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ORIGINAL ARTICLE

Effect of quantity and quality of dietary protein on choline acetyltransferase and nerve growth factor, and their mRNAs in the cerebral cortex and hippocampus of rats

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Abstract The brain protein synthesis is sensitive to the dietary protein; however, the role of dietary protein on biomarkers including choline acetyltransferase and nerve growth factor (NGF) for the function of cholinergic neurons remains unknown in young rats. The purpose of this study was to determine whether the quantity and quality of dietary protein affects the concentration of NGF and activity of choline acetyltransferase, and their mRNA levels in the brains of young rats. Experiments were carried out on five groups of young rats (4 weeks) given the diets containing 0, 5, 20% casein, 20% gluten or 20% gelatin for 10 days. The activity of choline acetyltransferase in the cerebral cortex and hippocampus declined gradually with a decrease in quantity and quality of dietary protein. The concentration of NGF in the cerebral cortex and the mRNA levels of choline acetyltransferase in the cerebral cortex and hippocampus did not differ among groups. However, the concentration and mRNA level of NGF in the hippocampus was significantly lower in rats fed with lower quantity of protein or lower quality of protein. In the hippocampus, the mRNA levels of NGF significantly correlated with the NGF concentration when the quantity ($r = 0.704$, $P < 0.01$) and quality ($r = 0.682$, $P < 0.01$) of dietary protein was manipulated. It was further found that a significant positive correlation existed between the NGF concentration and the activity of choline acetyltransferase in the hippocampus (dietary protein quantity, $r = 0.632$, $P < 0.05$; dietary protein quality, $r = 0.623$, $P < 0.05$). These results suggest that the ingestion of lower quantity and quality of dietary protein are likely to control the mRNA level and concentration of NGF, and cause a decline in the activity of choline acetyltransferase in the brains of young rats.

Keywords Dietary protein · Choline acetyltransferase · Nerve growth factor · Brain · Rats

Abbreviations
NGF Nerve growth factor
GH Growth hormone

Introduction

Nerve growth factor (NGF) is one of the neurotrophic factors that stimulate differentiation and growth of basal forebrain cholinergic neurons (Hefti et al. 1989; Lindsay et al. 1994). The highest concentrations of NGF mRNA were found in the cortex and hippocampus, which are the major targets of the NGF-responsive cholinergic neurons of the basal forebrain (Shelton and Reichardt 1986). Choline acetyltransferase, the biomarker for the function of cholinergic neurons, is induced by NGF (Gnahn et al. 1983),

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and the concentration and mRNA level in hippocampal NGF are correlated with the density of cholinergic innervation (Korsching et al. 1985). NGF was able to affect survival of central cholinergic neurons after axonal transections in adult rats (Hefti 1986). Alzheimer's disease is associated with loss of cholinergic neurons (Kosik 1992), which are essential for learning and memory processes, not withstanding the exact mechanism of response. Sherwin (1994) reported that there was a beneficial effect of estrogen on memory tasks in postmenopausal women. Data in ovariectomized rats indicated that soy isoflavones and estrogen increased the mRNAs of choline acetyltransferase and NGF mRNAs in the brain (Pan et al. 1999). We also reported that estrogen and dietary genistein increased the brain protein synthesis in ovariectomized female rats (Hayase et al. 2001; Lyou et al. 2002). These data suggest the possibility that the choline acetyltransferase mRNA level and the concentration and mRNA level of NGF may play a role in determining the choline acetyltransferase activity in the brain regions.

On the other hand, several investigations have suggested that the brain protein synthesis is also sensitive to the dietary protein, and that the brain function was also affected by the dietary protein (Yokogoshi et al. 1992). The biomarkers including choline acetyltransferase and NGF have been shown to be important for the function of cholinergic neurons (Gibbs et al. 1994). However, the role of dietary protein in maintaining NGF and choline acetyltransferase in the brains of young rats remains unknown. Therefore, the possible effects of the dietary protein on the concentrations of NGF and activity of choline acetyltransferase in the brains of young rats are of nutritional importance in understanding the role of nutrition in the brain function in mammals.

The purpose of our study was to determine whether the quantity and quality of dietary protein affect the NGF concentration and choline acetyltransferase activity, and their mRNA levels in the brains of the young rats. Three questions were considered in the present study: (1) whether the quantity and quality of dietary protein might affect the concentrations of NGF and activitiy of choline acetyltransferase in the brains of young rats, (2) whether the mRNA level of choline acetyltransferase in the brains might control the choline acetyltransferase activity when the quantity and quality of dietary protein was manipulated, and (3) whether the decreased mRNA level of NGF in young rats fed with lower quantity and quality of dietary protein might result in the decreased NGF concentration and the lower choline acetyltransferase activity in the brain regions compared with rats fed with higher quantity and quality of dietary protein.

