Research Report

Prenatal restraint stress impairs learning and memory and hippocampal PKCbeta1 expression and translocation in offspring rats

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ABSTRACT

Prenatal stress results in various learning, behavioral and emotional alterations observed in later life. However, the mechanisms underlying these effects of prenatal stress are not fully understood. In the present study we examined the impact of prenatal stress (an unpredictable restraint stress) during gestational days 13 to 20 on the performance in Morris water maze and passive avoidance training in 1- and 3-month-old rat offspring. The expression and translocation/activation of protein kinase C (PKC) beta1 in the hippocampus of prenatally stressed offspring were also investigated. One-month-old female and male and 3-month-old female prenatally stressed offspring showed longer latency to find the platform and used the inefficient search strategy in the water maze task and showed lower memory score in the passive avoidance training compared with controls. The expression of PKCbeta1 protein and mRNA in the hippocampus of prenatally stressed offspring was dramatically weakened. In the control offspring hippocampus, passive avoidance training induced the PKCbeta1 translocation from the cytosol to the membrane, which, however, was not observed in prenatally stressed offspring. Our results suggest that deficient signal transduction of PKCbeta1 in the hippocampus resulting from prenatal restraint stress may play an important role in the impairment of learning and memory abilities of offspring.

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1. Introduction

Increasing evidence in humans indicates a high incidence of developmental lags and behavioral/emotional problems in children born from mothers who experienced chronic, psychological stress during pregnancy (Glover and O'Connor, 2002; Mulder et al., 2002). Animal studies have also shown that prenatal stress leads to impairments in brain structure, function and behavior in the offspring (Weinstock, 1997; McEwen, 2000; Blanchard et al., 2001). Learning and memory, as an important function of the brain, have been impaired in prenatally stressed offspring, such as slower learning in the water maze, and deficits in delayed alteration learning, passive avoidance conditioning and operant conditioning (Lordi et al., 1997; Fleming et al., 2002).
Although prenatally stressed animals exhibit increased secretion of adrenocorticotropic hormone and corticosterone (McCormick et al., 1995; Weinstock et al., 1998; Schneider et al., 2004), indicating elevated neuroendocrine responses to stress, the mechanism by which prenatal stress affects learning and memory is still not fully understood. Protein kinase C (PKC) is a phospholipid-dependent enzyme that exhibits a central role in activity-dependent neuronal plasticity and the early stage of hippocampal long-term potentiation (LTP) (Ben-Ari et al., 1992). PKC is composed of at least 12 different isoforms and enzyme activation is often accompanied by enzyme translocation from the cytosol to the plasma membrane. PKC signaling pathways are strong candidates for mediating the biochemical changes that underlie learning since specific PKC substrates play critical roles in neuronal physiology and activation of PKC can increase release of specific neurotransmitters from neurons in the hippocampus and throughout the nervous system (Majewski and Iannazzo, 1998). The hippocampus has an important role in many types of learning and memory. Some evidence indicates that PKC pathways are involved in hippocampal-mediated learning. PKC appears to be activated in hippocampal cells following learning (Bernabeu et al., 1995; Cammarota et al., 1997; Colombo et al., 1997) and translocated from the cytosol to the membrane (Routenberg et al., 2000). PKC beta 1 is one of behaviorally relevant PKC isoforms that was demonstrated to participate in the early synaptic events responsible for the acquisition and consolidation of an inhibitory avoidance learning (Paratcha et al., 2000). Furthermore, prenatal exposure to heroin blocked the translocation of PKC beta 1 in the hippocampus of mice (Huleihel and Yanai, 2006). Taken together, PKC beta 1 might play a role in the learning impairment observed in prenatally stressed animals.

In this study, we first investigated the effects of prenatal restraint stress on spatial learning and memory and on the ability to learn and avoid an electric shock of 1- and 3-month-old offspring rats. We next measured the expression of PKC beta 1 in the hippocampus of the prenatally stressed and control offspring. Finally, we examined the activity of PKC beta 1 after passive avoidance training to evaluate hippocampal PKC beta 1 translocation/activation of animals in both groups. Our study sought to reveal a possible mechanism by which prenatal stress impairs learning and memory of the offspring.

