical to the home cage for a 24-hour period. This procedure had been shown to increase the amount of social behavior in a social interaction test (Cirulli et al., 1996). On PND 35 all subjects underwent a 30 min period of social interaction with an unfamiliar partner of the same sex, age and strain that had not undergone any experimental procedure. The unfamiliar pair was placed in a cage identical to the home cage with new sawdust as bedding. Testing age was chosen taking into account previous literature suggesting that a high degree of social interactions characterize rodents around PND 35 (Terranova et al., 1999; Spear, 2000).

### **Behavioral observations**

Behavioral testing of unfamiliar animals took place in an experimental room maintained at the same temperature and humidity conditions as the housing room, from 10:00 -14:00 h. Testing time was counterbalanced between experimental groups. Behavior of each pair was video-recorded by means of a video camera connected to a professional Sony videocassette recorder V0-5800PS. A "focal animal, all occurrences" sampling method was used (Altmann, 1974). Social behaviors were scored as previously described (Terranova et al., 1999; Venerosi et al., 2003).

### **Statistical analysis**

Effects of NH, EH and DEP on the density of CBP reactive neurons in the

whole hippocampus, PVN, basal and lateral amygdala were analyzed by one-way ANOVA. The dentate gyrus and Ammon's horn were further analyzed independently, including condition (NH/EH/DEP) as between-subjects factor and repeated measures (layers; molecular/granular/polymorphic for the dentate gyrus and lacunosum-molecular/radiatum/pyramidal/oriens for the Ammon's horn) as fixed within subject factor. Each individual cell layer was further analyzed by one-way ANOVA (Table 1). Post hoc comparisons were carried out with Tukey HSD test. To assess volumetric changes in different experimental groups, we carried out a comparison between the areas of the brain regions previously analyzed and measured on Cresyl Violet- and CBP-stained sections. ANOVA comparison of area estimates revealed no significant difference between experimental groups in any region analyzed and no significant interaction between treatment and hippocampal cell layers. Statistical analyses of behavioral data were performed by the BMDP statistical software (version BMDP/dynamic 7.0, Berkeley, CA, USA). Social interaction test data were analyzed by ANOVA for repeated measures including condition (NH/EH/DEP) as between litter factor and repeated measures (Time, three timeblocks of 10 min) as fixed within subject factor. Litter effects were assessed in each behavioral test for each behavioral response. Since no litter effects were found, all subjects were considered as individual statistical units. A value of  $P \leq 0.05$  was accepted as statistically significant.

### **Preparation of figures**

Digital images were processed by using Adobe Photoshop 7.0 (Adobe Systems Incorporated, USA), and assembled into montages using Corel-Draw 11 (Corel Corporation, Eden Prairie, MN, USA). Only general adjustments of color, contrast, and brightness were made. The images were not otherwise manipulated.

## **RESULTS**

### **CBP immunoreactivity inof the hypothalamus**

Densities of CR, CB containing neurons were analyzed in the hypothalamic paraventricular region (see Experimental Procedures). One-way ANOVA revealed a significant main effect of maternal separation on the density of both CR ( $F(2,13)=6.25$ ;  $P=0.013$ ) and CB ( $F(2,13)=7.29$ ;  $P=0.008$ ) positive neurons. Post hoc comparison revealed that both CR and CB positive neurons densities were decreased in DEP (CR  $P=0.012$ ; CB  $P=0.006$ ) when compared with EH rats (Fig. 1). Reduction percentages in DEP rats were 58% and 55% respectively for CR and CB.

Whereas the statistical analysis showed no significant differences between DEP and NH controls, a trend to a reduction in both CR and CB neuronal density was detected (CR,  $P=0.051$ ; CB,  $P=0.054$ ).

High densities of CR and CB containing neurons were found in regions immediately surrounding the PVN (Figs. 1, 2). Since GABAergic innervation from PVN-adjacent areas can modulate function of CRF positive cells in the PVN (Herman et al., 2002a,b; Miklos and Kovacs, 2002), we determined whether CR and CB might colocalize with GABA in the PVN itself and in the surrounding regions.

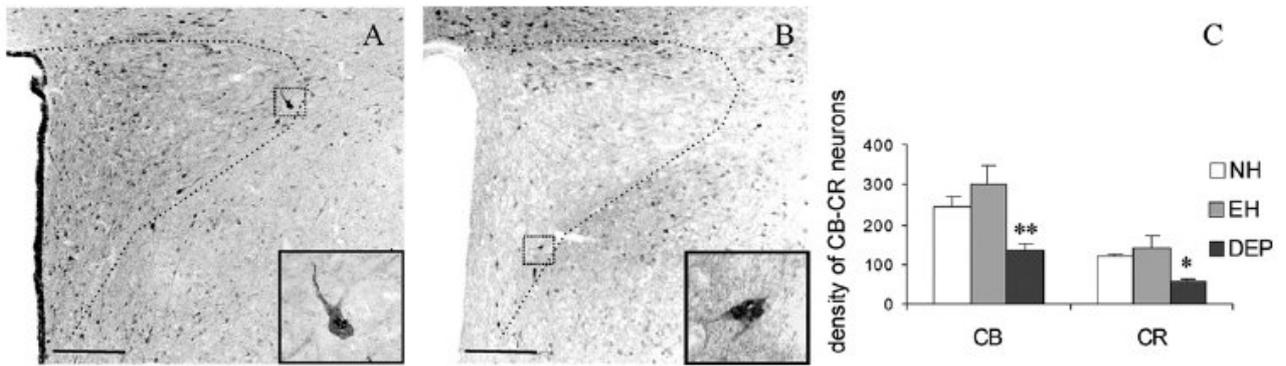
We found that 25% of CR- and 30% of CB-containing neurons are GABA immunoreactive (Fig. 2A-C) thus supporting the hypothesis of the inhibitory nature of a subpopulation of these cells. Moreover, 30% of CB positive cells are also CR positive, indicating that these two neuronal populations partially overlap in the PVN area. Since CB-positive neurons are the largest population