Therefore, we examined concentrations of NGF and activity of choline acetyltransferase, and their mRNA levels in the cerebral cortex and hippocampus. Gelatin and gluten are known to be lower quality protein than casein because of their deficiency in several essential amino acids (e.g. tryptophan) or in lysine, respectively, and because of lower nitrogen retention (Steele and Harper 1990). Particularly, because the gelatin contained less of many essential amino acids the body weight decreased in young rats fed with 20% gelatin diet (Yokogoshi et al. 1992). Thus, in this experiment, 20% gelatine, 20% gluten, and 20% casein diets were chosen to investigate the effect of dietary protein quality on neurotrophic factors in young rats.

Materials and methods

Chemicals

1-^{14}C-AcetylCoA (1.85 GBq/mmol) was obtained from GE Healthcare Bio-Sciences Co. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical (Osaka, Japan).

Animals and diets

Young male Wistar rats (4 weeks, Japan SLC, Hamamatsu, Japan) were individually housed at 24°C in a room with a 12-h light–dark cycle. The rats were switched to experimental diets containing 0, 5, 20% casein, 20% gluten or 20% gelatin (Table 1) after consuming a commercial

Table 1 Composition (g/100 g diet) of experimental diets

Ingredient	20% Gelatin	20% Gluten	20% Casein	5% Casein	0% Casein
Casein			20.0	5.0	
Gluten		20.0			
Gelatin	20.0				
Cornstarch[a]	21.8	21.8	21.8	26.8	28.4
Sucrose[a]	43.5	43.5	43.5	53.5	56.9
Corn oil	5.0	5.0	5.0	5.0	5.0
AIN-93G mineral mix[b]	3.5	3.5	3.5	3.5	3.5
AIN-93VX vitamin mix[b]	1.0	1.0	1.0	1.0	1.0
Cellulose[a]	5.0	5.0	5.0	5.0	5.0
Choline chloride	0.2	0.2	0.2	0.2	0.2

[a] Supplied by Oriental Yeast, Tokyo, Japan

[b] Supplied by Nihon Nosan K.K., Yokohama, Japan (American Institute of Nutrition, 1993)

nonpurified diet (MF; Oriental Yeast, Tokyo, Japan) for 2 days. All rats were individually housed and given free access to food and water. The approval of Aichi University of Education Animal Care and Use Committee was given for our animal experiments.

Experimental design

The experiment was carried out with 30 rats being divided randomly into five groups. All rats were fed with experimental diets for 10 days. After the 10-day feeding period, the rats were decapitated, and the cerebral cortex and hippocampus (Reinstein et al. 1979) were quickly removed. The effects of quantity and quality of dietary protein on the activity of choline acetyltransferase and concentrations of NGF, and mRNA levels of choline acetyltransferase and NGF in the brains were investigated.

Determination of NGF by enzyme immunoassay

The tissue samples were homogenized with 20 volumes of cold 20 mmol/l Tris–HCl buffer (pH 8.0) containing 137 mmol/l NaCl, 1% Triton X100, 10% glycerol, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mmol/l sodium vanadate. Homogenates were centrifuged at 15,000×*g* for 15 min at 4°C. The supernatant was used for the measurements of NGF. The NGF protein was measured by enzyme immunoassay (EIA; NGF E_{max} System; Promega Co., Madison, USA).

Determination of choline acetyltransferase activity

The activity of choline acetyltransferase in the brains was measured by Fonnum (1975). The tissue samples were homogenized with 20 volumes of cold 50 mmol/l sodium phosphate buffer (pH 7.4) containing 0.01 mol/l EDTA and 0.5% Triton X100. The homogenate was incubated with 50 mmol/l sodium phosphate (pH 7.4), 0.2 mmol/l acetylCoA, 14.8 MBq/l 1-^{14}C-acetylCoA, 0.8 mmol/l choline chloride, 20 mmol/l EDTA, and 0.1 mmol/l physostigumine sulphate at 37°C in 300 μl. After 15 min, all reaction mixtures were quickly transferred to the scintillation vial by cold 10 mmol/l sodium phosphate (pH 7.4). The radioactivity was determined by adding the toluene scintillation mixture containing 0.05% diphenyloxazole and 0.02% 1,4-bis-(5-phenyloxazole-2-yl)benzene, and acetonitrile containing 0.5% Kalignost (LS 5000TD, Beckman Japan, Tokyo, Japan).

