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Neuroscience Letters 399 (2006) 135–140
Neuroscience Letters
www.elsevier.com/locate/neulet

Effects of food restriction on synthesis and expression of brain-derived neurotrophic factor and tyrosine kinase B in dentate gyrus granule cells of adult rats

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Received 28 November 2005; received in revised form 13 January 2006; accepted 24 January 2006

Abstract

We have previously found that the dendritic trees of dentate gyrus granule cells are selectively vulnerable to food restriction but there are reorganizational morphological events that minimize functional impairments. As the neurotrophin brain-derived neurotrophic factor (BDNF) and the cognate receptor tyrosine kinase B (TrkB) are involved in the maintenance of the structure of dendritic trees, we thought of interest to verify if there are alterations in its synthesis and expression in granule cells. To investigate this issue, 2-month-old rats were submitted to 40% caloric restriction for 6 months and compared to controls fed ad libitum. The numbers of granule cells containing BDNF and TrkB proteins were estimated from immunostained sections and the respective mRNA levels of individual neurons evaluated using nonradioactive in situ hybridization. After dietary treatment there was a 15% reduction of BDNF-immunoreactive granule cells with no changes of the number of TrkB-immunostained neurons. No alterations were found in the levels of BDNF and TrkB mRNAs of individual granule cells. As caloric restriction extends the lifespan of animals, the restrictive dietary regimens are generally regarded as beneficial to the organisms, but the present results suggest that caution is needed when extrapolating to some neuronal populations.

Keywords: Hippocampal formation; Granule cells; Food deprivation; BDNF; TrkB; Stereology

Dietary restriction regimens involving a daily reduction of food intake or periodic fasting have been shown to extend by up to 50% the mean and maximum lifespans of rodents and monkeys [2,9,16,17]. The restriction of calories has also been demonstrated to reduce or delay the incidence of several diseases in adulthood and senescence [2,9,16,17], to increase the resistance to epileptic seizures [6,8] and to protect neurons after metabolic and ischemic insults [2,8].

The mechanisms underlying the beneficial effects of dietary restriction in the central nervous system (CNS) are not well understood although some hypotheses have been advanced, including reduction of oxidative damage and stimulation of the expression of genes that encode cytoprotective proteins such as heat-shock protein 70 and neurotrophins [2,10,16,17]. Neurotrophins are abundant in the rodent hippocampal formation, which contains one of the highest brain levels of brain-derived neurotrophic factor (BDNF) [7,22]. This neurotrophin promotes neurogenesis, increases cell survival and enhances activity-dependent synaptogenesis mediated through the high-affinity receptor tyrosine kinase (TrkB) that triggers multiple intracellular signaling pathways [31]. Nevertheless, the effects of dietary restriction on BDNF protein estimated using biochemical methods are controverse since intermittent fasting has been shown to increase its global levels in whole hippocampal homogenates of rodents [10,16,17], whereas no alterations were found using a prolonged model of daily caloric restriction [25]. Also, the available informations regarding the BDNF and TrkB levels in specific neuronal populations of the hippocampal formation after dietary restriction are very sparse [16]. However, we have previously shown that a prolonged period of daily 40% caloric restriction induced some regressive but subtle alterations in the dendritic trees of the dentate gyrus granule cells with no change of its total number [1]. As it is known that BDNF and TrkB are related to the complexity of the dendritic arborization of these neurons [1,31], we thought of interest to estimate the total number of granule cells immunoreactive for BDNF and TrkB

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doi:10.1016/j.neulet.2006.01.043

using unbiased morphometric methods. Also, nonradioactive in situ hybridization was used to evaluate BDNF and TrkB mRNA levels in individual granule cells. Using those techniques, we sought to examine whether or not daily 40% caloric restriction can affect the synthesis and expression of BDNF and TrkB in the dentate gyrus granule cells of the adult rat.

The studies were carried out in 2-month-old male Wistar rats. Animals were individually housed and maintained throughout the experiment under room temperature (20–22 °C) and a daily photoperiod of 12 h (lights on at 07:00 h). Control rats ($n = 10$) were fed for 6 months ad libitum with standard rodent laboratory chow (Letica, Spain) containing casein (17%), supplemented with lysine (0.7%), methionine (0.3%) and cystine (0.5%), carbohydrates (57%), fat (4%) and salts (7%). All rats had free access to water throughout the experimental period and were weighted at 2-week intervals. Food consumption of controls was monitored daily and food-restricted rats ($n = 10$) were fed 60% of the mean amount of food consumed by control animals every day for 6 months. These animals were fed once a day at 08:00 h and they consumed the totality of their diet in less than 2 h. Both groups of rats were supplemented with Diet Vitamin Fortification Mixture (MP Biomedicals, USA). The handling and care of the animals were carried out according to the EU guiding principles in animal research (86/609/UE) and Portuguese Act no. 129/92.