**Table 1.** Effects of maternal separation on the density of CB (1), CR (2) and PV (3) reactive neurons in the dentate gyrus (A) and Ammon's horn (B)

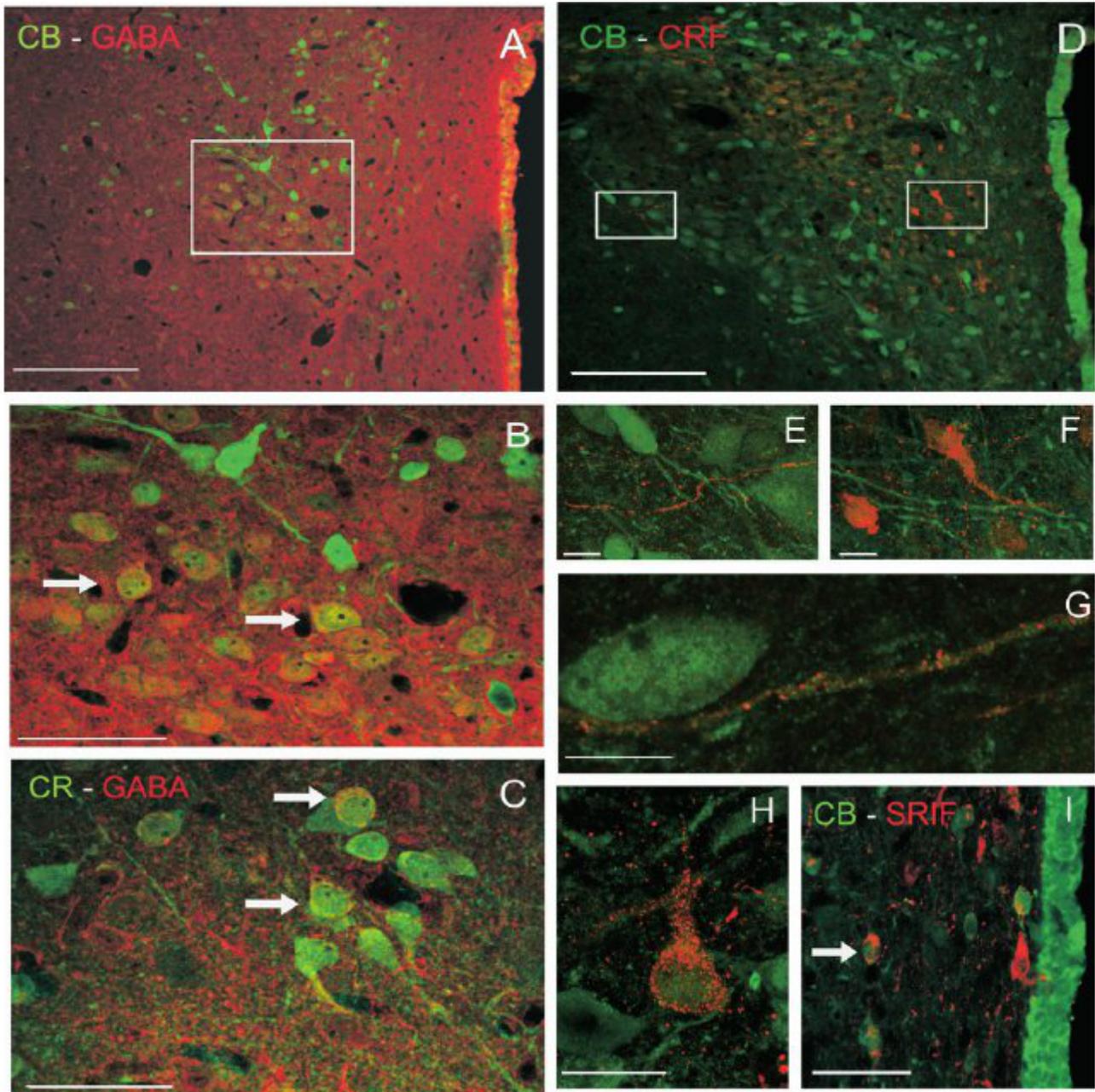
|                          | NH              | EH               | DEP             |
|--------------------------|-----------------|------------------|-----------------|
| 1) A. Dentate gyrus      |                 |                  |                 |
| Molecular layer          | 1.02 (0.19)     | 2.64 (0.86)      | 3.12* (0.28)    |
| Granule cell layer       | 3424 (182.99)   | 4059.14 (306.63) | 3941.58 (67.64) |
| Polymorphic layer        | 14.07 (2.52)    | 17.54 (3.09)     | 16.62 (2.04)    |
| B. Ammon's horn          |                 |                  |                 |
| St. lacunosum moleculare | 1.32 (0.12)     | 1.93 (0.45)      | 2.2 (0.61)      |
| St. radiatum             | 7.4 (0.58)      | 9.58 (3.69)      | 6.82 (0.77)     |
| St. pyramidale           | 694.25 (28.929) | 845.32* (35.68)  | 781.28 (40.34)  |
| St. oriens               | 9.75 (0.77)     | 11.9 (1.43)      | 14.1* (0.96)    |
| 2) A. Dentate gyrus      |                 |                  |                 |
| Molecular layer          | 5.22 (0.91)     | 5.98 (0.95)      | 7.94 (0.62)     |
| Granule cell layer       | 28.27 (5.3)     | 42.32 (7.88)     | 46.62 (7.95)    |
| Polymorphic layer        | 81.87 (18.68)   | 84.74 (11.98)    | 75.40 (8.89)    |
| B. Ammon's horn          |                 |                  |                 |
| St. lacunosum moleculare | 19.13 (2.57)    | 30.38* (2.27)    | 27.10 (3.6)     |
| St. radiatum             | 10.5 (1.0)      | 17.98* (1.26)    | 18.28* (2.41)   |
| St. pyramidale           | 48.98 (2.63)    | 61.02 (6.62)     | 56.56 (4.43)    |
| St. oriens               | 19.47 (2.25)    | 22.44 (3.42)     | 30.76* (3.13)   |
| 3) A. Dentate gyrus      |                 |                  |                 |
| Molecular layer          | 0.5 (0.12)      | 0.77 (0.32)      | 0.89 (0.16)     |
| Granule cell layer       | 54.45 (7.04)    | 45.08 (3.9)      | 54.8 (15.03)    |
| Polymorphic layer        | 17.08 (12.08)   | 16.42 (10.34)    | 20.12 (8.25)    |
| B. Ammon's horn          |                 |                  |                 |
| St. lacunosum moleculare |                 |                  |                 |
| St. radiatum             | 2.36 (0.44)     | 2.74 (0.56)      | 3.34 (0.16)     |
| St. pyramidale           | 74.72 (18.08)   | 94.28 (25.18)    | 77.42 (10.45)   |
| St. oriens               | 18.9 (2.46)     | 22.12 (2.7)      | 23.76 (3.18)    |

