# Executive Functions of Postweaning Protein Malnutrition in Mice

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**It is well known that nutritional status during the fetal and/or lactation period is important for the development of the central nervous system (CNS). In contrast, the effect of malnutrition on postweaning development has not yet been thoroughly investigated. In the present study, we analyzed the behavioral and neuroanatomical effects of protein malnutrition (PM) postweaning in mice. Starting at 20—21 d of age, male ddY mice were maintained on a 5% casein diet (PM group) or 20% casein diet (control group) for 20 d. On the 20th d, body and brain weights of PM mice were lower than those of the control group. PM mice exhibited excessive alertness and spontaneous activity under novel conditions in the Irwin test. In addition, PM mice showed increased open arm exploration in the elevated plus maze compared to control mice. These results suggest that hyperactivity and reduced anxiety behavior or higher impulsiveness in PM mice could be due to an immature brain.**

**Key words** protein malnutrition; reduced anxiety; hyperactivity; brain atrophy; mouse

An inadequate supply of nutrition is one of the main non-genetic factors affecting brain development.[1)] Liu *et al.*[2)] demonstrated that children with malnutrition signs at three years of age tend to have low IQ, excessive motor activity and antisocial behavior in adulthood. In the framework of such nutritional inadequacy, early protein malnutrition (PM) adversely affects central nervous system (CNS) maturation, resulting in long-lasting, or even permanent, deleterious effects.[3,4)]

Malnutrition is a common problem worldwide and occurs in both developing and developed countries. In developing countries malnutrition is associated with poverty or socioeconomic problems, whereas in developed countries, the weight of the newborn is influenced by the mothers diet[5)] and the weight at birth affects subsequent development.[6)] Also, in developed countries, severe malnutrition is commonly found in patients with eating disorders which frequently occurs during adolescence leading to interruptions of somatic and psychological development. The use of common weight control techniques by healthy weight adolescents can produce chronic undernutrition.[7)]

In rats, there are two principal types of PM experimental models: prenatal (*i.e.*, gestation period) and early postnatal (*i.e.*, lactation period) malnutrition. There are many studies focused on body and brain morphological affects of these PM models, such as body and brain weight,[3)] hippocampal effects,[8,9)] span of basilar dendrities[10)] and neocortical effects.[11)] The prenatal and early postnatal PM also causes various abnormal behaviors, such as increased exploratory behavior,[12,13)] reduced anxiety,[13,14)] decreased social interaction,[15)] increased depressive behavior,[16)] increased aggressive behavior,[17,18)] prepulse inhibition deficits[19)] and impairment of memory-related behavior.[20—22)] In contrast, there are remarkably few reports concerning postweaning PM effects on the CNS. Lukoyanov and Andrade[23)] found that eight months of PM in adult rats induced a marked loss of neurons and synapses in the hippocampal formation and that the morphological alterations led to noticeable impairments in open-field behavior and spatial learning in the water maze. Thus, even if PM begins in adult animals, abnormal behaviors are induced which take a long time to express.

The human brain develops rapidly in the last third of pregnancy and the first two years of life. Brain weight increases with age and achieves adult weight between six and 14 years of age. By two years of age the brain is approximately 80% of the adult weight and at one time it was believed that, to a large extent, brain development had ended. More recently it has become apparent that brain development continues through adolescence and even as adults the brain can adapt to changing circumstances. Adolescence is a particularly important time for brain development as more adult ways of thinking emerge: abstract thinking, deductive reasoning and the ability to solve problems.[24)] There is increasing evidence that dieting is beginning during childhood, even before the onset of puberty.[25)] Therefore, we focused on the effect of altered nutritional status during this period.

