Juvenile Emotional Experience Alters Synaptic Inputs on Pyramidal Neurons in the Anterior Cingulate Cortex

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Analogous to the experience-driven development of sensory systems, the functional maturation of limbic circuits is significantly influenced by early socio-emotional experience. In a combined light and electron microscopic study in the anterior cingulate cortex of *Octodon degus*, the densities of spine and shaft synapses on apical dendrites of layer III pyramidal neurons were compared in 45 day old (1) undisturbed control animals; (2) handled animals; (3) animals which were repeatedly maternally deprived during the first three postnatal weeks; (4) animals which were treated similarly to group 3 and thereafter kept in chronic social isolation. Animals in groups 2-4 showed significantly higher spine densities (up to 121%, 142% and 151% respectively) compared to control group 1. Group 3 displayed significantly longer apical dendrites compared to control group 1. The electron microscopic analysis in cortical layer II revealed significantly higher spine synapses in group 4 (up to 166%) and fewer shaft synapses in groups 3 and 4 (down to 53% and 65% respectively) compared to group 1. These results demonstrate that early traumatic emotional experience alters synaptic input of pyramidal neurons. Such experience-induced modulation of limbic cortex development may determine psychosocial and cognitive capacities during later life.

Introduction

It is generally accepted that environmental influences during certain sensitive periods of early postnatal life have a strong impact upon later development and behavior (Parker, 1993; Parker *et al*., 1995, 2000); however, the mechanisms whereby early experience induces such long-lasting and perhaps permanent effects upon behavior are still unclear. Variations in environmental complexity have been shown to induce changes in the anatomy of the brain (Walsh, 1981; Rosenzweig and Bennett, 1996; Schrott, 1997; Joseph, 1999), and such environmentally- and experience-induced changes of synaptic wiring patterns most likely accompany and modify behavioral development. An impoverished environment during early developmental stages results in decreased brain size, cortical thickness (Walsh, 1981; O'Kusky and Colonnier, 1982; Rosenzweig and Bennett, 1996; Schrott, 1997), dendritic tree complexity (Walsh, 1981; Rosenzweig and Bennett, 1996; Schrott, 1997; Jarvinen *et al*., 1998) and neuron numbers (O'Kusky and Colonnier, 1982), and at the synaptic level such chronic deprivation alters synaptic densities and ultrastructure (O'Kusky and Colonnier, 1982; Joseph, 1999). In most of these studies (Black *et al*., 1989; Martin *et al*., 1991; Rosenzweig and Bennett, 1996; Herschkowitz *et al*., 1997) the environmental changes were induced around the time of weaning from the mother, and the morphological changes were analyzed in sensory or motor cortical regions, thus mainly reflecting the effects of sensory stimulation or motor activities on cortical development. It appears likely that, analogous to this experience-driven development of sensory and motor systems, the functional maturation of higher associative cortical regions, in particular those that are part of the limbic system, is strongly influenced by emotional experience, especially when it occurs immediately after birth.

The most important environmental factor for the newborn individual is its interaction with its parents, leading to the earliest emotionally modulated learning process in life, which is classically termed filial imprinting (Lorenz, 1935; Gray, 1958). The parents, in particular the mother, control the infant's external environment, and a variety of physiological mechanisms in the infant respond to specific cues during the parent–infant interaction (Rosenfeld *et al*., 1991), which are critical for physical as well as psychological maturation. The critical impact of the early socio-emotional environment on behavioral development is well documented by observations from clinical studies, which showed that a disturbance or interruption of the child–parent interaction leads to the so-called 'hospitalism syndrome' (Spitz, 1945) and later can result in severe and permanent deficits in speech behavior (Brodbeck and Irwin, 1946), personality development (Lowrey, 1940; Parker, 1993; Parker *et al*., 1995, 2000), intellectual and social capacity (Skeels, 1966) and mental disturbances (Agid *et al*., 1999; Furukawa *et al*., 1998, 1999). Parallel experimental studies in non-human primates and in rodents revealed strikingly similar behavioral disturbances after different manipulations of the socio-emotional environment early in life (Joffe and Levine, 1973; Suomi, 1997; Anisman *et al*., 1998; Hall, 1998).

The remarkable stability of these deprivation-induced behavioral abnormalities may reflect dysfunctions of limbic circuits. We postulate that juvenile positive (formation of emotional attachment) or negative (e.g. maternal separation or loss) emotional experience may carve a permanent trace into a still developing neuronal network of immature synaptic connections, and thereby can extend or limit the functional capacity of the brain during later stages of life. Following this hypothesis we investigated in a newly established animal model, the precocious species *Octodon degus*, whether and in which way changes of the socio-emotional environment interfere with the development and refinement of synaptic input in a limbic cortical area, the anterior cingulate cortex (ACd).

Materials and Methods

Animals

The degus (Fig. 1) were bred in our colony at the Leibniz Institute for Neurobiology Magdeburg. Family groups consisting of an adult couple and their offspring were housed in standard rat cages (length × height × depth: 53 × 24 × 32 cm) and exposed to a light/dark cycle with 12 h light. Fresh drinking water and rat diet pellets, vegetables and fruits were available *ad libitum*. The rooms were air-conditioned with an average temperature of 22°C.

All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and according to the German guidelines for the care and use of animals in

Figure 1. A 2 day old *Octodon degus* pup.

laboratory research and the experimental protocols were approved by an ethical committee.

For light microscopic (LM) and electron microscopic (EM) analysis a total of 36 male degu pups, derived from eight (LM) and nine (EM) different litters, were analyzed at postnatal day (P) 45. The animals were divided into the following groups, the described manipulations were performed on all pups of a litter.

Group 1

Social controls: these pups were raised undisturbed under social conditions in their families (LM: $n = 4$; EM: $n = 6$).

Group 2

Handling controls: the animals were handled three times per day from P1 to P21 (the day of birth was defined as P0), handling consisted of briefly lifting each individual pup up for several seconds without removing it from the home cage, and then gently placing it back to the cage floor. After weaning at P22 these animals remained with their families until P45 (LM: $n = 4$; EM: $n = 4$).

Group 3

Maternal separation: these pups were exposed to repeated maternal separation from P1 to P21. For separation the pups were removed from the parents and siblings and kept individually in small rodent cages (length × height × depth: 37 × 11 × 8.5 cm) for 1 h, this procedure was repeated three times per day. During separation no visual, but auditory and olfactory contact to the siblings was allowed. From P22 to P45 the animals remained undisturbed with their families (LM: $n = 3$; EM: $n = 4$).

