Glucose regulates AMP-activated protein kinase activity and gene expression in clonal, hypothalamic neurons expressing proopiomelanocortin: additive effects of leptin or insulin

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Abstract

The mammalian hypothalamus comprises an array of phenotypically distinct cell types that interpret peripheral signals of energy status and, in turn, elicits an appropriate response to maintain energy homeostasis. We used a clonal representative hypothalamic cell model expressing proopiomelanocortin (POMC; N-43/5) to study changes in AMP-activated protein kinase (AMPK) activity and glucose responsiveness. We have demonstrated the presence of cellular machinery responsible for glucose sensing in the cell line, including glucokinase, glucose transporters, and appropriate ion channels. ATP-sensitive potassium channels were functional and responded to glucose. The N-43/5 POMC neurons may therefore be an appropriate cell model to study glucose-sensing mechanisms in the hypothalamus. In N-43/5

POMC neurons, increasing glucose concentrations decreased phospho-AMPK activity. As a relevant downstream effect, we found that POMC transcription increased with 2·8 and 16·7 mM glucose. Upon addition of leptin, with either no glucose or with 5 mM glucose, we found that leptin decreased AMPK activity in N-43/5 POMC neurons, but had no significant effect at 25 mM glucose, whereas insulin decreased AMPK activity at only 5 mM glucose. These results demonstrate that individual hypothalamic neuronal cell types, such as the POMC neuron, can have distinct responses to peripheral signals that relay energy status to the brain, and will therefore be activated uniquely to control neuroendocrine function.

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Introduction

The hypothalamus is critical for the regulation of homeostatic processes, such as feeding and energy expenditure (Schwartz *et al.* 2000). Within the hypothalamus, energy homeostasis is controlled by two opposing neuronal systems, through orexigenic or anorexigenic mechanisms. These neurons are modulated by circulating hormones, nutrient-derived signals, and cytokines. Specific neurons from the hypothalamus can sense nutrient-derived signals like glucose, and respond appropriately by changing the levels of neuromodulators in the brain. Two important peripheral signals are leptin, the product of the obesity (Ob) gene, which is secreted mainly from adipocytes, and insulin, produced mainly in the pancreas, which also signals energy status to the hypothalamus (Zhang *et al.* 1994, Niswender & Schwartz 2003). Leptin and insulin modulate neurons within the arcuate nucleus and are thought to act through their specific receptors on melanocortin neurons (Niswender & Schwartz 2003, Breen *et al.* 2005). The two main opposing neurons in the melanocortin system express either the orexigenic hormones, NPY and

AgRP, or the anorexigenic hormones, proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART). These neuropeptides are ultimately modulated by peripheral signals that lead to changes in signaling cascades and alteration of intracellular second messengers. These cellular changes affect gene expression and neuropeptide secretion, which in turn affects feeding behavior.

Glucose is a critical fuel necessary for the survival of brain cells, which may be why the brain itself evolved a mechanism to sense and respond to peripheral glucose changes (Burdakov *et al.* 2005). It is likely that the brain integrates information both locally, through neuronal glucose-sensing machinery, and from peripheral sensors. The knowledge that neurons respond to changes in glucose was generated from exquisite electrophysiological experiments, which indicated that neurons can be either inhibited or excited by extracellular glucose changes (Anand *et al.* 1964, Oomura *et al.* 1964, Routh 2002, Wang *et al.* 2004). The hypothalamus is a recognized brain center involved in the central control of glucose homeostasis, with the lateral, arcuate, and ventromedial

hypothalamic regions linked to glucose sensing (Levin *et al.* 2004). However, how individual hypothalamic NPY or POMC neurons sense glucose levels and respond with unique neuronal signals is not yet understood due to the lack of appropriate cell models.

