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Original Article

Effects of Soft-diet Feeding on BDNF Expression in Hippocampus of Mice

Tetsu Yamamoto, Akihiko Hirayama*, Nobuo Hosoe, Masaru Furube*** and Shusuke Hirano*****

Department of Physiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
* *Laboratory of Radioisotopes, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan*
** *Department of Internal Medicine, Toho University Sakura Medical Center, 564-1 Shimoshizu, Sakura, Chiba 285-8741, Japan*
*** *The Nukada Institute for Medical and Biological Research, 5-18 Inage-cho, Inage-ku, Chiba 263-0035, Japan*

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Abstract

Our previous study showed that mice fed a soft diet after weaning had reduced synaptic connections in the hippocampal formation and impaired spatial learning ability after 3 months of age. We hypothesized that soft-diet feeding during development reduced levels of brain-derived neurotrophic factor (BDNF) protein in the hippocampus, resulting in lower synaptic densities in this region. Male pups of C57BL/6 mice were fed either a solid (hard-diet group) or powdered diet (soft-diet group), starting at weaning. Expression of BDNF protein in the hippocampus and cerebral cortex was evaluated quantitatively with enzyme-linked immunosorbent assay (ELISA) at 1, 3 and 6 months of age. Reduction in BDNF protein levels due to soft diet was detected markedly in the hippocampus of 3- and 6-month-old mice. On the other hand, a soft diet showed no significant effect on BDNF content in the cerebral cortex throughout the ages investigated. Immunohistochemistry of hippocampal formation in 3-month-old mice revealed that intensities of BDNF immunoreactivity in the dentate gyrus granule cell layer and CA1 and CA3 pyramidal cell layers appeared diminished in mice fed the soft diet compared with mice fed the hard diet. These results indicate that insufficient mastication activity during development reduces BDNF protein levels in the hippocampus and influences synaptic plasticity in this region.

Key words: Mastication—Synaptogenesis—Neurotrophin

Introduction

Insufficient mastication activity during development affects maturation of the synaptic connections in the hippocampal formation. Our previous study[21] showed that mice fed a powdered diet after weaning exhibited lower synaptic densities in the cerebral cortex at 3

months of age compared with mice fed a pelleted diet. A reduction in synaptic density was evident in the dentate gyrus and CA3 subfield of the hippocampus. These hippocampal regions are known to play a role in the formation of working memory through a neurotrophin-induced cellular mechanism[9]. Poor formation of synaptic contacts in the hippocampus may be caused by lower secretion of neurotrophic factors, especially brain-derived neurotrophic factor (BDNF). BDNF is a member of the neurotrophin family, which is widely distributed throughout the cortex[6,14], with the highest levels found in the hippocampal formation[6,7,22]. Among neurotrophins, BDNF is important for enhancing neurite outgrowth[5] and promoting formation of synaptic connections[1,19]. Recently, a molecular mechanism through which BDNF promotes the formation of new synapses was shown to be induced by morphological rearrangement of individual presynaptic compartments[2]. Indeed, mutant mice expressing reduced levels of BDNF exhibited a decrease in both number of active synaptic vesicles per synapse and synaptic connections in the hippocampus[8,17]. We hypothesized that insufficient mastication activity due to soft-diet feeding during development would cause a lower level of BDNF protein in the hippocampal formation, which would subsequently result in both lower synaptic density in this region and the inferior learning abilities in radial maze tests[21]. In this study, a quantitative comparison of BDNF expression in the hippocampus was made between mice fed a soft diet and mice fed a hard diet.

Materials and Methods

1. Animals

Male pups of C57BL/6 mice were weaned at 3 weeks after birth and fed either a pelleted (hard-diet group) or powdered chow (soft-diet group) containing the same ingredients (Certified diet, MF, Oriental Yeast, Tokyo), with free access to water. Mice were housed in groups of 4 to 5 in standard polyethylene cages (30 cm × 20 cm × 14 cm) with a bedding of wood shavings, and placed in an air-conditioned room (22 ± 1°C) under a constant light-dark cycle (12:12 hr) with lights on at 06:00. Animals were treated in accordance with the principles of the Council of the Physiological Society of Japan and in compliance with the guidelines of the Japanese government.

