Anatomical Science International (2007) **82**, 211–217 doi: 10.1111/j.1447-073x.2007.00186.x

Original Article

Prenatal stress induces learning deficits and is associated with a decrease in granules and CA3 cell dendritic tree size in rat hippocampus

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Abstract

Exposure to gestational stress impairs hippocampal-dependent learning in offspring. In spite of the known decisive role of hippocampal dendritic architecture in learning and memory, there has been no study to date that examines the effect of prenatal stress on the morphology of the hippocampal neurons. Therefore we performed a quantitative morphological analysis of the dendritic architecture of Golgi-impregnated hippocampal neurons in prenatally stressed rats. Subjects included male rat offspring (2 months old) for which the mothers had been restrained for 1 h/day during the last week of gestation. Spatial learning performance levels using Morris water maze and changes in the morphology of hippocampal dendritic trees were studied. Results indicated that the study group had lower spatial learning capabilities along with decreased length and number of dendritic segments, branching of granules and cornu ammonis (CA)3 pyramidal cells. There was no change in the dendritic morphology of CA1 pyramidal cells. These results suggest that prenatal stress in rat results in spatial learning deficits and profound alterations in the neurites of the hippocampal cells of male offspring.

Key words: dendrites, hippocampus, prenatal stress, spatial learning.

Introduction

Exposure of pregnant female rats to stress provides an important model to investigate the influence of early stressful experiences on brain development and alterations in behavior of offspring later in life. It is well documented from animal studies that stress during pregnancy induces significant neurobiological and endocrinological changes in adult offspring (Takahashi, 1998; Maccari *et al*., 2003).

There is growing evidence that prenatal stress produces hippocampal-dependent learning and memory impairment (Aleksandrov *et al*., 2001), but the cellular and morphological basis of memory decline remain largely unknown.

The hippocampus, which is a sensitive region of the brain with high plasticity, plays a critical role in certain aspects of learning and memory. The hippocampal circuit integrity is crucial for spatial learning and memory (Rusakov *et al*., 1997). The dendritic systems are the functional core of neuronal ensembles because they represent approximately 90% of the receptive surface of neurons. There is also substantial evidence that organization of neuronal receptive surfaces is crucial for integration and transfer of information at the synaptic level (Horner, 1993). In this way, a precise knowledge of the dendritic organization of neuronal population is of great importance in evaluation of the morphological substrate available for establishment of synapses, thus providing valuable data about the overall receptive area of the neuron.

On the basis of this background, it seems reasonable to postulate that altered dendritic morphology might be responsible for prenatal stress-associated hippocampal-dependent learning and memory deficits.

To test this hypothesis, learning and memory functions were evaluated using Morris water maze procedures that have been widely used previously to examine the effect of hippocampal damage on learning and memory performance (Garrud *et al*., 1982). Subsequently, quantitative analysis of dendritic arborizations of the main neuronal populations of the hippocampal formation, that is, the dentate granule

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Received 11 March 2007; accepted 9 May 2007.
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cells, cornu ammonis (CA)3 and CA1 pyramidal cells from prenatally stressed (PS) and control rats was done using Golgi-impregnated material.

While there are no quantitative Golgi studies focussing on the morphology of neurons in the hippocampal formation after prenatal stress exposure, those performed in adult rats are abundant (Watanabe *et al*., 1992; Magarinos & McEwen, 1995; Sousa *et al*., 2000; for review Conrad, 2006). Most of these studies report that chronic stress exposure leads to dendritic retraction within the hippocampal CA3 region (Watanabe *et al*., 1992; Magarinos & McEwen, 1995), although some of them found that dentate gyrus neurons and CA1 cells too express dendritic retraction (Sousa *et al*., 2000).

Method and material

Animals

Virgin female Wistar rats weighing 230–250 g (procured from animal house of Isfahan Medical Faculty, Iran) were housed in the presence of a sexually experienced Wistar male weighing 450–500 g. Vaginal smears were obtained each morning to detect mating. The pregnant animals were individually housed with free access to rat feeds (Khorak Daam Pars, Iran) and water in a temperature-controlled (22 ± 2°C) animal room, on a 12 h light–dark cycle (light on at 07.00–19.00 hours).

The pregnant dams were assigned randomly to control and study groups. Animal care and handling was performed in accordance with rules approved by the local research council of Shahid Sadoughi Medical University of Iran.