Values are expressed as mean cell density per mm<sup>2</sup> and SEM in parentheses. Data were analyzed by one-way ANOVA in each layer.\* P<0.05 vs. NH.

among CBP-positive cells in the PVN area (see Fig. 1), we quantified the percentage of CB/GABA colabeled cells in all experimental groups. This study showed no significant difference between groups (NH, 28%; EH, 31%; DEP, 31%:  $F(2,6)=0.203$ ;  $P=0.821$ ). This result, considering that the number of CB positive neurons was almost halved in DEP rats compared with EH rats (Fig. 1), supports the idea that the CB/GABA double positive population is reduced in DEP rats. Moreover, about 10% of CB positive neurons colocalize with the PVN growth hormone-releasing hormone inhibitory factor somatostatin (SRIF) cell population (Fig. 2I). Interestingly, in the rat, SRIF inhibits CRH-stimulated ACTH release from isolated pituitary cells (Lamberts et al., 1989) and CRH release from the hypothalamus in vitro (Tizabi and Calogero, 1992). Finally, CB- and CR-positive cells and processes are spatially juxtaposed to CRF positive elements in the PVN (Fig. 2E, F), and some CRF/CB double-labeled cells were detected in the PVN (Fig. 2H), indicating that a subpopulation of CRF positive neurons expresses CB. Taken together, these data support a role of CB and CR containing neurons in the regulation of CRF positive cells in the PVN. In agreement with previous studies (Arai et al., 1993), no PV positive neurons were observed in the PVN region in any experimental group.



**Fig. 1.** Effect of EH, DEP and NH on CB and CR immunoreactive cells in the PVN region. CB immunoreactive cells are abundant in the PVN area (A), whereas CR positive cell are less numerous (B). Density of CR and CB immunoreactive cells is reduced in DEP rats (C). Data are mean density of labeled cells per mm<sup>2</sup> (+S.E.M.). \* Significant difference from EH. Dotted lines delineate the PVN. Scale bars=200  $\mu$ m; insets 20  $\mu$ m.