One important cellular energy gauge is 5'-AMP-activated protein kinase (AMPK; Carling 2005, Kahn *et al.* 2005). In general, AMPK is activated in response to low levels of ATP that results in an increase in the AMP:ATP ratio. Once activated, AMPK turns on catabolic pathways to generate ATP and turns off biosynthetic pathways requiring ATP consumption (Hardie 2004). In peripheral tissues, AMPK controls a number of metabolic processes, including glucose and lipid metabolism (Kahn *et al.* 2005). The activity of AMPK is acutely regulated in the hypothalamus by peripheral nutrient signals, and may therefore act as a central regulator of energy balance (Kim & Lee 2005). AMPK activity is regulated in certain regions of the hypothalamus by indicators of nutrient status, including fasting/refeeding, leptin, insulin, glucose, fatty acids, and ghrelin (Andersson *et al.* 2004, Kim *et al.* 2004, Minokoshi *et al.* 2004). AMPK activity has also been shown to be modulated by ciliary neurotrophic factor (Steinberg *et al.* 2006), or the anti-diabetic drug, metformin (Chau-Van *et al.* 2006), both linked to weight loss. However, the exact mechanisms of AMPK action are not well defined in the hypothalamus, and it is not yet known what specific neuropeptides linked to the control of energy homeostasis are involved in this process.

The hypothalamus is a heterogeneous population of cell types, each expressing a specific complement of peptides, receptors, and neuromodulators. Since the glucose sensing and AMPK studies to date have analyzed the entire or specific regions of the hypothalamus, it is difficult to determine exactly which cell types directly respond to peripheral signals to change AMPK activity. Since AMPK activation results in the modulation of downstream events, such as changes in neuropeptide gene expression and/or secretion ultimately affecting overall energy homeostasis, it is necessary to define the cell types involved in this process. We generated a number of clonal, immortalized hypothalamic cell lines that express neuropeptides associated with energy homeostasis. These cell models were generated by retroviral transfer of T-antigen into embryonic mouse primary hypothalamic cell cultures, with subsequent subcloning to ensure pure clonal cell lines (Belsham *et al.* 2004). We used these cell lines to determine the effects of peripheral signals on gene expression and secretion (Belsham *et al.* 2004, Cui *et al.* 2005, Titolo *et al.* 2006). Using one of these cell models expressing POMC, we demonstrate the expression of functional glucose-sensing machinery, that these cells specifically respond to glucose, and that glucose itself can regulate neuropeptide gene expression. We were also able to define regulation of AMPK activity by specific nutrient signals, including glucose, leptin, and insulin.

Materials and Methods

Cell immortalization and subcloning

Immortalized cell lines were generated as described (Belsham *et al.* 2004, Cui *et al.* 2005). Briefly, hypothalami were harvested and dissected from mice at embryonic day 15 (E15), E17, and E18. The primary cell cultures were infected with retrovirus containing the intact cDNA sequence for simian virus (SV40) large T-antigen and neomycin resistance gene, harvested from a confluent culture of ψ 2 cells (psitex cells) producing a replication-defective, recombinant murine retrovirus. After 48 h in culture medium with the retrovirus, the cells were incubated with medium containing Geneticin (G418) with a selective concentration (400–600 μ g/ml for initial selection; 250 μ g/ml for cell maintenance). Mixed populations of hypothalamic cells were further subcloned by successive dilution of the trypsinized cell populations into 96-well plates. Cell colonies were allowed to grow and then successively split into 24-well plates, then into 6-well plates, and finally into 60 mm plates. Each cell line was purified three to four times. The N-43 cell line was subsequently purified after the initial report, in order to be confident of clonality. In this study, N-43/5 (N-43, clone 5) was used because it expressed detectable levels of POMC (Fig. 1A).

