Acta Biochim Biophys Sin 2011, 43: 601–606 | © The Author 2011. Published by ABBS Editorial Office in association with Oxford University Press on behalf of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. DOI: 10.1093/abbs/gmr057.
Advance Access Publication 8 July 2011

Original Article

The effects of prenatal stress on expression of CaMK-II and L-Ca^{2+} channel in offspring hippocampus

Qing Cai[1*], Boli Zhang[2], Shuyun Huang[2], Tao Wang[3], and Tao Zhou[1]

[1]Department of Physiology, Tianjin University of Traditional Medicine, Tianjin 300193, China
[2]Tianjin University of Traditional Medicine, Tianjin 300193, China
[3]Department of Histoembryology, Tianjin University of Traditional Medicine, Tianjin 300193, China
*Correspondence address. Tel: +86-22-59596201; Fax: +86-22-59596222; E-mail:qingcai@tjutcm.edu.cn

The purpose of the present study was to characterize the expressions of phosphorylated Ca^{2+}/calmodulin-dependent protein kinase II (p-CaMK-II), total CaMK-II, and L-type Ca^{2+} channel in offspring hippocampus that was induced by prenatal restraint stress. Pregnant rats were divided into two groups: the control group and the prenatal stress (PNS) group. Pregnant rats in the PNS group were exposed to restraint stress on day 14–20 of pregnancy three times daily for 45 min. Adult offspring rats were used in this study. The results demonstrated that prenatal restraint stress induced a significant increase in the expression of p-CaMK-II, total CaMK-II, and L-Ca^{2+} channel by western blot analysis in offspring hippocampus. The immunohistochemistry results revealed that PNS increased the expressions of CaMK-II and L-Ca^{2+} channel in the hippocampal CA3 of offspring rats. These data suggest that PNS can have long-term neuronal effects within hippocampal structure involved in the feedback mechanisms of the hypothalamo-pituitary-adrenal axis.

Keywords CaMK-II; hippocampus; CA3; L-Ca^{2+} channel

Received: February 24, 2011 Accepted: April 22, 2011

Introduction

Chronic or repeated stress during human prenatal brain development has been associated with various learning, behavioral, and/or mental disorders in later life [1]. In studies using the animal model, stress exposure during pregnancy can program adult behavior and hypothalamo-pituitary-adrenal (HPA) axis stress responsiveness [2,3]. The hippocampus has the highest density of glucocorticoid receptors in the brain. It involves in the regulation of the HPA and the behavioral response to stress. Studies in non-human primates and rodents have demonstrated the alteration in the structure and function of the hippocampus as a consequence of prenatal stress (PNS) [4,5]. PNS in mid-pregnancy also affects the spine density and basal dendrites of pyramidal neurons of hippocampus [6].

Our previous studies have demonstrated that PNS can increase the expression of extracellularsignal-regulated kinase and p38 mitogen-activated protein kinase in offspring hippocampus neurons [7,8]. Recent data have indicated that PNS can increase glutamate concentration in the hippocampus and cause apical dendritic atrophy of pyramidal neurons of hippocampal CA3 in offspring rats [9]. It has been reported that restraint stress promotes the phosphorylation of CaMK-II in the hippocampus [10]. A previous study also showed that p-CaMK-II was enhanced shortly after swim stress [11]. Whether PNS causes the changes of L-Ca^{2+} channel and CaMK-II in offspring hippocampus neurons is unknown. In the present study, western blot analysis and immunohistochemistry were carried out to examine the effects of PNS on the expression of L-Ca^{2+} channel and CaMK-II in offspring hippocampus.

