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Short communication

Glucokinase inhibitor glucosamine stimulates feeding and activates hypothalamic neuropeptide Y and orexin neurons

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\textbf{A B S T R A C T}

Maintaining glucose levels within the appropriate physiological range is necessary for survival. The identification of specific neuronal populations, within discreet brain regions, sensitive to changes in glucose concentration has led to the hypothesis of a central glucose-sensing system capable of directly modulating feeding behaviour. Glucokinase (GK) has been identified as a glucose-sensor responsible for detecting such changes both within the brain and the periphery. We previously reported that antagonism of centrally expressed GK by administration of glucosamine (GSN) was sufficient to induce protective glucoprivic feeding in rats. Here we examine a neurochemical mechanism underlying this effect and report that GSN stimulated food intake is highly correlated with the induction of the neuronal activation marker cFOS within two nuclei with a demonstrated role in central glucose sensing and appetite, the arcuate nucleus of the hypothalamus (ARC) and lateral hypothalamic area (LHA). Furthermore, GSN stimulated cFOS within the ARC was observed in orexigenic neurons expressing the endogenous melanocortin receptor antagonist agouti-related peptide (AgRP) and neuropeptide Y (NPY), but not those expressing the anorectic endogenous melanocortin receptor agonist alpha-melanocyte stimulating hormone (α-MSH). In the LHA, GSN stimulated cFOS was found within arousal and feeding associated orexin/hypocretin (ORX) expressing neurons. Our data suggest that GK within these specific feeding and arousal related populations of AgRP/NPY and ORX neurons may play a modulatory role in the sensing of and appetitive response to hypoglycaemia.

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The detection and maintenance of physiologically appropriate levels of glucose is paramount to mammalian viability. Multiple physiological systems, therefore, operate to detect fluctuations in glucose levels and to prompt the induction of apposite counter-regulatory responses, including ingestive behaviour. Glucose, as the primary fuel source in mammals, is a key indicator of nutritional state, with low or falling blood glucose levels triggering defensive physiological responses and hunger. However, for glucose to be able to influence feeding behaviour, whether as an emergency response to glucoprivation or within a more normal appetitive context, the brain must be able to accurately and rapidly detect oscillations in interstitial glucose levels. In this regard, the hypothalamus has been identified as a key component of the centrally regulated energy homeostasis network.

Within the basomedial hypothalamus, the melanocortin system in the arcuate nucleus (ARC) plays a critical role in energy balance; the orexigenic endogenous melanocortin receptor antagonist agouti-related peptide (AgRP) is co-expressed with orexigenic neuropeptide Y (NPY), while the anorectic endogenous melanocortin receptor agonist alpha-melanocyte stimulating hormone (α-MSH) is co-expressed with anorectic cocaine and amphetamine regulated transcript (CART)\cite{1}. These basomedial populations may form part of an integrated homeostatic network with neurons of the lateral hypothalamic area (LHA) that express orexin/hypocretin (ORX) or melanin concentrating hormone (MCH). In controlling energy balance, these neurons respond to a complex series of nutritional cues, including those communicated by peripherally derived circulating factors or relayed by visceral nerves. In addition, some neurons are able to influence energy homeostasis in response to...
directly sensed changes in the levels of specific brain substrates within their local environment [2,3]. In this regard, AgRP/NPY, α-MSH, ORX and MCH neurons sense changes in extracellular glucose concentration [4–8]. Such neurons can exhibit excitatory (glucose-excited, GE) or inhibitory (glucose-inhibited, GI) firing responses to rising glucose levels [3]. Indeed, approximately 40% of AgRP/NPY expressing neurons in the ARC and 90% of ORX neurons in the LHA demonstrate hyperpolarisation on elevation of extracellular glucose concentration [4,5]. Thus, activation of these neurons under hypoglycaemic conditions is thought to induce counter-regulatory responses, including arousal and hunger symptoms aimed at stimulating a protective feeding reaction.