2. Results

2.1. Morris water maze

The prenatally stressed and control offspring rats were separately tested at the age of 1 and 3 months for spatial learning in a water maze. The average latencies in 9 sessions of various groups are shown in Fig. 1. All animals were able to improve their performance \( F_{8,496} = 173.457, P < 0.01 \) as shown by the reduction of escape latencies. The average latencies of the prenatally stressed groups were significantly longer than those of the control groups in 1-month-old female \( F_{1,15} = 33.85, P < 0.01 \) and male \( F_{1,15} = 16.73, P < 0.01 \), and 3-month-old female rats \( F_{1,16} = 4.591, P < 0.05 \), however, there was no significant difference in 3-month-old male rats \( F_{1,16} = 3.509, P > 0.05 \).

At the beginning of training, marginal and random strategies were mainly used by rats in searching the platform. With training, tendency and straight strategies were adopted. Although some differences in the search-escape strategy were observed in 1-month-old male and female and 3-month-old female offspring, the 1-month-old female ones were more persuasive (Fig. 2). The occurrence times of the straight strategy, the most efficient one, in prenatally stressed 1-month-old female animals was significantly reduced \( P < 0.05 \).

2.2. Passive avoidance conditioning

One- and three-month-old offspring rats were trained in the step-through passive avoidance procedure, and the retention session was performed after 24 h. The results (Table 1) indicate that the differences in T1 values were not significant between the control and prenatally stressed offspring of each age and sex subgroups. Except 3-month-old male rats \( U = 24.5, P > 0.05 \), the memory scores \( T2 - T1 \) of prenatally stressed rats in other groups were significantly lower than those of the control rats \( U = 10.5, P < 0.01 \) in 1-month-old female rats; \( U = 17, P < 0.01 \) in 1-month-old male rats; \( U = 15, P < 0.01 \) in 3-month-old female rats. In addition, the animals entering the dark compartment in the prenatally stressed groups during the retrieval test were significantly more than those in the control groups \( \chi^2 = 6.6, P < 0.01 \) in 1-month-old female rats; \( \chi^2 = 5.51, P < 0.05 \) in 1-month-old male rats; \( \chi^2 = 4.8, P < 0.05 \) in 3-month-old female rats, except for 3-month-old male offspring \( \chi^2 = 0.8, P > 0.05 \).

2.3. Expression of PKC beta 1 protein and mRNA

PKC beta 1 immunoreactivity is widely distributed in the hippocampus. In the control offspring rats, the neuropil of the stratum oriens and stratum radiatum was moderately stained in the hippocampal CA2 region, but heavily stained in the CA1 and CA3 regions (Fig. 3A). The crowded pyramidal cells of the CA3 and some of granular cells of the dentate gyrus were moderately labeled with a peripheral soma and process staining (Figs. 3C, D), whereas cell staining in the CA1 and CA2 was weak. The distribution of PKC beta 1 immunoreactivity in the prenatally stressed rats was similar to that of controls, however, the staining was very weak in all regions examined (Fig. 3B). PKC beta 1 mRNA detected by the in situ hybridization was mainly located in the cytoplasm of pyramidal cells of the CA1-CA3 regions and in the granular cells of the dentate gyrus. The PKC beta 1 mRNA signal was weaker in the prenatally stressed group vs. the control group in all hippocampal regions (Fig. 4). There were no marked differences in PKC beta 1 immunostaining and mRNA signal between male and female and between 1-month-old and 3-month-old offspring.

2.4. Translocation of PKC beta 1 after passive avoidance training

Western blot analysis displayed similar results in both 1- and 3-month-old offspring. The levels of PKC beta 1 protein in Triton-soluble membrane fractions of the hippocampus in the
Fig. 1 – The latency for offspring rats finding the platform in the Morris water maze test. The latency per testing session represents the average of four trials of all animals in each group. Generally, the latencies of all rats in both control and prenatally stressed groups are reduced with training ($P<0.01$). The latency of 1-month-old female ($n=9$) (A) and male ($n=9$) (B) and 3-month-old female ($n=10$) (C) prenatally stressed rats is significantly longer than that of respective control rats ($n=8$ each) ($P<0.01$, $P<0.01$, and $P<0.05$, respectively). However, there is no significant difference between 3-month-old male (D) prenatally stressed rats ($n=10$) and control rats ($n=8$).

