Original Article

The effects of perinatal protein malnutrition on spatial learning and memory behaviour and brain-derived neurotrophic factor concentration in the brain tissue in young rats

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This study aimed to investigate the effects of perinatal protein malnutrition on brain derived-neurotrophic factor (BDNF) concentration in brain tissue and spatial learning and memory performance in young rats. Nine pregnant Wistar rats were assigned into three groups. Rats in one group were fed with a control diet containing 20% protein. Rats in remaining two groups were fed with a diet containing 6% protein from gestation day eight and day 15 respectively till four weeks after birth. At four weeks of age, the rat pups were evaluated for spatial learning ability using Morris Water Maze (MWM) task. At the end of the behaviour tests, rat pups were sacrificed and the brain tissue samples were collected for measurement of total protein and BDNF concentrations. It was found that rat pups fed the low protein diet had lower body weight and slightly lighter brain compared to the control pups. Total protein levels in hippocampus and cerebral cortex were significantly lower in malnourished pups than the controls. The concentration of BDNF in the hippocampus was also significantly lower in rat pups suffered protein malnutrition from early pregnancy than in the controls. MWM tests showed that perinatal protein deprivation, particularly from early pregnancy, significantly impaired learning and memory ability. The results of the present study indicate that perinatal protein malnutrition had adverse influence on spatial navigation and brain BDNF levels in rats. The decreased hippocampal BDNF concentration might partially contribute to the poor learning memory performance in the protein deprived rats.

Key Words: BDNF, protein malnutrition, Morris Water Maze, spatial learning, rat

Introduction

Protein malnutrition is a severe problem in developing countries, especially for the young population. Dietary protein deprivation during early life is known to have adverse effects on brain anatomy, physiology and biochemistry,[1] and even permanent brain damage.[2] Rapid brain growth known as rain growth spurt occurs from the third trimester of pregnancy to 24 months after birth in humans and it occurs from gestation day 15 up to 21 days after birth in rats.[3] If dietary depletion happens at this stage, the deficits in brain structure and function may be the consequence.

Numerous studies have revealed a causal linkage between protein malnutrition and poor brain development in animals[4,5] and humans.[6, 7] 5owever, the mechanism of impaired learning and memory by perinatal protein malnutrition is still incompletely understood. Various morphological alterations have been found in the brain in association with perinatal protein malnutrition. Granados *et al*[8] reported that the total area of the mossy fibres plexus was decreased significantly in prenatal protein malnourished rats. Another study showed a decreased cortical neuronal density in perinatal protein malnourished rats at early age.[9] It was also noted that prenatal protein deprivation decreased hippocampal granule cell numbers, which could not be recovered by nutrition rehabilitation in rats.[10] In severe malnutrition infants, short apical dendrites, fewer spines, and dendritic spine abnormalities were observed.[11] There are also reports showing disturbances in neuronal transmission in association with perinatal protein malnutrition in rats. Mokler *et al*,[12] found an enhanced serotonin release in prenatal protein malnourished rats and Galler *et al*,[3] reported a decreased turnover and an increased reuptake of norepinephrine, and a reduction in β-adrenergic receptor numbers in the cerebral cortex. It has also been reported that perinatal protein malnutrition impaired long-term potentiation (LTP), one of the important cellular bases for learning and memory.

Little is known about the relationship between perinatal protein malnutrition and brain BDNF level. BDNF is one of the neurotrophins, a group of structure and function related proteins, which play important roles in neuronal survival, differentiation, and specification.[13]

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Neurotrophins also regulate neuronal plasticity and LTP formation[14], influence neurotransmitter release, and stimulate the growth of dendritic branches. It has been reported that the level of neurotrophins in the brain, especially the BDNF level is related to the learning performance. It has also been reported that the BDNF concentration can be affected by nutritional status.[15, 16] However, there were no reports showing the changes of BDNF concentration under perinatal protein malnutrition. The current study aimed to assess the effects of perinatal protein malnutrition on brain BDNF levels and on learning and memory behaviour.