RT-PCR analysis

RNA was extracted from the cerebral cortex and the hippocampus by the method of Chomczynski and Sacchi (1987). Total RNA (5 μg) was reverse-transcribed by M-MLV reverse transcriptase using an oligo dT primer. The cDNA encoding choline acetyltransferase, NGF, or glyceraldehyde-3-phosphate dehydrogenase was amplified by real-time PCR (ABI Prism 7300 System, Applied Biosystems Japan, Tokyo, Japan) using specific primers and probes (TaqMan Gene Expression Assays, Applied Biosystems Japan, Tokyo, Japan). The primers and probes were as follows: choline acetyltransferase, Rn01453446_m1; NGF, Rn00824646_m1. The cDNA was quantified using ABI Prism 7000 Sequence Detection Systems (Applied Biosystems Japan, Tokyo, Japan) from the cycle number for threshold signal detection. We used the glyceraldehyde-3-phosphate dehydrogenase mRNA level quantified with TaqMan Rodent GAPDH Control Regents (Applied Biosystems Japan, Tokyo, Japan) to normalize the choline acetyltransferase and NGF mRNA levels.

Statistical analysis

The means and SEM are reported. Duncan's multiple range test was used to compare means after a one-way ANOVA (Duncan 1955; Snedecor and Cochran 1967). Differences were considered significant at $P < 0.05$. A linear regression analysis was used to assess the relationship between the concentration and mRNA level of NGF, and choline acetyltransferase (Snedecor and Cochran 1967).

Results

Effects of the quantity of dietary protein on the activity and mRNA level of choline acetyltransferase, and on the concentration and mRNA level of NGF in the brain regions

The body weight gain and food intake decreased significantly with the 5% casein diet and still more with 0% casein diet compared with the 20% casein diet (Table 2). There was no difference among the three groups in terms of the weights of the cerebral cortex and hippocampus (Table 2). The activity of choline acetyltransferase declined gradually with the decreasing quantity of dietary protein in the cerebral cortex and hippocampus (Table 3). The mRNA levels of choline acetyltransferase in the cerebral cortex and hippocampus did not differ among groups (Table 3). The concentration of NGF in the cerebral

cortex did not differ among the experimental groups. However, the concentration and mRNA level of NGF in the hippocampus was significantly lower in rats fed with 5% casein diet or 0% casein diet than in those fed with 20% casein diet (Table 4).

In the hippocampus, the concentrations of NGF significantly correlated with the mRNA levels of NGF ($r = 0.704$, $P < 0.01$) and choline acetyltransferase activity ($r = 0.632$, $P < 0.05$), respectively. The mRNA levels of NGF in the cerebral cortex were not detected by RT-PCR analysis.

Effects of the quality of dietary protein on the activity and mRNA level of choline acetyltransferase, and on the concentration and mRNA level of NGF in the brain regions

The body weight gain decreased significantly with the 20% gluten diet and still more with 20% gelatin diet compared with the 20% casein diet alone (Table 2). Compared with rats fed with 20% casein or 20% gluten diets, rats fed with 20% gelatin diet had food intake that was significantly lower. The weights of the cerebral cortex and hippocampus were not affected by the dietary protein quality (Table 2). The activity of choline acetyltransferase in the cerebral cortex decreased significantly with casein, gluten, and gelatin diets in that order (Table 3). The choline acetyltransferase activity in the hippocampus was significantly lower in rats fed with 20% gluten diet or 20% gelatin diet than in those fed with 20% casein diet (Table 3). The mRNA levels of choline acetyltransferase in the cerebral cortex and hippocampus did not differ among groups (Table 3). The concentration of NGF in the cerebral cortex did not differ among the groups. In the hippocampus, the concentration and mRNA level of NGF decreased significantly with the 20% gluten diet and the 20% gelatin diet compared with the 20% casein (Table 4). The correlation between the concentration and mRNA level of NGF was significant in the hippocampus ($r = 0.682$, $P < 0.01$). It was further found that a significant positive correlation existed between the NGF concentration and the activity of choline acetyltransferase in the hippocampus ($r = 0.623$, $P < 0.05$).