Fig. 2 – The times of various search strategies in 36 trials (9 sessions, 4 trials/session) used by offspring rats in the Morris water maze test. One-month-old female (A) and male (B), and 3-month-old female (C) prenatally stressed rats tend to use inefficient random strategy compared with the respective control rats ($^{*}P<0.05$, $^{**}P<0.01$). There is no significant difference in using search strategies between 3-month-old male prenatally stressed and control rats (D).
1- and 3-month-old training control rats were increased by 66.0±12.2% and 55.6±5.6%, respectively, both significantly higher (P<0.05) than those in the non-training control rats, indicating a translocation of PKCbeta1 (Fig. 5A). There was no significant difference in the level of PKCbeta1 protein in Triton-soluble membrane fractions of the hippocampus between the training and non-training prenatally stressed rats of both 1- and 3-month-old groups (Fig. 5B). No effect of passive avoidance training on PKCbeta1 level in cytosol fractions of the hippocampus was found in the training control and prenatally stressed rats.

3. Discussion

It is suggested from animal studies that prenatal stress may result in a general susceptibility to psychopathology, leading to behavioral alterations observed throughout postnatal life (Huizink et al., 2004). Numerous prenatal stressors, such as foot shock, cold water swim, heat stress and restraint or placement in a novel environment, are applied in such animal studies. In the present study prenatal restraint stress, which is likely to mimic the psychosocial stress that humans encounter in daily life, was utilized during the late gestation, a period critical for synaptogenesis. We found that 1-month-old prenatally stressed offspring, no matter female or male, showed longer latency to find the platform in the water maze task and lower memory score in the passive avoidance training compared with controls. These results indicate that prenatal restraint stress impairs the offspring’s abilities for spatial learning and memory tasks, and memory retention for noxious stimuli. However, in 3-month-old offspring, such effect was observed only in females, suggesting that prenatal stress-induced behavioral impairment may be reversible and that the memory ability of adult male offspring has recovered in a sense. Although mild prenatal stress and stress of short duration are shown to facilitate brain development and learning performance in offspring (Fujioka et al., 1999, 2001), most studies on prenatal stress of long duration during the late pregnancy have revealed changes in brain morphology and impaired learning and memory of offspring (Lemaire et al., 2000; Coe et al., 2003). Furthermore, the sex and age differences in learning and memory deficits induced by prenatal stress have also been reported in the literature with different results. Prenatal stress significantly affected the performance of female juvenile rats in the step-through type of passive avoidance procedure (Gue et al., 2004) and caused a high anxiety level in female offspring (Bowman et al., 2004). Jiang et al. (2004) found that prenatal exposure to a magnetic field induced impaired performance of female rats at a specific age in the Morris water maze. These findings are similar to our results. Lordi et al. (1997), however, found that the memory capability of offspring from stressed mothers in passive avoidance conditioning was impaired, but only adult male offspring were involved. It seems that different results are

![Fig. 3 - Immunohistochemical staining for PKCbeta1 in the hippocampus of 3-month-old offspring rats. PKCbeta1 immunoreactivity is intensely located in the stratum oriens (o) and stratum radiatum (r) of the CA1-CA3 (A) and pyramidal cells (arrows) of the CA3 (C) and granular cells (arrows) of the dentate gyrus (D) in the control rat. However, the staining is dramatically reduced in the prenatally stressed rat (B). Scale bar=500 μm (A and B), 50 μm (C and D).](image-url)
present in different studies. Maybe, it is because of the difference in starting time, duration and prediction degree of prenatal stress, the age and sex of offspring, and even the strain of animals.