Materials and methods

Animals and diets

The experimental protocol was approved by the Committee on the Use of Live Animal for Teaching & Research of The University of Hong Kong. During the whole experimental period, nine pregnant Wistar rats and their offspring were kept at 22-24^{o}C in a 12:12 hr dark-light cycle. The animals were allowed free access to food and water. Prior to the experiment, the rats were maintained on the standard rat chow (Teklad, Madison, WI, USA). The pregnant rats were randomly divided into three dietary treatment groups of three animals each. One group of pregnant rats were fed on a control diet containing 20% protein from day eight of pregnancy till four weeks after given birth (CTR group), and the remaining two groups were fed on a low protein diet containing 6.0% protein from day eight (PM8 group) and day 15 (PM15 group) of pregnancy respectively till four weeks after given birth. The two experimental diets were isocaloric and were prepared based on AIN purified diet.[17] All the pregnant rats were housed individually in solid bottom plastic cages with soft wood chip bedding. On the day of birth, the size of all litters was adjusted to eight pups by culling extra pups in the litter. The rat pups were weaned at postnatal day 24 and were then separated by gender and maintained on the same diet in a group of three or four rats per cage. The rat pups were evaluated for spatial learning and memory ability using Morris Water Maze test at four weeks of age. During eight-day behaviour test period, all rats were fed on the control diet containing 20% protein.

Morris Water Maze test

The water maze was a circular pool of 182 cm in diameter and 50 cm in depth with water temperature at 23 ± 2^{o}C. The water pool was made opaque by adding 100 g of non-dairy creamer coffee mate. The water pool was divided into four equal quadrant zones. An escape platform of 15 cm by 15 cm was centrally placed in one quadrant of the pool and submerged 1.5–2 cm below the water surface during the training and long term memory trials. The pool was surrounded by several extra-maze cues visible by the rats from the swimming in the pool. The swim paths were recorded with a digital video camera and analysed by Noldus software (Noldus Information Technology).

One day before the training trials, all the rat pups were allowed to swim freely for two minutes to get habitation with the water pool. During the training period, the rat pups underwent a training session consisting of six trials per day for four consecutive days. In each trial, the rat pups were released into the pool, with the nose pointing toward the wall, at one of the assigned three starting points and allowed to swim freely until they found the platform. The trial starting points were the middle of each quadrant edge excluding the quadrant with platform. The starting points were ordered in a random manner between trials. Upon reaching the platform, the rat was allowed to remain on it for 20 seconds, followed by a 30 seconds rest outside the pool. If the rat failed to locate the platform within two minutes, it was manually guided to the platform and allowed to stay there for 20 seconds. To test the short-term memory retention, a probe trial without the escape platform in the pool was conducted for each rat after completing the 24th training trial. To assess the long-term memory retention, six trials were carried out for each rat three days after the completion of training trials in the same method as the training trial used. During the probe trial, the rats were allowed to swim freely for one minute, and the times of rats passed through the target quadrant were recorded.

Brain sample processing

Within three hours of finishing the behaviour test, the rats were sacrificed and around 50mg to 100mg tissue samples were dissected from the cerebral cortex, hippocampus and cerebellum respectively. The tissue samples were suspended in 10 volumes of lysis buffer containing 137 mM NaCl, 20 mM Tris HCl and 10 % glycerol with freshly added protease inhibitor cocktail III (Calbiochemical CA, USA). The tissue samples were then homogenized by ultrasonication at 60 duty cycle for about 40 seconds on ice using BrNSON Sonifier 250 (VWR Scientific, VWR Company). The tissue homogenate was centrifuged (Eppendorf centrifuge 5415D, Eppendorf AG, Germany) at 9000 rpm 4^{o}C for 20 minutes and the supernatant was collected and kept at -80^{o}C for future protein and BDNF assays.

