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BRAIN RESEARCH

Research Report

Lack of paternal care affects synaptic development in the anterior cingulate cortex

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ARTICLE INFO

Article history:
Accepted 28 July 2006
Available online 1 September 2006

Keywords:
Synapses
Prefrontal cortex
Paternal care

ABSTRACT

Exposure to enriched or impoverished environmental conditions, experience and learning are factors which influence brain development, and it has been shown that neonatal emotional experience significantly interferes with the synaptic development of higher associative forebrain areas. Here, we analyzed the impact of paternal care, i.e. the father's emotional contribution towards his offspring, on the synaptic development of the anterior cingulate cortex. Our light and electron microscopic comparison of biparentally raised control animals and animals which were raised in single-mother families revealed no significant differences in spine densities on the apical dendrites of layer II/III pyramidal neurons and of asymmetric and symmetric spine synapses. However, significantly reduced densities (–33%) of symmetric shaft synapses were found in layer II of the fatherless animals compared to controls. This finding indicates an imbalance between excitatory and inhibitory synapses in the anterior cingulate cortex of father-deprived animals. Our results query the general assumption that a father has less impact on the synaptic maturation of his offspring's brain than the mother.

1. Introduction

Recent studies have provided convincing evidence that, in addition to sensory stimulation and physical exercise, socio-emotional stimulation, in particular with the mother, exerts a critical impact on the normal development of endocrine functions, brain wiring and behavior (Matthews et al., 1996; Hall, 1998; Ladd et al., 2000; Ruedi-Bettschen et al., 2005). Quantitative morphological studies in different rodent species revealed significantly altered densities of dendritic spines in limbic brain areas, including the anterior cingulate cortex, hippocampus and amygdala in response to maternal or parental separation (Helmeke et al., 2001a,b; Ovtscharoff and Braun, 2001; Poeggel et al., 2003; Bock et al., 2005). In these experiments, the synaptic changes were most pronounced in the limbic anterior cingulate cortex (ACd), a subregion of the rodent medial prefrontal cortex, which is critically involved in emotional as well as cognitive function (Fuster, 2002). Analogous to observations in human and non-human primates (MacNeilage, 1998), the rodent ACd is involved in vocal communication and social interaction between the pups and the dam (Poeggel and Braun, 1996).

In order to identify the impact of paternal care on neuronal and synaptic maturation, we analyzed the biparental trumpet-tailed rat *Octodon degus*, a semi-precocial rodent which displays the same principal brain anatomy as common laboratory rodents. Degus are characterized by complex family and social structures, highly active play behavior and elaborated vocal

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0006-8993/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2006.07.106

communication (Wilson, 1982). It has been shown that male degus invest enormous efforts in the upbringing of their offspring, and while the mother–pup contacts gradually decrease within the first postnatal weeks, the father–pup contacts increase with the offspring's age (Wilson, 1982). The elaborated paternal behavior includes huddling, licking and grooming, body nosing, nose–nose contacts, nosing-the-nape-of-the-neck and riding on the fathers back (Reynolds and Wright, 1979, own observations). Thus, to a similar degree as the mother, the father is a source of a variety of sensory as well as emotional stimulation for his offspring and thereby provides an "enriched environment" to stimulate and optimize brain development, in particular the emotionally relevant limbic circuits. The mothers, in the presence or absence of their spouses, display a strikingly similar behavioral repertoire (plus nursing) towards their offspring as the fathers. Since we observed that single degus mothers spend approximately the same degree of maternal care (including nursing), with their pups as they do when their mate is present, thus they do not compensate the absence of their mate by increasing their maternal attention towards their pups (C. Helmeke, unpublished observations), we can assume that the pups raised by a single mother are in fact partly emotionally deprived.

The aim of the present study was to test our prediction that father-deprived degus should develop different (retarded?) synaptic wiring patterns in the ACd, compared to biparentally raised animals.

