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Hypothalamic neurons innervating fat tissue in the pig express leptin receptor immunoreactivity

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Abstract

While leptin receptors have been found in both the autonomic ganglion neurons and the hypothalamic nuclei, studies dealing with the projections from the central nervous system to the adipose tissue have been conducted mainly in laboratory animals. Therefore, the purpose of our study was to establish whether hypothalamic neurons are transsynaptically connected to adipose tissue depots in the pig, and if these neurons express leptin receptor immunoreactivity. Pseudorabies virus (PRV; Bartha's K strain) was introduced in perirenal or subcutaneous adipose tissue depots in domestic pigs. On day 9, animals were euthanized and hypothalami were collected and processed immunohistochemicaly with primary antisera against PRV and leptin receptor (OBR). PRV-labeled neurons were localized in paraventricular nucleus, supraoptic nucleus and arcuate nucleus following injections in both the perirenal and the subcutaneous adipose tissue depots. Ventromedial nucleus, dorsomedial nucleus and preoptic area-labeled neurons were observed after injection of the PRV into the perirenal adipose tissue, while in the lateral hypothalamic area-labeled neurons projected only to the subcutaneous adipose tissue. The majority of the PRV-labeled neurons simultaneously expressed OBR-immunoreactivity. Our results provide the morphological data on multisynaptic projections from hypothalamus to the fat tissue in the pig and demonstrate that these neurons, located in areas involved in reproductive processes and feeding behavior, may regulate fat tissue metabolism.

Keywords: Fat tissue; Innervation; Hypothalamus; Tracing; Pseudorabies virus; Pig

Leptin secreted mainly from the fat tissue and acting at the brain to increase energy expenditure and alter endocrine activity has been previously reported in the pig [4,14]. Hypothalamic centers receive and integrate the leptin signal through leptin receptors to control appetite and energy expenditure [1]. Leptin mediates the metabolic and neuroendocrine responses to fasting [6]. Moreover, it is implicated in the reproductive process as well as in glucose and lipid metabolism [2,4]. Several reports indicate that central actions of leptin in regulation of energy balance and neuroendocrine function are mediated by hypothalamic peptides such as neuropeptide Y (NPY), melanocyte-stimulating hormone (MSH) and agouti-related protein (AGRP) [18,24]. For example, the rise in circulating leptin concentrations associated with overfeeding and obesity is likely to inhibit appetite by decreasing the expression of orexigenic peptides such as NPY. In contrast, the associated decline in leptin secretion with fasting may promote feeding behavior by increasing hypothalamic NPY concentrations and decreasing anorexigenic peptides [10]. Low blood leptin levels may modulate the fasting-induced suppression of thyroid and reproductive axes, and influence the expression of hypophysiotropic peptides, such as thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH), and GnRH [4]. While this regulatory pathway is well established in rodents, there are many questions about its applicability to body weight and reproduction in large domestic animals. The above-mentioned studies demonstrate that leptin provides a link between adipose tissue and the brain and illustrates the importance of adipose tissue in regulation of energy balance and reproduction. Furthermore, leptin receptors have been found in both the autonomic ganglion neurons and the hypothalamic nuclei involved in reproductive processes and the control of energy balance [11,18]. The introductions of transsynaptic viral tracing techniques [9] have made identification of multisynaptic pathways linking adipose tissue depots to the brain possible to approach. Pseudorabies virus Bartha's K strain (PRV) is one of several viruses frequently used for

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transsynaptic tracing and has been widely employed for analysis of multisynaptic circuits following peripheral injections [7,13]. Recent morphological studies using viral tracing revealed that neurons within the CNS are transsynaptically connected to different organs including fat tissue [17,27]. An additional, advantage of transsynaptic viral tracing is the ability to perform double immunohistochemical staining and identify the labeled neurons [3,20,27].

Therefore, the objective of the present study was to first establish, by means of retrograde viral tracing and immunofluorescence, if hypothalamic neurons innervate adipose tissue depots in the pig and second, if these neurons express leptin receptor (OBR) immunoreactivity.

All experimental procedures were in accordance with the NIH "Guide for the Care and Use of Laboratory Animals" (Publication No. 86-23, revised 1985). Injections or PRV, and subsequent housing and handling of animals, took place in a Biosafety Level II facility (USDA, Athens, GA) in accordance with Health and Human Services Publication No. 88-8395, "Biosafety in Microbiological and Biomedical Laboratories".