2. BDNF ELISA analysis

For enzyme-linked immunosorbent assay (ELISA) analysis of endogenous levels of BDNF in the hippocampal formation and cerebral cortex, mice were anesthetized with sodium pentobarbital and decapitated at 1, 3, or 6 months after birth (n = 10 mice/each group). Fresh hippocampal and cortical tissues were rapidly removed and homogenized in lysis buffer containing complete protease inhibitor cocktails (Complete Lysis-M reagent, #04719956001, Roche Diagnostics, Mannheim, Germany), followed by centrifugation at 2,000 × g for 20 min at 4°C. Levels of BDNF protein in the supernatants were assessed using the BDNF E-Max Immunoassay System (ELISA kit; Promega, Madison, WI) according to the manufacturer's instructions. Briefly, samples were diluted in 0.1 M phosphate buffered saline (PBS, pH7.4) and processed by acidification and subsequent neutralization. Ninety-six well plates were coated with mouse anti-BDNF monoclonal antibody (Chemicon, Temecula, CA) and blocked with the block provided and sample buffer for 1 hr at room temperature, followed by incubation with samples in triplicate in each plate and BDNF standards in duplicate for 2 hr at room temperature. A standard curve was established using serial dilutions of known amounts of BDNF. Plates were washed with 0.1 M Tris-buffered saline (TBS, pH7.4) containing Tween 20 (TBS-T), followed by incubation with rabbit anti-BDNF polyclonal antibody (Chemicon) for 2 hr at room temperature. After washing with TBS-T, a hydrogen peroxide solution was added to the wells with a peroxidase substrate; wells were then incubated for 10 min at room temperature. Reactions

were stopped with 1 M phosphoric acid, and absorbance at 450 nm was measured with an automated microplate reader. Triplicates were averaged, and values were corrected for total amount of protein in the sample. BDNF levels (pg/mg protein) were expressed as total BDNF concentration (pg/ml) divided by total protein concentration (mg/ml).

3. BDNF immunohistochemistry

At 3 months after birth, mice (n = 5 mice/each group) were anesthetized with sodium pentobarbital and perfused transcardially with 4% paraformaldehyde in PBS. The brains were removed, postfixed in the same fixative, and cryoprotected in 30% sucrose in PBS. Serial coronal sections (40 μm) were cut using a cryostat-microtome. Free-floating sections were washed in TBS and treated with 0.3% H_2O_2 in TBS for 10 min. After rinsing with TBS, sections were incubated in TBS containing 0.25% Triton X-100, 10% normal goat serum (NGS), and 2% bovine serum albumin (BSA) for 1 hr. Staining was performed by incubating sections with 200 ng/ml rabbit anti-BDNF polyclonal antibody (Chemicon) in TBS-T containing 1% NGS and 2% BSA for 48 hr at 4°C. After several washes with TBS-T, the sections were processed for avidin-biotin-horseradish peroxidase immunohistochemistry (ABC kit, Vector Labs, Burlingame, CA). Subsequently, sections were mounted on slides and observed under a light microscope.

4. Statistics

All data are presented as mean ± standard deviation. Statistical differences were analyzed using an analysis of variance (ANOVA) with Fisher's PLSD tests for post-hoc comparisons. The analysis was carried out using the Stat-View software (SAS Institute, Cary, NC). $p<0.05$ was considered statistically significant.

Results

Before the ELISA analysis, the body weight of the mice in the hard-diet and soft-diet groups was measured. Mean body weight in the hard-diet and soft-diet mice at 1, 3 and 6 months of age (n = 10 for each group) was 13.9 ± 0.7 and 14.3 ± 0.9 g, 27.0 ± 1.4 and 32.1 ± 3.6 g, and 33.9 ± 1.7 and 37.3 ± 2.5 g, respectively. Increase in body weight due to soft-diet feeding was significant at 3 ($p<0.001$) and 6 months after birth ($p<0.005$). A two-way ANOVA on body weight in the two groups revealed significant effects for soft diet ($F_{1,54}=31.1$, $p<0.001$) and age ($F_{2,54}=580.0$, $p<0.001$).

Quantitative investigation of BDNF protein with ELISA was made on hippocampal formation and the cerebral cortex at 1, 3 and 6 months after birth. Average BDNF protein levels in the hippocampus decreased significantly from 1 to 3 months of age in both the hard-diet ($p<0.001$) and soft-diet groups ($p<0.001$), thereafter showing no remarkable change (Fig. 1). A two-way ANOVA on hippocampal BDNF levels in the two groups revealed significant effects for soft diet ($F_{1,54}=30.6$, $p<0.001$) and age ($F_{2,54}=140.1$, $p<0.001$). Reduction rates in BDNF content due to soft-diet feeding at 3 and 6 months of age were 17.2% ($p<0.005$) and 16.2% ($p<0.005$), respectively. BDNF protein levels in the cerebral cortex also decreased significantly between 1 and 3 months after birth in the hard-diet ($p<0.001$) and soft-diet groups ($p<0.001$), and then remained at similar levels (Fig. 1). A two-way ANOVA on BDNF levels in the cerebral cortex revealed a significant effect for age ($F_{2,54}=135.6$, $p<0.001$), but no significant effect for soft diet ($F_{1,54}=3.9$, $p>0.05$).