We have previously reported that mice fed the PM diet for 20 d during the prepubertal period (postnatal days 20—21; early postweaning) showed memory deficits and a decrease in the choline acethyltransferase (ChAT) protein in the hippocampus. In that report, postpubertal mice (postnatal days 56—60) were also fed the PM diet for 20 d. Despite the fact that postpubertal PM mice showed a decreased body weight, these mice did not show an impairment of memory-related behavior. We also examined the nutritional rehabilitation effect on the impairment of memory-related behavior observed in the PM mice. Memory function measured on the 20th d in the rehabilitation group (the group fed a standard protein diet for 10 d after PM feeding for 10 d) remained at the control group level. However, the 5 d rehabilitation group (the group fed a standard protein diet for 5 d following 15 d of PM feeding) showed the impairment of memory-related behavior. Thus, there is a possibility that the impairment of memory-related behavior induced by PM may result from neuronal degeneration of the CNS in prepubertal mice.[26)] Nevertheless, the neuropharmacological profiles of postweaning PM animals have not yet been studied. The aim of the present work is to examine the effects of PM on behaviors deter-

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Table 1. The Neurobehavioral Observation Battery

| Observations | Range; Normal score | Descriptions of severity for scores |
|---|---|---|
| Alertness | −4 to +4; 0 | −4: No reactivity, reduced or absent vibrissae activity; −2: markedly reduced reactivity and head or body movement, reduced vibrissae activity; 0: normal reactivity, calm appearance or slight freezing, normal vibrissae activity; +2: excessive reactivity, active freezing or rapid, sudden and sharp head or body movements, increased vibrissae activity; +4: exaggerated excitement, escape from the cage. |
| Stereotypy | 0 to +4; 0 | 0: No stereotypy behavior (circling, self-destructive biting and restless sniffing); +2: occasional stereotypy behavior; +4: exaggerated and continuous stereotypy behavior. |
| Grooming | −4 to +4; 0 | −4: No grooming; −2: slight grooming; 0: moderate grooming; +2: marked grooming; +4: extreme and continuous grooming. |
| Irritability | 0 to +4; 0 | 0: No reaction to head touch; +2: escape behavior to head touch; +4: reflex biting when touched. |
| Spontaneous activity | −4 to +4; 0 | −4: Absence of spontaneous locomotor activity even when stimulated; −2: slowed head or body movements but increased spatial spontaneous locomotor activity; 0: normal head or body movements and spatial spontaneous locomotor activity; +2: normal head or body movements but increase spatial spontaneous locomotor activity; +4: continuous spontaneous locomotor activity. |
| Pain response | −4 to 0; 0 | −4: No motor response when tail is pinched; −2: slowed motor response when tail is pinched; 0: rapid motor response (without flight tail) when tail is pinched. |
| Startle response | 0 to +4; 0 | 0: Positive reaction to a hand clap, without jerk; +2: moderate increase in startle response (fleeing reaction) without or with jerk; +4: marked increase in startle response with marked jerk. |
| Grip strength | −4 to 0; 0 | −4: Unable to grip the horizontal wire; −2: decrease in grid–gripping performance; drop within 20 s; 0: normal. |

mined in the Irwin and elevated plus maze tests.

## MATERIALS AND METHODS

**Animal Treatment** Male ddY mice (Japan SLC, Hamamatsu, Japan) weighing 10—12 g (postnatal days 20—21) at the beginning of the experiment, were used. The animals were housed under conditions of constant temperature (23±1 °C) and humidity (55±5%), on a 12 h/12 h light–dark cycle (light from 9 to 21 h; dark from 21 to 9 h). The mice were housed in plastic cages (31×21×13 cm) and divided into two different dietary groups for the experiment. The PM group was provided with a 5% casein diet (powdered low protein diet, Ajinomoto Co., Inc., Kawasaki, Japan) daily. The control group was given a 20% casein standard diet (powdered normal diet, Ajinomoto Co., Inc.). The detailed composition of the diet has been previously reported.[26] The mice had free access to food and water throughout the experimental period. Body weight and food intake (corrected for spillage) of each mouse were recorded daily throughout the experiment.

**Irwin Test** Each subject was first observed in a transparent box (24×17×12 cm) for any signs of abnormal behavior, changes in body posture, walking and tremor. Mice were then exposed to a battery of basic neurological tests as described by Irwin.[27,28] A description of each separate measurement is contained in Table 1. Behavioral scoring was performed on a "blind" basis by a trained experimenter. Each behavioral measure was scored using a points system (0 to +4, −4 to 0 or −4 to 4).