Twenty-two crossbred gilts (50 ± 2 kg b.w.) were used in the study. The Pseudorabies virus (Bartha's K strain, 1.0×10^8 plaque forming units per milliliter) combined with green fluorescing peptide (GFP) was injected into perirenal ($n = 9$) or subcutaneous ($n = 9$) adipose tissue depots under pentobarbital anesthesia (Vetbutal, Biovet, 30 mg/kg b.w.). PRV was introduced in ten injections of 2 μl/injection, injection sites were evenly distributed in the right perirenal fat tissue depot using a Hamilton syringe by way of the paralumbar fossa. In subcutaneous fat, PRV was injected into 15 sites on the right side of the body through incisions in the skin exposing the subcutaneous fat tissue. Sites of injection consisted of three incision of five sites per incision. The first incision started at the iliac spine and ended at the caudal angle of the scapula; the second incision was from the ischial spine in parallel to the first incision and the third incision was from the side fold to the brachial joint. Special care was taken to avoid introducing the virus into surrounding tissues. In order to evaluate specificity of the tracing, four control pigs underwent the same surgical procedure as described above. In the control animals perirenal ($n = 2$) and subcutaneous ($n = 2$) adipose tissue depots were injected with 0.1 M phosphate-buffered saline (PBS; pH 7.4). The volume of injected virus and survival period were based on our previous studies [27]. Animals were sacrificed 9 days after injections and perfused transcardially with 4% paraformaldehyde in 0.1 M PBS. Brains were collected and hypothalami were dissected out after making the following cuts: rostral to the optic chiasm, rostral to the mammillary bodies, lateral to the hypothalamic sulci, and ventral to the anterior commissure. Tissue blocks were then postfixed in 4% paraformaldehyde in PBS for 2–3 h at 4 °C and transferred into 30% sucrose and 0.01% NaN_3 until they sank.

Every third section of 10 μm thick frozen serial sections were mounted on slides and the presence of GFP-PRV-infected neurons was examined with a Zeiss Axiophot fluorescence microscope. Every third section containing infected neurons was processed for double-immunofluorescence with primary antisera against PRV (Mouse # P01510, dilution 1:800, Institut Pourquier, France) and OBR (Goat # sc-1833, dilution 1:400, SC Biotechnology, USA). Anti PRV antibody enables the PRV causal agent of Aujeszky's disease to be identified with indirect immunofluorescence in cell culture and reacts in Western blot with the PRV glycoprotein, a major protein of this virus. The sensitivity of this antibody has been verified on different European and American PRV isolates. Specificity of the antibody demonstrated no cross-reactivity with bovine virus (B-RSV, P.I.3) and the herpes virus (BHV-1). The OBR antiserum was an affinity-purified goat polyclonal antiserum raised against the corresponding amino acids 877–894 mapping to the C terminus of the mouse Ob-R. This antiserum has been extensively tested and was found to bind to both the short and long isoform of OBR in transfected cells [23].

Sections were air-dried at room temperature for 30 min, rinsed three times for 5 min each with PBS, preincubated with a blocking mixture containing 0.25% Triton X-100, 1% bovine serum albumin, and 10% normal goat serum (NGS) in PBS for 1 h at room temperature, and incubated overnight at room temperature in a humid chamber with a mixture of primary antisera. Then, sections were incubated with biotinylated anti-mouse IgG for 1 h at room temperature, followed by a mixture of Texas Red-conjugated streptavidin and anti-goat FITC-conjugated IgG for 1 h at room temperature, and mounted with Vectashield (Vector, CA).

Consecutive sections to those that were double-stained were processed for immunohistochemistry with primary antibody against PRV (Rabbit # PA1-081, dilution 1:1000, Affinity BioReagents, USA). PA1-081 has been successfully used in Western blot and immunohistochemistry procedures [8]. With Western blot, this antibody detects proteins in the range of 80–115 kDa representing various PRV proteins. Briefly, sections were washed in cold PBS and treated for 15 min with PBS containing 0.5% sodium borohydride. Then, they were treated for 15 min with PBS containing 0.1% hydrogen peroxide. Next, sections were preincubated with a blocking solution of 5% NGS and 0.5% bovine serum albumin (BSA) in PBS. Primary antibody incubations were performed overnight in a PBS containing 1% NGS and 0.1% BSA at 4 °C. Detection of primary antibody was done by incubating the sections with peroxidase-labeled goat anti-rabbit IgG that had been pre-absorbed with rat serum (Kirkegaard and Perry Laboratory, MD, dilution 1:100) for 3 h at room temperature. Visualization of the peroxidase was done by incubating with 3,38-diaminobenzidine (DAB; Sigma) in 50 mM Tris HCl buffer (pH 7.4) including 0.9% NaCl and 0.005% hydrogen peroxide. The specificity of primary antisera was tested with pre-absorbed controls. In brief, 1 μM of the respective peptide completely abolished fluorescence and there was no immunostaining observed in the absence of primary antisera. Sections were examined and photographed with a Zeiss Axioplan 2 imaging photomicroscope equipped with a digital camera (Axio Cam) and filters for Texas Red and FITC, while the DAB chromagen product was visualized using the light microscopy. With the aid of a computer, the captured images were evaluated with the Axio Vision Imaging System (Carl Zeiss, Germany).