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Effects of Leptin on NADPH-d Reactivity in the Dentate Gyrus of Rats

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Leptin is a peptide hormone regulating food intake and body weight. The effects of leptin are mediated via leptin receptors present in the central nervous system (including hippocampal regions and gyrus dentatus). It is known that leptin induces phosphorylation of the neuronal isoform of nitric oxide synthase (nNOS) in defined hypothalamic regions. Identification of specific extrahypothalamic sites of leptin-induced activation of nNOS has been largely ignored. The present study was therefore undertaken to investigate the effects of leptin on NO expression in gyrus dentatus of rats. Six male Wistar rats were injected i.p. with either leptin (0,5 mg/kg) or saline (control group) and anesthetized 45 min later. Serial coronal sections were stained with the histochemical nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) technique and examined with a light microscope. Our results demonstrated that leptin-treated animals had a significant increase in NADPH-d positive neurons in the dentate gyrus compared to that seen in the control group. These data suggest that leptin results in increased expression of NO in dentate gyrus of rats. We speculate that leptin may exert an effect on the hippocampal neurogenesis or neuroprotective properties by activating the endogenous nitric oxide synthase system.

Key words: leptin, nitric oxide, NADPH-d, dentate gyrus, rats

Introduction

Leptin is an adipokine, expressed and synthesized by the adipocytes [28]. It has a role in regulating energy homeostasis and neuroendocrine function [20, 25]. Different isoforms of the leptin receptor were found, but only the long form of the receptor, LepRb, appears to be the critical receptor for leptin action [3]. LepRb is expressed in different areas of the central nervous system (CNS) such as hypothalamus, cerebellum, cerebral cortex and hippocampus, including dentate gyrus (DG) [11].

The dentate gyrus (DG) is located mainly in the antero-medial temporal lobe, referred to allocortex [9]. It is main part of hippocampal formation and is specialized in associative memory (consolidating events and what is happening) [22]. The dentate gyrus (DG) is composed by three layers: molecular layer (primarily interneurons – the axo-axonic cells and the MOPP cells (molecular layer perforant path-associated cells),

the dendrites of the granule cells, pyramidal basket cells and mossy cells and the terminal axonal arbors from the entorhinal cortex), granular layer with the subgranular zone (the granule cells and the pyramidal basket cells), and the polymorphic zone (hilum, containing the mossy cells, a number of fusiform cells and also multipolar or triangular cells) [24]. The granule and the mossy cells project axons to make excitatory synapses on the dendrites of CA3 pyramidal neurons. The only cortical structure from which DG gets direct inputs is the entorhinal cortex.

It has been found that the effects of leptin in the CNS are mediated by nitric oxide (NO) [2]. NO is a freely diffusible gaseous neurotransmitter. It is biosynthesized endogenously from the amino acid L-arginine and oxygen, by nitric oxide synthase (NOS) [12]. Nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) is routinely used as a histochemical marker for NOS [15, 26]. Since DG neurons are known to express LepRb, we investigated the effects of leptin on NADPH-d reactivity in the DG of male Wistar rats.

Material and Methods

Animals: Male Wistar rats, with average body weight 250-300 g, were housed in a temperature-controlled room (20–22°C) on a 12:12-h light-dark cycle (07:00 to 19:00 h). They were divided in 2 groups (3 rats per group): the first one treated with leptin and the second one (control group) – with saline. Rats had ad libitum access to standard pelleted chow and water. All experiments were conducted in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and approved by the Ethical Council of the Bulgarian Food Safety Agency.

Leptin stimulation and NADPH-d histochemistry: On the day of the experiment, rats were injected i.p. with leptin (0.5 mg/kg) or vehicle (saline) and anesthetized 45 min later with thiopental (40 mg/kg i.p.). Transcardial perfusion was performed using 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. The brains were removed and postfixed overnight in the same fixative solution at 4ºC. Serial coronal sections from septal part of dentate gyrus (from bregma -2.12 mm to -3.30 mm) on a freezing microtome (Reichert-Jung) at a thickness of 40 μm were done. Every fifth section was processed for NADPH-d histochemical examination.

The DG levels were identified according to their stereotaxic coordinated in the rat brain atlas (Paxinos and Watson, 2007). Sections were then stained with the NADPH-d-technique using 0.1–0.2 mg/ml of nitroblue tetrazolium, 1 mg/ml β-NADPH and 0.3–0.5 % Triton X-100 in 0.1 M TRIS – HCl buffer (pH 7.4) at 37 °C for 30–60 min. Afterwards, the sections were given three consecutive 5-min rinses in the same phosphate buffer and mounted on gelatin-coated glass slides. The slides were air dried overnight at room temperature, rinsed three times with distilled water, dried again and cover-slipped with Entellan (Merck, Germany).