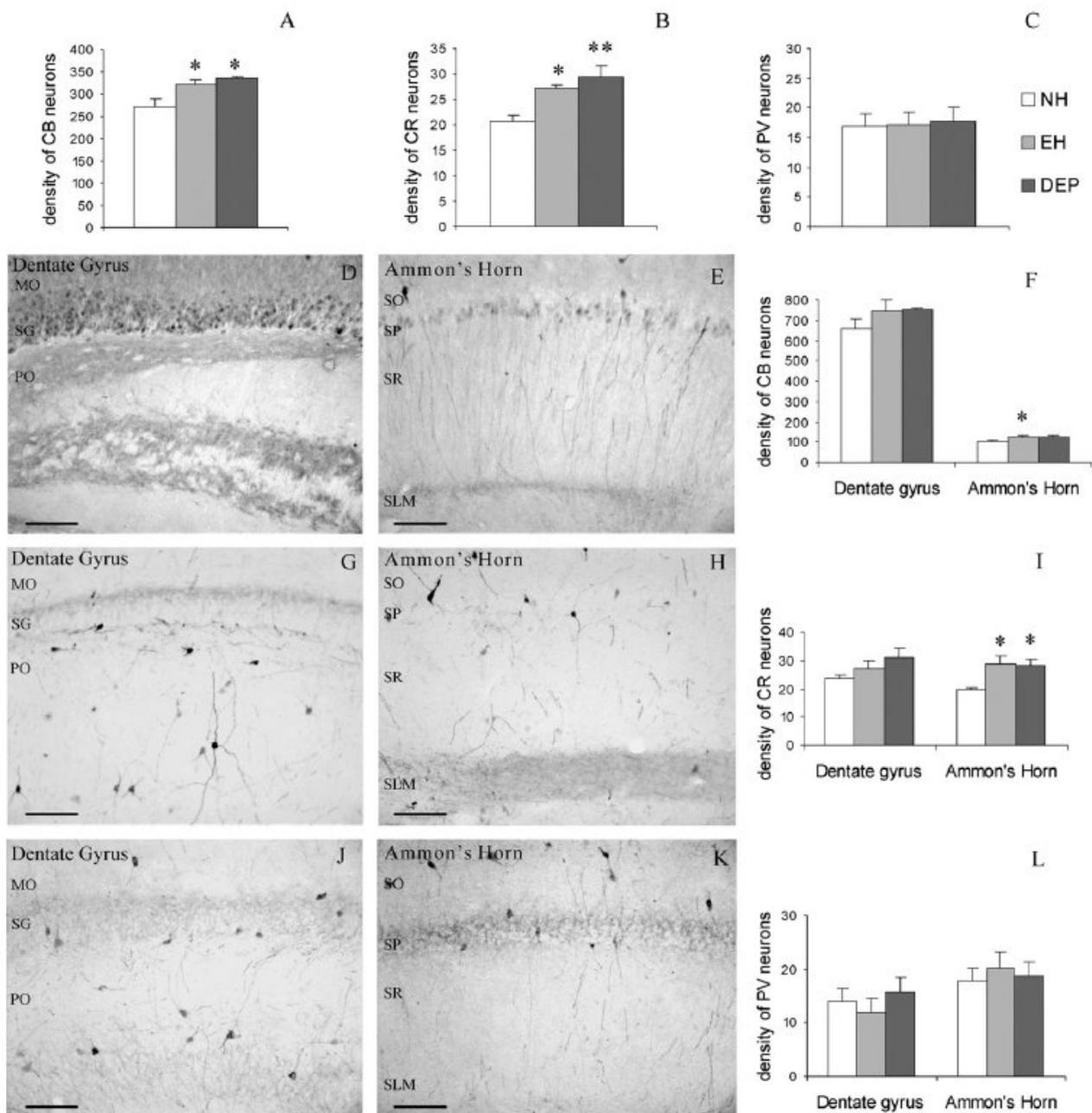
**Fig. 2.** Double labeling for CB/GABA, CR/GABA, and CB/CRF and SRIF in the PVN region. Both CB (green) and CR (green) colocalize with GABA-(red) labeled cells in the PVN area (A-C, arrows in B, C). Numerous CB positive cells (green) surround the area where CRF positive cells (red) are located (D). CB and CRF positive cells and processes are often in close contact (E, F). Some CRF immunoreactive cells (H) and processes (G) are faintly CB positive. SRIF positive cells (red) colocalize with CB-labeled cells (green) in the PVN region (I, arrow). Scale bars=A, D, 200  $\mu$ m; B, C, 50  $\mu$ m; E-H, 20  $\mu$ m; I, 50  $\mu$ m.

### **CBP immunoreactivity in the hippocampus**

Densities of CR, CB and PV containing neurons were first analyzed in the whole hippocampus (Fig. 3). One-way ANOVA revealed a significant main effect of maternal separated DEP rats ( $P=0.01$ ) compared with NH controls (Fig. 3) on both CR ( $F(2,13)=9.46$ ;  $P=0.003$ ) and CB ( $F(2,13)=6.83$ ;  $P=0.009$ ) neuronal densities, whereas no effect was detected for PV neuronal density.

Post hoc comparison revealed overall densities of CR and CB positive neurons had increased in both EH (CR,  $P=0.024$ ; CB,  $P=0.043$ ) and DEP (CR,  $P=0.003$ ; CB,  $P=0.011$ ) compared with NH rats (Fig. 3A, B). Increased percentages in the hippocampus for CR and CB were 32% and 19% in EH rats and 43% and 24% in DEP rats, respectively.