Materials and Methods

Animals and procedure

A total of 15 female (230–250 g) and five male (280–350 g) Sprague–Dawley rats were used. The animals were provided by the experimental animal center of Tianjin University of Traditional Medicine. The rats were housed in an animal room with controlled temperature (22 ± 2°C) and controlled humidity (60%) on a 12 h light/dark cycle (with the light on from 8 a.m. to 8 p.m.) with free access to food and water throughout the experiment. Virgin female rats were placed overnight with adult male rats (3:1) for mating. Vaginal smears were examined on the following morning. Pregnancy was determined by the presence of vaginal plugs (embryonic day 0, E0). Each pregnant rat was then housed separately. A total of 12 pregnant

rats were randomly assigned to the PNS group ($n = 6$) and the control group ($n = 6$). The pregnant rats in the PNS group were exposed to restraint stress three times daily for 45 min during the last week of pregnancy (E14–E21) as previously described [7]. The pregnant rats of the control group were left undisturbed. On Day 21, all offspring were weaned, then male and female pups were separated and every four pups were housed in one cage, respectively, until being tested at 1 month of age.

Western blot analysis

Six adult offspring rats in each group were deeply anesthetized with chloral hydrate and decapitated immediately. The hippocampus was dissected out on the petri dish filled with ice and immediately frozen in liquid nitrogen, then kept in an −80°C freezer until later experimental use. Each hippocampus was homogenized with a glass tissue grinder in 500 μl cold phosphate-buffered saline (PBS) buffer containing protease inhibitors (2 μg/ml aprotinine, 1 μg/ml leupeptine, and 1 mmol/l PMSF). The samples were centrifuged at 12000 *g* for 20 min at 4°C after homogenization. The total protein concentration in each sample was determined using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, USA). The sample proteins of hippocampus (50 μg/lane) were separated by electrophoresis performed on 10% sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE, Bio-Rad, Barcelona, Spain) at room temperature. The primary antibodies against L-type Ca^{2+} channel (Santa Cruz, USA, 1:1000), p-CaMK-IIα (Thr 286)-R (Santa Cruz, 1:1000), and total CaMK-II (Santa Cruz, 1:1000) were used. After washing three times (10 min each) with 75 ml TBST buffer, the membranes were incubated for 1 h with horseradish peroxidase conjugated secondary antibody (Promega, Madison, USA, 1:5000) in TBST buffer. After washing three additional times with TBST buffer, and three times with tris-buffered saline (TBS) [20 mM Tris–HCl (pH 7.5), 150 mM NaCl], the enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK) was used for signal detection. β-actin was detected with mouse anti-rat β-actin antibody (Santa Cruz) as the control. For the densitometric analysis, the protein bands on the blot were measured by Gel Doc200 systems and software.

Immunocytochemistry staining

Six adult offspring from each group were dissected for immunocytochemistry as previously described [7]. Under deep anesthesia with chloral hydrate, the rats of each group were perfused transcardially with ~200 ml of 0.1 M PBS (pH 7.4), followed by approximately cold 400 ml of 4% paraformaldehyde in phosphate buffer (PB) (pH 7.4). The brains were removed, cut into blocks containing the hippocampus, and post-fixed in the same solution for 24 h at 4°C. Subsequently, the tissue blocks were dehydrated, processed for paraffin embedding, and then cut into frontal sections (4 μm thickness). Brain sections were deparaffinized in xylene and hydrated through graded alcohols to TBS, washed with TBS three times (5 min each time). They were then treated with freshly made 3% H_2O_2 in TBS for 30 min. After being washed several times in TBS, they were then treated with 0.1% Triton X-100. Non-specific binding sites were blocked by incubation with normal goat serum 1 h at 37°C. The sections were incubated with polyclonal antibodies of anti-total CaMK-II (Santa Cruz, 1:200) and L-type Ca^{2+} (Santa Cruz, 1:1000) in 0.1 M TBS containing 0.5% bovine serum albumin (BSA), and 0.05% sodium azide at 4°C. After overnight incubation, sections were rinsed and incubated with biotinylated secondary antibody at a dilution of 1:200 in 0.1 M PBS containing 0.5% BSA for 1 h at room temperature. After rinsing, the sections were incubated with ABC reagent diluted at 1:50 in TBS for 1 h at room temperature, and then rinsed with TBS followed with 0.1 M PB. Finally, sections were incubated in SIGMA FAST DAB kit (Sigma-Aldrich, St. Louis, USA), until the desired stain intensity developed. Sections were rinsed with 0.1 M PB and then mounted on gelatin-coated slides, and dehydrated through alcohols and xylene. The optical density (OD) analysis was performed on high resolution and analyzed with Scion Image Software based on NIH image (NIH Image, Bethesda, USA). OD value was calculated as described: OD value = average of OD values of sample-average of OD of background values. Background values were obtained from the neighboring white matter. The average values from three sections for each subject were used for statistical analysis.

