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Chronic overproduction of ghrelin in the hypothalamus leads to temporal increase in food intake and body weight

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ABSTRACT

Ghrelin is known to be a critical stimulator of feeding behavior mainly via actions in the hypothalamus. However, its functional contribution to the control of energy homeostasis under chronic elevated conditions is unknown. Here we show that overproduction of ghrelin via an AAV viral delivery system in the hypothalamus leads to an increase in food intake associated with increases in body weight. However, this increase in food intake is only temporary and is diminished and no longer significant after 3 weeks. Analysis of brain sections of mice 6 weeks after AAV–ghrelin virus injection demonstrates unaltered neuropeptide Y levels but strongly up-regulated pro-opiomelanocortin levels indicating that a compensatory mechanism has been activated to counter regulate the feeding stimulatory actions of ghrelin. This demonstrates that control mechanism exists that is activated under conditions of prolonged high ghrelin levels, which could potentially be utilized to control feeding and the development of obesity.

1. Introduction

Ghrelin, derived from the stomach, is the first known peripheral hormone that shows orexigenic effects through its action on hypothalamic appetite-regulating pathways (Nakazato et al., 2001). However, there are also reports that ghrelin is being produced by hypothalamic neurons themselves and the role of ghrelin produced by this source is much less understood. Ghrelin exists in two forms, the active (acyl-ghrelin) and the inactive (des-acyl ghrelin) isoforms. This modification of ghrelin with a medium length fatty acid occurs through the enzyme ghrelin O-acyltransferase (GOAT) that adds an O-n-octanoylated serine in position 3 to the pro-ghrelin peptide (Gutierrez et al., 2008). This posttranslational modification is essential for ghrelin to bind to its receptor, the growth hormone secretagogue receptor (GHS-R) type 1a. GHS-R is found in a variety of tissues in the periphery, but importantly is abundantly expressed in neurons of the hypothalamus (Papotti et al., 2000).

Ghrelin levels change depending on energy levels and when elevated stimulate neuropeptide Y (NPY)/Agouti-related peptide (AgRP) neurons in the arcuate nucleus of the hypothalamus (ARC) (Kohno et al., 2003). This subsequently induces the upregulation of NPY, which in turn stimulates appetite and inhibits energy expenditure by suppressing pro-opiomelanocortin (POMC) controlled neuronal pathways. There is also evidence that ghrelin signalling can reach the ARC via vagal afferents (Date et al., 2002) and regulate food intake via this pathway. Importantly, double knockout mice lacking both NPY and AgRP completely lack the orexigenic action of ghrelin, confirming that these two neuropeptides are essential for the orexigenic effect of ghrelin (Chen et al., 2004).

Ghrelin levels are dependent on energy status and altered ghrelin levels have been found in obese subjects (Geliebter et al., 2008) as well as in patients with anorexia nervosa (Otto et al., 2001). In addition to its prominent food stimulatory action, ghrelin is also known for its powerful effect on growth hormone (GH) release from somatotroph cells of the anterior pituitary (Korbonits et al., 2004); thereby significantly influencing the GH axis and insulin-like growth factor production. Ghrelin also appears to be a peripheral counterpart of insulin and leptin, contributing to the long-term regulation of energy homeostasis (Cummings, 2006) via peripheral mechanisms. For example, ghrelin reverses the down-regulating effect of insulin on phosphoenolpyruvate carboxykinase mRNA levels, the rate-limiting enzyme of gluconeogenesis (Murata et al., 2002). Furthermore, ghrelin has been shown to be able to reduce glucose-stimulated insulin secretion in humans and rodents (Dezaki et al., 2004; Tong et al., 2010) that can lead to increases in blood glucose levels and impairs glucose tolerance. Ghrelin is also known to reduce the use of fat as fuel source and promotes an increase in adipose tissue and body weight (Tschop et al., 2000). Increases in ghrelin can induce abdominal obesity, independently of its central orexigenic activity, via GHS-R-dependent lipid retention (Davies et al., 2009).

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To study the functional role of ghrelin several transgenic and knockout models have been generated (De Smet et al., 2006; Sun et al., 2008). Acute studies with central injection of ghrelin peptide have also been used to investigate the downstream mechanisms that are controlled by ghrelin action. However, little is known about the chronic elevation of ghrelin levels and signalling in the hypothalamus. We therefore generated an adeno-associated virus (AAV)-construct that allows for the simultaneous generation of ghrelin as well as GOAT, which is essential to convert ghrelin into the active form. The effect of chronic ghrelin overproduction has then been analysed in mice.

2. Materials and methods

2.1. Animals

Research procedures and animal care were approved by Garvan Institute/St. Vincent's Hospital Animal Ethics Committee, which were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Mice were housed under conditions of controlled temperature (22 ± 1 °C as room temperature) and illumination (12-h light–dark cycle, lights on at 07:00 am and off at 07:00 pm). Normal chow diet (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate, and 2.6 kcal/g; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia) was used to feed these experimental mice *ad libitum*. No water was restricted in the process of this study. The age-matched male mice on a mixed genetic background (C57/B16-129SvJ) were randomly separated into the AAV–ghrelin injected group and the AAV–GFP (green fluorescent protein) injected group in this study. To identify the potential differences in the experimental animals, the body weight of the mice with overexpressed hypothalamic ghrelin and the controls was intensively monitored before and after the operation, followed by a weekly examination for 6 weeks.