To identify whether changes of CBP neuronal densities were layer specific in each hippocampal region, we analyzed separately the dentate gyrus and Ammon's horn, separation being the independent variable and cellular layers the repeated measures. ANOVA for repeated measures of CR neuronal density revealed a significant main effect of separation in the Ammon's horn ( $F(2,13)=7.537$ ;  $P=0.007$ ) but no interaction between treatment and cellular layers. No significant effects were detected in the dentate gyrus. Post hoc comparison for the Ammon's horn indicated an increased CR density in both EH ( $P=0.022$ ) and DEP rats ( $P=0.01$ ) compared with NH controls (Fig. 3I). For CB neuronal densities a significant main effect of separation ( $F(2,13)=5.171$ ;  $P=0.022$ ) and an interaction between treatment and cellular layers ( $F(6,39)=4.84$   $P=0.001$ ) were detected in the Ammon's horn. Post hoc comparison revealed an increased cellular density for CB only in EH rats compared with NH controls ( $P=0.019$ ; Fig. 3F). Further analysis by one-way ANOVA on individual cell layers revealed a significant increase in CB positive cells in the pyramidal layer of EH rats and in the oriens layer of DEP rats compared with NH controls (Table 1). No main effect of treatment was detected for CB in the dentate gyrus. However, we found an interaction between treatment and layers ( $F(4,26)=2.78$ ;  $P=0.048$ ) which probably reflects an increase in labeled neurons in the molecular layer of DEP rats compared with NH controls (Table 1). No changes between treatments were detected for PV in any hippocampal region and layer analyzed (Fig. 3J-L, Table 1).

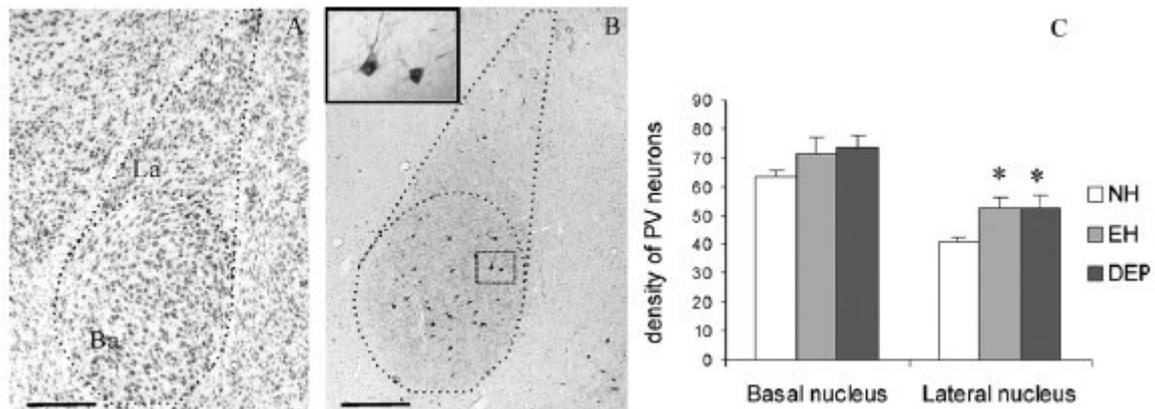


**Fig. 3.** Effect of EH, DEP and NH on CB, CR and PV positive cells in the hippocampus. Significant increase of CB (A) and CR (B), but not PV (C) immunoreactive cells in EH and DEP rats. CB (D-E), CR (G-H) and PV (J-K) immunoreactivity were further analyzed in dentate gyrus (D, G, J) and Ammon's horn (E, H, K), independently. The density of CB positive cells is increased in the Ammon's horn, but not in the dentate gyrus, of EH rats (F). The density of CR positive cells is increased in the Ammon's horn, but not in the dentate gyrus, of both EH and DEP rats (I). PV positive cells were not affected by maternal separation (L). Data are mean density of labeled cells per mm<sup>2</sup> (+S.E.M.). \* Significant difference from NH. Scale bars=100  $\mu$ m.

### Quantification of CBP containing neurons in the amygdala

Densities for CBP immunoreactive cells were analyzed focusing on the basal and lateral nuclei of the amygdala (Fig. 4) where the majority of amygdalar PV and CB positive GABAergic neurons are found (Yilmazer-Hanke et al 2002). One-way ANOVA revealed a significant main effect of separation of PV neuronal density in the lateral nucleus ( $F(2,25)=5.14$ ;  $P=0.014$ ) but not in the basal

