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Effects of Transient Restraint Stress on the Plasticity of the Cortical Areas of the Brain and Cognitive Functions in Adult Rats with Normal and Disturbed Embryogenesis

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The aim of the present work was to analyze changes in the numbers of labile synaptopodin-positive dendritic spines in the parietal cortex and hippocampal field CA1 and the ability of adult rats to remember after repeated transient restraint stress (5 min daily for 10 days). These parameters were compared in animals with normal development and in those subjected to hypoxia during embryogenesis (E14, 7% O_2, 3 h). Short-term and long-term memory were degraded in adult rats with normal embryogenesis subjected to restraint stress, and the number of labile spines was decreased in hippocampal field CA1 (by 17.3 ± 10.4%, $p \le 0.05$) and increased in the molecular layer of the parietal cortex (by 36.9 ± 9.2%) compared with intact control animals. Rats subjected to prenatal hypoxia, regardless of whether or not they had been subjected to restraint stress at the adult stage or not, showed impairments to both short-term and long-term memory, along with decreases in the numbers of labile spines in the hippocampus (by 22.9 ± 10.5%) and the parietal cortex (by 28.1 ± 9.3%). These results lead to the conclusion that increases in plasticity, supporting adaptive behavior in the animals, occur in neural networks in the neocortex in response to repeated transient stress only after normal brain formation during embryogenesis, while impairments to embryogenesis led to decreases in plasticity and the adaptive potentials of the nervous system in further ontogeny.

Keywords: stress, ontogeny, memory, synaptopodin, dendritic spines, synaptic plasticity, rats.

Acute stress, including during the prenatal period of development, can induce impairments to cognitive functions and behavior in animals. Existing data identify a link between cognitive impairments and decreases in the adaptive potential of the brain and its plasticity [2, 11, 13]. On the other hand, repeated mild stress can lead to adaptation of the organism, often as a result of increases in nervous system plasticity [2, 10]. The literature includes descriptions of the effects of compensatory increases in the expression of a number of synapse-associated proteins after exposure to stressors [9]. Changes in the levels of the stress hormone corticosterone have been shown to affect the expression of actin-associated spine apparatus protein synaptopodin [5] which is associated with supporting the plasticity of neural networks due to rearrangements of the cytoskeleton of labile spines [3, 7, 14]. Synaptopodin is regarded as necessary for maintaining long-term potentiation, which underlies the molecular-cellular mechanisms of memory [14]. We have previously demonstrated a reduction in the number of labile synaptopodin-positive dendritic spines (henceforth labile spines) in the cortical areas of the brain, which is accompanied by cognitive dysfunctions, in normal aging and after prenatal pathology [1]. Compensatory increases in the number of labile spines after repeated mild stresses may occur, though experimental confirmation remains lacking. There are grounds for suggesting that the action of postnatal stressors on nervous system plasticity and cognitive functions in animals may depend on the features of prenatal development. The aim of the present work was to analyze changes in brain tissue plasticity (assessed in terms of the