Based on the above findings, distribution of BDNF protein in the hippocampus of the 3-month-old mice was examined by immunohistochemistry. Under a light microscope, BDNF-immunoreactive structures were detected in the dentate granule cell layer (Fig. 2A, C) and CA1 and CA3 pyramidal cell layers (B, D) in both the hard-diet (A, B) and soft-diet groups (C, D). Although overall expression of BDNF protein appeared in these cell layers, BDNF immunoreactivity was conspicuous in the neuronal cell somas in the dentate gyrus (A, C) and CA3 subfield (B, D). The intensities of

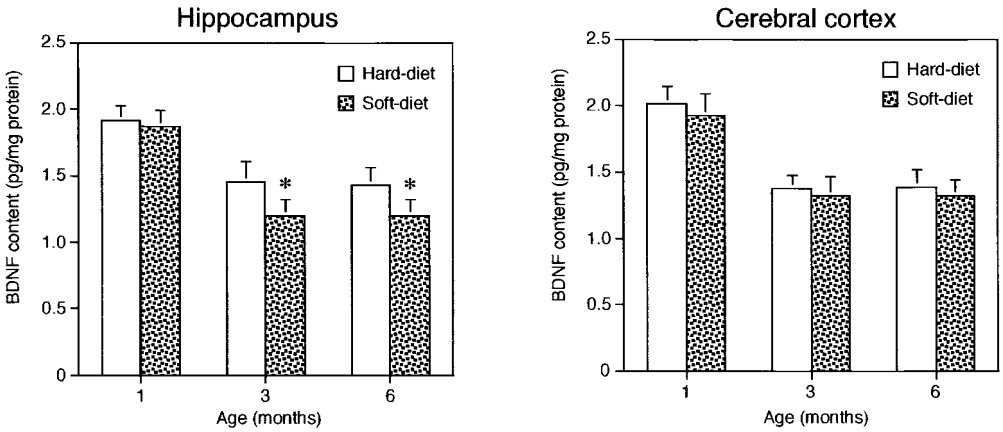

Fig. 1 Expression of BDNF protein in hippocampal formation as measured with ELISA. Soft diet feeding from weaning period significantly reduced BDNF protein in hippocampal formation at 3 and 6 months after birth. No remarkable reduction in BDNF content due to soft diet feeding was observed in cerebral cortex throughout ages investigated. Data are expressed as mean ± SD (n = 10 mice/each group). *: $p<0.005$ compared to hard-diet group.