Data analysis: NADPH-d-labeled neurons were visualized using a 20 X objective on a light microscope (Nikon Eclipse 80i microscope). The analysis started with digitally capturing (a digital camera Nikon DMX 1200) and storing the areas of interest. The density of nerve cell bodies and the amount of the cells in the DG areas were estimated using the same Nikon's NIS Elements Digital Imaging software. DG sections were used for cell counts and the average of the cells was calculated. The neuronal densities of the selected brain areas were quantified by determining the percentage of the measurement grid occupied by stained cells. Data were statistically assessed by one-way analysis of variance (ANOVA) and Holm–Sidak post hoc test. All values are presented as mean ± standard error of the mean (SEM). A p-value equal to or less than 0.05 was considered to be statistically significant.

Results

Our results have shown NADPH-d reactivity in the DG of both saline treated and leptin – treated rats (**Fig. 1**).

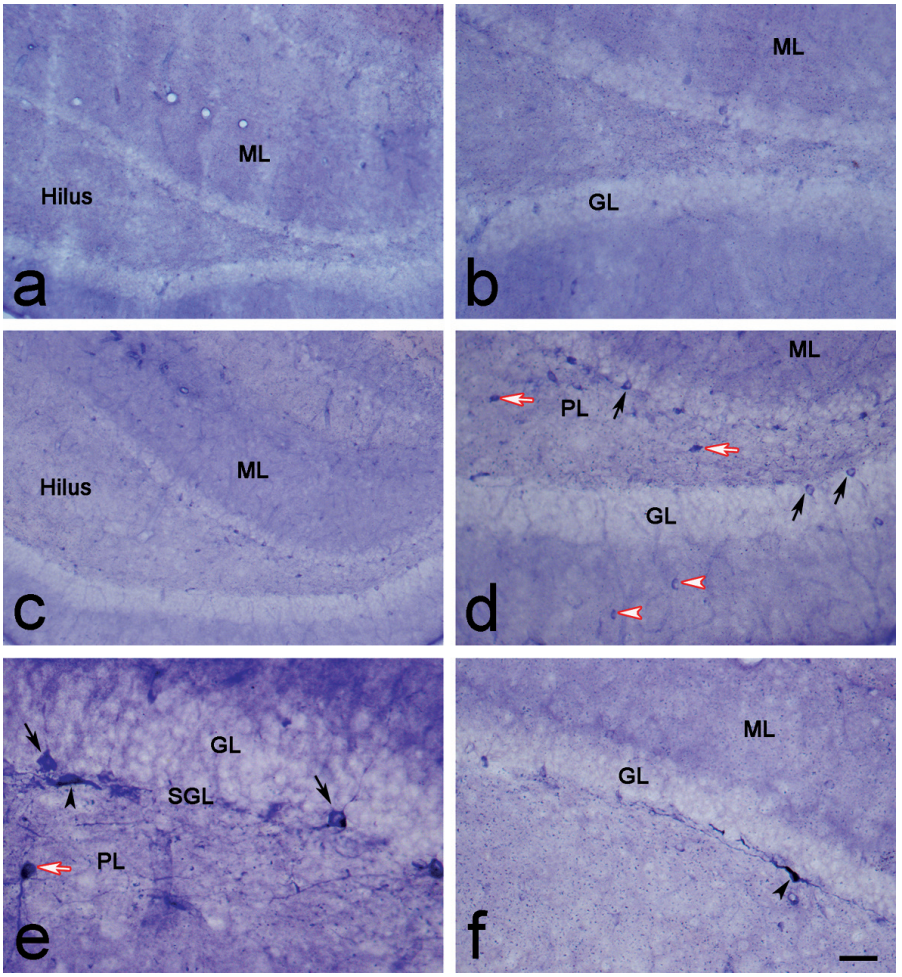

Fig. 1. NADPH-diaphorase reactivity of DG neurons in the control (a, b) and leptin treated groups (c-f). ML – molecular layer, GL – granular layer, SGL – subgranular layer, PL – polymorphic layer. The pyramidal basket cells – black arrows, the mossy cells – white arrows, the elongated cells on the border of subgranular zone – black arrowheads, the neurons in molecular layer – white arrowheads. Scale Bar: 100 μm (a, c), 50 μm (b, d, f), 25 μm (e).

There was statistically significant increase in the number of NADPH-d positive neurons in leptin-treated rats compared to that observed in the control group ($p<0.01$, **Fig.2**). NADPH-d reactivity was seen as cytoplasmic staining into cell bodies and their branches. The cytoplasm was diffusely filled with NADPH-d reaction products unlike the nucleus that was stain-free. Based on the cell body shape, the neurons were multipolar, pyramidal, bipolar, oval, and pear-shaped. Representation by zones included

round or fusiform NADPH-d reactive neurons in molecular layer, single cone-shaped or oval cells in granular layer, larger pyramidal-basket cells in the subgranular zone and some mossy cells in the hilus.