Cell culture and treatments

Immortalized cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen), 20 mM glucose, and penicillin/streptomycin and maintained at 37 °C with 5% CO₂. N-43/5 cells were grown overnight to 80–90% confluency in 60 mm dishes. Cells were starved for 2 h in Krebs–Ringer bicarbonate HEPES (KRBH) buffer, a minimal buffer containing no glucose, before treatments, and then replaced with 2.8 or 16.7 mM glucose added to the KRBH. Cellular RNA was harvested at 1-, 2-, 4-, 6-, and 8-h time points. At each time point, RNA from a time-matched control plate with no treatment was also harvested. For the initial AMPK studies, the medium was replaced with KRBH buffer without glucose for 1 h before treatment. KRBH buffer was used during treatments with 0, 5, 10, 15, or 25 mM glucose individually for 30 min. Cells were then treated with the indicated concentrations of leptin (R&D Systems, Minneapolis, MN, USA) or insulin (Novo Nordisk Canada, Inc., Mississauga, Ontario, Canada; DIN 02024233), in combination with 0, 5, or 25 mM glucose for 30 min before harvesting protein to assay AMPK activity.

Reverse transcriptase PCR (RT-PCR) for screening

Total cellular RNA was isolated by Trizol reagent, based on the guanidinium thiocyanate–phenol–chloroform extraction method, following the manufacturer's instructions

Figure 1 N-43/5 POMC phenotypic profiles. (A) Phase contrast micrograph of immortalized mouse N-43/5 cells. (B) RT-PCR and western blotting analysis (calcium channels) confirms that glucose-sensing machinery was expressed in a number of clonal hypothalamic cells expressing NPY and POMC. These include glucokinase; glucose transporters 1–4; ATP-sensitive K⁺ channel subunits Kir 6.2, and Sur 1 and 2; and calcium channel subunits, Cav1.2 and Cav2.2.

(Invitrogen). Two hundred nanograms RNA of each cell line were used as a template for one-step RT-PCR using the Qiagen one-step RT-PCR kit according to the manufacturer's instructions (Qiagen). The primers were as follows: NPY, sense 5'TAG GTA ACA AGC GAA TGG G3' and

anti-sense 5'ACA TGG AAG GGT CTT CAA GC3' (282 fragment); POMC: sense 5'ATG CCG AGA TTC TGC TAC AGT CG3' and anti-sense 5'TTC ATC TCC GTT GCC AGG AAA CAC3' (191 bp). The primer sequences for the glucose-sensing machinery are described in Table 1.

Table 1 Primer sequences used in marker screening

	Primer sequences	Annealing temperature (°C)	No. of cycles	Amplicon size (bp)	Crosses intron
Marker					
Glucokinase (GCK)	S: gtgtaaagatgtgtccacc AS: ccacattctgcatctcctc	60	40	442	Yes
Glucose transporter 1 (Glut1, slc2a1)	S: tggatcccagcagcaagaa AS: taggtgacacctctccacat	57	40	493	Yes
Glucose transporter 2 (Glut2, slc2a2)	S: ggaagtcagggcaaagaaaa AS: acgtaactcatccaggcgaat	57	40	279	Yes
Glucose transporter 3 (Glut3, slc2a3)	S: tgttcgccgtgactgttg AS: tccaaaccaaagatctgagct	57	40	915	Yes
Glucose transporter 4 (Glut4, slc2a4)	S: tgcccatgtatgtgggagaa AS: cggtcaggggctttagact	57	40	823	Not
Kir6.1	S: gtcttctaggaggacgcgtg AS: tatcatcacgggggctacgc	58	40	531	Yes
Kir6.2	S: aagaaaggcaactgcaacgt AS: ccccatagaatctcgtcagc	50	40	654	Yes
Sur 1	S: gtgagttcctgtccagtga AS: gtgatgttctctccaccgt	65	40	493	Yes
Sur 2	S: gagacggaagacattgccat AS: ctatgatccagtcagcgtgc	58	40	279	Yes

S, sense; AS, antisense.