Total protein and BDNF quantification

Protein concentration in the tissue supernatant was estimated by Lowry's assay. [18] BDNF concentration was quantified using ELISA kit followed the manufacture's protocol (BDNF EMAX® ImmunoAssay System kit, Promega Inc., Madison, WI, USA). In brief, a 96-well plate was coated with BDNF primary antibody overnight at 4^{o}C. After washing and blocking, a set of serially diluted BDNF standard solutions in duplicate was added into the wells of the first two columns. The tissue supernatant was diluted five times with DPBS containing 0.2 g KCl/L, 8.0 g NaCl/L, 0.2 g KH_2PO4/L, 1.15 g Na_2HPO4/L, 133 mg $CaCl2{\cdot}2H_2O$/L, and 100 mg $MgCl_2{\cdot}6H_2O$/L (pH 7.35). The preparation was acidified by adding 1N HCl at a ratio of 1:50 (HCl:sample, v/v) and incubated at room temperature for ten minutes followed by neutralization with equal volume of 1 M NaOH. The acidified sample preparation was then added to the rest of the wells in duplicates. After incubation and washing, BDNF secondary antibody was added, followed by addition of anti-IgY HRP conjugates. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) solution was added to develop blue color. After ten minutes of color development, 100 µl of 1 N HCl was added to stop the

reaction. The plate was read by micro-reader (Bio-Rad Laboratories, Hercules, CA, USA) at 495 nm wavelength within 30 minutes after stopping the reaction. The result was calculated based on the standard curve. The value was expressed as pg BDNF per 100 mg tissue.

Data analysis

The experimental results were analysed by one way analysis of variance (ANOVA) using the computer program SPSS 12.0 (SPSS Inc., USA). The significant level was set at $p<0.05$.

Results

Rat pups in the perinatal protein malnutrition groups had significant lower body weight and slightly reduced brain weight than the control rats (Fig 1). The brain to body weight ratio was significant higher in rats of PM8 group than the control group. There was no significant difference in brain to body weight ratio between control and PM15 groups.

As shown in Figure 2, protein malnourished rat pups had significant lower protein concentrations in the cerebral cortex and the hippocampus than that in the control animals. Protein concentrations in the cerebellum tissue did not markedly differ among the three dietary treatment groups. The rat pups in both protein malnutrition groups had lower hippocampal BDNF levels than that in the controls, but the significant difference was observed only between CTR and PM15 groups (Fig 3).