Discussion

Cholinergic neurons are essential for learning and memory processes (Kosik 1992; Singh et al. 1994). Biomarkers including choline acetyltransferase and NGF have been shown to be important for the function of cholinergic neurons (Gibbs et al. 1994). NGF is one of the neurotrophic factors that induces choline acetyltransferase. In previous studies, we found that the rate of brain protein synthesis in young rats given with gluten or gelatin diets is lower than in rats given with casein diet, and that the brain function may be affected by the dietary protein (Yokogoshi et al. 1992). Therefore, the possible effects of the dietary protein on the concentrations of NGF and activity of choline acetyltransferase in the brains of young rats are of interest nutritionally. We hypothesized that the NGF concentration and choline acetyltransferase activity in the brain decreased in young rats given with lower quantity and quality of protein.

The NGF has been shown to affect survival of central cholinergic neurons after axonal transections in adult rats (Hefti 1986). The higher concentrations and higher mRNA level in NGF were found in the cortex and hippocampus, which are the major targets of the cholinergic neurons of the basal forebrain (Shelton and Reichardt 1986). However, the NGF receptor mRNA was observed in the cells of the basal forebrain of rats, not in the hippocampus (Ayger-LeLievre et al. 1988). Thus, NGF is considered to be selectively taken up by cholinergic nerve terminals in the cortex and hippocampus and transported in a retrograde manner to the basal nuclei of the forebrain (Schwab et al. 1979; Seiler and Schwab 1984). In the present experiments, the concentration of NGF in the cerebral cortex did not differ among groups (Table 4). However, the NGF

Table 2 Effects of the quantity and quality of dietary protein on the body weight gains and tissue weights in the brains of rats

	0% Casein	5% Casein	20% Casein	20% Gluten	20% Gelatin
Initial body weight (g)	103.4 ± 2.7	103.2 ± 2.2	103.0 ± 1.4	102.4 ± 1.7	102.8 ± 1.8
Body weight gain (g/10 days)	−20.2 ± 1.6a	9.0 ± 2.6b	48.0 ± 2.5d	25.6 ± 0.9c	−17.8 ± 1.1a
Food intake (g/day)	10.1 ± 0.6b	14.5 ± 0.7c	16.4 ± 0.3d	17.0 ± 0.4d	10.3 ± 0.8b
Tissue weight (g)					
Cerebral cortex	0.26 ± 0.009	0.26 ± 0.006	0.28 ± 0.010	0.27 ± 0.014	0.26 ± 0.014
Hippocampus	0.092 ± 0.007	0.092 ± 0.007	0.096 ± 0.005	0.094 ± 0.005	0.085 ± 0.003

Values are means ± SEM, $n = 6$. Means in a row without a common letter differ: $P < 0.05$

Table 3 Effects of the quantity and quality of dietary protein on the activities and relative mRNA levels of choline acetyltransferase in the brains of rats

	0% Casein	5% Casein	20% Casein	20% Gluten	20% Gelatin
Choline acetyltransferase[a] (U/g tissues)					
Cerebral cortex	4,040 ± 120c	4,610 ± 80d	5,130 ± 150e	4,620 ± 60d	4,200 ± 80c
Hippocampus	3,920 ± 170c	4,430 ± 40d	5,100 ± 250e	4,460 ± 30d	4,170 ± 60c,d
Choline acetyltransferase mRNA[b]					
Cerebral cortex	0.955 ± 0.047	1.005 ± 0.077	1.000 ± 0.071	1.108 ± 0.021	1.127 ± 0.105
Hippocampus	0.753 ± 0.095	0.525 ± 0.083	1.000 ± 0.392	0.710 ± 0.083	0.753 ± 0.249

Values are means ± SEM, $n = 6$. Means in a row without a common letter differ: $P < 0.05$

[a] Unit of enzyme activity: nanomoles of acetylcholine produced per hour

[b] The relative mRNA levels was shown as the ratio to the value of 20% casein group

Table 4 Effects of the quantity and quality of dietary protein on the concentrations and relative mRNA levels of NGF in the brains of rats

	0% Casein	5% Casein	20% Casein	20% Gluten	20% Gelatin
NGF[a] (ng/g tissues)					
Cerebral cortex	4.95 ± 0.43	4.82 ± 0.47	5.26 ± 0.39	5.19 ± 0.35	4.63 ± 0.29
Hippocampus	5.60 ± 0.09c	5.50 ± 0.13c	6.43 ± 0.42e	5.49 ± 0.15c	5.62 ± 0.11c,e
NGF[a] mRNA[b]					
Cerebral cortex	ND[c]	ND	ND	ND	ND
Hippocampus	0.298 ± 0.094d	0.261 ± 0.149d	1.000 ± 0.303e	0.707 ± 0.258d,e	0.088 ± 0.088d