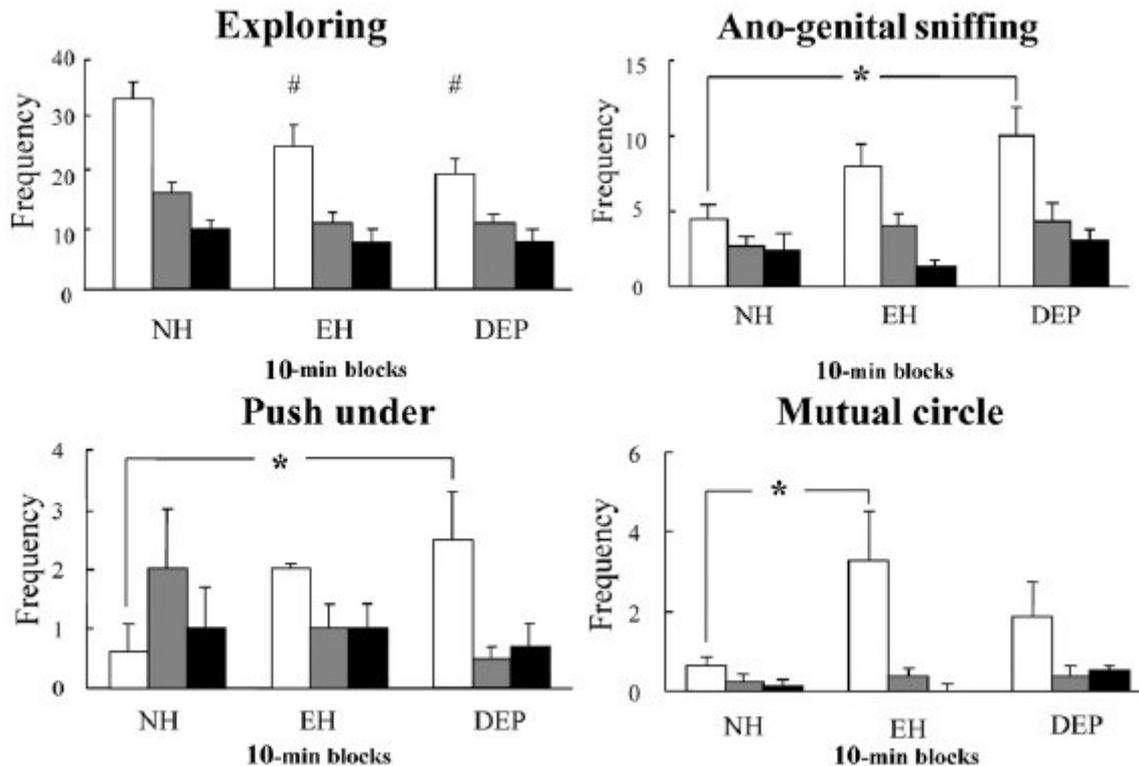
nucleus. Post hoc comparison revealed densities of PV positive neurons had increased (30%) in the lateral nucleus of both in EH and DEP rats compared with NH control rats ( $P=0.028$ ; Fig. 4). CR and CB neuronal densities did not change in any nucleus of the amygdala analyzed (not shown).



**Fig. 4.** Effect of EH, DEP and NH on PV immunoreactive cells in the basolateral amygdala. (A) Cresyl Violet staining (A) shows the boundaries of basal (Ba) and lateral (La) nuclei where PV positive cells (B) were quantified. The density of PV positive cells is increased in lateral nucleus of EH and DEP rats (C). Data are mean density of labeled cells per mm<sup>2</sup> (+S.E.M.). \* Significant difference from NH. Scale bars=200  $\mu$ m; inset, 30  $\mu$ m.

### Social interaction test

To assess whether neurochemical changes induced by maternal separation in stressor-sensitive brain regions were paralleled by behavioral effects, age-matched rats undergoing handling, deprivation or non-handling were tested for anxiety in the social interaction test. Social behaviors in adolescent male rats were affected by maternal separation (Fig. 5). A significant interaction between rearing condition and time blocks was found when investigative affiliative and play soliciting elements were analyzed. In particular, DEP subjects showed an increase of frequency of ano-genital sniffing ( $F(4,42)=2.53$ ;  $P=0.05$ ) while handling procedures resulted in a greater number of mutual circle episodes ( $F(4,42)=2.68$ ;  $P=0.04$ ) both during the first time block and when compared with the NH group (Tukey HSD  $P<0.01$ ). Furthermore, ANOVA revealed a condition per time blocks effect on push under behavior. In the first time block, in fact, DEP subjects performed both greater frequency ( $F(4,42)=4.38$ ;  $P=0.01$ ) and duration ( $F(4,42)=2.66$ ;  $P=0.048$ ) of push under behavior when compared with the NH group. A condition per time-blocks effect was also found in the allogrooming frequency ( $F(4,42)=3.51$ ;  $P=0.01$ ); post hoc comparisons showed that EH subjects performed higher grooming frequency when compared both to the DEP and the NH groups (Tukey HSD: handled vs. control  $P<0.01$  and handled vs. deprived  $P<0.05$ ). One of the main effects of deprivation was found in



**Fig. 5.** Effects of early manipulations on social behavior. On PND 35 all subjects underwent a 30 min period of social interaction with a same sex, same age and strain unfamiliar partner. Each session has been divided into three time blocks (of 10 min each). Data are mean occurrences of behaviors (+S.E.M.) in each time block. Inter- (\*) and intra-group (#) comparisons are shown.

nonsocial behaviors: the time spent by DEP animals in exploring activity was significantly lower than in the NH group ( $F(2,21)=6.17$ ;  $P=0.01$ ) and both deprived and EH subjects showed a lower number of exploring episodes when compared with this group ( $F(2,21)=5.63$ ;  $P=0.01$ ). No differences were found among the three rearing condition groups when follow, social inactive, social rest, social sniff, crawl over, rough-and-tumble play, digging, self-grooming and inactive were analyzed. The element crawl under was expressed too rarely and was therefore discarded from ANOVA.