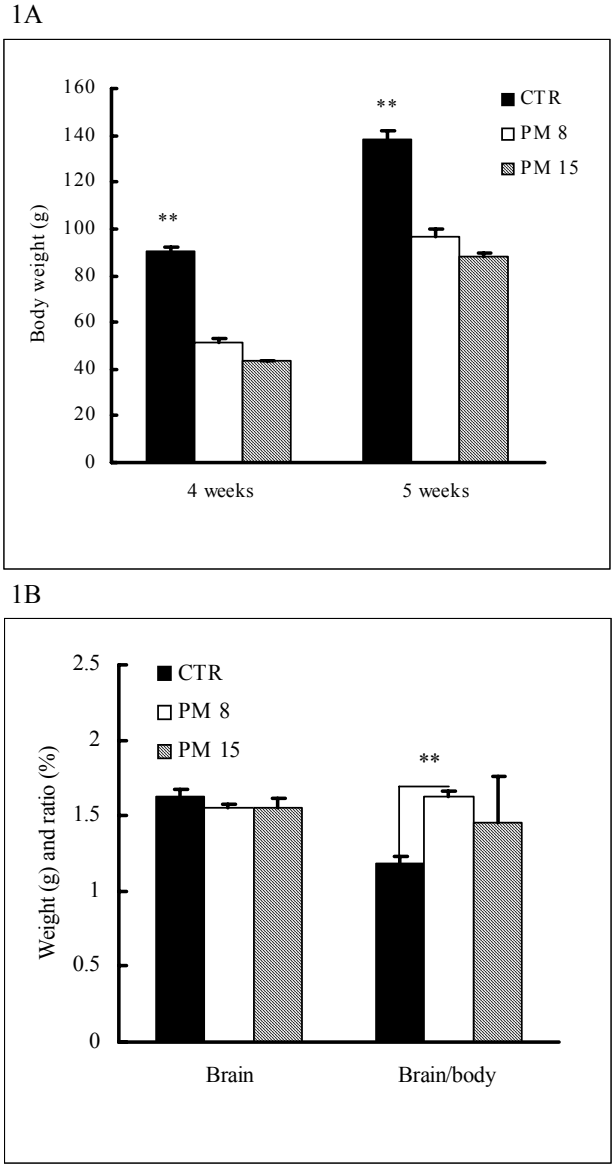

Figure 1. The effects of perinatal protein malnutrition on the brain and body weights of rats. In panel A, the left set of the bars represent the body weight of the experimental rats prior to the behaviour test at 4 weeks of age. The right set of bars represent the body weight of the experimental rats after the completion of the behaviour test at 5 weeks of age. Panel B presents the brain weight and the brain to body weight ratio of the experimental rats. Data presented as mean±SEM; $**p<0.01$; N=12 for each group. CTR: control group; PM8: group with dietary protein restriction from gestation day eight till four weeks after birth; PM15: group with dietary protein restriction from gestation day 15 till four weeks after birth.

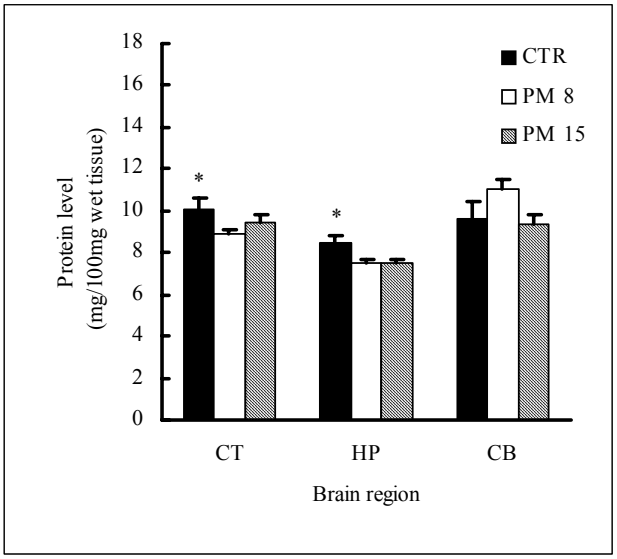

Figure 2. Protein concentrations in cerebral cortex (CT), hippocampus (HP) and cerebellum (CB) of the experimental rats. Perinatal protein malnutrition significantly reduced protein levels in CT and HP. Data presented as mean±SEM; $*p<0.05$; N=8 for each group; See the legend of Figure 1 for the dietary treatment of CRT, PM8 and PM15.

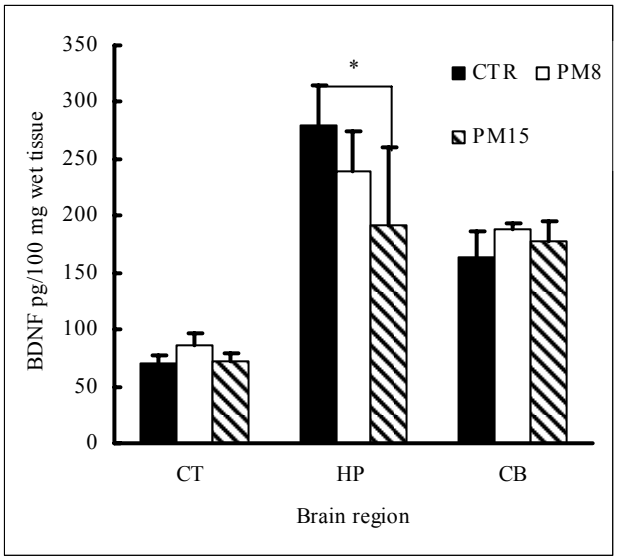

Figure 3. BDNF concentrations in cerebral cortex (CT), hippocampus (HP) and cerebellum (CB) of the experimental rats. Data presented as mean±SEM. $*p<0.05$, N=6 for each group; See the legend of Figure 1 for the dietary treatment of CRT, PM8 and PM15.