Group 4

Extended deprivation: these pups were exposed to repeated maternal separation as described for group 3 from P1 to P21. After weaning (P22) they were individually housed in opaque standard rat cages, where they had olfactory and acoustic, but no visual or physical contact to conspecifics (LM: $n = 4$; EM: $n = 5$).

Light Microscopy

After decapitation the unfixed brains were impregnated in Golgi solution for 14 days (Bock and Braun, 1999), sectioned at 150 μm and developed using a modified Golgi–Cox technique (Glaser and Van der Loos, 1981). Spines and dendritic lengths were measured using an Olympus BH-2 microscope at a final magnification of ×1000.

Pyramidal neurons (Fig. 2) in the pregenual ACd were analyzed using the image analysis system Neurolucida™ (MicroBrightField, Inc., Colchester, USA), which allows a quantitative three-dimensional analysis of microscopic images. For each animal and hemisphere five neurons whose cell bodies were located in the center of the 150 μm sections were selected for analysis, resulting in 30–40 pyramidal cells per experimental group. Only neurons that were impregnated in their entirety and displayed complete dendritic trees within the 150 μm section, and whose somata were located in layer III (Fig. 2*A*,*B*), were selected. An additional morphological criterion was the ramification pattern of their apical dendrite (cf. Fig. 2*B*): only neurons that had already started to ramify in layer II, which was analyzed in the EM part of this study, were selected. For each neuron the lengths and spine densities of the complete apical and basal (data not shown here) dendrites were analyzed. All protrusions, thin or stubby, with or without terminal bulbous expansions, were counted as spines if they were in direct continuity with the dendritic shaft. The different branches of the dendritic trees were numbered consecutively from proximal to distal. The average spine frequency (number of visible spines per 10 μm dendritic length) was calculated (i) for the complete dendrite and (ii) for branching orders 1–5 (Fig. 4*B*) by dividing the number of spines by the dendritic length. An average of 80 000 spines per group were counted. An attempt to correct for hidden spines (Feldman and Peters, 1979) was not made, since the use of visible spine counts for comparison between different experimental conditions had been validated previously (Horner and Arbuthnott, 1991).

The neurons in the left and right hemisphere were analyzed separately; since no significant differences were observed, the values for both hemispheres were pooled for statistical analysis.

Electron Microscopy

Perfusion and Fixation

Degus were deeply anesthetized with an i.p. injection (0.5 ml/100 g body wt) of Ketanest and Rompum 1:4 (Bayer AG, Leverkusen, Germany) and then transcardially perfused with 30 ml Tyrode buffer containing 1% Liquemin (Hoffman-La Roche AG, Germany), followed by 400 ml of a mixture of 2% paraformaldehyde and 3% glutaraldehyde fixative. The brains were removed and placed in the same fixative overnight. Vibratome sections (90 μm thick) were prepared for electron microscopy by postfixing in 1% osmium tetroxide for 1 h, dehydrated by a series of graded alcohols, and embedded in Spurr's resin (Ted Pella, Inc., USA). Layer II of the ACd was identified by LM observation of osmium-stained sections by its darker appearance compared to the adjacent layer I. Layer II was chosen for the EM analysis because the apical dendrites of the layer III pyramidal neurons, which were analyzed in the LM part of this study, ramify in this layer. A random block of the left hemisphere from each animal containing layer II of the ACd subregion was used to calculate synaptic density. Ultrathin sections of silver interference color were cut with a diamond knife (Diatome) using a microtome RMC with digital control setting and corrected with parameters suggested by Evans and Howard (Evans and Howard, 1989). Serial sections were collected on single-slot grids coated with Formvar support film (Plano, Wetzlar, Germany).

Quantitative Microscopy

We applied the disector method (Sterio, 1984; Gundersen, 1988) since it has been proposed as a standard technique for quantitative electron microscopy (Guillery and Herrup, 1997; Saper, 1997). According to Calverley *et al*. (Calverley *et al*., 1988) the numerical density of synaptic profiles is not significantly different when measured by the 'disector' or the 'unfolding' method, and similar findings were reported for the 'size–frequency' method (formula N_a/d) (De Felipe *et al*., 1999). The disector technique allows one to calculate the number of synaptic profiles per volume, and to estimate object numbers without the influence of section thickness, provided that this does not exceed one-quarter to one-third of the mean particle height (Gundersen *et al*., 1988b). Section thickness was estimated from the interference color of the sections from completely regular series (Guillery and Herrup, 1997), and, in addition, for some sections the thickness was reconfirmed by measurement after re-embedding (de Groot, 1988). From the pairs of serial sections, which were mounted on the same slot grid, one section was chosen at random (the nominated section) and the adjacent section became the reference section. The field of view was placed on a conspicuous object, e.g. a mitochondrion in the top left corner of the nominated section. Twenty-four fields, containing only neuropil and no somata, were selected for the sections from at least three grids at a magnification of