2. Results

2.1. *Light microscopy*

The absolute number (mean ± SEM; biparental controls: 722.4 ± 73.2, fatherless: 643.7 ± 52.6) and density of apical spines in the left ACd were not significantly different between father-deprived and control animals. However, a visible trend to lower apical spine densities (−18%) was observed in fatherless animals in comparison to controls (biparental controls: 8.4 ± 0.1/10 μm, fatherless: 6.9 ± 0.7/10 μm).

The length (biparental controls: 869.5 ± 34.4 μm, fatherless: 954.9 ± 85.9 μm) and complexity (biparental controls: 8.0 ± 0.6 nodes, fatherless: 7.6 ± 1.0 nodes) of apical dendrites were not significantly different between the two experimental groups. Branching order analysis revealed no significant differences in segment lengths, spine density and spine number in the branch orders 1–5 of father-deprived animals in comparison to controls.

2.2. *Electron microscopy*

The ultrastructural analysis of layer II of the ACd revealed no significant difference in overall synaptic density between the two animal groups (Fig. 1A). By dividing the synaptic subtypes according to the ultrastructural features of their postsynaptic elements (shaft/spine), the fatherless animals did not show significant differences in shaft and spine density (Fig. 1A). Dividing both spine and shaft synapses into symmetric and asymmetric synapses revealed significantly lower (−33%) densities of symmetric shaft synapses ($p = 0.04$) in the fatherless animals (Fig. 1B). The density of asymmetric shaft synapses and of asymmetric and symmetric spine synapses was not significantly different between the two animal groups (Fig. 1B).

2.3. *Brain weights*

No significant differences in brain weights were detected between the control (1.48 ± 0.03 g; mean value and SEM) and the paternally deprived (1.53 ± 0.01 g) group.

3. Discussion

Paternal behavior is also observed in other mammalian species, for example, prairie voles (*Microtus ochrogaster*) (Lonstein and De Vries, 1999) and California mouse (*Peromyscus californicus*) (Trainor and Marler, 2001). The presence of the sire enriches the care for the offspring and can also facilitate maternal behavior and thereby increase the chances of the offspring's survival (Wright and Brown, 2000). The present study has unveiled a significant contribution of paternal care on synaptic development in the anterior cingulate cortex of his offspring.

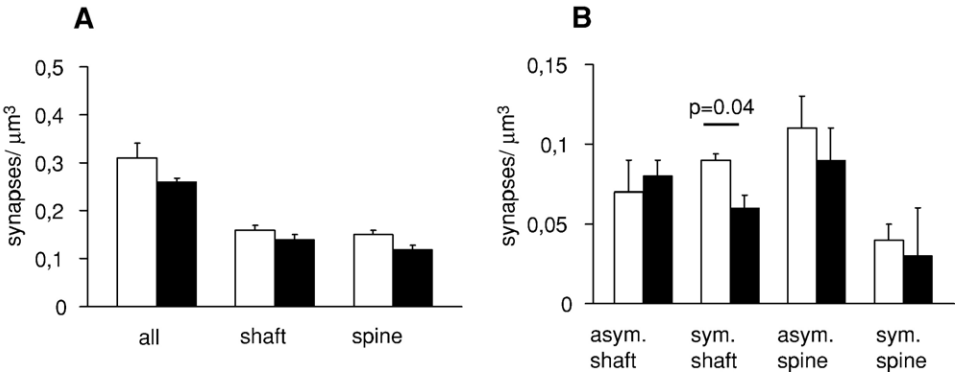

Fig. 1 – Synaptic density in layer II of the anterior cingulate cortex. (A) Overall synaptic density and shaft and spine synaptic density. (B) Further division into symmetric and asymmetric shaft and spine synaptic density. Bars represent mean values and SEM of social controls (white bars) and paternally deprived animals (black bars). *p* values for Student's *t* test are indicated.