Values are means ± SEM, $n = 6$. Means in a row without a common letter differ: $P < 0.05$

[a] Nerve growth factor

[b] The relative mRNA levels was shown as the ratio to the value of 20% Casein group

[c] Not detectable

concentration in the hippocampus was significantly lower in rats fed with 5% casein diet or 0% casein diet than in those fed with 20% casein diet (Table 4), and concentration of NGF decreased significantly with the 20% gluten diet compared with the 20% casein diet (Table 4). In the present study, the mRNA levels of NGF decreased with the decrease in the quantity and quality of dietary protein in the hippocampus (Table 4). Recently, data in DNA microarray technology demonstrated that feeding the gluten diet or the protein-free diet downregulated most of gene expression in the liver compared to the casein diet (Endo et al. 2002; Kato and Kimura 2003). The results suggest that the ingestion of lower quantity and quality of protein is likely to regulate the NGF mRNA level and decrease the NGF concentration only in the hippocampus, and that the function of cholinergic neurons is partly related to the concentration of NGF in the hippocampus.

In the cerebral cortex and hippocampus, the activity of choline acetyltransferase declined gradually with the decreasing quantity and quality of dietary protein (Table 3). In the liver, many enzyme activities such as the urea cycle and amino acid catabolism have been known to decrease in rats given with low quantity (Schimke 1962; Imai et al. 2003) and low quality (Das and Waterlow 1974; Tujioka et al. 2002) of protein. Therefore, in the present study, the quantity and quality of dietary protein may have controlled the activity of choline acetyltransferase in the brains of young rats, thus corroborating the findings in terms of the other enzymes in visceral organs (Imai et al. 2003; Tujioka et al. 2002). However, the mRNA levels of choline acetyltransferase in the cerebral cortex and hippocampus did not depend on the dietary protein quantity and quality (Table 3). Thus, it was found that the regulation of choline acetyltransferase activity in the brains was not mediated through changes in the mRNA levels of this enzyme.

Little information is available on the mechanism by which the dietary protein quantity and quality affect the activity of choline acetyltransferase in the brains. Deckwerth and Johnson (1993) reported that the programmed cell death (apoptosis) was induced in the neurons deprived of NGF. It is well known that NGF protects cholinergic neurons against cell death and that an elevated survival of cholinergic neurons correlates with enhanced

choline acetyltransferase activity. Korsching et al. (1985) indicated that the concentration and mRNA level of hippocampal NGF are correlated with the density of cholinergic innervation. Gnahn et al. (1983) reported that the NGF treatment increased the activity of choline acetyltransferase in vivo in the hippocampus and cortex of newborn rats. The lower mRNA level NGF in the hippocampus of rats fed with protein-free, 5% casein or 20% gluten diet might have regulated the NGF concentration in the hippocampus and reduced the activity of choline acetyltransferase in the brains. Therefore, the quantity and quality of dietary protein might have controlled the concentration of NGF and been one of the factors affecting choline acetyltransferase activity. In the present study, we did not determine the acetylcholinesterase activity in the brains. Acetylcholinesterase has been shown to play a key role in cholinergic transmission in the central nervous system of mammals (Appleyard et al. 1983; Inestrosa and Perelman 1990). In future studies, the effect of dietary protein on cell death and acetylcholinesterase activity in the brains will be measured.

A deficiency of growth hormone (GH) also affects brain function. Treatment of adult GH-deficient patients with human GH is reported to improve the cognitive efficiency and memory function (Deijien et al. 1998; Gibney et al. 1999). The GH has been found to facilitate the long-term memory and the extinction response as recorded in a behavioral assay in rats (Schneider-Rivas et al. 1995). The possibility that the hormone itself may pass the blood–brain barrier is supported by several studies (Burman et al. 1996). Recently, we suggested that the plasma concentration of GH decreased in rats fed with lower quantity or lower quality of protein (Ohsumi et al. 2006). However, the role of GH treatment in maintaining NGF and choline acetyltransferase in the brains of young rats remains unknown. This is another possibility to consider in further examination of the mechanism by which the dietary protein alters the activity of choline acetyltransferase in the cerebral cortex and hippocampus.

The present results indicate that the activity of choline acetyltransferase decreases with the decrease in the quantity and quality of dietary protein, and that the NGF concentration mediated through changes in the mRNA of NGF is at least partly related to the mechanism by which the dietary protein affects the choline acetyltransferase activity in the brains of rats. These findings are also of importance nutritionally in understanding the role of dietary protein in the brain function in mammals.

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