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A ROLE FOR VGF IN THE HYPOTHALAMIC ARCUATE AND PARAVENTRICULAR NUCLEI IN THE CONTROL OF ENERGY HOMEOSTASIS

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Abstract—The arcuate nucleus is the main receptive area of the brain for peripheral and central metabolic cues and its integrity is essential for the maintenance of energy homeostasis. In the arcuate nucleus, different neuronal populations process metabolic signals and transmit this information to other nuclei of the hypothalamus by means of neurotransmitters and a combination of neuropeptides whose expression is modulated by the nutritional status. Here we investigated the changes in expression and synthesis of the polypeptide VGF in the arcuate nucleus of rats, in relation to the two main categories of neurons that show colocalization with VGF: the orexigenic NPY-expressing cells and the anorexigenic POMC-expressing cells. The results show that fasting is the most important stimulus for VGF expression, and that the up-regulation of VGF mRNA is restricted to the NPY area of the arcuate nucleus. POMC neurons express VGF under all feeding conditions, but especially in *ad libitum*-fed and fasted-refed animals. We also show that VGF arcuate neurons project to the pre-autonomic neurons of the paraventricular nucleus of the hypothalamus, providing anatomical evidence suggesting VGF as a central modulator of the autonomic nervous system.

Key words: NPY, α-MSH, fasting, refeeding, autonomic nervous system.

INTRODUCTION

VGF (non-acronymic) is a neurotrophin-induced gene that is expressed in neuronal and neuroendocrine cells (Levi et al., 1985; Salton et al., 1991). The *vgf* gene encodes for a precursor polypeptide that is stored in dense core vesicles, cleaved by prohormone convertases typical of neuronal/endocrine tissues and then released after depolarizing stimuli (Possenti et al., 1989; Trani et al., 1995). *VGF* mRNA is widely expressed throughout the nervous system, both in embryonic and adult tissues (Snyder and Salton, 1998; Snyder et al., 1998). The highest expression of *VGF* has been detected in the ventromedial hypothalamus, especially in the arcuate nucleus (ARC) and in the suprachiasmatic nucleus (SCN) (Van den Pol et al., 1989; Van den Pol et al., 1994).

The participation of VGF in metabolism was discovered after development of *VGF* knock-out mice: these animals are smaller than their control littermates (both heterozygous and normal mice) and are lean throughout their lives. At birth, however, they weigh the same as the controls and, in proportion to their size, they eat more food than the controls (Hahm et al., 1999); they also show a high level of oxygen consumption and locomotor activity, indicating that an inappropriate increase in energy expenditure could be the cause of the lean phenotype. Currently, some VGF cleavage products are known for their role in the control of energy homeostasis. For example, the TLPQ-21 peptide has catabolic properties since central administration causes decrease in food intake, the mobilization of fat storage and the reduction of body weight (Bartolomucci et al., 2006; Jethwa et al., 2007), whereas the NERP-2 peptide stimulates food intake via the orexin system (Toshinai et al., 2010). Interestingly, *vgf* is required for the development of several forms of obesity (Hahm et al., 2002; Watson et al., 2005). For instance, Agouti mice (A^y/a) over-express the agouti polypeptide, thus have a decreased α-MSH satiety-signaling and develop obesity; in addition, they also

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Abbreviations: α-MSH, alpha-melanocyte stimulating hormone; ARC, arcuate nucleus; CTB, cholera toxin B; CTR, control group; FST, fasted group; DVC, dorsovagal complex; GABA, gamma-amino butyric acid; GTG, gold thioglucose; IR, immunoreactivity; ME, median eminence; MSG, monosodium glutamate; NPY, neuropeptide Y; Pha-L, phaseulus vulgaris leucoagglutinin; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus 3 h-RF, three hours refed group; 3V, third ventricle; 6 h-RF, six hours refed group.

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show a typical yellow-colored coat. When inbred with *vgf*−/− mice, A^{y}/a animals fail to become obese, but they still have a yellow coat, suggesting that *vgf* may be a key component of the metabolic melanocortin signaling specifically in the brain (Hahm et al., 2002; Watson et al., 2005). The chemical lesion of the ARC also indicates that *vgf* is essential for energy balance. In particular, gold thioglucose (GTG) is a substance that produces the loss of glucosensitivity in the ARC resulting in obesity (Bergen et al., 1998); the latter is totally prevented by *vgf* ablation (Hahm et al., 2002; Watson et al., 2005). Monosodium glutamate (MSG) is another neurotoxic agent that causes extensive injuries to the hypothalamus, including the removal of NPY and melanocortin signaling and the impairment of the sympathetic output (Pizzi and Barnhar, 1976). Conversely to GTG lesion, *vgf* gene inactivation in knock-out mice does not prevent MSG-induced obesity, suggesting that *vgf* acts on the hypothalamic autonomic outflow, and that this action of *vgf* is necessary for the development of the hypermetabolic phenotype (Hahm et al., 2002). More recently, Watson et al. (2009) demonstrated that the *vgf* knock-out mice display hyperactivation of the sympathetic system that can be responsible for the reduced accumulation of fat, together with an up-regulation of uncoupling proteins in brown adipose tissue.