**Elevated Plus Maze** Anxiety-related behavior was evaluated using the plus maze test, according to the method described by Kuribara *et al.*[29] with modifications. The plus maze consisted of two open arms (6×30 cm) opposite each other, crossed by two enclosed arms (6×30 cm) with an open roof and the central platform (9×9 cm). The maze floor and walls were constructed from acrylic plate and elevated 40 cm from the ground floor. The animals was placed on the central platform facing an enclosed arm and allowed to explore for 5 min. During this period, the behavior of each mouse was monitored using a video camera (SONY DCR-PC300K). The test area was cleaned with disinfectant between each testing. The time spent in the open arms and the number of entries into open arms was calculated as the percentage of the time spent in all four arms, and of the total arm entries, respectively. Latency time was estimated by measuring the length of time for the mouse to move from platform to open arm from the start of the test. Arm entry was defined as the entry of all four paws into one arm.

**Brain Weight** On the 20th d after commencement of PM or control feeding, one group of mice was sacrificed by decapitation. The brains were removed and vertical cuts were made to include the olfactory bulbs anterior to the frontal cortex, and the medullary region posterior to the cerebellum. The removed brain was immediately weighed.

All experiments were performed according to the Guide for Care and Use of Laboratory Animals at Tohoku Pharmaceutical University.

**Statistical Analysis** Statistical analyses were performed with the computer program, GraphPad Prism 5.01 (Abacus Concepts, Berkeley, CA, U.S.A.). Daily food intake and body weight data were analyzed using a repeated measures

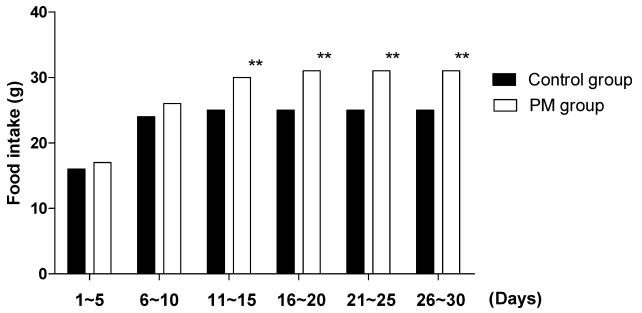


Fig. 1. The Food Intake (Corrected for Spillage) of Control and PM Groups over a 30 d Period

The bars represent 5 d blocks. Control group: This group was provided with a normal protein (20% casein) diet. PM group: This group was provided with a 5% casein diet. $n=4$ per group. Each mouse was housed individually. $^{**}p<0.01$ significant difference between control and PM groups (two-way ANOVA; Bonferroni *post hoc* test).

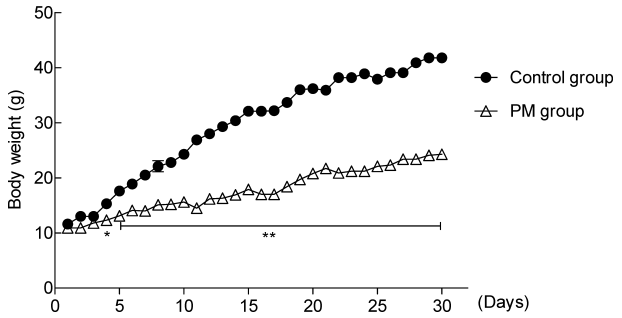


Fig. 2. Influence of PM on the Growth Curve for 30 d

Control group: This group was provided with a normal protein diet. PM group: This group was provided with a 5% casein diet. Vertical bars represent standard errors of means (S.E.M.). $n=10$ per group. $^{*}p<0.05$, $^{**}p<0.01$ significant difference between control and PM groups (two-way ANOVA; Bonferroni *post hoc* test).