Although the mechanistic underpinnings of neuronal glucose sensing remain poorly understood, it has been hypothesised that these neurons may employ a similar mechanism of detection to that seen peripherally. The low affinity hexokinase, glucokinase (GK), catalyses the phosphorylation of glucose to glucose-6-phosphate, but in pancreatic β-cells can also function as a glucose-sensor [9]. More recently, GK has also been identified within the brain and specifically in canonical glucose-sensitive nuclei, including the ARC, ventromedial nucleus and dorsomedial nucleus of the hypothalamus [10–12]. In the ARC, GK has been identified in AgRP/NPY and α-MSH/CART neurons [13]. Functional studies in hypothalamic neurons have shown that inhibition of GK function suppresses activity and/or blocks the ability of glucose to stimulate GE neurons and suppress GI neurons [14,15]. Indeed, recent species studies have demonstrated the importance of hypothalamic GK in the mediation of counter-regulatory responses to insulin-induced hypoglycaemia [16]. These data support the notion that GK represents a central ‘glucostat’ capable of regulating neuronal function, and downstream protective physiological responses.

Consistent with this, we recently reported that intracerebroventricular (i.c.v.) infusion of GK inhibitors such as glucosamine (GSN) [17] resulted in a rapid stimulation of protective feeding in rats [18]. Here we examine the underlying mechanism of this effect by assessing neuronal activation in chemically defined neurons induced by central GSN administration under normoglycaemic conditions.

Male Sprague–Dawley rats (Charles River) weighing 280–300 g were individually housed with ad libitum access to water and standard laboratory chow (Eurodent Diet, PMI Nutrition International). Animals were maintained in a light (12 h on/12 h off) and temperature controlled environment (21.5–22.5 °C). All procedures used were in accordance with the guidelines for the care and use of animals established by the UK Animals (Scientific Procedures) Act 1986.

Rats were anesthetized by intraperitoneal (i.p.) administration of ketamine (100 mg/kg, National Veterinary Supplies) and xylazine (20 mg/kg, National Veterinary Supplies) and a single-guide cannula (Plastics One, VA) was inserted into the third ventricle (coordinates from bregma anteroposterior – 2.2 mm, lateral 0.9 mm, dorsoventral 8.4 mm) and cemented in place with anchoring screws, as described previously [18]. Five to eight days post surgery, ad libitum fed animals received either aECF (n = 7, CMA Microdialysis AB distributed by Linton Instrumentation) or recombinant glucosamine (GSN; CMS Chemicals 15 or 150 nmol/min, n = 4 and 6, respectively) via the indwelling cannula for 60 min, starting at mid light cycle at an infusion rate of 0.3 μl/min, with a priming dose of 0.9 μl/min over the first 3 min. The GSN doses used were characterized in an earlier report [18]. Food intake was measured by weighing chow pellets two hours after the termination of aECF or GSN infusion. Animals were then anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.), and transcardially perfused with diethylpyrocarbonate (DEPC; Sigma)-treated 0.9% saline followed by phosphate-buffered 10% formalin, pH 7.0 (Sigma). Brains were removed, post-fixed in the same fixative for 4 h and then submerged overnight in 30% sucrose in DEPC-treated phosphate-buffered saline (DEPC-PBS). Brains were cut on a freezing microtome at 25 μm (1:6 series) and stored in an antifreeze solution containing 30% ethylene glycol and 20% glycerol in DEPC-PBS at –20 °C.

For quantitative assessment of neuronal activation, sectioned tissue was processed for immunohistochemical detection of cFOS immunoreactivity (FOS-IR). Each step listed below was preceded by PBS rinses for 15 min. Sections were pre-treated in 0.3% H2O2 (Sigma) for 1 h, blocked in 0.3% normal donkey serum (Sigma) in PBT (0.04% Triton X-100 (Sigma) in PBS) and then incubated with rabbit anti-FOS antibody (Calbiochem; 1:10,000) in 0.3% normal donkey serum and PBT-azide (0.02% sodium azide (Sigma) in PBT) overnight at room temperature. Sections were then incubated for 1 h with biotinylated donkey anti-rabbit serum (Jackson Laboratories; 1:500) in 0.3% normal donkey serum and PBT and then with avidin–biotin complex (ABC; Vector Elite kit; Vector laboratories; 1:250) in PBS for 1 h. The immunoperoxidase was developed in 0.04% 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.003% hydrogen peroxide in PBS. Sections were mounted onto polystyrene slides, air-dried for 30 min, counter-stained in cresyl violet (Sigma) for 1 min and dehydrated in an ascending ethanol series, before being cleared in xylene (VWR International) and coverslipped with mounting media (Micromount, Surgipath).