Synaptic remodeling and network refinement in the brain is achieved by synaptic proliferation and by the selective pruning and survival of synapses during specific critical time windows, events which play a key role in the specification of neuronal connections in response to the individual's environment (Granger et al., 1995; Segal, 2005). The majority of studies analyzing the effects of early socio-emotional experience in rodents were focused on disturbances of parent–offspring interaction, i.e. handling, maternal separation and chronic social isolation (Matthews et al., 1996; Hall, 1998; Ladd et al., 2000; Helmeke et al., 2001a,b; Ovtscharoff and Braun, 2001; Poeggel et al., 2003; Ruedi-Bettschen et al., 2005). In the present study, we analyzed the impact of paternal care, or paternal deprivation, respectively, on the synaptic development in the limbic ACd. The finding of reduced densities of symmetric shaft synapses, i.e. primarily inhibitory synapses on the dendritic shaft, in the ACd of father-deprived degus pups is in principle in line with similar findings in sensory cortical areas as result of sensory deprivation, induced by impoverished environment (Walsh, 1981) or by whisker trimming (Sadaka et al., 2003). It is interesting that the synaptic reduction (symmetric shaft synapses) in response to chronic deprivation (in the present study being raised by a single parent) is different to the effect of repeated, short periods of separation from both parents, after which elevated (spine) synaptic densities were observed in the ACd (Helmeke et al., 2001a,b; Poeggel et al., 2003). These differential effects emphasize the remarkable and quite deprivation- and synapse-specific sensitivity of the limbic anterior cingulate cortex. The different synaptic outcome of the different deprivation paradigms might be due to the fact that repeated exposure to the separation from the familiar environment is very stressful for the young immature animal (Gruss et al., 2006), and in addition may even to some extent be compared to an "enriched environment" paradigm, which in the visual cortex has been shown to result in elevated spine densities (Turner and Greenough, 1985). In contrast, the removal of the father at birth is less stressful, but considerably reduces the pups' opportunity for social interactions and partly deprives them of emotional input, which can be considered as "impoverished environment". Differential, yet to be identified endocrine and neurochemical changes evoked by these different manipulation might interfere with the maturation and reorganization of specific subsets of synaptic subpopulations. In the case of the paternal deprivation paradigm, these appear to be the (presumably inhibitory) symmetric shaft synapses, while in the case of repeated maternal or parental separation stress, the (presumably excitatory) spine synapses are affected. Our study revealed that the lack of paternal care alters the ratio of excitatory (asymmetric) to inhibitory (symmetric) synaptic inputs (biparental 58%: 42%; fatherless 66%: 33%) towards reduced inhibitory synapses within layer II of the ACd. For a functional interpretation, it is important to identify the nature of these symmetric shaft synapses, on which neuron types are they located and which input do they reflect? Concerning symmetric synapses, it has been shown in the rat and monkey prefrontal cortex that both dendrites of GABA and non-GABAergic neurons are contacted by dopamine- or TH-immunoreactive terminals, which form symmetric synapses (Sesack et al., 1995). Noradrenergic axons are known to form small symmetric synapses in rat and monkey prefrontal cortex, but rarely with pyramidal neurons (Smiley and Goldman-Rakic, 1993; Branchereau et al., 1996). GABAergic terminals, predominantly from local GABAergic neurons, form numerous symmetric synapses on somata and dendrites of pyramidal cells as well as on inhibitory interneurons (Melchitzky et al., 2005). Possible physiological consequences of the reduced symmetric innervation in the cingulate cortex of father-deprived animals have to be characterized in future studies by detailed transmitter specific EM analysis as well as electrical recordings on identified neurons.

On the behavioral level, a variety of studies in humans have reported a correlation between the absence of the father during childhood and the risk for alcoholism, poor educational performance, aggressiveness and criminality (Vaden-Kiernan et al., 1995; Baskerville, 2002). Ongoing extensive behavioral analysis in our animal model will determine the behavioral correlates of the synaptic changes observed in the fatherless animals. The analysis of animal models, in which the impact of distinct familial constellations on synaptic maturation can be identified and quantitatively characterized, may serve as a basis for understanding the endocrine and neuronal factors, which mediate experience-driven neuronal and synaptic malformations and at later life periods result in the development of behavioral and cognitive deficits as well as mental disorders.