Prenatal stress

During the last week of pregnancy; from day 15 until delivery, pregnant females in the study group were individually restrained for 1 h a day (08.00–09.00 hours). This stress procedure described by Ward and Weisz (1984) was chosen because it has an indirect influence on the fetus via direct stress on the mother. The restraint device was a transparent plastic tube (7 cm in diameter) with air holes for breathing and a closed end. The length could be adjusted to accommodate the size of the animals. Rats in the control group were undisturbed in their home cages.

All dams delivered their offspring vaginally. Only 8–12 offspring in each group similar in respect to sex were studied. On day 21 after all offspring were weaned, female and male offspring were separated and housed, four in each cage. A maximum of two male offspring were taken from each litter to remove any ‘litter effects’ (Chapman & Stern, 1978). The animals were exposed to normal animal room conditions until testing at 2 months of age.

Water maze test

Morris water maze consisted of a black circular pool (diameter, 180 cm; height, 60 cm) filled to 30 cm with water at room temperature. Animals placed in the water were able to escape only by finding a black platform (12 × 12 cm), which was hidden 1 cm under the surface in the center of one of the quadrants of the maze. Finding the platform within 120 s was the animals’ challenge. In case it did not succeed within the mentioned period, the animal was gently guided to the platform by hand. Each rat received four acquisition trials per day for 3 days. The time from placing the animal into the water until finding the platform was measured as the escape latency for each trial. After swimming, the rats were allowed to stay on the platform for 30 s. Animals were given 20 min rests between trials.

Blood sampling and corticosterone measurements

Corticosterone levels were measured in blood samples drawn from the tail vein on three occasions: before stress; 30 min after restraint; and 120 min after restraint between 09.00 and 12.00 hours. The rats were restrained in plastic cylinders identical to those used for the prenatal stress procedure. Blood corticosterone levels were determined by radioimmunoassay using kits purchased from ICN Pharmaceuticals (Carlsbad, CA, USA). Assays were conducted according to the protocols provided by the manufacturer.

Histological procedure

Rats were deeply anesthetized with urethan ($C_3H_7NO_2$-; Merk, Germany) and transcardially perfused with a phosphate-buffered solution (pH 7.2, 0.12 mol/L) of 4% formaldehyde and 1% glutaraldehyde. The brains were removed and hemisected by a midsagittal cut. The right hippocampal formations were post-fixed in the perfusate, overnight. Coronal sections of 100 μm thickness were cut serially with a calibrated vibratome (Bio-Rad H1200, UK) into a bath of 3% potassium dichromate in distilled water. Sections were then processed according to a modified version of the single-section Golgi impregnation procedure (Gabbott & Somogyi, 1994). Brain sections were incubated in 3% potassium dichromate in distilled water overnight. The sections were then rinsed in distilled water, mounted on plain slides and a coverslip was glued over the sections at four corners. These slide assemblies were incubated in 1.5% silver nitrate in distilled water overnight in darkness. On the following day, the slide assemblies were dismantled, tissue sections rinsed in distilled water and then dehydrated first in

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95% ethanol followed by absolute ethanol. The sections were then cleared in xylene, mounted onto gelatinized slides and coverslipped under Permount (Fisher Scientific, Pittsburgh, PA, USA). The slides were blind coded for quantitative analyses.

From the lateral (suprapyramidal) blade of the dentate gyrus granular layer, 10 granule cells were sampled and pooled per animal in a single group. Considering the fact that the dendritic tree form varies according to the location of the cell bodies within the height of the layer, granule cells at two-thirds of the depth of the granular layer were selected (Green & Jurska, 1985). From the hippocampal pyramidal cell layer, 10 pyramids of the CA1 and CA3 regions were selected and pooled per animal in a single group. The criteria used for selecting the neurons to be measured were as follows (De Ruiter & Uylings, 1987): (i) dark and consistent impregnation throughout the extent of dendrites; (ii) cell bodies located in the middle part of the section thickness in order to minimize branch segments cut off at the plan of the section; and (iii) relative isolation from neighboring impregnated cells in order not to have overlapping dendrites of adjacent cells. Because these criteria were fulfilled solely by apical dendrites, the basal dendritic trees of pyramidal cells were not included in the estimations.