Subsequent chemical identification of FOS-IR cells was achieved by dual-labelled immunofluorescence analysis. Sections were treated as described above and then incubated with goat anti-cFOS antibody (Santa Cruz, 1:1,000) and either sheep anti-α-MSH serum (Chemicon; 1:10,000), rabbit anti-ORX serum (Phoenix Pharmaceuticals; 1:10,000), or rabbit anti-MCH serum (Phoenix Pharmaceuticals; 1:10,000) in PBT overnight at room temperature. Following this, tissue was incubated with biotinylated donkey anti-goat antibody (Jackson Laboratories; 1:500) for 1 h, followed by incubation with Alexa Fluor-488 conjugated streptavidin (Molecular Probes; 1:1000) and Alexa Fluor-594 conjugated donkey anti-rabbit or anti-sheep (Molecular Probes; 1:1000) for 1 h. After mounting on polystyrene slides, the sections were coverslipped with anti-fade mounting medium for fluorescence (Vectashield, Vector).

To investigate the colocalisation of cFOS and NPY, a modified method combining immunofluorescence and fluorescent in situ hybridization histochemistry (FISH) was used [19]. Tissue was processed first for FOS-IR as described above using RNase-free methods. Following this, sections were rinsed thoroughly in PBS, equilibrated in 5× sodium saline citrate (SSC) for 30 min and transferred to hybridization buffer (HB) [19] for 2 h at 56 °C. A digoxigenin-labelled riboprobe (DIG-NPY) was generated from cDNA template specific to the rat NPY sequence by in vitro transcription with T7 polymerase, as previously described [20]. The DIG-labelled riboprobe (500 ng) was heated to 90 °C in 100 μl HB solution for 10 min, placed on ice for 5 min, and added to the tissue/HB mix and incubated for 12 to 16 hrs at 56 °C. Sections were then rinsed with 2× SSC and incubated with RNase A (Boehringer–Mannheim) in 0.5 M NaCl, 10 mM Tris–HCl, pH 8.0 and 0.5 M EDTA for 6 min at 37 °C. The sections were washed in 2× SSC for 1 h at 65 °C, and in 0.2× SSC for 1 h at 65 °C. After a brief equilibration in a solution of 0.1 M Tris–HCl, 0.1 M NaCl and 50 mM MgCl2, pH 7.5 (GB1) at room temperature, the sections were transferred to blocking solution, containing 0.1 M Tris–HCl, 0.15 M NaCl and 0.5% blocking regent (PerkinElmer). Immunological detection of the DIG-NPY probe was achieved by incubating the sections in GB1 solution containing sheep anti-DIG antibody (Roche, 1:100) at room temperature overnight. The next day, following a rinse in GB1 and equilibration in 0.1 M Tris–HCl, 0.15% NaCl and 0.05% Tween 20 (TNT), DIG-NPY was visualized by Cy3 fluorophore tyramide (PerkinElmer, 1:50) for 3–10 min. Sections were briefly washed in...
GSK significantly activates ARC NPY and LHA ORX neurons, but not ARC α-MSH or LHA MCH neurons. (a) In the ARC, GSK (150 nmol/min, i.c.v.) induced FOS-IR in less than 1% of ARC α-MSH-IR neurons, but induced FOS-IR in approximately one-third of NPY neurons. (b) In the LHA, GSK did not increase FOS-IR in MCH-IR neurons, but produced a significant increase in FOS-IR in ORX neurons. **p ≤ 0.01, ***p ≤ 0.001.
Fig. 3. Colocalization of FOS-IR with ARC NPY mRNA and α-MSH-IR neurons and with LHA ORX-IR and MCH-IR neurons in rats treated with GSN (150 nmol/min, i.c.v.). (a–d) are merged micrographs showing representative regions of FOS-IR and neuropeptide co-expression. (a1–a3, b1–b3, c1–c3, and d1–d3) are higher-power magnification of boxes area in a–d, respectively, with a1, b1, c1, and d1 illustrating FOS-IR positive cells (green); a2 illustrating NPY mRNA, b2 illustrating α-MSH-IR, c2 illustrating ORX-IR and d2 illustrating MCH-IR (red); and a3, b3, c3, and d3 illustrating merged photographs. Arrows indicate colocalization. Scalebar in a, 75 μm, also applies to b; scalebar in c, 100 μm, also applies to d; scalebar in d3, 25 μm, applies to all other images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2011.03.043.

References