Additional data indicate that in the fed state, *VGF* mRNA co-localizes with *POMC* mRNA in the lateral ARC, whereas, in the fasted state, the co-localization between *VGF* mRNA and POMC decreases, and *VGF* is expressed in NPY/AgRP cells (Hahm et al., 2002).

In the present work, we aimed to study two aspects of VGF's participation in the hypothalamic regulation of metabolism: we investigated the dynamic of VGF synthesis in α-MSH and NPY cells of the ARC according to the metabolic state, and we studied the neural pathways through which VGF of the ARC may exert a control on energy expenditure.

We first compared *VGF* mRNA expression and VGF protein synthesis with NPY and POMC or α-MSH in response to different feeding conditions, with the hypothesis that VGF distribution shifts from NPY to α-MSH according to a negative or positive metabolic state, respectively. In particular, we compared the changes of: (a) *VGF*, *NPY* and *POMC* mRNA; (b) VGF, NPY and α-MSH immunoreactivity (IR); (c) VGF immuno-co-localization in NPY and α-MSH neurons, in response to 48-h fasting; 48-h fasting followed by 3-h re-feeding; 48-h fasting followed by 6-h re-feeding.

Next we explored the possibility that pre-autonomic neurons within the hypothalamic PVN might be a target of VGF neurons of the ARC, by injecting an anterograde tracer in the ARC and a retrograde tracer in the Dorsovagal complex (DVC) of the brainstem, and observing the VGF projection by triple fluorescent immunohistochemistry.

The present results showed that VGF expression depends on the metabolic state and it could exert its effect on metabolism through pre-autonomic neurons in the PVN.

EXPERIMENTAL PROCEDURE

Male Wistar rats, 250–300-g body weight, were used for all experiments. Animals were housed in individual cages, for one week at 22 °C, under a 12:12 light–dark cycle, with free access to food (Teklad Global Diet 2018S, Harlan, Madison, WI, USA) and water, before either of the following experiments was carried out. All the procedures were approved by the committee for ethical evaluation at the Universidad Nacional Autónoma de México, in strict accordance with the Mexican norms for animal handling (Norma Oficial Mexicana NOM-062-ZOO-1999).

Experimental design

Animals were randomly divided into four groups ($N = 5$ animals/group): the control group was fed *ad libitum* throughout (CTR); the fasted group was food-deprived for 48 h (FST); the 3-h re-fed group was fasted for 48 h and then re-fed for 3 h immediately preceding sacrifice (3 h-RF); the 6-h re-fed group was fasted for 48 h and then re-fed for 6 h (6 h-RF). All animals were sacrificed at the same time of the day (ZT10). Before sacrifice, animals were deeply anesthetized with an overdose of sodium pentobarbital (Sedalpharma, Pet's Pharma, Mexico; 50 mg/kg) and perfused transcardially with 0.01 M phosphate-buffered saline (PBS; pH = 7.4) and 4% paraformaldheyde (Sigma–Aldrich Corp., St. Luis, MO, USA) in 0.01 M phosphate buffer.

In situ hybridization

Brains were rapidly removed, additionally post-fixed overnight by immersion in the same fixative at 4 °C, followed by cryoprotection in 30% sucrose in diethyl pyrocarbonate treated PBS at 4 °C. Series of 16-µm-thick coronal sections were cut throughout the rostrocaudal extent of the hypothalamus. Alternate sections were collected on Superfrost/Plus glass slides (Fisher Scientific Co., Pittsburgh, PA, USA) and stored at −80 °C. Sections were dried at room temperature for 2 h before overnight incubation at 6 °C in hybridization buffer [1× diethyl pyrocarbonate-treated 'salts' (200 mm NaCl, 5 mm EDTA, 10 mm Tris, pH 7.5, 5 mm $NaH_2PO_4{\cdot}2H_2O$, 5 mm Na_2HPO_4); 50% deionized formamide; 1× Denhardts (RNase/DNase-free; Invitrogen Corporation, Carlsbad, CA, USA); 10% dextran sulfate (Sigma–Aldrich)] containing 400 ng/mL digoxigenin-labeled RNA probes purified on Sephadex G-50 columns. Sense and antisense probes were generated by linearization or excision of plasmids with appropriate enzymes, and purified using QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA). Primers were synthesized by Sigma: sense 5′ TATCCCTGCTCGTGTGTTTG 3′ and antisense 5′ AGGCAGACTGGTTTCACAGG 3' for NPY; sense 5′ GACTGAAAATCCCCGGAAGT 3′ and antisense 5′ TCTTGATGATGGCGTTCTTG 3′ for POMC; sense 5′ ATGAAAACCTTCACGTTGCC 3′ and antisense 5′ GCGCTTAGCATTACTCGGAC 3′ for VGF.

The hybridization solution consisted of 50% formamide, 2× sodium phosphate, sodium chloride and