Gene expression analysis using quantitative real-time RT-PCR

Total RNA from N-43/5 cells was isolated by the guanidinium thiocyanate–phenol–chloroform extraction method. First strand cDNA was synthesized from 2 µg RNA in a total volume of 20 µl using High archive cDNA kit (Applied Biosystems, Inc., Streetsville, ON, Canada). POMC real-time PCRs were performed using the POMC1 Taqman Gene Expression Assay (Applied Biosystems, Inc.), according to the manufacturer’s instructions, and run on the Prism 7900 real-time PCR machine (Applied Biosystems, Inc.). Real-time PCR values were calculated by absolute quantity method and normalized to γ-actin mRNA levels at the corresponding time points. γ-Actin primer sequences are as follows: sense 5’CTT CCC CAC GCC ATC TTG3’, and anti-sense 5’CCC GTT CAG TCA GAT CTT CAT3’.

SDS-PAGE and western blot analysis

Cell protein was prepared essentially as described previously (Cui *et al.* 2005). Cell protein was collected in 1 × cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) with 1 mM phenylmethylsulfonylfluoride. Forty micrograms of each cell protein were resolved on SDS–PAGE gels and blotted onto Hybond-C nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The resulting blot was blocked with 5% skim milk in PBS containing 0.1% Tween 20, and incubated with primary antibody (1:1000) overnight at 4 °C, and then with horseradish peroxidase-labeled secondary goat anti-rabbit antisera (Amersham Biosciences, #NA934V) 1:5000 at room temperature for 1 h. Protein was visualized using the ECL advance western blotting detection kit (Amersham Biosciences). Cav1.2 or Cav2.2 was purchased from Chemicon International,

Inc. (Temecula, CA, USA) and phospho-AMPK-α (Thr172), phospho-acetyl CoA carboxylase (ACC; Ser79), ACC, or AMPK-α subunit antibodies were from Cell Signaling Technology.

Fluorescent measurements of Ca²⁺ mobilization

For fluorescent measurements, the incubation and perfusion buffer had the following compositions (in mM): 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 NaHCO₃, and 10 HEPES, pH 7.4. In selected experiments, to depolarize the cells, 50 mM NaCl were replaced with 50 mM KCl. Cells, plated onto glass coverslips coated with poly-L-lysine, were loaded with 2 µM Fluo-4 for 45 min in incubation buffer in the presence of 2 mM glucose at 37 °C and 5% CO₂–95% air. Fluorescent experiments were carried out using an Olympus BX51W1 fluorescent microscope fitted with 20×/0.95 water immersion objective and cooled CCD camera. For excitation, xenon lamp-based Delta Ram high-speed monochromator from Photon Technology International (PTI, Lawrenceville, NJ, USA) was used. For control of monochromator and video camera, as well as fluorescent imaging and collecting of data, the ImageMaster 3.0 software (PTI) was used. Coverslip with cells was transferred to an open chamber, placed on microscope stage, and perfused at a rate of 1 ml/min. Experiments were performed at 36–37 °C using TC-324B Heater Controller (Warner Instruments, Hamden, CT, USA). The fluorescence of Fluo-4 AM was excited at 480 nm and emission was measured with 525 bandpass filter using 505 nm beam splitter. The changes in intracellular Ca²⁺ ([Ca²⁺]_i) were calculated according to the equation

$$[Ca^{2+}]_i = K_d \left(\frac{F - F_{min}}{F - F_{max}} \right),$$

where F is the measured fluorescence intensity, $F_{\min}$ and $F_{\max}$ are the fluorescence at Ca^{2+} -free and -saturating conditions, and K_d is the dissociation constant of the indicator for Ca^{2+} (345 nm). Ca^{2+} -saturating and -free conditions were obtained by addition of 5 μM ionomycin to cells incubated with 5 mM Ca^{2+} , and by addition of 5 mM EGTA to cells incubated in Ca^{2+} -free medium.

Statistical analysis

Data were analyzed using one-way ANOVA by GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) and statistical significance was determined using Tukey's multiple comparison tests or Student's t -test with $P < 0.05$.