At the end of the experimental period, animals were deeply anesthetized with chloral hydrate (i.p., 1 ml/100 g body weight, 6% solution) and killed by transcardiac perfusion of a fixative solution containing 4% paraformaldehyde in phosphate buffer, at pH 7.6. Subsequently, the brains were rapidly removed from the skull, coded for blind processing and analysis, stored in the same fixative solution for 1 h and maintained overnight in RNAse-free 10% sucrose solution at 4 °C. Blocks containing the right and left hippocampal formations were mounted on a vibratome, serially sectioned in the coronal plane at 40 μm and collected in RNAse-free phosphate buffered saline [19,30].

Two independent sets of sections containing the hippocampal formations were selected, using a systematic random sampling procedure [14,21,33], from five rats of each experimental group. One of those sets was used for BDNF immunostaining using the rabbit polyclonal antibody BDNF-N20 (sc546, Santa Cruz Biotechnology, USA). The other set of sections was used for TrkB immunostaining using the rabbit polyclonal antibody TrkB-794 (sc12, Santa Cruz Biotechnology). Both antibodies were tested elsewhere for the specificity [13]. For immunocytochemical staining, all sections were processed simultaneously, using the avidin-biotin technique with diaminobenzidine as the chromogen as previously described [19,30]. The same procedure was followed for control sections also in parallel, which were incubated without primary antiserum. No immunostaining was observed in these sections.

For the analysis of BDNF and TrkB mRNA levels ($n = 5$ per group) in individual granule cells, sections containing the hippocampal formations were sampled in order to obtain two independent sets of sections from each brain. One set was used for detection of BDNF mRNA and the other for detection of TrkB mRNA. The BDNF probe used was a 46 bases long oligonucleotide [18] and the TrkB probe was a 48 bases long oligonucleotide [5], both 3′-tailed with digoxigenin (MWG-Biotech, Germany). Hybridizations were carried out under steady-state conditions overnight at 40 °C as previously described [19,30]. The probes were detected using the Digoxigenin Detection Kit (Roche Molecular Biochemicals, Germany) and the alkaline phosphatase activity demonstrated using 5-bromo-4-chloro-3-indolyl phosphatase and nitroblue tetrazolium (NBT/BCIP, Roche Molecular Biochemicals). The hybridization steps and the histochemical development of alkaline phosphatase were performed simultaneously in all sections. Pretreatment of the sections with RNAse and sections hybridized with the hybridization buffer alone or with an excess of unlabeled probe displayed no specific signal.

The total numbers of BDNF and TrkB-immunoreactive neurons in the dentate gyrus granular layer (Fig. 1A–D) were estimated using the optical fractionator method [21,30,33]. This sampling scheme ensured that all parts of the granular layer had an equal opportunity of being sampled and in each sampled area neurons were counted using optical disectors [21,30,33]. All the estimations were performed, using the C.A.S.T.-Grid system software (Olympus DK, Denmark) and a Heidenhain MT-12 microcator (Heidenhain, Germany). At magnification of ×2000, the presence of immunostaining of the perikaryal cytoplasm with an unstained nucleus was the criterion for the identification of BDNF- and TrkB-containing granular neurons (Fig. 1A–D). The coefficient of error (CE) of the individual estimates of BDNF- and TrkB-immunoreactive neurons, calculated as described by Gundersen et al. [14], was 9 and 8%, respectively.

The deep-blue purple precipitate indicative of the presence of BDNF or TrkB mRNAs was characterized by dense particulate labeling within the cytoplasm and a clear unlabeled nucleus in individual granule cells (Fig. 1E and F). The hybridization signals from these positive neurons were analyzed using a computer-assisted image analyzer (Optimas-Bioscan) fitted with a Leica axioplan microscope and a Sony digital video camera. Densitometry of digitized images was used to integrate and compare the optical densities of hybridization signals of a mean of 70 positive dentate granule cells per animal, and for each mRNA, under bright-field illumination. A random sampling scheme was used to select the positive neurons. The background signal was compensated and after correction the mean optical density was expressed in gray values of 1–256 [19,30]. All the estimations were performed in both sides of the brain and the data were pooled for each animal.

Data were analyzed using unpaired Student's *t*-test. *P*-values were adjusted to multiple comparisons and differences were considered to be significant if $P < 0.05$. Data were shown as means ± 1 standard deviation (S.D.).

Daily food intake, measured at 08:00 h every day, was 30.6 ± 1.25 g in control rats, leading to a consumption of 19.1 ± 1.33 g in the food-restricted group. On average, the body weight of food-restricted animals at the end of the experiment was 35% lower than that of control rats ($P < 0.0005$; Fig. 2). No significant difference ($P = 0.80$) was detected between the mean brain weights of control (1.54 ± 0.03 g) and food-restricted animals (1.54 ± 0.04 g) at the end of the experiment.