The presence of cut terminal segments on a neuron was not considered as a criterion for its exclusion from the estimations because the elimination of these neurons would have biased the sample towards smaller neurons (Uylings *et al*., 1986). And because we found that in the Golgi sections of experimental and control rats there was a similar percentage of cut branches (15%), the likelihood that these cut branches could have interfered with the final results is negligible (Uylings *et al*., 1986).

Morphometry

The dendritic trees of the dentate granule cells and apical dendritic trees of CA3 and CA1 pyramidal cells were drawn with the aid of a camera lucida, at a final magnification of ×640. The centrifugal ordering of dendritic trees was used to estimate the number of dendritic segments per cell (Uemura *et al*., 1995). Accordingly, order 1 was assigned to the dendrites arising from the soma and the successive orders were sequentially attributed to each branching point up to the terminals. In pyramidal neurons, the same criteria were used and therefore there was no distinction between oblique dendrites, apical shafts and terminal tufts. The total number of segments per cell was calculated by summing the number of dendritic segments of all orders. For metric analysis, the dendritic length was measured using a Zeiss interactive digitizing analysis system (Zeiss, Germany).

The branching density of dendritic trees was evaluated by applying the method of concentric rings (Uylings *et al*., 1986). The number of dendritic intersections crossing each concentric ring centered in the cell body was counted. The concentric rings were calculated at intervals of 20 μm for granule cells and 25 μm for CA3 and CA1 pyramidal cells. Whenever the dendrites extended beyond 375 μm (circle 15), they were included in circle 15.

Statistical analysis

Student's *t*-test was performed on data from the PS and control rats. Differences were considered to be significant for $P < 0.05$.

Results

Mean (± SEM) basal corticosterone levels in control (0.35 ± 0.07 μg/100 mL) and PS (1.60 ± 0.81 μg/100 mL) animals were not significantly different. Higher corticosterone values were observed in PS rats, both 30 min (control, 21.00 ± 3.36 μg/100 mL; PS, 38.17 ± 3.40 μg/100 mL; $P = 0.0016$) and 150 min after initiation of the stress procedure (control, 5.14 ± 0.79 μg/100 mL; PS, 19.78 ± 4.11 μg/100 mL; $P = 0.0147$).

Prenatal stress impaired spatial learning capability during the three acquisition days. As shown in Fig. 1, the time latency for finding the platform in the water maze test by the control group was significantly lower than that observed for the PS rats (37.5 ± 2.23 s vs 51.1 ± 4.66 s, $P = 0.000$).

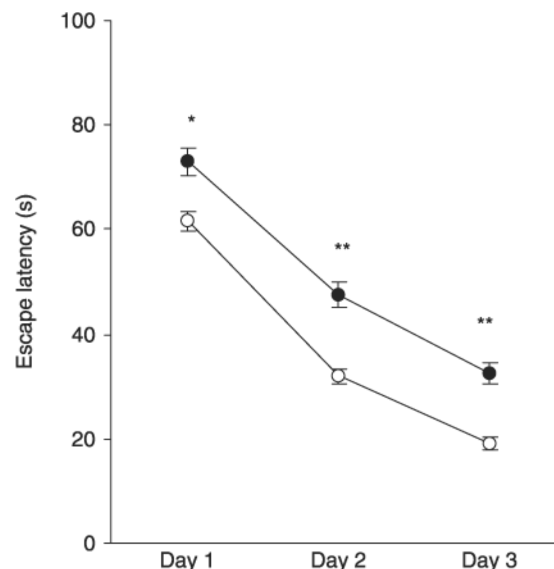

Figure 1. Effect of prenatal stress on learning performance in the Morris water maze test. (●) Prenatally stressed (PS) animals ($n = 10$) had weaker cognitive performance than (○) control animals ($n = 10$). Symbols represent mean ± SEM. *$P = 0.002$; **$P = 0.000$).