Statistical analysis

Results were presented as mean+SE. Statistical analysis was performed using SigmaStat 3.5 Statistical Software (Jandel Scientific, San Rafael, USA). The data were analyzed by two-way analysis of variance. When multiple comparisons were indicated, the Student–Newman–Keuls test was applied. Differences were considered to be significant at the $P < 0.05$ level.

Results

The effects of PNS on the expression of p-CaMK-II and total CaMK-II in offspring hippocampus

Phosphorylated CaMK-II and total CaMK-II activation in total hippocampal extracts were analyzed by western blot using specific antibodies. The levels of p-CaMK-II and total CaMK-II were normalized to the expression level of β-actin. Quantitative analysis showed that the level of p-CaMK-II in the PNS female offspring rats was

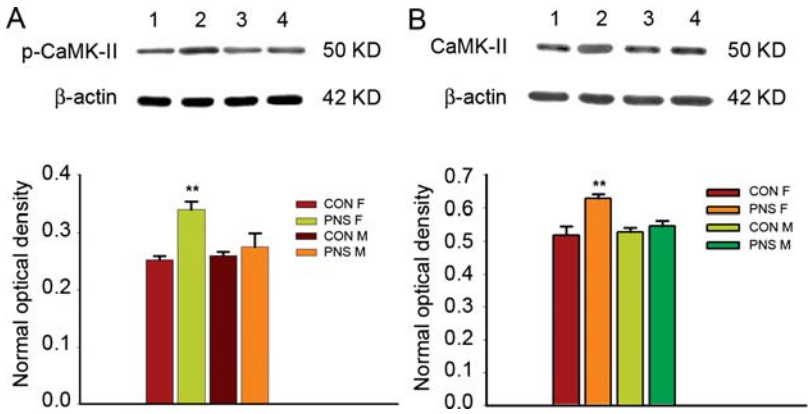

Figure 1 Effects of PNS on the expression of p-CaMK-II and total CaMK-II in offspring hippocampus (A) Effects of PNS on the expression of p-CaMK-II. Cytosolic fractions (50 μg protein/lane) from control female (lane 1), PNS female (lane 2), control male (lane 3), and PNS male offspring hippocampus (lane 4). CON F, control female; PNS F, PNS female; CON M, control male; PNS M, PNS male. (B) Effects of PNS on the expression of total CaMK-II in offspring hippocampus. Cytosolic fractions (50 μg protein/lane) from control female (lane 1), PNS female (lane 2), control male (lane 3), and PNS male offspring hippocampus (lane 4). CON F, control female; PNS F, PNS female; CON M, control male; PNS M, PNS male.

significantly increased (0.3393 ± 0.0138, $n = 6$) compared with that in the control female offspring rats (0.2528 ± 0.016, $n = 6$, $P < 0.01$). There was no significant difference in the level of p-CaMK-II between the male control group and PNS offspring rats (0.2597 ± 0.0186 and 0.2753 ± 0.0239, respectively) [**Fig. 1(A)**].