2.2. AAV mediated acyl ghrelin expression in the hypothalamus

The anaesthetized mouse was placed on a stereotaxic frame with ear bars (David Kopf Instruments, Tujunga, CA, USA). A 0.5 cm skin along the midline was opened rostrally to the lambda. A 2 µl Hamilton MicroliterTM injection syringe attached to Micro4 Micro Syringe Injection Unit (World Precision Instruments Inc., Sarasota) was adjusted over the desired injection site. A 25 G stainless needle was used to drill a hole on the skull to expose the brain and the injection syringe was then lowered to the injection site in the hypothalamus. The coordinates used for injection were recalculated by the size of the mouse brain according to the maps in the Mouse Brain in Stereotaxic Coordinates (2.0 mm rostrally to interaural line, 0.3 mm off the midline and 5.5 mm deep from the surface of the brain). A total of 0.5 µl AAV–ghrelin/AAV–GFP in saline was injected over 10 min into the targeting hypothalamic area unilaterally. The syringe was left in place for another 10 min after completion of the injection to avoid reflux along the needle and then slowly taken out. One suture was used to close the wound. The animal was kept on heating pad until waking up and returned to a single cage.

2.3. Feeding study

To minimize the effect of new housing environment to food intake, mice were acclimatized individually in respective cages since the surgery day. Accumulated spontaneous 24-hour food intake, water consumption and faeces were monitored over four consecutive weeks, starting one week after the surgery, which allows enough time for the experimental mice to recover. The energy intake was calculated by converting consumed food weight into calorie intake and normalized with body weight of individuals.

2.4. Tissue collection

Mice were culled by cervical dislocation followed by decapitation. The time between the first picking and the decapitation was less than 60 seconds to remain stressed at basal levels. Serum examples were collected. Fresh brains were removed from the skull and frozen on an aluminium plate on dry ice, then stored at –80 °C. The interscapular brown adipose tissue (BAT) and white adipose tissue (WAT), including inguinal white adipose tissue (WATi), epididymal white adipose tissue (WATe), retroperitoneal white adipose tissue (WATr) and mesenteric white adipose tissue (WATm), were collected and weighed. The organs including gonads, spleen, pancreas, kidney, liver and heart, as well as bones were also collected and stored at –80 °C for further analysis. The weight data were normalized as percentage of body weight.

2.5. Immunohistochemistry

In order to verify the expression of GOAT in the hypothalamus, mice were anaesthetized and perfused with 0.9% saline and 4% paraformaldehyde, followed by post-treatment in 4% PFA for 2 hours and 30% sucrose overnight. Brains were then sectioned at 35 µm interval and stored in cryoprotectant at –20 °C until use. Brain regions were identified according to the maps in the Mouse Brain in Stereotaxic Coordinates and the immunohistochemistry was implemented following the protocol described in Supporting Information Experimental Procedures. The area producing GOAT was determined by rabbit anti-GOAT antiserum (1:500; Catalog No.: H-032-12, Phoenix Pharmaceuticals, Inc. Burlingame, CA94010, USA) and examined with fluorescent microscope (Leica Microsystems Pty Ltd, North Ryde, Australia). Images were digitally captured with the attached camera.

2.6. In situ *hybridization and densitometry*

Fresh frozen brains were sectioned at 30 µm intervals and thaw-mounted on Superfrost Plus® glass microscope slides (Lomb Scientific Pty Ltd., NSW 2229, Australia). For *in situ* hybridization, the method has been described in Sainsbury et al. (2002). Briefly, matching sections of deletion and control mice through the ARC were assayed together with candidate mRNAs (listed below), which were labelled with [^{35}S] thio-dATP (Amersham Pharmacia or NEN) using terminal deoxynucleotidyltransferase (Roche, Mannheim, Germany). Silver grain densities of labelled mRNAs were analysed and compared by ImageJ image processing program.

DNA oligonucleotides include the ones complementary to the mRNAs of mouse ghrelin oligonucleotides (5′-ATGCTGCTGATACTGAG CTCCTGACAGCTTGATGCCAACATCG-3′); mouse NPY (5′-GAGGGT CAGTCCACACAGCCCCATTCGCTTGTTACCTAGCAT-3′); and mouse POMC (5′-TGGCTGCTCTCCAGGCACCAGCTCCACACATCTATGGAGG-3′).

2.7. Statistical analyses

All data are presented as means ± SEM. Difference between AAV-injected mice and the controls was analysed by t-test and two-way ANOVA. Statistical analyses were performed with SPSS for Mac OS X version 16.0.1 (SPSS Inc, Chicago, IL, USA). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Ghrelin viral construction and injection

In order to investigate the effects of long term elevated ghrelin levels in the hypothalamus on food intake and body weight we generated a viral vector that allows for the production of the active form