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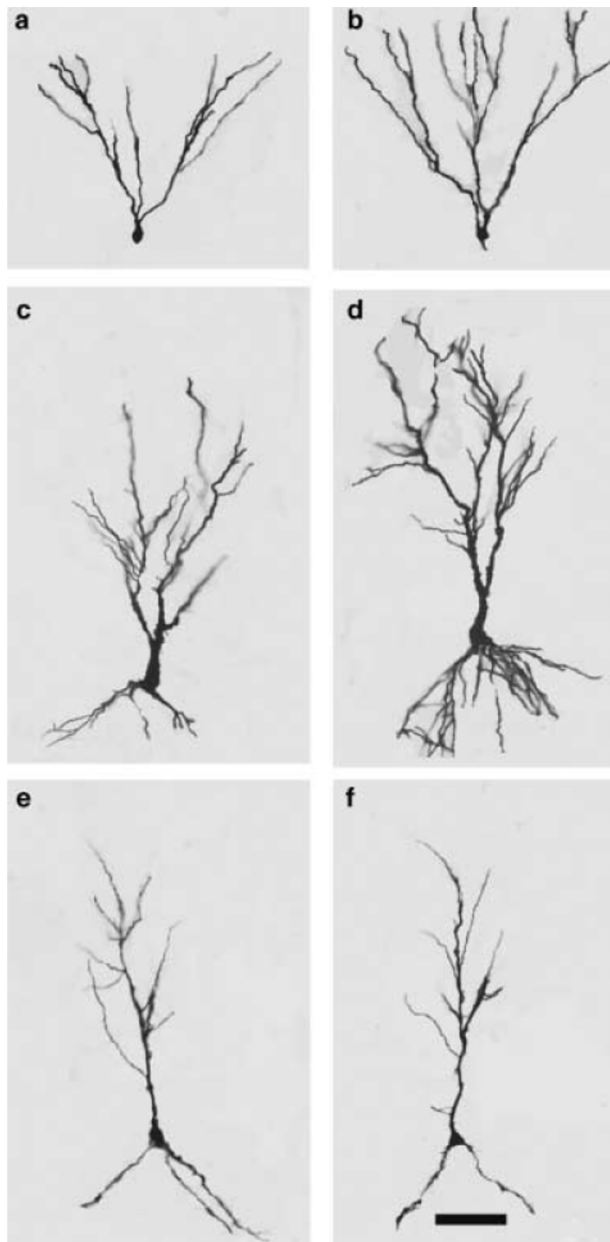

Figure 2. (a,b) Golgi-impregnated granule, (c,d) cornu ammonis (CA)3 and (e,f) CA1 pyramidal cells. Neurons from control rats are shown in the right column (b,d,f), from prenatally stressed rats in the left column (a,c,e). Granule and CA3 pyramidal cells from the (a,c) prenatally stressed rats display an obvious impoverishment of their dendritic arborizations, when compared to (b,d) similar neurons from control rats whereas there is no observable changes of (e,f) CA1 dendritic tree. Scale bar, 50 μm (applies to all frames).

A significant impoverishment of the dendritic trees of granules (Fig. 2a,b) and CA3 pyramidal cells (Fig. 2c,d) was present in sections from the study group as compared to the control group. There was no dendritic impoverishment of CA1 pyramidal cells (Fig. 2e,f) in sections of both the groups.

Comparison between the two groups found a significant effect of prenatal stress on the total number of dendritic segments per cell in the dentate granule and CA3 pyramidal cells ($P = 0.001$, $P = 0.011$, respectively), but there was no effect on the CA1 pyramidal cells (Table 1).

In the PS group, the total dendritic length of granule cells and CA3 pyramidal cells was significantly reduced by 32% and 26%, respectively (Table 1). Although there was an 11% reduction in the total dendritic length of CA1 pyramidal cells of the study group, it was not significant.

Study of the dendritic intersections showed that the effect of prenatal stress was significant for circles 11, 12 and 13 in granule cells ($P < 0.05$) and for circles 11, 12, 13, 14 and 15 in CA3 pyramidal cells ($P < 0.05$). The number of intersections in these circles in the study group was lower than in the control group. No significant prenatal stress-induced difference was detected in the dendritic branching density of CA1 pyramidal cells (Fig. 3).

Discussion

In the present experiment, exposure of pregnant female rats to restraint stress during the final week of gestation prolonged the plasma corticosterone response to acute stress in adult male offspring, consistent with earlier reports (Henry *et al.*, 1994; Barbazanges *et al.*, 1996; Koehl *et al.*, 1999; Szuran *et al.*, 2000).