The mechanism for impaired learning and memory of prenatally stressed offspring needs further investigation, and the hippocampus is the most studied target brain region. Learning and memory of spatial and contextual information depend on the hippocampus (McEwen, 1999, 2000). Many researches have demonstrated that prenatal stress induces a decrease in dendritic arborization and density of dendritic spines and synapses (Hayashi et al., 1998; Ishiwata et al., 2005; Barros et al., 2006), a reduction in cell proliferation and brain-derived neurotrophic factor content (Van Den Hove et al., 2006) and a lifespan reduction of neurogenesis and volume in the hippocampus of offspring animals (Lemaire et al., 2000; Coe et al., 2003). Moreover, the sex difference in hippocampal impairments of prenatally stressed offspring has also been found, showing that prenatally stressed females, but not males, had a decrease in the number of hippocampal neurons (Schmitz et al., 2002; Zhu et al., 2004). These data seem to well link the poor behavioral performance to altered hippocampal morphology and biochemistry. However, the signaling pathway is largely unknown. Increasing evidence indicates that prenatal stress impairs LTP in the hippocampus of offspring and PKC pathway is involved in hippocampal LTP and certain forms of learning and memory (Bernabeu et al., 1995; Yang et al., 2006). Thus our study focused on hippocampal PKCbeta1, which is a behaviorally relevant isoform of PKC and not yet widely studied in the field of prenatal stress. Our result showed that prenatal stress diminished the expression of PKCbeta1 at both protein and mRNA levels in the hippocampus of offspring, especially in the CA3, CA1 and dentate gyrus, the regions important in the entorhinal–hippocampal trisynaptic circuit (Jones, 1993). Unexpectedly, the sex and age difference in PKCbeta1 expression was not obvious in our study. This may be due to limited sensitivity of the methods used. On the other hand, the control of the behavior is complex and multiple factors such as glucocorticoids, excitatory amino acids and N-methyl-D-aspartate receptors are involved individually or cooperatively (McEwen, 1999). For example, the high response of the hypothalamic–pituitary–adrenal axis to prenatal stress is more marked and prolonged in females than in males (McCormick et al., 1995; Richardson et al., 2006), which might affect the behavior of offspring independently of the PKC pathway. Nevertheless our finding suggests that PKCbeta1 downregulation is, at least in part, responsible for impaired learning and memory of prenatally stressed offspring.

Basically, PKC is assumed to be present in an inactive form in the cytosol and Triton-soluble membrane fractions of offspring rats after passive avoidance training. (A) The control group. (B) The prenatally stressed group. The upper parts of both (A) and (B) show PKCbeta1 protein bands detected by Western blot, and the lower parts are the bar graphs showing the relative changes in PKCbeta1 protein levels. The protein levels in the non-training rats are ascribed 100%. Each bar represents the average of three separate assays. *P<0.05 compared with the non-training rats (n=5 in each subgroup).
stimulation of phospholipid lead to its translocation to the membrane, where it develops a physiologic activity. Accordingly, the translocation of PKC can reflect its activation. Translocation of PKC from cytosol to membrane has been reported after LTP and inhibitory avoidance learning in chicks and rats (Burchuladze et al., 1990; Bernabeu et al., 1995; Cammarota et al., 1997). The deficits of learning and memory which occurred in aging rats were accompanied by the impaired translocation of PKC (Battaini et al., 1995). Therefore, we presumed that, besides generally decreased expression of PKCbeta1 in the hippocampus, prenatal stress might also interfere with translocation/activation of PKCbeta1 during the learning procedure. In order to test this hypothesis, we investigated the content of hippocampal PKCbeta1 in cytosol and Triton-soluble membrane fractions by Western blot analysis in the prenatally stressed and control offspring 30 min after passive avoidance training. Our results indicate that, after training hippocampal PKCbeta1 in the control, but not in prenatally stressed, offspring is redistributed and the membrane-associated PKCbeta1 content is increased. This finding is consistent with a previous study, which showed that step-down inhibitory avoidance training resulted in a selective increase in the content of PKCbeta1 isozyme in hippocampal synaptic membrane, and bilateral microinjection of a selective inhibitor of PKCbeta1 isozyme into the CA1 of the dorsal hippocampus produced amnesia when given before or after training (Paratcha et al., 2000). Moreover, the translocation of PKCbeta could not be induced in the hippocampus of mice prenatally exposed to heroin, which was associated with behavioral deficits, such as impaired spatial discrimination learning (Shahak et al., 2003; Huleihel and Yanai, 2006). Thus our results of passive avoidance training indicate that prenatal stress results in a deficit in hippocampal PKCbeta1 translocation/activation during learning and memory.