The specific signals for total CaMK-II were quantified and plotted. As shown in **Fig. 1(B)**, there was no significant difference in the level of CaMK-II between the male control and PNS male offspring rats (0.5284 ± 0.0126 and 0.5465 ± 0.0155, $n = 6$). The level of total CaMK-II in female PNS offspring group was significantly increased compared with that in the female control group (0.5185 ± 0.0265 and 0.6288 ± 0.0128, respectively, $P < 0.01$).

The effects of PNS on the expression of L-Ca^{2+} channel in offspring hippocampus

The level of L-Ca^{2+} channel in the hippocampus was detected by western blot [**Fig. 2(A)**] and quantitatively analyzed [**Fig. 2(B)**]. The results showed that the level of L-Ca^{2+} channel in the PNS female offspring rats was significantly increased (0.2465 ± 0.0854, $n = 6$) compared with that in the control female offspring rats (0.1719 ± 0.0874, $n = 6$, $P < 0.01$). Western blot analysis also revealed that there was significant difference in the level of L-Ca^{2+} channel between the male control and PNS offspring rats (0.1721 ± 0.0856, 0.2070 ± 0.0739, respectively, $P < 0.05$) [**Fig. 2(B)**].

The effects of PNS on the CaMK-II-positive expression in the CA3 regions of offspring hippocampus

Immunohistochemistry was used to demonstrate the CaMK-II immunoreactive cells in the principal pyramidal neuron layer of hippocampus. **Figure 3(A)** showed the representative photographs of CaMK-II staining in control and PNS offspring hippocampus. We analyzed the pyramidal cell layer in CA3. The OD of CaMK-II was significantly increased in PNS female offspring hippocampus (68.4517 ± 2.6422, $P < 0.01$, $n = 6$) compared with that in the female control (59.9350 ± 2.9755, $n = 6$) [**Fig. 3(B)**]. The OD of CaMK-II in the CA3 region of the male PNS groups was significantly higher than that in the male control group (65.5967 ± 2.3877, 60.9667 ± 2.2574, respectively, $P < 0.05$) [**Fig. 3(B)**].

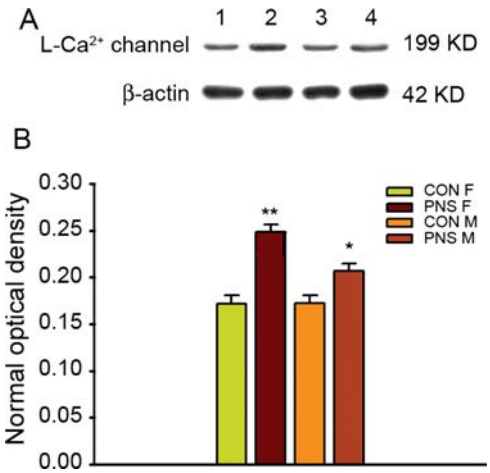

Figure 2 Effects of PNS on the expression of L-Ca^{2+} channel in offspring hippocampus (A) Western blot analysis. Cytosolic fractions (80 μg protein/lane) from control female (lane 1), PNS female (lane 2), control male (lane 3), and PNS male offspring hippocampus (lane 4). Representative immunoblot for β-actin to control protein loading (low lane). (B) Quantitative analysis of L-Ca^{2+} channel levels in the hippocampus. CON F, control female; PNS F, PNS female; CON M, control male; PNS M, PNS male.