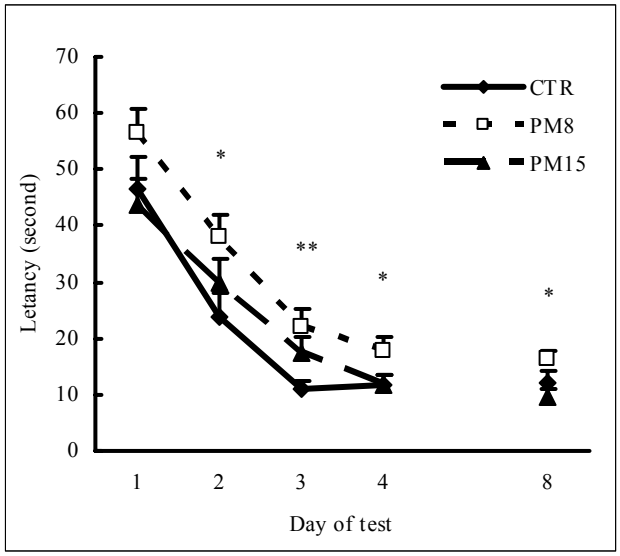

Figure 4. Escape latency of the experimental rats during the MWM tests. The results were expressed as the time taken (latency) by the rats to find the submerged platform during the training trials. The significant differences were observed between PM8 and CTR groups. Data presented as mean±SEM; *p<0.05; **p<0.01; N=12 for all groups; See the legend of Figure 1 for the dietary treatments of CRT, PM8 and PM15.

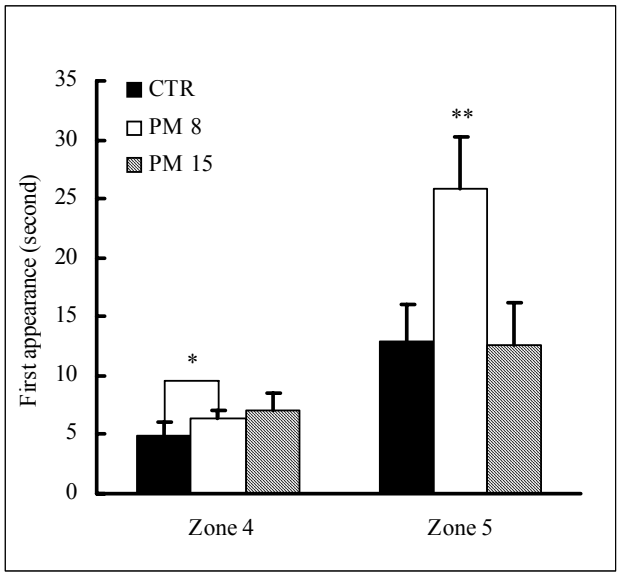

Figure 5. First appearance of the experimental rats in the target zone during the probe test for memory retention. The time between entering the pool and the first time getting into the target zone was defined as the first appearance in that zone. Zone 4 was the quadrant of the water pool where the escape platform was located during the previous training trials and zone 5 was the exact area of the water pool where the escape platform was located. Data presented as mean±SEM; *p<0.05; **p<0.01. The rats in PM8 group appeared in zone 5 (platform area) significantly later than the rats in other groups. See the legend of Figure 1 for the dietary treatments of CRT, PM8 and PM15.

The behaviour test showed that the rat pups suffered longer term of protein malnutrition in group PM8 spent significantly longer time to find the platform than the control rat pups did during the training and long-term memory retention trials (Fig 4). The rats suffered shorter term of protein malnutrition in PM15 group performed better than the ones in PM8 group, but poorer than the rats in the control group during the daily training trials, although the differences did not reach the significant level (Fig 4). During the probe tests for short-term memory retention, rat pups from malnourished groups tended to take longer time to reach the target zone (Fig 5). In comparison with the rat pups in the control and PM15 group, rats from PM8 group took significantly longer time to appear in the place where the escape platform was previously located during the training trials.