## DISCUSSION

### DEP decreases CBP immunoreactive neurons in the hypothalamic paraventricular region

The PVN is a core component of the HPA axis. Upon stimulation by stress, neuroendocrine neurons in the PVN regulate glucocorticoid secretion via release of CRF. CRF release is modulated by stressor-sensitive forebrain regions through interaction with GABA, glutamate and peptide-containing neurons located in the preoptic area and the hypothalamus (Herman and Cullinan, 1997; Herman et al., 2002b; Jessop, 1999). Previous studies have shown that EH decreases while DEP increases expression levels of paraventricular CRF (Avishai-Eliner et al., 2001; Husum and Mathe, 2002; Fenoglio et al., 2004; Ladd et al., 2005). Similarly, EH and DEP exert different effects on CBPs in the hypothalamic PVN area, since we found that DEP

decreased the density of CR and CB containing neurons, compared with EH rats, which did not differ from NH controls.

Our results also indicate that some CR and CB positive neurons express GABA and SRIF, thus suggesting that they contribute to the PVN local inhibitory system which regulates CRF secreting neurons (Herman et al., 2002a,b; Jessop, 1999; Miklos and Kovacs, 2002). This is further suggested by the presence of CB positive processes juxtaposed to CRF-labeled elements. Moreover, we found some CRF/CB double-labeled cells in the PVN. Whether CBPs also colocalize with local glutamatergic regulatory neurons (Herman et al., 2002b) is at present still unknown. Considering that CBP content may influence firing and synaptic plasticity (Gall et al., 2003; Gurden et al., 1998; Schiffmann et al., 1999; Schurmans et al., 1997; Schwaller et al., 2002), we suggest that the decrease in CR and CB in the PVN of DEP rats alters local regulatory signaling and function of CRF-containing neurons.

### **Maternal separation differently affects CR, CB and PV in hippocampal fields**

In the hippocampus, we have shown that maternal separation differently affects CBP-positive interneuronal subpopulations. In fact, the densities of CR and CB containing neurons increased in the hippocampus of both EH and DEP rats compared with NH rats while the density of PV containing neurons did not change in any experimental group. These effects are region specific because CR and CB increased only in the Ammon's horn and not in the dentate gyrus. While the effects of maternal separation on CR were uniformly distributed in the hippocampal Ammon's horn, the effects on CB positive cells seemed to be more marked in specific CA layers.

In the hippocampus, CR, CB and PV are associated to largely non-overlapping subsets of GABAergic interneurons that control hippocampal output (Freund and Buzsaki, 1996). CB is also expressed by some non-GABAergic hippocampal neurons (Freund and Buzsaki, 1996). Colocalization of CB and CR had been previously detected in the stratum oriens (Wouterlood et al., 2001), suggesting that the increase in these two CBPs in this layer might reflect changes of the same neuronal population. By contrast, the increase in CB and CR in the other layers of the Ammon's horn, where colocalization of these proteins is rare (Wouterlood et al., 2001), is likely to represent an effect of maternal separation on different neuronal populations. Finally, the increase in CB in the pyramidal layer of EH rats, where this protein also labels some pyramidal cells in the CA1 subfield (Freund and Buzsaki, 1996), suggests that maternal separation might also affect CBP positive principal neurons.

Previous studies showed that maternal separation can induce alterations of the cytoarchitecture and neurochemistry of the hippocampus (Vaid et al., 1997; Matthews et al., 2001; Huot et al., 2002; Husum and Mathe, 2002; Lehmann et al., 2002; Mirescu et al., 2004). In particular, the population of nitric oxide-producing neurons in the Ammon's horn, which significantly overlaps with CR and CB containing neurons (Megias et al., 1997; Jinno and Kosaka, 2002), is increased in EH rats (Vaid et al., 1997) suggesting a relationship between CBPs and the hippocampal NO-system.

The increase in CR and CB positive neurons in EH and DEP rats might represent a differential response to increased growth factors expression. In fact, maternal separation can induce the expression of nerve growth factor

and brain-derived neurotrophic factor (BDNF) in different brain regions, including the hippocampus, in preweaning and adolescent rats (Cirulli et al., 2003; Kuma et al., 2004; Roceri et al., 2004). Moreover, BDNF supports differentiation of CR and CB immunoreactive nonpyramidal neurons in organotypic slice cultures from the early postnatal rat hippocampus (Marty et al., 1996).

### **Maternal separation increases the density of PV-positive interneurons in the lateral amygdala**

The amygdala is a brain region involved in the activation of the HPA axis and implicated in the control of emotional behavior (Herman and Cullinan, 1997; LeDoux, 2000). Subpopulations of GABAergic neurons in the amygdala express different CBPs and neuropeptides (Yilmazer-Hanke et al., 2002). We demonstrated that the density of PV containing neurons was significantly increased in the lateral nucleus of the amygdala of both EH and DEP rats compared with NH rats. This suggests an increase in the GABAergic inhibitory tone. In fact, PV-positive neurons in the basolateral complex are interneurons receiving excitatory synaptic input from amygdaloid projections and form local inhibitory circuits on pyramidal projection neurons (Smith et al., 2000). Accordingly, GABAergic tone in the basolateral amygdala can reduce emotional arousal in the social interaction test (Sanders and Shekhar, 1995a,b; File and Seth, 2003) and increased PV neuronal densities in the basolateral complex have been previously implicated in reduced anxiety behavior in the motility test (Yilmazer-Hanke et al., 2002). Thus, the changes of GABAergic circuits involving PV positive interneurons in the lateral nucleus correlate with the expression of reduced anxiety we observed in EH and DEP rats (see below).