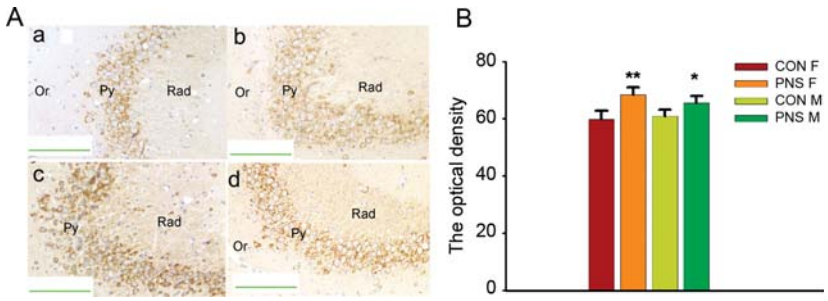

Figure 3 Effects of PNS on the expression of CaMK-II in offspring hippocampal CA3 region (A) Representative photographs of CaMK-II staining in offspring hippocampal CA3 region. a: control female; b: PNS female; c: control male; d: PNS male. Or, oriens layer; Py, pyramidal layer; rad, radiatum layer. The scale bars=50 μm. (B) Quantitative analysis of the expression of CaMK-II in hippocampal CA3 area. CON F, control female; PNS F, PNS female; CON M, control male; PNS M, PNS male.

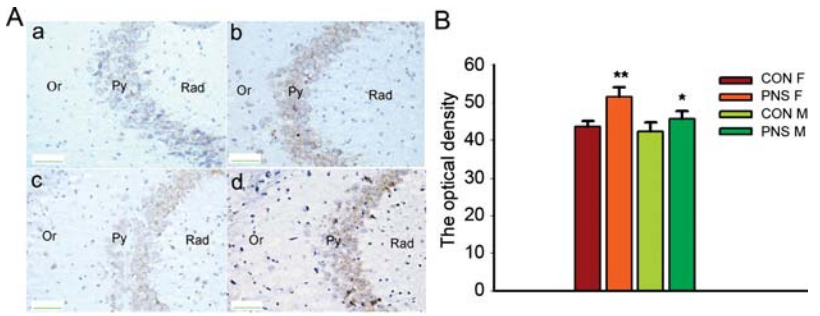

Figure 4 Effects of PNS on the expression of L-Ca^{2+} channel in offspring hippocampal CA3 region (A) Representative photographs of L-Ca^{2+} channel staining in offspring hippocampal CA3 region. a: control female; b: PNS female; c: control male; d: PNS male. Or, oriens layer; Py, pyramidal layer; rad, radiatum layer. The scale bars=25 μm. Six animals from each group were dissected for immunohistochemistry. (B) Quantitative analysis of the expression of L-Ca^{2+} channel in hippocampal CA3 area. CON F, control female; PNS F, PNS female; CON M, control male; PNS M, PNS male.

The effects of PNS on the L-Ca^{2+} channel-positive expression in the CA3 regions in offspring hippocampus

Immunohistochemistry results demonstrated that L-Ca^{2+} channel immunoreactive cells existed in the principal pyramidal layer of hippocampus. **Figure 4(A)** showed representative photographs of L-Ca^{2+} channel staining in control and PNS offspring hippocampus CA3 region. The OD of L-Ca^{2+} channel was significantly increased above female control (43.6017 ± 1.5084, $n = 6$) in PNS female offspring hippocampus (51.5283 ± 2.5070, $P < 0.01$, $n = 6$) [**Fig. 4(B)**]. The OD of L-Ca^{2+} channel in the CA3 region of male PNS groups was significantly higher than that in the male control group (42.2933 ± 2.3541, 46.3683 ± 1.9768, respectively, $P < 0.05$) [**Fig. 4(B)**].

Discussion

The current study demonstrated that the prenatal restraint stress induced a significant increase in the expression of p-CaMK-II, total CaMK-II, and L-Ca^{2+} channel by western blot analysis in offspring hippocampus. The immunohistochemical results revealed that the exposure of pregnant rats to restraint stress during the last week of gestation resulted in an increase of CaMK-II and L-Ca^{2+} channel levels in the hippocampal CA3 of offspring.

