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The role of leptin and ghrelin in appetite regulation in the Australian Spinifex hopping mouse, *Notomys alexis*, during long-term water deprivation

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ABSTRACT

Water deprivation of the Spinifex hopping mouse, *Notomys alexis*, induced a biphasic pattern of food intake with an initial hypophagia that was followed by an increased, and then sustained food intake. The mice lost approximately 20% of their body mass and there was a loss of white adipose tissue. Stomach ghrelin mRNA was significantly higher at day 2 of water deprivation but then returned to the same levels as water-replete (day 0) mice for the duration of the experiment. Plasma ghrelin was unaffected by water deprivation except at day 10 where it was significantly increased. Plasma leptin levels decreased at day 2 and day 5 of water deprivation, and then increased significantly by the end of the water deprivation period. Water deprivation caused a significant decrease in skeletal muscle leptin mRNA expression at days 2 and 5, but then it returned to day 0 levels by day 29. In the hypothalamus, water deprivation caused a significant up-regulation in both ghrelin and neuropeptide Y mRNA expression, respectively. In contrast, hypothalamic GHSR1a mRNA expression was significantly down-regulated. A significant increase in LepRb mRNA expression was observed at days 17 and 29 of water deprivation. This study demonstrated that the sustained food intake in *N. alexis* during water deprivation was uncoupled from peripheral appetite-regulating signals, and that the hypothalamus appears to play an important role in regulating food intake; this may contribute to the maintenance of fluid balance in the absence of free water.

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1. Introduction

Most mammals have access to free water and compensate for the water lost by evaporation and excretion by drinking free water. However, many mammals live in deserts where free drinking water is absent and survive by gaining preformed and/or metabolic water from food and employing a suite of physiological and behavioural adaptations to reduce water loss (see detailed reviews in Degen, 1997; Schmidt-Nielsen, 1965; Schwimmer and Haim, 2009; Donald and Pannabecker, 2015). Small desert rodents and marsupials in particular are renowned for their ability to produce a small volume of concentrated urine in the range of 6000–9000 mOsm kg^{-1} (see Bozinovic and Gallardo, 2006; Degen, 1997; Gordge and Roberts, 2008; Schmidt-Nielsen, 1965; Donald and Pannabecker, 2015). The ability to achieve such a high urinary concentration is due to a renal architecture that maximises water reabsorption in the kidney, and a low glomerular filtration rate (Donald and Pannabecker, 2015). In addition, the gastrointestinal system of desert mammals has anatomical specialisations that increase the intestinal surface area to enhance the reabsorption of water and nutrients (Degen, 1997; Donald and Pannabecker, 2015).

In addition to reducing water loss, desert mammals also have strategies to gain water by maximising consumption of preformed water from food and the generation of metabolic water from the oxidative metabolism of food substrates (Degen, 1997; Walsberg, 2000; Donald and Pannabecker, 2015). However, given the importance of food as a direct and indirect source of water, little is known about how desert mammals can regulate appetite to increase consumption of preformed water and substrate provision for metabolic water production. Two important peripheral hormones in the control of appetite are leptin and ghrelin. In laboratory mammals, leptin functions as a satiety signal to reduce food

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intake in response to increased adiposity caused by a positive energy balance, and the concentration of plasma leptin has a strong positive correlation with body and adipose tissue mass (Allison and Myers, 2014; Chi and Wang, 2011; Park and Ahima, 2015). However, the anorexigenic action of leptin is not always observed in free-living animals confronted with changing environments where fattening is adaptive prior to a seasonal food shortage or hibernation, and plasma leptin concentration is uncoupled from white adipose tissue mass (see e.g. Kronfeld-Schor et al., 2000; Schradin et al., 2014). Ghrelin is primarily synthesised in the stomach and, in contrast to leptin, generally promotes food intake and increased body mass in both laboratory and free-living animals (Cabral et al., 2015; Florant and Healy, 2012; Mason et al., 2014; Schradin et al., 2014; Schellekens et al., 2015).

In mammals, both leptin and ghrelin act on the arcuate nucleus (ARC) of the hypothalamus via leptin receptor b (LepRb; Park and Ahima, 2015; Schneeberger et al., 2014) and growth hormone secretagogue receptor type 1a (GHSR1a; Lin et al., 2014; Mason et al., 2014), respectively. The ARC neurons contain either orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) or anorectic proopiomelanocortin (POMC)/cocaine and amphetamine-regulated transcript (CART; Schneeberger et al., 2014; Sobrino Crespo et al., 2014). LepRb is highly expressed on ARC neurons, and leptin signalling activates the POMC/CART neurons and inhibits the AgRP/NPY neurons, respectively, to suppress appetite drive (Schneeberger et al., 2014; Sobrino Crespo et al., 2014). GHSR1a is also expressed on ARC neurons, but in contrast to leptin, ghrelin signalling inhibits the POMC/CART neurons and activates the AgRP/NPY neurons, respectively, to promote appetite drive (Mason et al., 2014; Schneeberger et al., 2014; Sobrino Crespo et al., 2014).