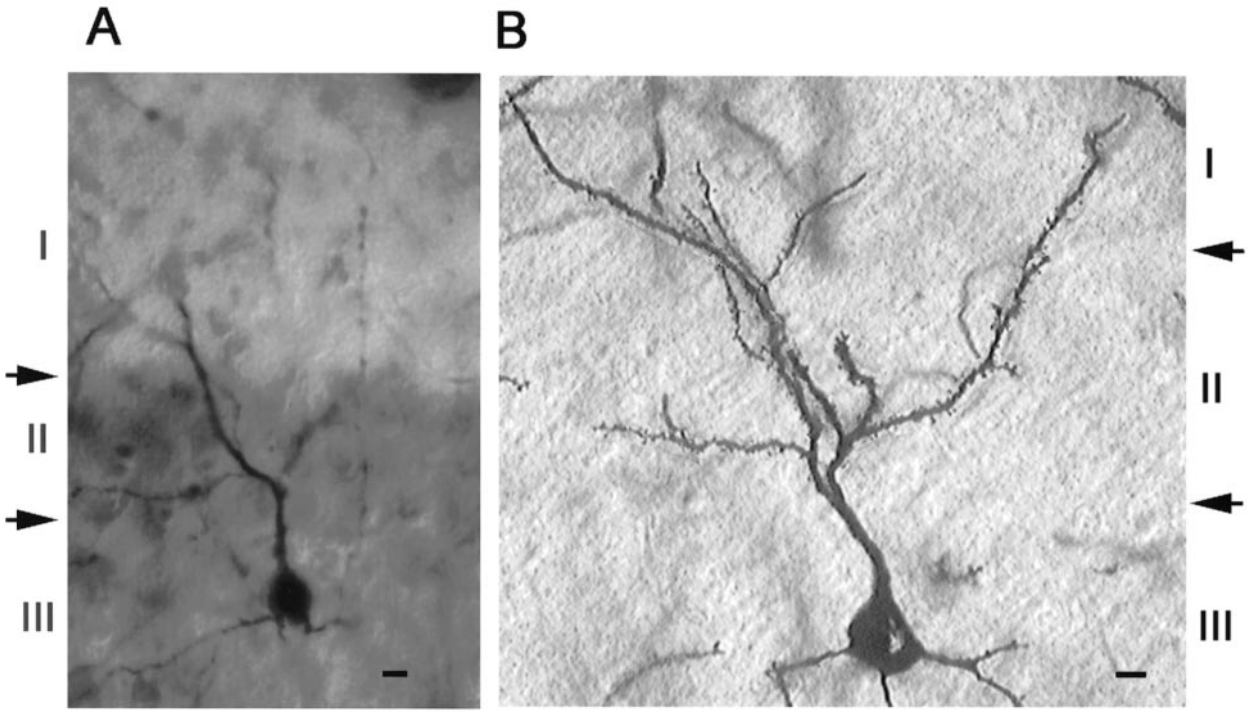

Figure 2. (*A*) Low-power micrograph of a pyramidal neuron in the anterior cingulate cortex, illustrating the demarcation between the cortical layers I, II and III in a section which was counterstained for Nissl substance with cresyl violet. Due to the section thickness (150 μm) the Golgi-impregnated neuron is not entirely in focus. Scale bar = 12 μm. (*B*) Photomontage of a Golgi–Cox impregnated pyramidal cell of similar morphological type as the neuron shown in (*A*) of the ACd. The soma is located in layer III and the spiny apical dendrite ramifies in layers I and II (top of the figure). Due to the section thickness the dendrites, in particular the basal dendrites are not entirely in focus. Scale bar = 7 μm

3000× and micrographs were taken at 4150× using a high-resolution slow scan camera (Gatan, Inc., Pleasanton, USA) and DigitalMicrograph 3.3. software (Gatan, Inc.). This sampling resulted in 48 micrographs for pairs/grid, which were then enlarged twofold by software to improve visual acuity of the structures. Unbiased counting frames were placed on the micrographs for synaptic counts, and an average of 100 disector scores were made to obtain reliable estimates of a (Q^-) (Gundersen and Jensen, 1987). Synaptic profiles were counted when they were present in the nominated section and absent in the reference section. Identification of synaptic profiles was made on the basis of (i) an apposition of pre- and postsynaptic densities, (ii) a visible synaptic cleft and (iii) vesicles situated near the presynaptic membrane or at presynaptic dense projections. This definition excluded axons, axonal growth cones and axon varicosities apposed to neuronal surfaces without any presynaptic specialization from quantitative analysis. Depending on the postsynaptic target site, synapses were further subdivided (Peters *et al.*, 1991) into shaft synapses, i.e. terminating directly on the dendritic shaft (compare Fig. 3*C*) and spine synapses, i.e. small spherical postsynaptic protrusions of the dendrite (cf. Fig. 3*C*) filled with a fluffy material, which consists of fine and indistinct filaments, without microtubules and neurofilaments and sometimes with a visible spine apparatus. On the basis of the disposition of the cytoplasmatic density on each side of the synaptic junction (Gray, 1969), synapses were classified into symmetric (presumed inhibitory) or asymmetric (presumed excitatory) synapses (Colonier, 1968). Asymmetric synapses resembling so-called perforated synapses were rare and therefore not counted separately. In cases where a clear classification of synapse type could not be achieved in the disector section pair, confirmation was achieved on the adjacent sections and sometimes on the next pair. Calculation of mean synaptic numerical density (N_v syn) per μm^3 was done using the following formula:

$$N_v \text{ syn} = \Sigma Q^- \text{ syn}/A_t$$

where ΣQ^- syn is the total number of counted synaptic profiles that appear only in the nominated section, t is the section thickness, and A is the area of the counting frame. The area of the counting frame and the separation of disector were corrected for magnification (previously checked with grating replica, 2160 lines/mm). Separate counts were made for morphological subtypes of synapses. Additional measurements were taken for the size of the postsynaptic density, the mean projected synapse height (Calverley *et al.*, 1988) according to the formula:

$$H \text{ syn} = (\Sigma Q \text{ syn}/\Sigma Q^- \text{ syn})t$$

ΣQ syn is the total number of synaptic profiles in the nominated and reference fields. For more detailed description of the mathematical background for this formula can be found elsewhere (Sterio, 1984; Gundersen, 1988).

Numerical densities of synapses were only measured in tissue containing neuropil and indicated as number of profiles per 1 μm^3 area. Neuropil was defined as a portion of tissue that does not include the lumen and wall of blood vessels and any cell nuclei. The sum of area (neuropil) per animal, which was measured in μm^2 from micrographs, was further applied to the formula for N_v syn to give the stereologically correct density in μm^3. Measurements of synapse height were made from all the micrographs used for counting the synaptic density, but no distinction between morphological subtypes was attempted.

All data were loaded onto a Macintosh computer and synaptic profiles were counted using NIH Image software.

Statistical analysis for the LM and EM data was carried out using SIGMA-STAT software (Jandel Scientific, Erkrath, Germany). For comparison among all four experimental groups, a Kruskal–Wallis ANOVA test was performed; if the difference was significant (the significance level was set at $P \leq 0.05$), the Mann–Whitney *U*-test was used for pairwise comparisons. All quantifications were performed by an experimenter unaware of the experimental condition of the animals.

Brain and Body Weights

As control measurements the body weights (n = 111, which includes data from animals used for parallel studies) and fresh brains (n = 25, which includes the brains used for this Golgi study and from animals from

parallel studies) were taken. Cortical thickness of the ACd (Fig. 3*A*) was measured in Nissl-stained serial sections from perfused animals (n = 15, which includes data from animals used for parallel studies). For each animal and hemisphere measurements along the lines 'a' and 'b', were made according to Uylings *et al*. (Uylings *et al*., 1990) and Poeggel *et al*. (Poeggel *et al*., 1999) (see also Fig. 3*A*), for the entire cortical thickness, and measurements of layer II were taken from five 50 μm thick sections, 200 μm apart; the respective values were averaged and compared between the experimental groups. Layer II was distinguished by its darker staining, caused by the more densely packed Nissl stained nuclei and cell bodies, compared to layers I and III (Fig. 3*B*). Shrinkage of tissue was assumed to be identical since the fixation protocol was the same for all animals.