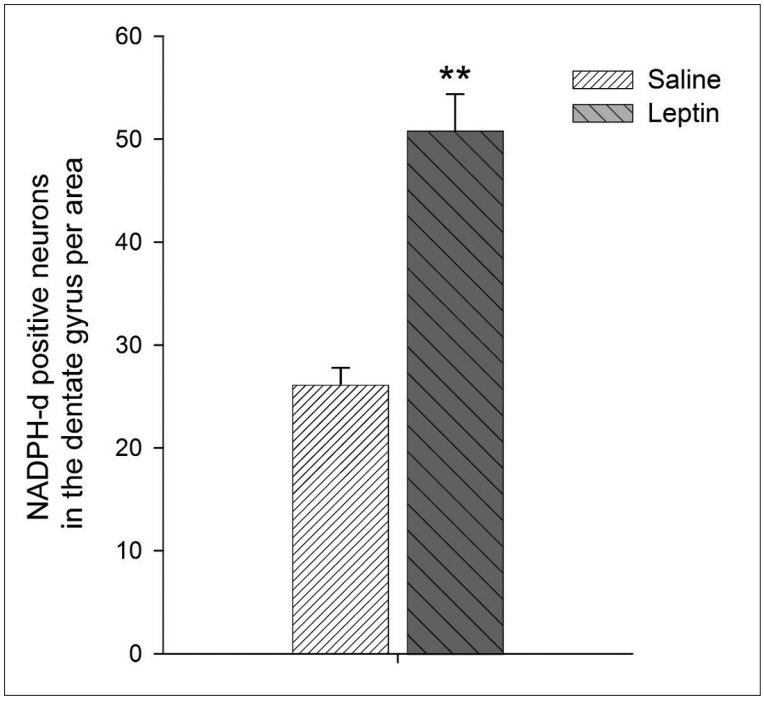

Fig. 2. The number of NADPH-d positive cells in the dentate gyrus of saline- and leptin-treated rats. Animals were fasted overnight and injected with leptin or saline and euthanized 45 min later. One of five series from each animal was analyzed. Statistical significance compared with saline group: **P<0.01 (n=3 per group). Values represent the mean ± SEM

There was a number of branched pyramidal-basket cells from subgranular zone which single aspiny apical dendrites traverse the granular layer, between the cell bodies of granule neurons stretch to the molecular layer. These cells had also several basal dendrites that extend into the polymorphic cell layer. The pyramidal-shaped neurons were larger than the granule cells. The granule cell layer had single NADPH-d reactive oval or elliptical-shaped neurons. The NADPH-d reactive cells were located mainly on the border between granular, subgranular and polymorphic layers.

The cells from polymorphic layer were different in shape and size. The main type of cells we seen in this layer were the mossy cells, who had got triangular or multipolar-shaped large cell bodies. Some of cell with elongated shape were situated along to the axis of subgranular layer. Two or more thick long dendrites originated from their cell body and run parallel to layer's border. They could be divided into primary and secondary spiny dendrites.

Discussion

Our results have shown that systemic administration of leptin resulted in increased NADPH-d positive cell number in the DG. In hippocampal neurons (areas CA1, CA3 and the dentate gyrus), leptin receptors are located at both presynaptic and postsynaptic sites [23]. As a neurotrophic growth factor, leptin promotes synaptogenesis, synaptic plasticity, axon growth and neuronal migration in the hippocampal formation [14]. It has a key role in improving memory formation and retention [13]. Studies have demonstrated deficiencies in brain myelin, reduced neuronal soma size, altered dendritic orientation in ob/ob and db/db mice [4].

The neural stem cells (NSCs) are pluripotent cells, located in CNS [10]. They may differentiate into neurons, astrocytes, and oligodendrocytes depending on the received signals for differentiation and maturation [6]. In adult CNS, NSCs are found in the subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the hippocampal formation. In SGZ, the NSCs maturate into the granule cells [21]. Increasing studies indicate that NO has a substantial role for the proliferation of NSCs. Recently, Carreira et al. have demonstrated that extracellular NO participates in the regulation of NSC proliferation [5]. They found that treatment with 10 μM of NO donor NOC-18 for 24 h increases NSC growth, whereas higher concentrations (100 μM) decrease cell growth. Moreover, Luo et al. have shown that NO, produced from nNOS, plays an important role in NSC proliferation [17]. Collectively, we suggest that leptin-induced NO production in the DG may affect the neurogenesis occurs in the hippocampal formation.

It has been found that leptin has neuroprotective effects under a variety of neurotoxic conditions [7, 27, 29]. Using primary cultured hippocampal neurons, Martins et al. have demonstrated that leptin reduces amyloid-β (Aβ) oligomers-induced production of superoxide and mitochondrial membrane depolarization, improving cell survival, and inhibit cell death through a receptor-dependent mechanism, thus highlighting its potential therapeutic role in Alzheimer‘s disease [18]. Additionally, in organotypic slices from rabbit hippocampus, exogenous leptin increases the basal expression levels of insulin-like growth factor-1 (IGF-1), a neuroprotective and neurotrophic factor, and reverses the Aβ-mediated decrease in IGF-1 levels [19]. Few studies have been published on neuroprotective properties of NO, caused through induction of the cGMP pathway [1, 8, 16]. Thus, we hypothesize that leptin, inducing NO synthesis in DG neurons, may have neuroprotective effects.

Conclusions

Our results have shown that leptin increases NADPH-d reactivity in the DG of male Wistar rats. We speculate that leptin may exert an effect on the hippocampal neurogenesis or possess neuroprotective properties by activating the endogenous nitric oxide synthase system. Additional studies are needed to examine this hypothesis.

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