Discussion

According to Dobbing's theory, rats belong to the category of postnatal brain developer and their brain growth spurt occurs at early postnatal period.[19, 20] During this period severe nutrient restriction could induce irreversible brain damage.[21] Numerous studies reported the effects of postnatal dietary insult on brain function.[4, 22] However, the neuronal multiplication in rats takes place during late gestation period, which is prior to the brain growth spurt. Therefore, dietary insults introduced in prenatal or perinatal period might have stronger impact on brain development than the insult during postnatal period only. The effects of prenatal protein deprivation on brain development reported in the literature were inconsistent. Cintra *et al*,[23] showed deficit in mossy fibre plexus in rat hippocampus by prenatal protein malnutrition, but others reported that impaired hippocampus was only caused by postnatal rather than prenatal protein malnutrition.[8] Permanent impairment on brain functional development was produced by pre- and postnatal protein malnutrition.[24]

The brain growth spurt in the rat is in the first three weeks of postnatal life, but the early gliogenesis starts in the second week of gestation and the macroneurogenesis occurs in the third week of gestation.[25] It implies that the brain development may be vulnerable to perinatal nutrition insults. The present study showed that perinatal protein deprivation not only reduced the brain total protein level, but also significantly decreased hippocampal BDNF concentration, which in turn may further interfere the neuronal growth and function. The impaired learning and memory performance may be the consequence of hippocampal neuron dysfunction in the malnourished rats. The results of this study demonstrated that perinatal protein restriction had adverse effect on functional brain development in rats, but the severity was varied upon timing and duration of the dietary insult.

During the period of behaviour tests in the present study, the rats were switched to the adequate protein diet, which gave the malnourished rats a recovery period. The malnourished rats had considerable body weight gain during this period (Fig 1A right bars). The body weight was almost doubled after eight days of rehabilitation in the malnourished rats, no matter the timing and duration of prenatal malnutrition. At the same time, rats in the control group only gained about 30-40% of body weight. Protein malnutrition also reduced brain weight in rats. The differences between the control and the protein malnourished rats were about 5-7%. From these results, it is clear that reduced body weight by protein malnutrition was not difficult to recover after a period of rehabilitation, but brain weight recovery was not as easy as the body weight. Gressens[19] reported an almost complete recovery in body weight in adult rats with severe prenatal protein malnutrition for a short period; the finding was in agreement with the present study.

Previous studies also showed that reduction in brain protein synthesis was more severe in rats subjected to perinatal protein malnutrition than that subjected to protein deprivation in prenatal or postnatal period only.[26] The results of the present study agree with the previous report. Total protein levels in the cerebral cortex and the hippocampus were significant reduced in perinatal protein restricted rats, which might be considered as direct effect of dietary protein deprivation. BDNF is a secretary protein synthesized by neurons and plays important roles in neuronal cell proliferation, differentiation, synapse genesis and brain function.[27, 28] The present study was the first investigation in the effects of perinatal protein malnutrition on brain BDNF level. Data from this study showed that brain BDNF level was deceased and the learning memory performance was concomitantly impaired in rats suffered perinatal protein malnutrition. Rats in both protein malnourished groups, particularly those suffered more severe protein malnutrition in PM8 group, spent longer time to find the escape platform.

Many earlier studies conducted protein malnutrition in whole gestation period and/or continued through the lactating period. Some studies even initiated the low protein diet five weeks before mating. The present study demonstrated that even started from the second week of gestation, moderate protein malnutrition could cause brain functional impairment in rats. Although the brain growth spurt for rats occurs in the postnatal period, the neural differentiation and proliferation start from early gestation. One report on postnatal protein malnutrition in rats showed that only memory was disturbed, but learning performance was not markedly influenced,[29] which implied that malnutrition during postnatal period only had less severe effect on brain functional development. The present study showed similar results and moderate protein malnutrition from late gestation period had only mild impact on brain function.

Summing up the results of the present study, it is obvious that maternal protein malnutrition not only reduced brain weight and total brain protein level, but also significantly decreased brain BDNF level. Spatial learning and memory performance was also remarkably affected by perinatal protein malnutrition in young rats. The longer the protein deprivation was, the more severe the brain function was affected and the more difficult the recovery would be. The impaired learning and memory performance might be the consequence of reduced brain BDNF level.

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