Results

The LM analysis of spine densities on the apical dendrites of layer III pyramidal neurons revealed increased spine densities after all three environmental manipulations (groups 2–4) compared to the undisturbed normal control group 1 (Fig. 4*A*,*B*). The EM analysis revealed no significant differences of the overall mean synaptic density (taking spine and shaft synapses together) between the four animal groups (Fig. 5*A*). When synapses were divided according to their postsynaptic target into spine and shaft synapses, the density of shaft synapses in the normal control animals (group 1) was higher than that of spine synapses (Fig. 5*A*). This balance between shaft and spine synapses was altered by the two maternal separation paradigms applied in groups 3 and 4, whereas the size of synaptic profiles, measured as synaptic height, was not affected by any treatment (Fig. 5*C*).

Effect of Handling (Group 2)

On the LM level, handling induced significant differences of apical spine densities compared to undisturbed normal control animals (group 1; Fig. 4*A*,*B*). Comparing the spine densities of the complete apical dendrites, the handled pups displayed higher spine densities (up to 120.9%; $P < 0.001$) than normal control pups (Fig. 4*A*). Branching order analysis showed higher spine densities in the handled pups on the third to the fourth apical branching orders compared to social controls (Fig. 4*B*). On the EM level no differences of shaft synapses and a non-significant trend towards increased spine synapse density was found in the handled animals (group 2) compared to the normal control group 1 (Fig. 5*A*). Handling did not affect dendritic length (Fig. 4*C*,*D*).

Effect of Repeated Maternal Separation (Group 3)

The LM analysis revealed that repeated maternal separation during the first three postnatal weeks lead to significantly higher spine densities on the complete apical dendrite (up to 142%; $P < 0.001$) compared to normal control animals (Fig. 4*A*). Branching order analysis revealed higher spine densities on the second to the fifth apical branching orders in the separated pups (Fig. 4*B*). The EM analysis revealed a significant decrease of shaft synapses (P = 0.01, down to 53%) after repeated maternal separation (group 3) compared to the normal controls (group 1) (Fig. 5*A*); however, the increase of spine density, which was seen in the LM part of the study in group 3 compared to group 1, was not significant on the EM level.

Compared to the handling controls (group 2) the LM analysis revealed significantly higher spine densities on the complete apical dendrites (up to 117.5%; P = 0.005) (Fig. 4*A*) in group 3. Branching order analysis, comparing groups 2 and 3, revealed in group 3 elevated spine densities along the third to the fifth apical branching order compared to group 2 (Fig. 4*B*). In contrast, the EM comparison between handling controls (group 2) and group 3 revealed no significant differences in spine or shaft synapse densities (Fig. 5*A*).

Dendritic length of the complete apical dendrites was significantly increased after maternal separation (group 3, up to 128.9%; P = 0.002; Fig. 4*C*) compared to normal controls (group 1). Branching order analysis showed significantly elongated fifth branching order segments in the pups of group 3 compared to normal controls (Fig. 4*D*). The comparison between the maternally separated (group 3) and handled (group 2) pups revealed no difference in the complete apical dendritic lengths (Fig. 4*C*), but branching order analysis showed elongated first and fifth branching order segments in group 3 (Fig. 4*D*).

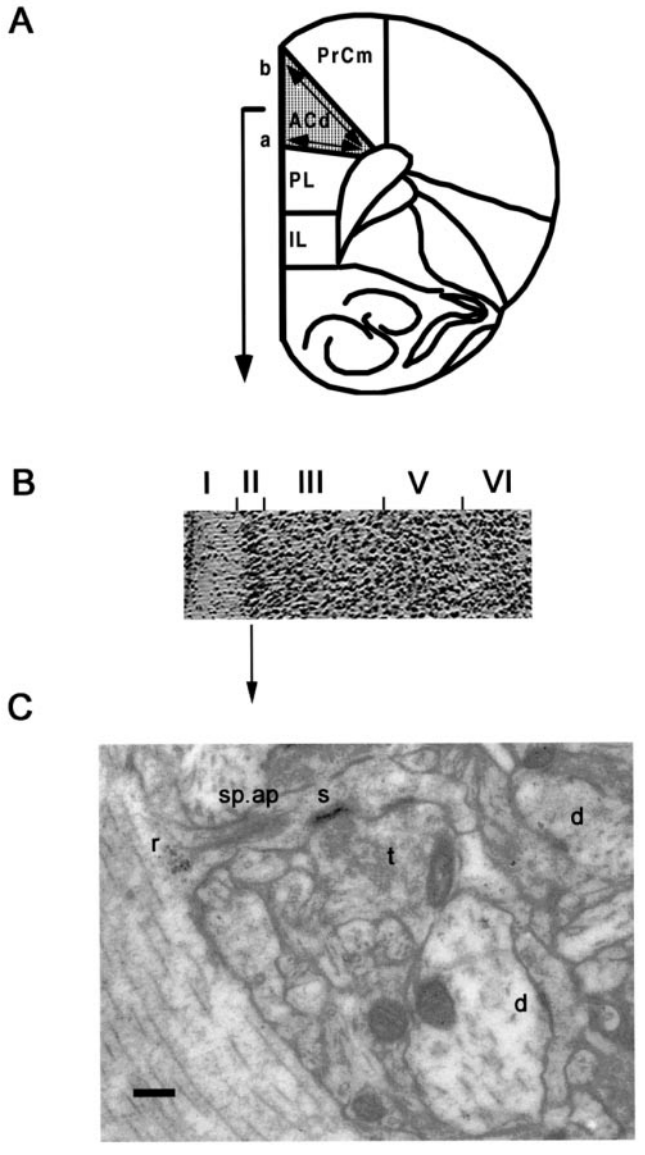

Figure 3. (*A*) Schematic illustration of the location of the pregenual anterior cingulate cortex (ACd) in a frontal section. Measurements of thickness of the anterior cingulate cortex were taken along the lines 'a' and 'b'. (*B*) Cortical layers of the ACd are shown at higher magnification in a Nissl-stained 50 μm thick section. Layer II, which was analyzed in the EM study, is marked by lines. (*C*) The electron micrograph illustrates shaft and spine synapses in layer II of the ACd. Scale bar = 0.2 μm. d, shaft; t, terminal bouton; s, spine; sp.ap, spine apparatus; r, polyribosomes.