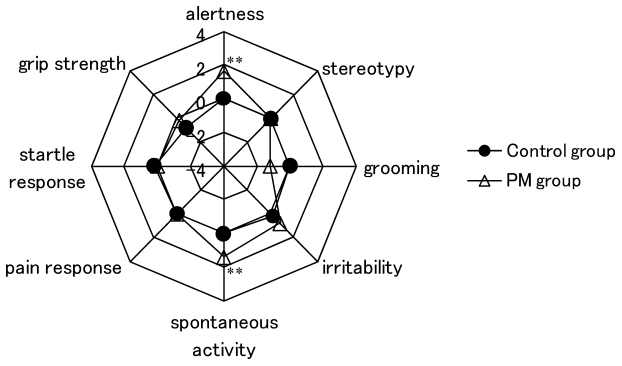


Fig. 3. The Behavioral Spectrums of the Normal and PM Groups

The Irwin test was performed on the 20th d of feeding. Axial elements show the difference from normal values. $^{**}p<0.01$ significant difference between control and PM groups (Mann–Whitney *U* test).

ANOVA. The Bonferroni *post hoc* test was used to determine individual group differences. For the effect of the PM diet on the Irwin test was determined by a Mann–Whitney *U*-test, elevated plus maze and brain weight were determined using a *t*-test. A difference of $p<0.05$ was considered significant. All results are given as mean±S.E.M.

## RESULTS

**Growth Curves During PM Feeding** Food intake of each group increased until the tenth day, but after this, control mice maintained a constant weight. The food intake of PM mice significantly increased from the 11th d compared to the mice on the normal protein diet (Fig. 1). Whereas food intake increased in PM mice, body weight was lower than the normal protein group. The suppression of growth curves was evident from the fourth day after commencement of PM feeding (Fig. 2).

**Irwin Test** Eight separate observations were performed to predict the behavioral effect of the PM diet on the 20th d. Mice were subjected to novel conditions in the cage. The alertness and spontaneous activity under novel conditions (habituation) was higher in PM mice than in normal mice ($p<0.01$, Fig. 3). PM mice tended to have an increase in the touch–escape response which is an index of irritability and a decrease in grooming frequency. However, there were no statistical significant differences. There were no differences in stereotypy, pain response, startle response, or grip strength in the Irwin test.

**Anxiety-Related Behavior (Elevated Plus Maze)** PM mice exhibited a significantly greater proportion of open arm entries than control mice ($p<0.05$). PM mice tended to increase the ratio of time spent on the open arms to total arm time and reduced the latency time to open arm, however, there was no significant differences (Table 2).

Table 2. Influence of PM on Exploratory Behaviors in the Elevated Plus Maze

| Groups | Number of entries [open/(open+enclosed)] (%) | Time spent [open/(open+enclosed)] (%) | Latency (s) |
|---|---|---|---|
| Control group | 11.8±3.2 | 7.1±2.5 | 149.7±38.5 |
| PM group | 29.3±7.2* | 19.6±6.3 | 93.2±27.0 |

Data are represented as mean±S.E.M.. $n=10$ per group. $^{*}p<0.05$ significant difference between the control and PM groups (unpaired *t*-test).

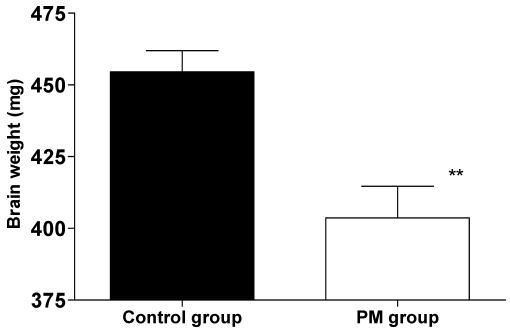


Fig. 4. Brain Weight of Control and PM Groups

$^{**}p<0.01$ significant difference between control and PM groups (unpaired *t*-test).

**Brain Weight** The mean brain weight of PM mice on the 20th d was significantly lower than the weight of control mice brains ($p<0.01$, Fig. 4).

## DISCUSSION

In the present study, early PM mice showed significant suppression in postweaning growth. Interestingly, food intake in mice on a PM diet (5% casein) was higher whereas their body weight was lower than that of control mice after the experimental period (20 d). Du *et al.*[30] showed that food intake

was dependent on the level of dietary protein. They reported that food intake increased in rats with 5—15% casein feeding compared to those on a 20% casein control diet. It has been suggested that when the protein in the diet is restricted, food intake is primarily determined by the animal's attempt to meet its protein requirement.[31] However, despite the increase in food intake, the total protein intake in these rats was still lower than that of the control group.[30] Thus, our result concerning and food intake and growth in PM mice are in agreement with previous findings.