4. Experimental procedures

4.1. *Animals*

The degus 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 wire cages (length × height × depth: 53 cm × 70 cm × 43 cm) equipped with little burrows and climbing scaffolds. The animals were exposed to a light/dark cycle with 12 h light. Fresh drinking water, rat diet pellets and vegetables 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 24th Nov. 1986 (86/609/EEC) and according to the German guidelines for the care and use of animals in laboratory research, and the experimental protocols were approved by an ethical committee.

4.2. *Experimental groups*

For light microscopic (LM) and electron microscopic (EM) analysis, male degus pups were studied at postnatal day (PND) 21. Brain weights were measured in both experimental groups at PND 21. The average litter size was 5–8 pups of both sex, and the families were assigned into two groups:

Biparental controls: these pups were raised undisturbed in families with both parents (LM: $n=4$ animals; EM: $n=5$ animals).

Fatherless animals: pups were raised undisturbed in single-mother families, i.e. without the sire, who was removed from the family at PND 1 to 21 (LM: $n=4$ animals; EM: $n=4$ animals).

To avoid litter effects, the pups were collected from 5 (LM) and 6 (EM) different litters and maximal 2 animals of a litter were taken for analysis. The comparison of the morphological parameters within each group revealed no significant or otherwise obvious higher similarity between siblings from the same family or non-related animals from different families.

4.3. *Light microscopy*

After decapitation, the unfixed brains were developed using the Golgi–Cox technique as described previously (Helmeke et al., 2001a,b). Neurons were analyzed using an Olympus BH-2 Microscope at a final magnification of ×1000. Morphological measurements were performed using the image analysis system Neurolucida™ (MicroBrightField, Inc., Colchester, USA), which allows a quantitative 3D analysis of microscopic images. Layer II/III pyramidal neurons in the left anterior cingulate cortex were analyzed (Fig. 2A). Five pyramidal neurons per animal were selected for analysis. For each neuron, the lengths, number of nodes, number of spines and the spine densities of a complete apical dendrite and its first five branch orders were analyzed. Only neurons which were impregnated in their entirety and displaying complete dendritic trees within the 150 μm section were selected. 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 by dividing the number of spines by the dendritic length.

4.4. *Electron microscopy*

Perfusion, fixation and embedding were performed as described in Helmeke et al. (2001a). We focused the EM analysis on layer II of the ACd (left hemisphere) since this layer includes the apical dendrites of layer II/III pyramidal cells, which were analyzed in the light microscopic part of this study. After polymerization at 60 °C, layer II of the anterior cingulate cortex was identified by light microscopic observation of osmium-stained sections by its darker appearance compared to the adjacent layer I. Cortical layer II was dissected out using a fine scalpel, glued on a sectioning block and serial sections were cut and collected on single-slot grids coated with Formvar support film (Plano, Wetzlar, Germany). We applied the disector method (Gundersen and Jensen, 1987) being a standard technique for quantitative electron microscopy. Twenty fields from nominate sections and same amount from reference sections were taken (Sterio, 1984). These fields represent counting frames which were taken from at least three grids, with serial sections. This sampling results in several thousand square micrometers counting area per animal. Approximately 100 disector scores