Previous work from our laboratory has demonstrated the effects of PNS on the capability of learning and memory and the levels of corticosterone. Corticosterone level was significantly higher in the stress group than in the control group [12]. The mechanisms by which PNS can program neuronal development with long-term consequences are not well understood. Within the developing brain, the limbic system (primarily the hippocampus) is particularly sensitive to endogenous and exogenous corticosterone during the development. PNS can increase the plasma levels of cortisol and corticotrophin releasing hormones in the mother and fetus, which may contribute to insulin resistance and behavior disorders in their offspring such as attention and learning deficits, generalized anxiety, and depression. The changes in behavior can be induced by PNS in laboratory rodents and non-human primates. The appearance of such changes is associated with structural changes in the hippocampus.

It can be assumed that the effects of PNS on the disturbance in the HPA axis activity could result in changing the density of glucocorticoid receptors in specific brain

regions, most probably in the hippocampus. The corticosterone level of offspring and dam induced by PNS is positively correlated [13]. Several plausible mechanisms by which PNS up-regulates the phosphorylation of CaMKII can be considered. It is well known that the elevation of intracellular Ca^{2+} levels induces Ca^{2+}-dependent phosphorylation of CaMK-II, and subsequently leads to autophosphorylation of CaMK-II, Ca^{2+}-independent activation. A previous study has shown that PNS cannot significantly change the expression of p-CaMK-II, while the acute challenge reduces p-CaMK-II (Thr286) levels in antenatally stressed rats at hippocampal level [14]. This result is inconsistent with our study. Such discrepancy could be because of specific experimental conditions of PNS as well as the different time-point for examining (i.e. 80 days of rat age in Fumagalli's study versus 30 days of rat age in our study), which may contribute to specificities of p-CaMK-IIa expression.

In this study, we showed the prenatal restraint stress induced a significant increase in the expression of CaMK-II and L-Ca^{2+} channel in female offspring hippocampus. PNS females had a higher overall adrenocorticotropic hormone response to restraint stress than female controls, whereas PNS males showed faster recovery of stress-induced elevations in adrenocorticotropic hormone than non-stressed males [15]. It was reported that there is a phase shift toward peak secretion of corticosterone in the light cycle in both sexes after PNS. Only female had significant elevations of corticosterone during all phases of the light/dark cycle [16]. One of the mechanisms that could explain the differential effects in prenatally stressed males and females could be the gender difference in placental transport of glucocorticoids from the mother to the female fetus. Higher baseline serum corticosterone concentrations were found in female fetuses than in male fetuses, which is due to a greater transport of corticosterone from maternal blood across the placenta of females and a greater binding of corticosterone in the placenta of male fetuses [17]. Pivina *et al*. found that prenatal social stress increased the basal level of corticostcrone in prenatal stressed female rats that have high stress reactivity of the HPA axis, as well as a more profound effect on anxiety level and oestrous cycle [18]. These reports indicated that effects of PNS on the HPA axis may be more pre-dominant in female than in male offspring.

In summary, the present results showed that the prenatal restraint stress induced a significant increase in the expression of CaMK-II and L-Ca^{2+} channel in offspring hippocampus, especially, in offspring hippocampus CA3. These data suggest that PNS can have long-term neuronal effects within the hippocampal structure involved in the feedback mechanisms of the HPA axis. Further studies are needed to elucidate the mechanism by which the activation of CaMK-II and L-Ca^{2+} channel leads to PNS-induced memory alteration, which may promote our understanding of stress-related disorders.

Funding

This work was supported by the grants from the National Natural Science Foundation of China (30772881) and the Science Development Foundation of Tianjin Institute of Education (20070301).