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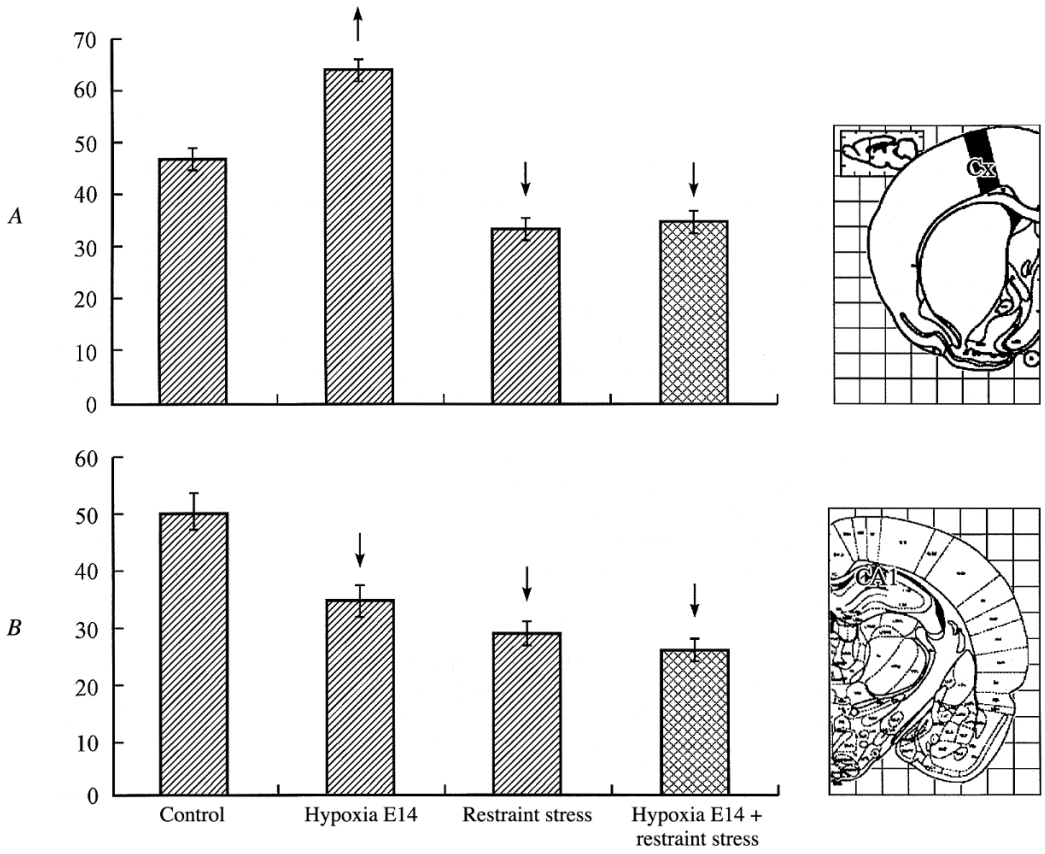

Fig. 1. Distribution of synaptopodin-positive spines in parietal cortex nervous tissues (*A*) and hippocampal field CA1 (*B*) in rats subjected to hypoxia on day E14 and short-term restraint stress (5 min/day, 10 days). The ordinates show the mean number of spines/10000 μm^2, presented as mean ± error of the mean; the abscissas show groups of animals: controls ($n = 25$), rats subjected to prenatal hypoxia (hypoxia E14; $n = 21$); rats with normal brain development after series of stresses (restraint stress, $n = 18$); rats subjected to prenatal hypoxia after analogous series of episodes of postnatal stress (hypoxia + restraint stress, $n = 8$). Arrows show statistically significant differences ($p \leq 0.05$) compared with controls. Diagrams to the right show the locations of the nervous tissue study areas – the molecular layer of the parietal cortex (Cx) and the stratum radiatum-moleculare of hippocampal field CA1 (CA1) in rats (modified from [15]).

number of labile spines) and the ability to remember in rats after repeated transient restraint stress in both normal development and after hypoxia during the prenatal period.

Methods

Experiments on rats were performed in compliance with the European Communities Council Directive #86/609 for the Care of Laboratory Animals. On day 14 of pregnancy, female Wistar rats were subjected to hypoxia (3 h at an O_2 content of 7%) in a 100-liter chamber with an oxygen control system, ventilation, and exhaled CO_2 adsorption. Control females were kept in the same conditions but with a normal O_2 concentration. Studies were performed on adult (three months) males from the litters of these females. Adult rats were subjected to transient restraint stress by placing them in transparent boxes providing maximal restriction of mobility for 5 min. This procedure was repeated at the same time of day for 10 days. During the last two days, short-term and long-term memory were evaluated 2 h before imposition of stress using a modified novel object recognition test [8, 12]. Memory and the distribution of labile spines in tissues in the cortical areas of the brain were performed in rats of the following cohorts: 1) animal-house control ($n = 25$); 2) animals subjected to prenatal hypoxia ($n = 21$); 3) animals with normal embryogenesis subjected to transient restraint stress ($n = 18$); and 4) animals exposed to hypoxia in the prenatal period and restraint stress as adults ($n = 8$). At the beginning of testing, animals were adapted to the test conditions for 15 min by placing them in a special arena of 100 × 100 cm surrounded by transparent walls 20 cm high. At 2 h after adaptation, animals received training presentations for 10 min in the experimental arena using two objects (No. 1 and No. 2). On subsequent testing, object No. 2 was replaced with new objects and object No. 1 remained unaltered, though its position was changed. Short-term memory was evaluated 10 min after training by presenting the animal with a pair of objects consisting of a familiar object (No. 1)