Results

Expression of POMC and other hypothalamic markers in N-43/5 neurons

We used a representative cell model from the cell lines that have been characterized in our laboratory (Fig. 1A). The cell line was specifically chosen as a representative neuronal cell model, as they expressed detectable levels of POMC (Fig. 1B). The mixed cell cultures were originally generated through dispersed whole hypothalamic primary cell culture; therefore, we cannot be certain of the exact region of origin without further characterization of other cellular markers. We analyzed the expression of an extensive list of neuropeptides, neuropeptide receptors, and enzymes associated with the synthesis of neurotransmitters in the N-43/5 cells. We found that POMC and CART were expressed in N-43/5 cells, but NPY was not expressed at detectable levels, as expected. Receptor profiles were analyzed for the N-43/5 cells, and the neurons expressed receptors for estrogen receptors α and β , androgen, leptin, insulin, melanocortin concentrating hormone (MCH), melanocortin (MC4), glucocorticoid, and serotonin. The cells also expressed tyrosine hydroxylase, the rate-limiting enzyme in the synthesis of catecholamines, dopamine and norepinephrine, and dopamine transporter. They also exhibited evidence for γ -aminobutyric acid synthesis, through glutamate decarboxylase expression. The phenotypic profiles reflected by determination of over 100 markers in the two cell lines indicate that the most likely origin of these clonal cells was the arcuate nucleus of the hypothalamus.

Analysis of functional glucose-sensing machinery in N-43/5 cells

The pancreatic β -cell senses glucose through specific cellular proteins (Schuit *et al.* 2001). Treatment of β -cells with stimulatory concentrations of glucose resulted in an increase in the ATP:ADP ratio, thereby inhibition of ATP-sensitive potassium channels (K_{ATP}), leading to depolarization of the plasma membrane and activation of voltage-dependent

calcium channels (VDCC). These same mechanisms have been hypothesized to function in specific cell types from the hypothalamus. We therefore confirmed by either RT-PCR or western blotting that glucose-sensing machinery was expressed in a number of NPY-expressing and POMC-expressing cells, including N-43/5 cells (Fig. 1B). Glucose transporters 1–4, glucokinase (GK), the subunits of the ATP-sensitive potassium channels (K_{ATP}), and the subunits of the VDCC (Cav1.2, Cav2.2) were all expressed in the cell lines at highly detectable levels.

We tested the effect of high glucose (16.7 mM), a specific K_{ATP} channel antagonist sulfonylurea tolbutamide (250 μM), and depolarizing concentrations of potassium chloride (KCl, 50 mM) on intracellular calcium mobilization ($[\text{Ca}^{2+}]_i$). We

Figure 2 Calcium mobilization in N-43/5 POMC neurons. N-43/5 cells loaded with Fluo-4 AM were perfused with the various reagents indicated. (A) Representative result of time-dependent changes in $[\text{Ca}^{2+}]_i$ in N-43/5 POMC neurons. Cells were perfused with 0.5 mM glucose, then with 16.7 mM glucose, followed by 50 mM KCl (left panel); cells were perfused with 0.5 mM glucose, then with 250 μM tolbutamide, followed by 50 mM KCl (right panel). (B) The mean values of $[\text{Ca}^{2+}]_i$ changes in N-43/5 POMC neurons after the indicated treatments. Lane 1, basal levels ($n = 17$); lane 2, with glucose added ($n = 9$); lane 3, with KCl added ($n = 10$); lane 4, with tolbutamide added ($n = 4$).

found that in N-43/5 neurons, these stimuli evoked a rapid increase in $[Ca^{2+}]_i$. The representative traces and quantification of the results from at least nine individual neurons for N-43/5 POMC cells are illustrated (Fig. 2A and B). All of the treatments (perfusion with high glucose, tolbutamide, or KCl) presumably led to the depolarization of plasma membrane and activation of Ca^{2+} channels, and as a result, increased Ca^{2+} influx into the cells. This indicates that the neurons possess

functional ATP-sensitive potassium channels and are responsive to glucose through changes in calcium mobilization.