In agreement with earlier research studies (Lemaire *et al.*, 2000; Aleksandrov *et al.*, 2001; Li *et al.*, 2003), there were significant prenatal stress-related hippocampal-dependent memory learning and memory deficits. Quantitative morphological analysis of dendritic architecture of Golgi-impregnated hippocampal neurons indicated that prenatal stress induces dendritic retractions in the granule dentate neurons and CA3 pyramidal neurons, whereas CA1 pyramidal cell morphology remained unaffected.

The findings of the present research can be explained by the fact that rats exposed to restraint stress during the last week of pregnancy have significant elevations in maternal levels of plasma corticosterone (Maccari *et al.*, 2003). It is also known that glucocorticoids, being very liposoluble, easily cross placental and blood–brain barriers and enter the fetal circulatory system and brain (Zarrow *et al.*, 1970).

Glucocorticoids receptors are first expressed during the third week of fetal rat development on embryonic day 18 (Cintra *et al.*, 1994). It is well established that prenatal stress results in reduction of hippocampus corticosteroid receptors (Barbazanges *et al.*, 1996; Koehl *et al.*, 1999; Szuran *et al.*, 2000), which are the principal substrate of the negative feedback

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Table 1. Comparison of dendritic trees (mean ± SD)

	Control ($n = 8$)	Prenatal stress group ($n = 8$)	P
Granule cells			
Total no. segments	20.9 ± 2.0	16.5 ± 2.0	0.001
Total dendritic length (μm)	1521 ± 151	1337 ± 94	0.011
CA3 pyramidal cells			
Total no. segments	33.3 ± 2.9	28.8 ± 3.2	0.011
Total dendritic length (μm)	2040 ± 162	1745 ± 167	0.003
CA1 pyramidal cells			
Total no. segments	41.0 ± 3.6	39.1 ± 3.6	0.314
Total dendritic length (μm)	2228 ± 207	2086 ± 167	0.152

CA, cornu ammonis.

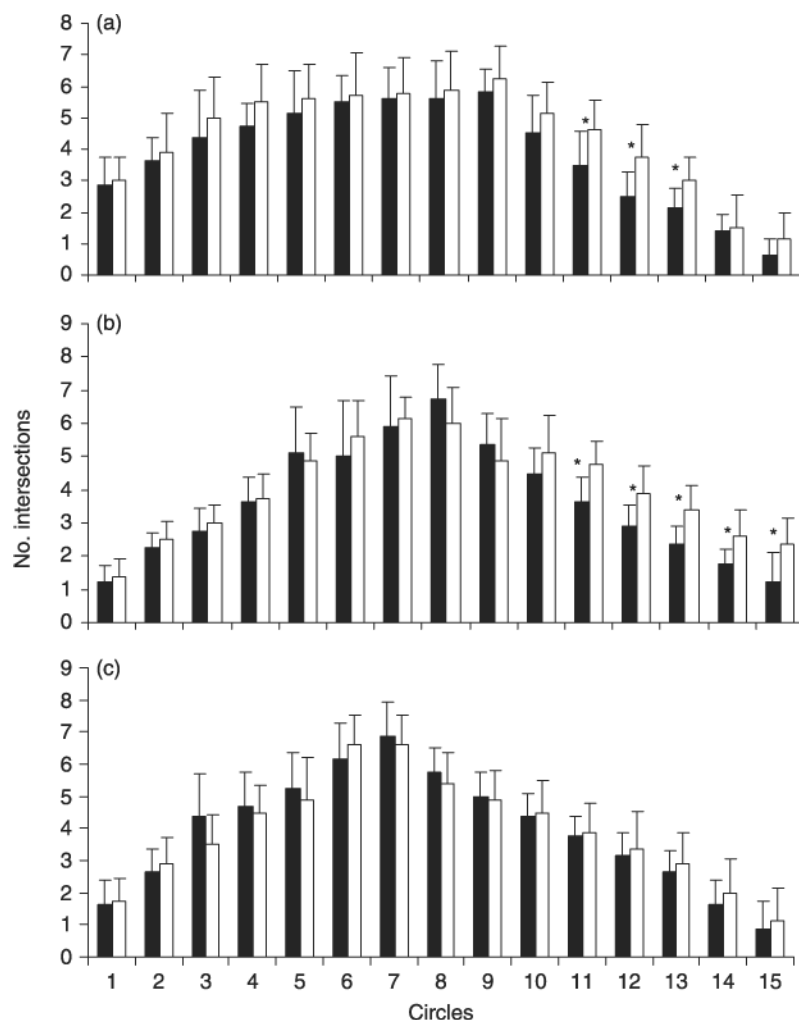

Figure 3. Dendritic branching density of hippocampal neurons for (■) prenatally stressed and (□) control animals. Vertical bars represent SD. (a) Dendritic trees of granule cells. Circles 11, 12 and 13, $P < 0.05$. (b) Apical dendritic trees of cornu ammonis (CA)3 pyramidal cells. Circles 11, 12, 13, 14 and 15, $P < 0.05$. (c) Apical dendritic trees of CA1 pyramidal cells.