In summary, our results not only demonstrate a sex- and age-dependent impairment in learning and memory of prenatally stressed offspring rats but also provide evidence that PKCbeta1 signaling is related to the behavioral deficits. Both the decreased expression and impaired translocation/activation of hippocampal PKCbeta1 induced by prenatal restraint stress may be an important profile contributing to the impairment in learning and memory ability in the offspring.

4. Experimental procedures

4.1. Animals and treatment

Sprague–Dawley rats, provided by the Xi’an Jiaotong University School of Medicine, were housed with free access to food and water and in 12 h light/12 h dark, 20–22 °C. The care and treatment of animals were approved by the Institutional Animal Care and Research Advisory Committee at the Xi’an Jiaotong University School of Medicine. Three mature females and two males were put together in a cage overnight and the vaginal smear was examined on the following morning. The presence in the smear of both vaginal cells typical of the estrous stage and spermatozoids indicated day 1 of pregnancy. Individual pregnant rats were separated in a plastic cage and either left undisturbed or stressed. Restraint stress was done by placing the rat in a transparent plastic tube (6 cm in diameter and adjustable length) for 45 min, 3 times/day at random intervals during gestational days 13 to 20.

After weaning offspring rats of the same sex were group housed. Only a single offspring from each litter was randomly selected to use in the following behavioral and neurochemical analyses.

4.2. Morris water maze

Offspring were divided into 8 groups: the prenatally stressed 1-month-old female and male (n=9 each), and 3-month-old female and male (n=10 each); the control 1-month-old female and male (n=8 each), and 3-month-old female and male groups (n=8 each).

The equipment for the water maze is the same as described by Jiang et al. (2004). For the place navigation (spatial learning acquisition) test, animals were subjected to two test sessions (four trials each) per day for 4.5 consecutive days. Each trial consisted of placing the rat in water facing the wall of the pool at one of the four starting locations (North, East, South and West) in a random order. The rat was allowed to search the platform for a maximum of 120 s. If an animal did not find the platform in 120 s, it was gently lifted up and placed onto the platform for 5 s before being returned to the cage. The escape latency (the duration for finding the platform) and swim path were automatically recorded by a video/computer system and the software developed by the Institute of Psychology, Chinese Academy of Sciences. The average latency of each session was calculated from the four trials of each rat in the group. The swim paths are defined as 4 swim strategies by the software: (1) marginal, swimming along the pool edge; (2) random, swimming randomly; (3) tendency, swimming around toward the platform area; (4) straight, swimming straight toward the platform. The first two strategies are inefficient and the last one is the most efficient in searching for the platform. The times of each strategy used by each rat in 36 trials were recorded and average times in each group were then calculated.

4.3. One-trail passive avoidance conditioning

Offspring were divided into 8 groups in the same way as in the water maze and trained a one-trial passive avoidance task and tested for retention 24 h later. The experiment was conducted in a shuttle box separated into a lighted compartment and a dark one by a wall with a hole permitting animals to pass from one compartment to the other. The dark compartment is equipped with a grid floor, to which scrambled footshocks (30 V, 4 mA and 50 Hz) can be delivered.

On the first testing day the rat was put into the lighted compartment, and they then entered the dark compartment by themselves. When the whole body of the rat entered the dark compartment, the footshock was delivered for about 5 s until the animal escaped to the lighted compartment, and was allowed to stay there for 1 min before it was returned to its cage. The step-through latency (T1) that elapsed from the
moment the rat entered the lighted compartment to the moment when it entered the dark compartment was measured. After 24 h, the animals were subjected to a retrieval test given exactly in the same way as the initial conditioning, except that the rats did not get electric shocks when entering the dark compartment. The step-through latency (T2) was recorded (up to 300 s). The number of animals entering the dark compartment was also counted. The memory score (T2–T1) would reflect the ability to remember the nociceptive experience. If the T2–T1 difference is zero or small, this indicates that the animals have forgotten their initial nociceptive experience (Lordi et al., 1997, 2000).