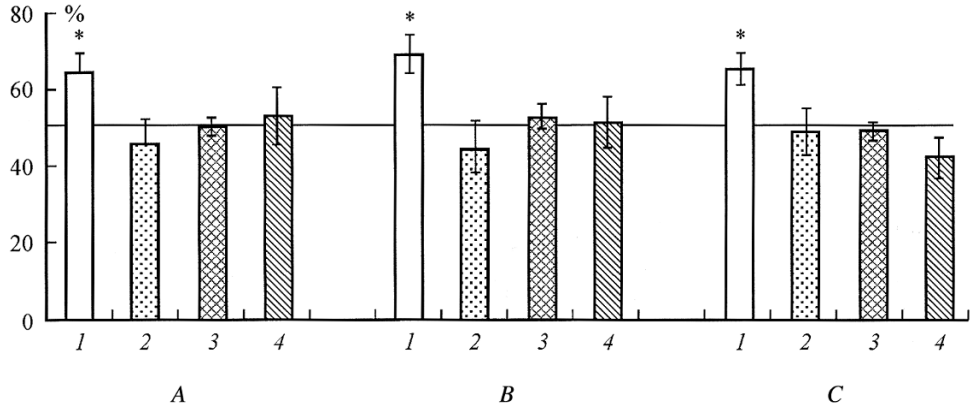

Fig. 2. Novel object recognition times at 10 min (*A*), 1 h (*B*), and 24 h (*C*) after training presentations of pairs of objects. The ordinate shows mean (± error of the mean) recognition time expressed as percentages of recognition times for familiar and novel objects for control animals (*1*, $n = 25$) and animals subjected to prenatal hypoxia (*2*, $n = 21$), as well as after series of episodes of stress in control animals (*3*, $n = 18$) and rats subjected to prenatal hypoxia (*4*, $n = 8$). Horizontal lines show the 50% level, which corresponds to object recognition times being equal for objects with the same level of attractiveness for the animals. *Significant differences between recognition times for familiar and novel objects, $p < 0.001$.

and a novel object (No. 3). Analysis of long-term memory was performed 60 min and 24 h after training, by presenting rats with a new combination of pairs of familiar and novel objects – Nos. 1–4 and Nos. 1–5, respectively. The time spent in immediate tactile or olfactory contact with each object was measured and expressed as a proportion of the total time spent investigating the pair of objects. All objects were made of thick glass and after each presentation they and the experimental arena were rinsed with 50% ethanol solution. The identical experimental scheme was used to test all four groups of animals.

At 2 h after the last restraint session, animals were decapitated and nervous tissue from the brain was studied. Brain tissue was fixed in 10% neutral formalin solution in 0.1 M phosphate buffer pH 7.4 and sections were cut on a Leica cryostat. Nervous tissue from the parietal cortex (at the level of +0.20 mm from the bregma, [15], Fig. 1, *A*) and hippocampal field CA1 (at the level of –3.30 mm from the bregma, Fig. 1, *B*) was studied using the Nissl light optical method. Frontal sections were stained with cresyl violet and examined using an ImagerA light microscope (Zeiss, Germany). The distribution of synaptopodin protein was studied by an immunofluorescence method (S9567 antibody, Sigma) and confocal microscopy. Synaptopodin immunolabeling was performed with FITC- or phytoerythrin (PE)-conjugated monoclonal secondary antibodies against rabbit IgG (Sigma, diluted 1:200). Immunofluorescent studies were performed using a DMR Leica microscope fitted with a Leica TCS SL confocal scanner (Leica Microsystems, Germany). Fluorochrome excitation was with an Ar/He laser at a wavelength of 488 nm. FITC fluorescence was detected at a wavelength of 496–537 nm and phytoerythrin at 652–690 nm. The immunofluorescence method detected separate labile synaptopodin-positive spines as a diffuse distribution of points about 1 μm in diameter with a more than five-fold difference in brightness from background. Total numbers of synaptopodin accumulations (labile synaptopodin spines) were counted in microscope fields of 10000 μm^2). The density of labile spines was measured as the ratio of the number of spines to the area of nervous tissue studied. Labile spine densities were calculated in different layers of the cortex and hippocampus. Labile spine density in the hippocampus was determined in the stratum radiatum-moleculare and the stratum oriens, separated by the stratum pyramidale, in which no immunohistochemical reaction was seen. Data were analyzed statistically in Statistica 6.0 for Windows (StatSoft). Significant differences between results from control and experimental animals were identified using the Mann–Whitney U test as the cohorts being compared did not have normal distributions or equal overall dispersions.