Glucose effects on AMPK and ACC

To determine whether glucose affected cellular function, we analyzed AMPK activity due to its known role as a nutrient sensor in many divergent cell types. We utilized the N-43/5 cell model to analyze the effects of nutrient signals on AMPK activity. We exposed the cells to an increasing concentration of glucose for 30 min to determine changes in AMPK α -subunit phosphorylation. Using a phospho-specific antibody against AMPK, we found that increasing glucose concentrations had a repressive effect on AMPK activity in the POMC cell line (Fig. 3A). Specifically, increasing glucose concentrations from 5 to 25 mM significantly decreased AMPK activity in N-43/5 POMC neurons (Fig. 3A; 5 mM, 0.84 ± 0.07 ; 10 mM, 0.80 ± 0.05 ; 15 mM, 0.73 ± 0.06 ; 20 mM, 0.79 ± 0.06 ; 25 mM, 0.69 ± 0.12 ; each $P < 0.05$, $n = 7-8$). To confirm the activity of AMPK in the N-43/5 neurons, we also measured the phosphorylation of ACC, a downstream substrate of pAMPK (Minokoshi *et al.* 2002, Yamashita *et al.* 2004, Targonsky *et al.* 2006). Decreased phosphorylation of ACC indicates increased activity leading to an increase in malonyl-CoA, a potential regulatory component of the energy-sensing system (Wolfgang & Lane 2006). We found that pACC phosphorylation, as measured by the western blot analysis using a phospho-specific antibody to ACC, mimicked that of pAMPK as expected (Fig. 3B). pACC phosphorylation was normalized to both total ACC and β -actin to confirm, since the total ACC antibody reactivity was not optimal.

Regulation of POMC mRNA expression by glucose

In order to define potential downstream effector molecules regulated by glucose, we decided to examine POMC transcription. We found that *POMC* gene expression in N-43/5 cells significantly increased at 4 h with 2.8 mM glucose (2.31 ± 0.57 ; $P < 0.05$, $n = 4$), with an increasing trend from 2 to 4 h (Fig. 4A). Stimulation with 16.7 mM glucose also significantly increased *POMC* gene expression at 4 h (Fig. 4B; 3.22 ± 0.91 ; $P < 0.05$, $n = 3$). These results not only substantiate these neurons as appropriate glucose-sensing models, but also indicate that glucose alone can have a major effect on the synthesis of neuropeptides directly involved in energy homeostasis.

Leptin effects on AMPK activity

Neurons are typically exposed to combinations of nutrient signals, and whether the effects are additive is not yet known. Using the results from the initial glucose experiments, we decided to analyze the effects of leptin (1 ng/ml or 0.062 nM) in the medium that contained 5, 25 mM, or no glucose, as these were the most relevant concentrations from the initial AMPK

Figure 3 Glucose regulates AMPK and ACC activity in N-43/5 POMC neurons. N-43/5 POMC neurons were stimulated with or without glucose for the indicated concentrations for 0.5 h. Cell lysates were examined by western blot with phospho-specific AMPK or ACC and normalized using total AMPK or ACC antibodies. The summary of all experiments performed ($n \geq 3$) are presented in the respective graphs as means $\pm$ S.E.M., indicated points significantly different (* $P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$) compared with the untreated control; (A) pAMPK and (B) ACC. (C) Immunoblots shown are representative of an experiment performed at least thrice.