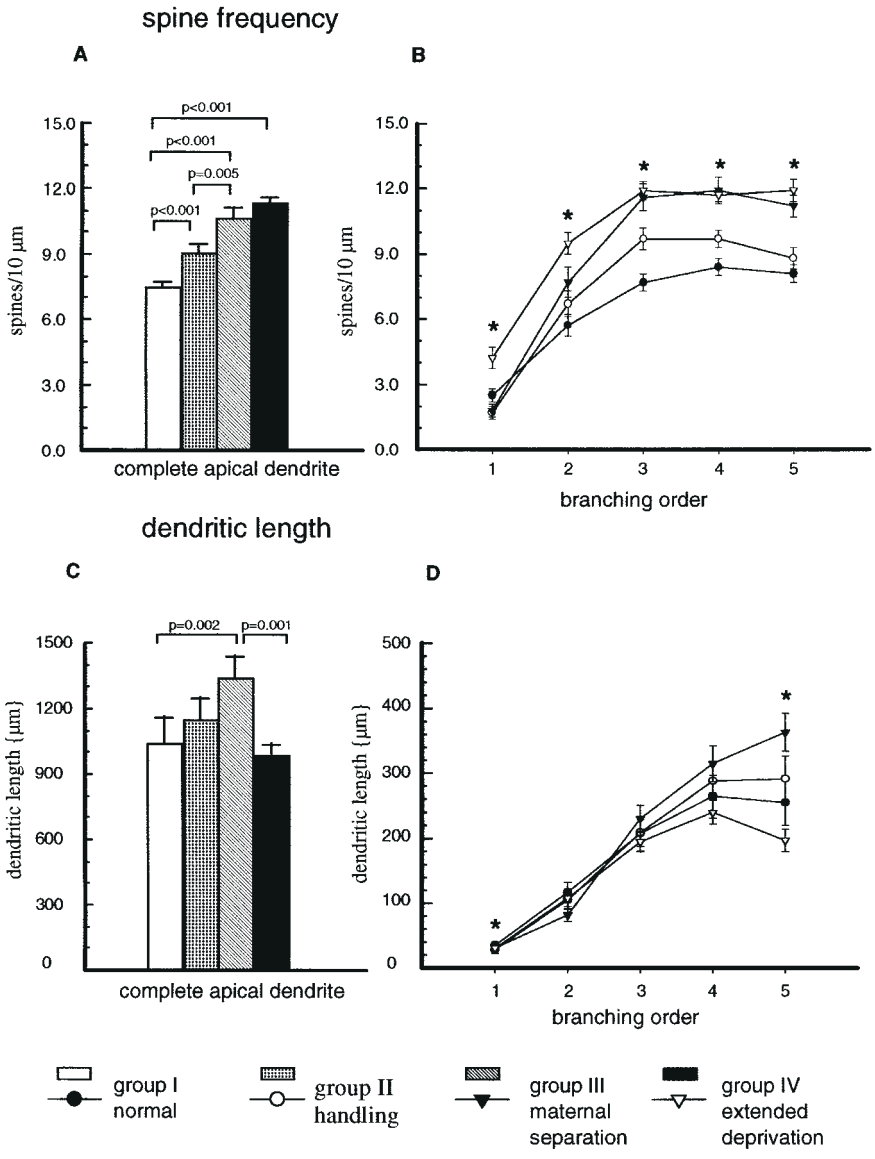

Figure 4. Changes of spine frequencies of layer III pyramidal cells of the ACd in response to different socio-emotional conditions, in (*A*) for the complete apical dendrite and in (*B*) for each apical dendritic branch separately. Bars and plots show mean values and SEM, *P*-values for Mann–Whitney *U*-test are indicated. (*B*) Analysis of spine frequencies for each apical dendritic branch. Asterisks (*) indicates $P < 0.05$ for the Kruskal–Wallis ANOVA test. The *P*-values for Mann–Whitney *U*-test for the pairwise comparison within the different branch orders are: first order: $1 > 2$ ($P = 0.032$), $1 < 4$ ($P = 0.011$); $3 < 4$ ($P < 0.001$); second order: $1 < 3$ ($P = 0.033$), $1 < 4$ ($P < 0.001$), $3 < 4$ ($P = 0.035$); third order: $1 < 2$ ($P = 0.004$), $1 < 3$ ($P < 0.001$), $1 < 4$ ($P < 0.001$), $2 < 3$ ($P = 0.009$); fourth order: $1 < 2$ ($P = 0.02$), $1 < 3$ ($P < 0.001$), $1 < 4$ ($P < 0.001$), $2 < 3$ ($P = 0.001$); fifth order: $1 < 3$ ($P < 0.001$), $1 < 4$ ($P < 0.001$), $2 < 3$ ($P = 0.002$). (*C*) Changes of dendritic lengths shown for the complete apical dendrite of layer III pyramidal cells of the ACd in response to different socio-emotional conditions. Bars and plots show mean values and SEM, *P*-values for Mann–Whitney *U*-test are indicated. (*D*) Analysis of dendritic length for each dendritic branch. Asterisks (*) indicates $P < 0.05$ for the Kruskal–Wallis ANOVA test. The *P*-values for Mann–Whitney *U*-test for the pairwise comparison within the different branch orders are: first order: $2 < 3$ ($P = 0.005$); fifth order: $1 < 3$ ($P = 0.014$), $2 < 3$ ($P = 0.033$), $4 < 3$ ($P < 0.001$).

Effect of Maternal Separation Followed by Post-weaning Chronic Social Isolation (Group 4)

Similar to the findings after pre-weaning maternal separation (group 3), this extended deprivation paradigm induced significantly higher spine densities on the complete apical dendrites (up to 151.4%; $P < 0.001$) compared to age-matched social controls (group 1) (Fig. 4*A*). Branching order analysis revealed this effect along the first to the fifth branching orders (Fig. 4*B*). In line with these LM findings the EM study revealed a significant increase ($P = 0.004$, up to 166%) of the density of spine synapses after extended deprivation (group 4) compared to the normal controls (group 1) (Fig. 5*A*). In addition, the density of shaft synapses was significantly decreased after extended deprivation (group 4) compared to the normal controls (group 1, $P = 0.03$, down to 65%) (Fig. 5*A*).