control of glucocorticoid secretion and is accompanied by prolonged corticosterone secretion in response to stress (Henry *et al.*, 1994; Barbazanges *et al.*, 1996; Koehl *et al.*, 1999; Szuran *et al.*, 2000). Some studies also report a change in basal adrenocorticotropic hormone or corticosterone secretion in offspring of PS rats (Fride *et al.*, 1986; Ward *et al.*, 2000). Exposure to elevated glucocorticoid concentrations has a neurotoxic effect on hippocampal neurons and lowers the threshold for hippocampal neuronal degeneration (Sapolsky, 1999).

In the literature there are reports indicating that prenatal stress induces hippocampal neuronal loss (Lemaire *et al.*, 2000; Schmitz *et al.*, 2002; Zhu *et al.*,

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2004), but there has been no previous study examining the effects of prenatal stress on the morphology of hippocampal neurons. However, extensive animal studies show that prolonged exposure to stress (Watanabe *et al*., 1992; Magarinos & McEwen, 1995; Sousa *et al*., 2000) and glucocorticoids (Woolley *et al*., 1990) produces dendritic atrophy, particularly in the CA3 subfield in adults (for review Conrad, 2006).

Although many reports have shown that chronic stress reduces dendritic arbors only in CA3 pyramidal cells, Sousa *et al*. (2000) also reported dendritic retraction in granules and CA1 neurons following 1 month of unpredictable stress in adult rats. However, their study demonstrated that of all neurons in the hippocampal formation, CA1 neurons are the least vulnerable to stress. Although the results of the present study are consistent with reports that postnatal chronic stress results in dendritic retraction in CA3 and granule cells, it does not confirm reports that claim that exposure to chronic stress leads to neuronal atrophy in CA1 pyramidal cells.

On the whole, it can be proposed that prenatal stress and prolonged stress during adulthood produce the same adverse effects on the dendritic arborization of hippocampal neurons.

Several lines of evidence could help to explain why granule neurons of dentate gyrus (DG) and particularly pyramidal neurons of CA3 are more vulnerable to prenatal stress. Granule cells of dentate gyrus contain high levels of both type I and type II corticosteroid receptors (Reul & De Kloet, 1985) and project heavily towards CA3 pyramidal cells via mossy fibers (Amaral & Witter, 1989). Because these cells have been shown to be highly dependent on corticosterone (Gould *et al*., 1990), it seems possible that increased levels of corticosterone exerts a stimulatory effect on these neurons, which become hyperactive and promote damage of the pyramidal cells of the CA3 region. The trophic role played by neuronal afferents in promoting the survival and maintenance of their target should be borne in mind (Cunningham, 1982). It is also interesting to note that in the present study, there were no morphological effects of prenatal stress on CA1 pyramidal cells, a region to which the dentate gyrus has no known projection in the rostral hippocampus of rat (Gaarskjaer, 1986).

The changes in dendritic morphology we have observed are likely to have profound consequences for hippocampal neuronal function. Differences in branching patterns and in amount of dendritic material (Purves, 1988) have been suggested to modulate the spatial/temporal integration of synaptic input to an individual neuron. Decreases in these parameters could result in smaller total dendritic surface area available for synaptic contact. These changes in hippocampal dendritic morphology may be involved in the impairment of hippocampal-dependent memory in PS rats.

In conclusion, results of the present study show that prenatal stress induces major alterations in neurites of the hippocampal cells. Because integrity of the hippocampal circuitry is crucial for hippocampal-dependent memory, it is reasonable to conclude that the structural changes in the neurons might lead to disruption of this circuitry and lead to deficits in spatial memory and learning in PS rats.

Acknowledgments

This study was funded by the research department of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The authors gratefully acknowledge Dr M. T. Yavari for the English editing of the article.

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