It is important to consider that modifications of CBPs described in all the regions studied may have resulted from either changed protein expression or number of cells. This latter possibility is consistent with the effects exerted by maternal separation on hippocampal cell proliferation (Mirescu et al., 2004) and with the existence of postnatal neurogenesis in several CNS regions including the hypothalamus (Kokoeva et al., 2005).

### **EH and DEP are less anxious in the social interaction test**

In order to support the functional relevance for the changes of CBPs in stressor-sensitive brain regions analyzed, in parallel we tested age-matched maternally separated rats in the social interaction test at adolescence. This test is an ethologically based test of anxiety (File and Seth, 2003) which is also used to study the behavioral effects of neurotransmission in the basolateral amygdala of rodents (Sanders and Shekhar, 1995a,b; Sajdyk et al., 1999a,b; File and Seth, 2003). Changes in social behavior are easily identifiable at adolescence, a critical period for the emergence of affiliative-agonistic behavior, spanning in rodents from PND 35- 40 (Terranova et al., 1999; Spear, 2000) and characterized by dramatic rearrangements in brain structure and function (Spear, 2000). Moreover, early brain dysfunctions may predispose for late development of psychological disorders with symptomatology typically increasing during adolescence (Spear, 2000). We found that both EH and DEP periadolescent subjects exposed to a social challenge readily engaged in social

interactions with an unfamiliar conspecific, while controls were more prone to explore the cage rather than the unfamiliar partner, especially at the beginning of the session. Higher frequency of play soliciting behaviors, such as mutual circle and push under, characterized both EH and DEP subjects, indicating reduced neophobia in a social situation. These data are consistent with previous findings in periadolescent mice undergoing the same separation protocol (3 h from PND 2–14) (Venerosi et al., 2003) which showed higher levels of aggressive social interactions, compared with unmanipulated controls, an effect that could depend upon reduced social phobia by DEP subjects (Venerosi et al., 2003). These data overall suggest that early maternal separations reduce emotionality in both rats and mice during the critical period of periadolescence.

Results from the present work and other studies point out that manipulations of the mother-infant interaction can induce in the offspring neurochemical changes in various brain regions that might result in long term effects on behavior (Cameron et al., 2005). Here, we show that both DEP and EH lead to changes of CBP containing neurons in brain areas involved in the regulation of the HPA axis and stress response, thus confirming and extending the role played by early experiences in shaping the development of neuronal circuits involved in emotional response. Despite the appealing associations we have found, especially between amygdalar PV-positive neurons and reduced neophobia, a causal relationship will require future designed functional studies.

We cannot exclude that CBP changes at periadolescence could be a consequence, and not a cause, of early modifications in the neuronal populations analyzed. However, we suggest that the differences in the CBPs between experimental groups are a long-term effect of the rearing condition, not the effect of contingent changes in behavior at periadolescence. Indeed, this view is supported by previous data showing that maternal separation may impact CR and CB brain content in rats very early during postnatal development (Lephart and Watson, 1999).

In this study we found some differences between EH and DEP rats (CBPs in the PVN region) but our data also indicate that these two manipulation procedures can lead to similar neuroanatomical (CBPs in hippocampus and amygdala) and behavioral (social interaction test) outcomes at periadolescence. This is apparently in contrast to several studies suggesting that brief versus long maternal separations result in different effects, respectively decreasing or increasing fear-related behavior and endocrine responses to stress (Plotsky and Meaney, 1993; Francis and Meaney, 1999). It must be pointed out, however, that the dichotomy long vs. brief maternal separation does not always hold (Pryce and Feldon, 2003). As an example, other reports have described that both handling and DEP have the same effects on the formation of neural circuits providing limbic and cortical control over autonomic emotional motor output (Card et al., 2005). In addition, data in the literature suggest that both manipulations are able to stimulate maternal care to a similar degree (Pryce et al., 2001; Lonstein et al., 1998). An interesting finding is that the lack of any manipulation (NH) appears to be the most dramatic form of deprivation. Considering that early postnatal non-handling may implicate a general chronic state of under-stimulation (Pryce and Feldon, 2003) and can induce some neurochemical changes in the brain comparable to those induced by prenatal stress (Vaid et al., 1997), our results

may also be interpreted as a detrimental effect of non-handling. Indeed, it has already been suggested that under-stimulation during infancy is rather detrimental, resulting in increased stress responsiveness and emotionality at adulthood (Levine et al., 1957).

Thus, we suggest that insufficient stimulation during early postnatal life (non-handling) may impair the development of CBP-containing neuronal populations in different brain regions, possibly leading to consequences on behavioral plasticity.

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