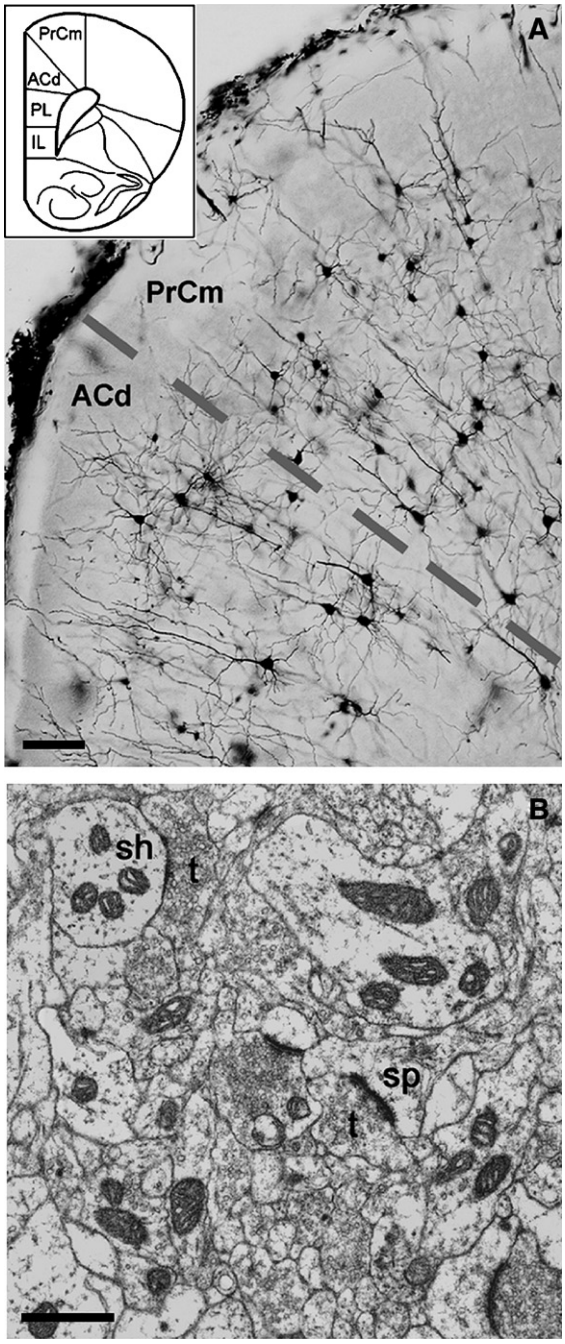

Fig. 2 – Light and electron microscopic images of the anterior cingulate cortex. (A) Composite image (Neurolucida Executable, Confocal Virtual Slice, MicroBrightField, Inc.) of Golgi-impregnated neurons in the medial prefrontal cortex of a 21-day-old *Octodon degus*. Scale bar, 60 μm. The location of the pregenual anterior cingulate cortex (ACd) which was analyzed in the light microscopic part of this study is illustrated on a schematic frontal section (inset). Prelimbic cortex (PL), infralimbic cortex (IL), precentral medial cortex (PrCm). (B) Electron micrograph illustrating different types of synaptic contacts. Shaft synapse (sh), spine synapse (sp) and axon terminal (t). Scale bar, 1 μm.

were made to obtain reliable estimates of (Q–) (Gundersen and Jensen, 1987). Data of numerical densities of synapses were given as number of profiles per 1 μm^3 of neuropil section. All data were loaded on a Macintosh computer, and synaptic profiles were counted using NIH-Image software. For detailed description, see Ovtscharoff and Braun (2001) and Helmeke et al. (2001a).

Only those synaptic profiles were included in the quantitative analysis where a clear identification was possible on the basis of (a) an apposition of pre- and postsynaptic densities, (b) a visible synaptic cleft and (c) 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, only those synapses were included in the quantitative analysis, which could be further subdivided into shaft synapses (i.e. terminating directly on the dendritic shaft) and spine synapses (Fig. 2B), i.e. small spherical postsynaptic protrusions of the dendrite filled with a fluffy material, which consists of fine and indistinct filaments, without microtubules and neurofilaments. On the basis of the disposition of the cytoplasmatic density on each side of the synaptic junction, synapses were classified into symmetric or asymmetric synapses.

4.5. *Statistics*

The values for cells (LM) or neuropil (EM) were averaged for each animal, statistical analysis for histological parameters was carried out using Sigma Stat software. For comparison between the two groups, A student's *t* test (normality tests passed) was applied.

Acknowledgments

We would like to thank Ute Kreher for technical assistance. This work was supported by a grant from the state of Saxony-Anhalt.

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