The light (complete apical dendrite) and electron microscopic comparison of synaptic densities between the two deprived groups (groups 3 and 4) revealed no significant differences for spine (Figs 4*A*, 5*A*) and shaft (Fig. 5*A*) synapses. However, LM branching order analysis showed elevated spine densities on the dendritic segments of the first two apical branching orders in group 4 compared to group 3 (Fig. 4*B*).

The lengths of the complete apical dendrites did not differ significantly between group 4 and the normal control group 1 (Fig. 4*C*). The comparison of the complete apical dendritic lengths between the two deprived groups (groups 3 and 4)

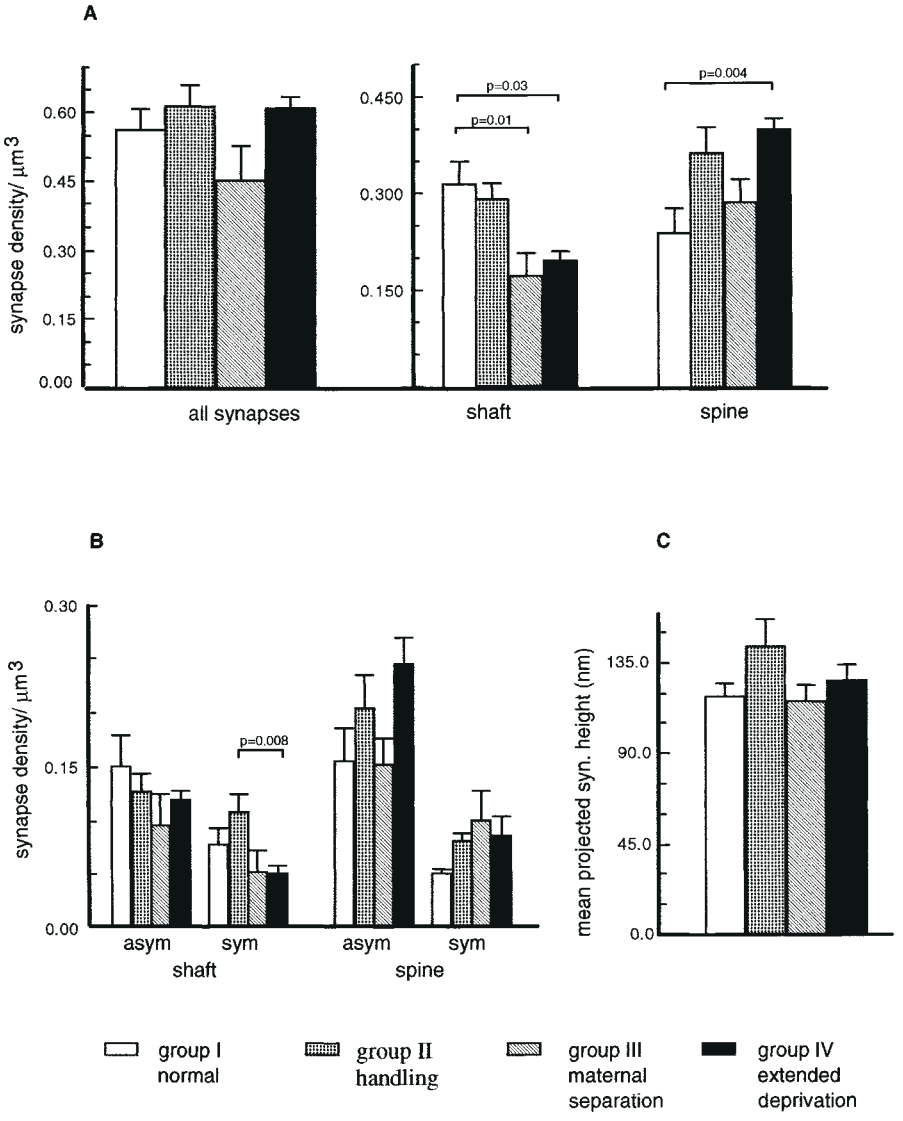

Figure 5. Electron microscopic analysis of synaptic changes in response to different socio-emotional conditions. (*A*) Taking all synaptic subpopulations together, no difference in the mean densities was found. Repeated maternal separation (group 3) and extended deprivation (group 4) induced a significant decrease of shaft synapses compared to normal control animals (group 1). The density of spine synapses was increased only after extended deprivation (group 4) compared to normal controls (group 1). (*B*) Decrease of symmetric shaft synapses after extended deprivation (group 4) compared to the handled controls (group 2). (*C*) No difference was observed in other synaptic subpopulations or in the mean projected height (i.e. size of terminal bouton).

revealed significantly longer (up to 137.1%; $P = 0.001$) dendrites in group 3 compared to pups of group 4 (Fig. 4*C*). Branching order analysis revealed that this effect is due to elongated dendritic segments of the fifth branching order in the animals of group 3 (Fig. 4*D*).

Body and Brain Weights

Significant differences between the four experimental groups were found in body ($P < 0.001$) and brain weight ($P = 0.018$). The body weights (mean values ± SE) of animals in group 3 (114.8 ± 2.2 g) were significantly higher than in group 1 (104.8 ± 1.6 g; $P < 0.001$), group 2 (100.8 ± 1.2 g; $P < 0.001$) and group 4 (103.7 ± 1.6 g; $P < 0.001$). The brain weights of animals in group 3 (1.9 ± 0.07 g) were significantly higher compared to group 4 (1.7 ± 0.02 g). Compared to group 1 (normal controls; 1.6 ± 0.1 g) no significant differences of brain weights were found in groups 2 (1.9 ± 0.05 g), 3 and 4.

Cortical Thickness

In normal animals (group 1) the mean values (± SE) were: vertical thickness (line 'a' in Fig. 3*A*) = 1316.8 ± 33.26 µm; diagonal thickness (line 'b' in Fig. 3*A*) = 2075.8 ± 35.96 µm and thickness of layer II = 42.8 ± 2.02 µm. No significant difference of the total vertical thickness of the ACd (layers I–VI; $P = 0.419$) and of the diagonal thickness of the ACd ($P = 0.091$) was seen between the four animal groups. Comparison of the thickness of layer II of the ACd revealed significant differences between the groups (Kruskal–Wallis $P = 0.029$). Layer II was significantly wider in animals in group II (65.6 ± 1.01 µm) compared to group 1 (normal controls, Mann–Whitney $P = 0.029$) and group 3 ($P = 0.029$; 46.1 ± 2.02 µm).