There are many reports that have shown that gestational and/or lactational malnutrition results in hyperactivity in open field tests.[32] Additionally, reduced anxiety behavior has been observed in the light–dark transition,[33] elevated T maze[14,34] and elevated plus maze.[12,13] In the present study, postweaning PM also produced hyperactivity in the Irwin test and lower anxiety levels or higher impulsiveness in the elevated plus maze, as indicated by high frequencies of entries into the open arms. A high percentage of anorexia nervosa (AN) subjects exercise intensively during the acute phase of the disorder.[35] Our findings suggest a possible role of low protein in AN induced hyperactivity.

The hyperactivity and lack of anxiety observed in our PM mice are similar to behaviors observed in juvenile stroke-prone spontaneously hypertensive rats (SHR). SHR exhibit deficits in attention, impulsivity and hyperactivity, which are thought to reflect attention deficit/hyperactivity disorder (ADHD)-like characteristics (for review see Sagvolden).[36] ADHD is the most common neurodevelopment disorder of childhood affecting between 3% and 5% of school-aged children.[37] Recently, Shaw *et al.*[38] compared the cortical thickness in children with and without ADHD by using magnetic resonance imaging (MRI). They found a marked delay in ADHD in attaining peak thickness throughout most of the cerebrum. Brain weight loss of our PM mice suggests a delay in brain development. Furthermore, in human studies, low protein/ high carbohydrate diets are thought to be one of the risk factors for ADHD.[39—41] Thus, PM from the juvenile stage may be a risk factor for ADHD.

In the present study, brain and body weights were decreased in postweaning protein malnutrition on the 20th d. The mean total body weight of the control group is 36.2 g, whereas that of the PM group is 20.8 g (Fig. 2). The brain weight of the control group is 455 mg, and that of the PM group is 404 mg (Fig. 4). When the levels of the limiting amino acids are below their requirement, endogenous body proteins are degraded to supply the limiting amino acids for the synthesis of vital proteins.[30] During malnutrition, the brain shows minimal weight reduction, in contrast to body weight which reflects the dietary nutritional status.[42]

The effect of protein malnutrition during the gestation and/or lactation period on brain weight loss has been reported.[32,43] Wolf *et al.*[32] measured the body and brain weights of PM (6 or 8% casein diet) and well-fed (25% casein) rats. The body weight of the PM (6 and 8% casein diet) rats was significantly decreased compared with well-fed rats. And the brain weight of the 6% casein group rats was also significantly decreased compared with well-fed rats, although this was not the case for the 8% casein group. Wolf *et al.* also measured behavior using an eight-arm radial-maze. The 6% casein group rats showed hyperactive behavior in open field tests and made more entries and re-entry errors than the well-fed rats in the eight-arm radial maze. However, the 8% casein group did not show abnormal behaviors.[32] These data suggest that abnormal behaviors may be associated with changes in brain weight in rodents.

In human studies, atrophy of the brain has been reported in patients with severe protein (or protein energy) malnutrition[44] and anorexia nervosa (AN).[45] MRI studies have found a positive relationship between the amount of weight loss and the degree of atrophic changes.[46—49] In addition, studies employing magnetic resonance spectroscopy have demonstrated various alterations in cerebral metabolism in patients with acute AN (for review see Frank *et al.*).[50] These changes may be related to decline of cognitive functioning.[51] There are reports that impairment of memory-related behavior and reduced anxiety behavior results from PM in rats.[12,20—22] We have also previously reported alterations in cognitive function in PM mice.[26] Taken together, these findings may suggest a relationship between brain weight loss and deficits in executive function.

Impairment of somatic and brain development as well as behavioral alterations were observed due to the insufficient supply of protein during the postweaning period. It can be concluded that early postweaning PM altered the behavior of the animals in the Irwin test and elevated plus maze as well as inducing hyperactivity, lower anxiety and/or higher impulsiveness of the animals. The immature brain resulting from PM may contribute to the impairment of executive functions. Our findings suggest that an adequate supply of protein may help protect executive functions.

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