4.4. Immunohistochemistry

One- and three-month-old control (n=8 each) and stressed (n=10 each) offspring (half males and half females) were deeply anesthetized with amobarbital, and all efforts were made to minimize the suffering of animals. Animals were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), and brains removed and immersed in the same fixative overnight. Serial coronal sections containing the dorsal hippocampus were cut at 40 μm with a vibratome. Sections from each rat were immunostained free-floating with a polyclonal antibody to PKCβ1 (1:400, Santa Cruz) and the avidin–biotin–peroxidase complex (ABC, Vector) method. PKCβ1-immunoreactive product was visualized in a chromogen solution containing 0.05% diaminobenzidine (DAB, Sigma) and 0.01% H2O2 in 0.1 M PB. Substitution of normal rabbit serum for PKCβ1 antibody in the negative control completely eliminated the immunohistochemical staining.

4.5. In situ hybridization

Animal grouping and perfusion were the same as for immunohistochemistry except that the fixative solution contained 0.1% diethyl pyrocarbonate (Sigma). Serial coronal paraffin sections containing the hippocampus were cut at 8 μm. Dewaxed and dehydrated sections were first pretreated with proteinase K (Sigma) followed by incubation with prehybridization solution. A synthetic oligonucleotide of PKCβ1 mRNA labeled with digoxigenin (Santa Cruz) was used as a probe, which was applied to tissue sections overnight at 37 °C. After washing in a series of standard saline citrate (SSC), sections were incubated with biotinylated secondary antibody (Santa Cruz) to digoxigenin and ABC, respectively. Coloration reaction was developed in DAB–H2O2 solution containing nickel ammonium sulfate. Incubation of sections in the prehybridization solution without the probe was taken as the negative control.

4.6. Western blot

Thirty minutes after passive avoidance training in the first testing day, twenty 1- and 3-month-old offspring were sacrificed by decapitation under deep anesthesia. Twenty control and prenatally stressed offspring not subjected to passive avoidance training were also used. The offspring rats in each age were divided into 4 groups (n=5 each): the non-training control, training control, non-training stressed and training stressed. The brain was rapidly removed, and hippocampus dissected, frozen in liquid nitrogen, and stored at −80 °C. Frozen hippocampus was homogenized in 20 mM Tris–HCl buffer (pH7.4) containing 0.32 M sucrose, 2 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride and 20 μg/ml leupeptin and centrifuged at 26,000×g for 30 min at 4 °C. The supernatant represented the cytosol fraction, while the pellet was resuspended in the same volume of buffer containing 0.2% Triton X-100 for 45 min at 4 °C, sonicated and centrifuged. This supernatant represented the Triton-soluble membrane fraction. The total protein concentration in both cytosol and Triton-soluble membrane fractions was determined with the Bradford method. The samples (30 μg protein/lane) were electrophoresed on 10% SDS–PAGE, electroblotted to nitrocellulose membranes, blocked with Tris buffered saline (TBS) containing 0.05% Tween 20 (TBST) and 5% non-fat dry milk for 2 h. Then membranes were incubated with antibody to PKCβ1 (1:100 in TBST containing 3% bovine serum albumin) overnight at 4 °C followed by alkaline phosphatase conjugated goat anti-rabbit IgG (1:500, Santa Cruz) for 1 h. Immunoreactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium (Sigma). Coomassie blue-stained gels were used to assess equal loading of protein into gel lanes. A digital gel image analysis system was used for semi-quantification of PKCβ1 immunoreactivity. PKCβ1 immunoreactivity of the non-training control and stressed groups was taken as a standard (100%) and that of the training control and stressed groups expressed as a percentage of the standard, respectively.

4.7. Statistical analysis

All data were expressed as mean±SD. The escape latency in the water maze was analyzed with repeated-measure analysis of variance (ANOVA). Treatment, age and sex were the three between-subject factors. Mann–Whitney non-parametric test was applied to analyze swimming strategies. In passive avoidance conditioning, comparison of T1 was made according to the Wilcoxon test and comparison of (T2–T1) to the Mann–Whitney tests. The difference in number of animals entering dark compartment was analyzed by chi square test. The relative expression levels of PKCβ1 in Western blot analysis were analyzed by the t test. Difference was regarded as significant at P<0.05.

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