Discussion

Our previous results as well as the data presented here illustrate that *O. degus* provides an ideal model for investigating the

neuroanatomical and neurochemical basis of mother–infant attachment and the consequences of maternal separation and social isolation (Poeggel and Braun, 1996; Braun *et al*., 2000). This diurnal species, which is becoming increasingly popular as a laboratory animal for a variety of studies (Krajnak *et al*., 1997; Thomas and Tillein, 1997; Goel *et al*., 1999), is closely related to the guinea-pig and chinchilla and displays the same principal brain anatomy as the common laboratory rodents (Wright and Kern, 1992). For studies on experience-induced changes of behavior and brain structure and function, some physiological and behavioral features make this species a superior model, in particular if they are to be interpreted in relation to data obtained in human and non-human primates (Reynolds and Wright, 1979; Poeggel and Braun, 1996; Braun and Scheich, 1997; Kenagy *et al*., 1999; Poeggel *et al*., 1999; Braun *et al*., 2000). For instance, similar to primate newborns, the maturity of their sensory systems allows them to perceive and respond to familiar and novel stimuli from their environment immediately after birth. While common laboratory rodents appear not to exhibit a filial attachment of the kind shown, for example, by young primates (Gubernick 1981), degus have been shown to develop a strong attachment to both parents. Similar to human babies (DeCasper and Fifer, 1980), newborn degu pups learn to recognize and to respond to their mother's vocalizations within the first days of life (Poeggel and Braun, 1996; Braun and Scheich, 1997), and this vocal communication appears to be an important component for the establishment and maintenance of the emotional attachment to the parents (Ziabreva *et al*., 2001).

Influence of Maternal Separation and Social Isolation on the Establishment of Cortical Synaptic Input

In contrast to previous studies, which demonstrated an influence of enriched or impoverished environment on synaptic development in sensory and motor systems, we focused on the effect of very early postnatal traumatic emotional experience on synaptic development in the limbic cortex. The environmental changes include, in addition to the exposure to an unfamiliar environment, separation from parents and siblings. Since these events are accompanied by heightened arousal and emotional stress, as reflected by the pups' behavioral responses (stereotyped distress calls, vigorous escape behaviors), we suspect that the emotional component is the critical factor that modulates synaptic development; however, this interpretation requires further analysis. The most intriguing finding of our study was the increased spine (light microscopy) and the decreased shaft (electron microscopy) synaptic densities in the ACd of the deprived animal groups (groups 3 and 4) compared to undisturbed normal controls (group 1). Previous studies have shown environmental influences on brain development; however, it is difficult to compare our results with these studies, since they investigated different isolation/enrichment procedures, different developmental time windows and brain regions, and the prefrontal cortex was not included in their analysis. It was shown that chronic exposure to an impoverished environment after weaning led to an increase of asymmetric synapses (spine and shaft) in the motor cortex of cats (Beaulieu and Colonnier, 1989) and an increase of mean synaptic density in rats (Diamond *et al*., 1975). Long-lasting isolation resulted in twice as many symmetric synapses in the impoverished cortex, whereas the density of asymmetric synapses remained unaltered (Beaulieu and Colonnier, 1987). However, increased synaptic densities were also found after exposure to enriched environmental conditions, e.g. in the occipital cortex (Volkmar and Greenough, 1972; Diamond *et al*., 1975; Turner and Greenough, 1985; Rosenzweig and Bennett, 1996), medial preoptic area (Sanchez-Toscano *et al*., 1991) and in the striatum (Comery *et al*., 1995, 1996) of rats.

The EM part of this study confirmed the LM observation of increased spine density in the deprived animal group 4 compared to undisturbed control animals (group 1). However, the LM observed elevated spine densities in group 3, which experienced only preweaning maternal separation without subsequent chronic social isolation, was not seen in the EM analysis. These seemingly discrepant results may be due to the sampling and analysis techniques: whereas in the LM study complete apical dendrites of individually identified neurons were analyzed, the EM analysis in layer II included the analysis of these apical dendrites, and in addition synapses located on other neurons, e.g. non-pyramidal neurons, layer V pyramidal neurons and from spindle cells in layer VI.

Another finding unique to our study was that early emotional experience modifies the balance between synaptic subtypes, i.e. the ratio of shaft/spine synapses. Periodic maternal separation without (group 3) or with subsequent chronic social isolation (group 4) reversed the shaft/spine ratio seen in the normal control pups (55:45%) towards lower shaft and higher spine synapse densities (group 3 = 37:63%; group 4 = 33:67%). The direction and extent of these synaptic changes appears to be specific for the ACd, since other prefrontal subregions change in a different way (Ovtscharoff and Braun, 2001).

Our LM results indicate that it is the earliest emotional event, i.e. periodic maternal separation starting immediately after birth, which most dramatically interferes with synaptic and dendritic development in the ACd, whereas the subsequent chronic social isolation appears not to add significantly to these early induced changes of spine density. However, the results from the EM analysis indicate that additional chronic social isolation, as applied in group 4, which showed the most dramatic synaptic changes, also contributes to the overall synaptic changes, but perhaps on different neuron populations. The observed synaptic changes are not due to malnutrition or general physical underdevelopment of the experimentally manipulated animals. The comparison of brain and body weights between the four animal groups revealed that only group 3 animals showed higher body weights than the other three groups; however, their brain weights did not differ from the normal control animals.

It is important to note that the early induced synaptic changes are still present several weeks after the animals were returned to undisturbed social conditions, indicating that these changes of synaptic connectivity are lasting and perhaps remain throughout life.

Influence of Maternal Separation and Social Isolation on Dendritic Growth

In order to assess if the elevated spine density in the deprived animals is a secondary effect of dendritic shrinkage, e.g. caused by a reduction of inter-spine distances, the lengths of pyramidal dendrites were compared between the four animal groups. This comparison revealed that pre- and postweaning environmental manipulations seem to modify dendritic growth in a different, perhaps opposite direction. Only those animals that experienced maternal separation during the preweaning phase (group 3), but not those that underwent additional postweaning chronic social deprivation (group 4), displayed elongated apical dendrites. Thus, the first three postnatal weeks, during which the apical and basal dendrites elongate (unpublished observations), represent a time window in which environmental

changes can stimulate or accelerate dendritic growth. This interpretation is supported by other studies, which showed that preweaning environmental manipulations induce increased, and postweaning environmental impoverishment can result in reduced dendritic fields. For instance, the effects of preweaning sensory and motor stimulation on dendritic development were found in layer III pyramidal neurons of the occipital cortex of rats (Venable *et al.*, 1989), and an elongation of basal dendrites has been found in layer III pyramidal neurons of the motor and visual cortex (Pascual and Figueroa, 1996).