References

1 Weinstock M. The long-term behavioural consequences of prenatal stress. Neurosci Biobehav Rev 2008, 32: 1073–1086.
2 Szymańska M, Budziszewska B, Jaworska-Feil L, Basta-Kaim A, Kubera M, Leśkiewicz M and Regulska M, *et al*. The effect of antidepressant drugs on the HPA axis activity, glucocorticoid receptor level and FKBP51 concentration in prenatally stressed rats. Psychoneuroendocrinolog 2009, 34: 822–832.
3 Brunton PJ and Russell JA. Prenatal social stress in the rat programmes neuroendocrine and behavioural responses to stress in the adult offspring: sex specific effects. J Neuroendocrinol 2010, 22: 258–271.
4 Coe CL, Kramer M, Czeh B, Gould E, Reeves AJ, Kirschbaum C and Fuchs E. Prenatal stress diminishes neurogenesis in the dentate gyrus of juvenile rhesus monkeys. Biol Psychiatry 2003, 54: 1025–1034.
5 Son GH, Geum D, Chung S, Kim EJ, Jo JH, Kim CM and Lee KH, *et al*. Maternal stress produces learning deficits associated with impairment of NMDA receptor-mediated synaptic plasticity. J Neurosci 2006, 26: 3309–3318.
6 Martínez-Téllez RI, Hernández-Torres E, Gamboa C and Flores G. Prenatal stress alters spine density and dendritic length of nucleus accumbens and hippocampus neurons in rat offspring. Synapse 2009, 63: 794–804.
7 Cai Q, Zhu Z, Huang S, Li H, Fan X, Jia N and Zhang B, *et al*. Sex and region difference of the expression of ERK in prenatal stress offspring hippocampus. Int J Dev Neurosci 2007, 25: 207–213.
8 Cai Q, Huang S, Zhu Z, Li H, Li Q, Jia N and Liu J. The effects of prenatal stress on expression of p38 MAPK in offspring hippocampus. Int J Dev Neurosci 2008, 26: 535–540.
9 Jia N, Yang K, Sun Q, Cai Q, Li H, Cheng D and Fan X, *et al*. Prenatal stress causes dendritic atrophy of pyramidal neurons in hippocampal CA3 region by glutamate in offspring rats. Dev Neurbiol 2010, 70: 114–125.
10 Suenaga T, Morinobu S, Kawano K, Sawada T and Yamawaki S. Influence of immobilization stress on the levels of CaMKII and phospho-CaMKII in the rat hippocampus. Int J Neuropsychopharmacol 2004, 7: 299–309.
11 Ahmed T, Frey JU and Korz V. Long-term effects of brief acute stress on cellular signaling and hippocampal LTP. J Neurosci 2006, 26: 3951–3958.
12 Li H, Li X, Jia N, Cai Q, Bai Z, Chen R and Song T, *et al*. NF-kappaB regulates prenatal stress-induced cognitive impairment in offspring rats. Behav Neurosci 2008, 122: 331–339.
13 Takahashi LK, Turner JG and Kalin NH. Prolonged stress-induced elevation in plasma corticosterone during pregnancy in the rat: implications for prenatal stress studies. Psychoneuroendocrinology 1998, 23: 571–581.
14 Fumagalli F, Pasini M, Frasca A, Drago F, Racagni G and Riva MA. Prenatal stress alters glutamatergic system responsiveness in adult rat prefrontal cortex. J Neurochem 2009, 109: 1733–1744.
15 McCormick CM, Smythe JW, Sharma S and Meaney MJ. Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. Brain Res Dev Brain Res 1995, 84: 55–61.

16 Koehl M, Darnaudery M, Dulluc J, Van Reeth O, Moal ML and Maccari S. Prenatal stress alters circadian activity of hypothalamo-pituitary-adrenal axis and hippocampal corticosteroid receptors in adult rats of both gender. J Neurobiol 1999, 40: 302–315.

17 Montano MM, Wang MH and Vomsaal FS. Sex-differences in plasma-corticosterone in mouse fetuses are mediated by differential placental transport from the mother and eliminated by maternal adrenalectomy or stress. J Reprod Fertil 1993, 99: 283–290.

18 Pivina SG, Shamolina TS, Akulova VK and Ordian NE. Sensitiveness to social stress in female rats with alteration of the pituitary-adrenal axis stress reactivity. Ross Fiziol Zh Im I M Sechenova 2007, 93: 1319–1325.