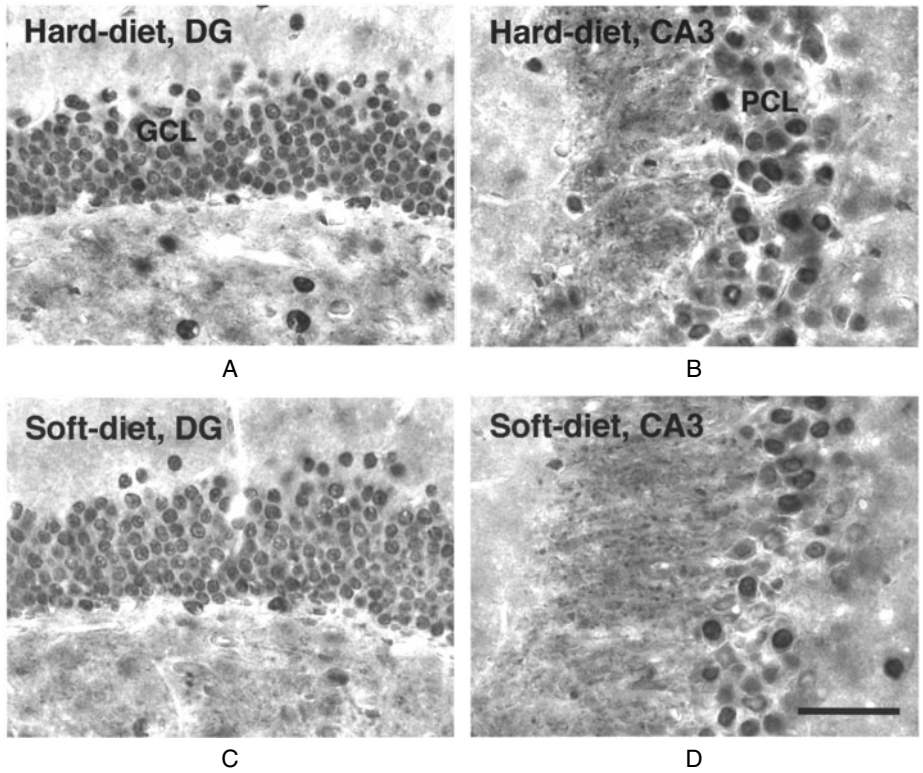

Fig. 2 BDNF immunoreactivity in dentate gyrus (A, C) and CA3 subfield (B, D) of hippocampus of hard-diet (A, B) and soft diet group mice (C, D) at 6 months after birth. Expression of BDNF protein was prominent in neuronal cell somas in granule cell layers (GCL) and pyramidal cell layers (PCL). Reduction in BDNF immunoreactivity was observed in dentate gyrus (C) and CA3 neurons (D) of mice fed on soft diet. Scale bar: 50 μm for all panels.

BDNF immunoreactivity in these cell somas appeared diminished in the soft-diet mice compared with in the hard-diet mice.

Discussion

We demonstrated that mice fed a soft (powdered) diet during development exhibited reduced levels of BDNF protein in the hippocampus after 3 months of age. A reduction in BDNF protein in the soft-diet group could cause lower synaptic densities in the hippocampal formation[21]. Synaptogenesis in the brain is regulated by neurotrophins, including nerve growth factor, neurotrophin-3, neurotrophin-4/5 and BDNF[12]. Among others, BDNF is essential for the maintenance of the function and structural integrity of synaptic connections[5,13], and effectively modulates synaptic densities in the adult hippocampus[4,19]. Endogenous signaling through the TrkB receptor, the specific tyrosin kinase receptor for BDNF, is required for maturation of synaptic contacts and synaptic vesicles[16].

Reduction in BDNF protein levels in the hippocampus due to soft diet could not be considered to result from inferior nutrition, as mean body weight in the soft-diet mice was heavier than that in the hard-diet mice at 3 and 6 months of age. Possible mechanisms of reduction in BDNF protein may involve chronic emotional stress or reduced activity of sensory pathways, or both. Functional disorders of the mouth and dentition, including missing teeth, are not only problems in eating, but a source of psychological discomfort[11]. In the present study, mice fed a powdered diet did not perform sufficient mastication activity during their lifetimes. Accordingly, they showed signs of emotional stress, such as overeating, biting non-edible substances, and loss of hair glossiness. Heavier body weight in the soft-diet mice seemed to be caused by such overeating. It has been reported that chronic stress decreases expression of BDNF mRNA in the hippocampus, especially in the dentate gyrus of rat[18]. Stress-induced decrease in BDNF mRNA is mediated by high levels of corticosterone[18].

Another hypothesis is based on the effect of diminished activity of sensory pathways from the jaws to the central nervous system. Mastication originates through coordinated activities of the masticatory muscles. Such movements produce various types of stimulation to sensory receptors in the oro-facial regions, such as the periodontal membrane, oral mucosa and masticatory muscles. Quantitative changes in afferent impulses from the mechanoreceptors in those regions might produce a reduction in BDNF levels in the hippocampus. Expression of BDNF in the brain is responsive to a variety of stimuli that enhance neuronal activity[3,10]. Exercise in the form of voluntary running results in an increase in the level of BDNF mRNA and protein in the hippocampus of rat[15,20]. In contrast, abrupt deprivation of habitual running leads to long-lasting decreases in BDNF mRNA expression in rat hippocampus[20]. Likewise, it is considered that less activity in terms of masticatory movements during development leads to decreased levels of BDNF protein in the hippocampus in adulthood. Such down-regulation of BDNF protein may diminish the number of synaptic connections in the hippocampus[21].

In conclusion, mice fed a soft diet after weaning exhibited lower levels of BDNF protein and lower densities of synaptic connections in the hippocampus in adulthood compared with mice fed a hard diet. These results indicate that insufficient mastication activity during development produces a reduction in BDNF protein levels in the hippocampus and influences synaptic plasticity in this region.

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Reprint requests to:
Dr. Tetsu Yamamoto
Department of Physiology,
Tokyo Dental College,
1-2-2 Masago, Mihama-ku,
Chiba 261-8502, Japan
E-mail: ttyamamo@tdc.ac.jp