These studies also revealed that not only dendritic length *per se*, but also the complexity of the dendritic ramification patterns can be altered by early environmental influences; however, no study on prefrontal cortical areas has been performed. In the present study the average lengths of the dendritic branches remained unchanged in the deprived animals of group 3, although their total dendritic length increased, which may reflect increased dendritic ramification. This 'stimulatory' effect of early socio-emotional deprivation on dendritic growth seen in group 3 appears to have been counter-regulated during postweaning chronic social deprivation in group 4, which did not show altered dendritic lengths although these animals had exactly the same experience as group 3 during the first three postnatal weeks. A 'suppressing' effect of postweaning social isolation on dendritic growth has been observed in rats, where postweaning social isolation resulted in reduced dendritic branching patterns in the visual cortex (Davies and Katz, 1983) and in granule cells of the dentate gyrus (Juraska *et al.*, 1985), and in the occipital cortex after postweaning social isolation/impoverished environment compared to enriched and super-enriched environments (Camel *et al.*, 1986).

Influence of Handling on the Establishment of Cortical Synaptic Input and Dendritic Growth

Repeated maternal separation involves daily handling by the experimenter, therefore a handling group had to be introduced to assess the contribution of separation to the observed synaptic changes. Since this group served as additional control group for separation-induced effects, the animals were neither removed from the home cage nor separated from the family, as opposed to the so-called handling paradigm applied by other groups (Meaney *et al.*, 1991; Denenberg, 1999). Handling was the only environmental manipulation in our study that induced a significant thickening of cortical layer II, but not of total cortex extension. Furthermore, handling alone accounted for almost 50% of the spine increase seen on the dendrites of the two deprived groups (groups 3 and 4). In line with our findings in the ACd, elevated spine densities have been found in the rat hippocampal CA1 pyramidal neurons after handling young rats during saline injections, i.e. a manipulation that is associated with stress and even painful sensation (Horner *et al.*, 1991). For a functional interpretation of these results it remains to be elucidated if and in which way this version of the handling paradigm can evoke changes of maternal behaviors (Bell *et al.*, 1971; Rosenfeld *et al.*, 1991; Liu *et al.*, 1997), which may have contributed to the observed synaptic changes.

Possible Functional Consequences of Altered Synaptic Composition

Our results demonstrate that juvenile socio-emotional challenges modulate or even regulate the establishment and maintenance of synaptic connections and may thereby shape the functional properties of limbic pathways. The cellular and molecular mechanisms for such neuronal adaptation mechanisms have yet to be determined. Separation from the family induces a reduction of metabolic activity in the ACd (Bock and Braun, 2000), which, when occurring repeatedly or chronically, may delay or suppress the activity-driven fine tuning of synaptic inputs on cortical neurons. Such synaptic refinement, which is achieved by the selective pruning and survival of synapses during certain critical phases of brain maturation (Huttenlocher, 1979; Bourgeois and Rakic, 1993; Granger *et al.*, 1995) and which is triggered by early emotional experience (Bock and Braun, 1998) and learning events (Wallhäußer and Scheich, 1987), is dependent on NMDA-receptor mediated synaptic activation (Lau *et al.*, 1992; Rabacchi *et al.*, 1992; Goodmann and Shatz 1993; Bock and Braun, 1999). Furthermore, the release of stress-related hormones, neurotransmitters, growth factors, and the induction of immediate early genes or transcription factors could be part of the molecular machinery that underlies these synaptic changes.

Which afferents of the ACd have been altered by the emotional experience, and how could this affect the functioning of the cingulate cortex within the limbic system? Dendritic spines of prefrontal cortical pyramidal neurons are the target of (i) thalamic fibers (Krettek and Price, 1977; Sarter and Markowitsch, 1984), (ii) callosal fibers (Carr and Sesack, 1998) and associational fibers (Bates and Goldman-Rakic, 1993), as well as (iii) fibers arising from the basolateral amygdala (Bacon *et al.*, 1996), (iv) fibers arising from the hippocampus (Carr and Sesack, 1996) and (v) fibers arising from the infralimbic cortex (Hurley *et al.*, 1991). Altered balances between spine and shaft synapses and between different neurotransmitter systems (Poeggel *et al.*, 1999, 2000; Braun *et al.*, 2000) can change the output characteristics of the pyramidal neurons into their limbic projection areas, including (i) the nucleus accumbens (Brog *et al.*, 1993); (ii) mediodorsal thalamic nucleus (Kuroda *et al.*, 1998); (iii) via the entorhinal cortex to the ventral striatum and the hippocampus (Joyce, 1993); and (iv) to the amygdala (Aggleton *et al.*, 1980). In order to determine if the deprivation-induced changes of synaptic development are beneficial or detrimental to the animal's ability to cope with its environment during later life, the morphological changes need to be correlated to behavioral and cognitive capacities. It remains to be elucidated if these synaptic changes reflect enhanced interhemispheric communication, or increased excitatory synaptic input from the amydgala, hippocampus or thalamus. For instance, increased afferent input from the amygdala may result in changes of anxiety behavior (LeDoux, 1998), or if the hippocampus–cingulate connections are affected, the outcome may be altered learning and memory formation. In socially deprived rats, monkeys and humans, altered or impaired behavioral and cognitive competence have been described (Spitz, 1945; Hall, 1998); however, no morphological analysis has been performed in these studies. In human and non-human primates disrupted mother–infant attachment and/or premature separation has been shown to promote vulnerability to stressors and the development of psychopathology later in life (Furukawa *et al.*, 1998, 1999; Agid *et al.*, 1999), and some psychopathological syndromes in humans are associated with increased spine and decreased shaft synapses in prefrontal cortical regions (Aganova and Uranova, 1990; Comery *et al.*, 1997; Weiler *et al.*, 1997). Since human infants can experience unavoidable separation from the parents, therapies for the prevention or amelioration of detrimental long-term behavioral consequences are greatly dependent on the identification of the underlying neurobiological substrate.

Notes

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