Results and Discussion

Light microscopic studies of the parietal cortex and hippocampal field CA1 in adults subjected to prenatal hypoxia and/or transient restraint stress revealed no significant difference in the structural organization of nervous tissue as compared with control animals. Immunohistochemical studies of the spine apparatus protein synaptopodin showed that the maximum density of labile spines in the parietal cortex was in the molecular layer in adult control rats (mean 47.4 ± 2.7 spines per area of tissue of 10000 μm^2). The greatest density of labile spines in hippocampal field CA1 was in the stratum radiatum-moleculare (mean 50.6 ± 3.1 spines/10000 μm^2).

Adult animals subjected to restraint stress showed a decrease in the mean number of labile spines in the stratum radiatum-moleculare of hippocampal field CA1 (by 17.3 ± 10.4%, $p \leq 0.05$) and an increase in the number of

labile spines in the molecular layer of the parietal cortex (by 36.9 ± 9.2%) as compared with intact control animals (Fig. 1, *A*). Rats subjected to prenatal hypoxia, regardless of whether or not they were subjected to restraint stress, showed decreases in the number of labile spines in parietal cortex from the control level (by 28.1 ± 9.3%) and hippocampal field CA1 (by 22.9 ± 10.5%). No differences were seen between rats subjected to hypoxia and rats subjected to hypoxia and transient restraint stress (Fig. 1). The changes in the numbers of labile spines seen here point to changes in the plasticity of nervous tissue, which in turn may reflect the animals' cognitive functions.

In the novel object recognition test, intact animals paid more attention to investigating novel objects (Fig. 2, *A1*, *B1*, and *C1*), while transient stress, like exposure to prenatal hypoxia, induced impairments to short-term and long-term memory regardless of the features of prenatal development. Rats subjected to prenatal, postnatal, or both of these stressors showed no preference to explore novel objects. These animals spent the same amounts of time (about 50%) investigating the familiar and novel objects (Fig. 2, *A*, *B*, and *C*). The impairments to cognitive functions seen here in adult animals after acute hypoxia on day E14 and after restraint stress may be linked with the decreases in the number of labile spines in hippocampal field CA1 and nervous tissue plasticity.

Our data on the decrease in the number of labile spines and the reduction in the adaptive potentials of the body in animals subjected to prenatal hypoxia are consistent with data from our previous studies [1] and published data obtained from other models of prenatal and neonatal pathology [16]. This phenomenon may be connected with the decreased content of synapse-associated proteins, including synaptopodin, described in a model of prenatal nonhypoxic stress [4]. An increase in the number of labile spines was seen in animals with normal embryogenesis with an enriched environment and training [7, 14]. The studies reported here also showed that increases in the plasticity of neural networks in the neocortex in response to sufficient repeated transient stress only occur in conditions of normal development and not in conditions of pathological development. Support for cognitive functions requires harmonious operation of several parts of the brain, particularly the neocortex and hippocampus. Disruption of the innervation of hippocampal field CA1 from the entorhinal cortex is known to be accompanied by changes in the content and distribution of synaptopodin [6]. We observed differently directed changes in the numbers of labile spines in the parietal cortex and hippocampus. These data may indicate that the increase in the number of labile spines in the parietal cortex with the decrease in the stratum radiatum-moleculare of the hippocampus (the cortico-hippocampal interaction zone) are insufficient to provide compensation for the impairments to cognitive function seen.

Thus, we report the first observation of an increase in the number of labile spines in the parietal cortex in response to transient stress, which may provide evidence of increases in the plasticity of neural networks. This increase was not seen in animals subjected to prenatal hypoxia, which may point to impairment to the mechanisms controlling labile axospinous synaptic contacts, leading to reductions in plasticity and the adaptive potentials of the nervous system. These data lead to the conclusion that the increase in plasticity supporting the animals' adaptive behavior occurs in cortical neural networks in response to repeated mild transient stress only on the background of normal brain formation during embryogenesis, while impairment of embryogenesis leads to decreases in the plasticity and adaptive potentials of the nervous system during further ontogeny.

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