We recently investigated food intake and the appetite control system during free water deprivation in the Spinifex hopping mouse, *Notomys alexis*, to determine if they are modulated in response to water restriction (Takei et al., 2012). During a 30 day water deprivation period, there was a biphasic pattern of food consumption in which an initial hypophagia was followed by a sustained food intake through the latter phase of water deprivation, which was above that of water-replete animals (Takei et al., 2012). In a subsequent experiment, the peripheral and central regulation of food intake was examined in the first twelve days of water deprivation during the cyclical pattern of food consumption. In brief, plasma leptin declined in parallel with the catabolism of body fat, plasma ghrelin was significantly increased, and the mRNA expression of the orexigenic and anorectic appetite regulating peptides in the hypothalamus was up- and down-regulated, respectively, in accordance with the accepted paradigm for appetite stimulation to drive food intake (Takei et al., 2012). The aim of the present study is to extend the previous research and examine the peripheral and central regulation of appetite during water deprivation of *N. alexis*, with emphasis on leptin and ghrelin during the sustained food intake that occurs after the initial hypophagia described by Takei et al. (2012).

2. Materials and methods

2.1. Animals and tissue sampling

Spinifex hopping mice (body mass 25–40 g), *Notomys alexis*, were obtained from a breeding colony at Deakin University. Under normal, water-replete conditions, the animals were housed in standard rat boxes containing straw, sawdust and paper towel for bedding, in a temperature controlled room (21 °C) with a 12:12 h light–dark cycle (lights on at 08:00). The animals received water *ad libitum*, and were fed millet seed, mixed grain, fruit and vegetables. All experimental procedures were performed according to the guidelines of the Deakin University Animal Ethics Committee. Animals were allocated into six groups (n = 7 animals per group) with access to water and millet seed for at least one week prior to the commencement of the experiment. The control group was water-replete animals that were sampled at day 0. Water was removed from the remaining 5 groups at day 0, which were provided with free access to millet seed only during the water deprivation period of 29 days. Animal mass and food consumption were recorded daily for the duration of the experiment. The hopping mice were humanely killed with isoflurane anaesthesia, and the brain, stomach, and skeletal muscle (*vastus lateralis*) were sampled at 5 time points (days 2, 5, 10, 17 and 29), respectively, and frozen in liquid nitrogen and stored at −80 °C until used. Blood samples were collected into microcentrifuge tubes containing aprotinin (500 kIU/mL) and disodium EDTA (1.25 mg/mL), and stored at 4 °C. To isolate the plasma, tubes were gently rocked and centrifuged at 1500×*g* for 15 min at 4 °C, and the plasma was transferred into new tubes and frozen at −80 °C until used. The plasma samples that were used for the ghrelin ELISA were treated with 1 M HCl at 10% of the plasma volume to maintain stability of the active (*n*-octoylation) form of ghrelin (Hosoda et al., 2000). Glucose concentration of plasma samples was determined using Accu-Chek Performa glucometer (Roche Diagnostics Aust. Pty. Ltd., Australia) and performed according to the manufacturer's protocol. The glucometer response time is approximately 5 s and the range of measurement is between 0.6 and 33 mM/L.

The body fat content of the carcass of each mouse was determined at days 2, 17 and 29 of water deprivation by dual energy X-ray absorptiometry (DEXA) using a Norlane Stratec DEXA Sabre XL (Stratec Medizintechnik GmbH) forearm densitometer.

2.2. Isolation of protein from skeletal muscle

Frozen skeletal muscle tissue was mechanically disrupted using a high-speed bench top homogenizer (MP Biomedical, CA, USA) in 1 mL of RIPA buffer (Sigma) containing 100 μL of protease inhibitor (Sigma). The suspension was centrifuged for 15 min at 10,000 rpm at 4 °C (Beckman Allegra 21R) to separate the lipid and protein components. The middle aqueous phase was transferred into a new tube, and was stored at −20 °C for further analysis. The protein concentration was determined using a bicinchoninic acid protein assay (Thermo Scientific Pierce) according to the manufacturer's protocol.

2.3. ELISA for ghrelin and leptin

Plasma ghrelin and leptin were measured using specific ELISA kits for each peptide. A rat/mouse ghrelin ELISA kit (LINCO Research, Missouri, USA) was used to determine plasma ghrelin levels by adopting a sandwich ELISA technique, according to the manufacturer's protocol. Each sample was tested in triplicate using a 96-well microplate, and the absorbance was read at 450 and 590 nm on a SpectraMax 340 PC384 plate reader. Leptin peptide levels in plasma and skeletal muscle were measured using the mouse leptin development kit (PromoKine). The leptin ELISA was performed in triplicate using a 1:10 dilution of non-treated plasma in a 96-well microplate as described by the manufacturer's protocol with the following modifications; 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma) was used instead of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), as recommended by the manufacturer for colour development. The reaction was inhibited using TMB stop buffer (Sigma) and was read at 450 nm using a SpectraMax 340PC384 microplate reader.

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