Figure 4 Glucose regulates *POMC* gene expression in N-43/5 POMC neurons. Analysis of *POMC* gene expression following 2.8 and 16.7 mM glucose stimulation. Total RNA was isolated at the indicated time points. Real-time RT-PCR was performed for *POMC*. mRNA levels were normalized to γ -actin mRNA levels. Only changes with treatments are represented in the histograms, whereas time-matched control values for each time point (vehicle alone), set to 1, are indicated by the dashed lines. Values are expressed as mean $\pm$ S.E.M. with statistical significance, * $P < 0.05$ ($n = 3-4$). (A) With 2.8 mM glucose, POMC mRNA increased at 4 h. (B) With 16.7 mM glucose, POMC mRNA also significantly increased at 4 h.

experiments. We found that in the absence of glucose, leptin suppressed AMPK activity in POMC neurons when compared with glucose-matched controls (Fig. 5A; 0.52 ± 0.09 ; $P < 0.05$, $n = 4$). At low glucose, AMPK activity was marginally suppressed by leptin in the POMC neurons (Fig. 5A; 0.73 ± 0.11 ; $P < 0.05$, $n = 5$), whereas the addition of leptin did not change the basal effect of 25 mM glucose alone.

Insulin effects on AMPK activity

Using the same parameters, we analyzed the effect of 100 nM insulin on AMPK activity in the N-43/5 cells. There was no change in phospho-AMPK levels in response to insulin stimulation in the cell line (Fig. 5B) in the absence of glucose in the medium. Similarly, at high glucose levels, there was no change in AMPK activity with insulin in N-43/5 POMC

Figure 5 Leptin and insulin regulate AMPK activity in N-43/5 POMC neurons. N-43/5 POMC neurons were stimulated with or without (A) leptin (1 ng/ml or 0.062 nM) or (B) insulin (100 nM) in combination with 5 or 25 mM glucose for 0.5 h. Cell lysates were examined by western blot with phospho-specific AMPK and total AMPK antibodies. The summary of all experiments performed ($n \geq 3$) are presented in the respective graphs as means $\pm$ S.E.M., indicated points significantly different (* $P < 0.05$, † $P < 0.001$) compared with the untreated glucose-matched control (0, 5, and 25 mM glucose alone). Only changes with treatments are represented in the histograms, whereas time-matched control values for each glucose concentration, set to 1, are indicated by the dashed lines. (C) Immunoblot shown is representative of an experiment performed at least thrice. Lep, leptin; Ins, insulin; C, control.

neurons. However, at low glucose levels, there was a significant decrease in AMPK activity with insulin treatment in the POMC neurons (Fig. 5B; 0.65 ± 0.12 ; $P < 0.05$, $n = 4$).

Discussion

Recent studies show that glucose-responsive hypothalamic neurons may use the same molecular mechanisms as the pancreatic β -cells to recognize and respond to extracellular glucose changes. As in the pancreatic β -cell, the ATP-sensitive potassium (K_{ATP}) channel is an important component of glucose sensing in glucose-excitable (also referred to as glucose-responsive) neurons (Ashford *et al.* 1990a,b, Miki *et al.* 2001, Minami *et al.* 2004), found predominantly in the ventromedial region of the hypothalamus, containing both the arcuate and ventromedial nuclei (Routh 2002). The mere presence of the K_{ATP} channel does not necessarily mean that a given neuron will be glucose sensing, since this channel is widely expressed throughout the brain. Furthermore, glucose-inhibited (also called glucose-sensitive) neurons most likely do not use the K_{ATP} channel to sense glucose (Kang *et al.* 2004, Levin *et al.* 2004). Thus, other regulatory components are also likely involved in neuronal glucose sensing. The control of both glycolysis and glucose transport has been proposed as a regulator of β -cell glucose sensing. In the pancreas, GK (also called hexokinase IV) regulates glycolytic flux and intracellular ATP production in both β - and α -cells and is a primary regulator of ATP production, K_{ATP} channel activity, and insulin secretion in the β -cells (Schuit *et al.* 2001). A large body of evidence also indicates that GK is a critical component of glucose sensing in neurons as well (Levin *et al.* 2004). GLUT2 has also been proposed as a regulator of β -cell glucose sensing (Thorens 2001), and some neurons do express GLUT2 (Kang *et al.* 2004). GLUT4 is a good candidate to regulate neuronal glucose sensing due to its localization in regions associated with glucose sensing, co-expression with the insulin receptor, and its optimal K_m for glucose within the physiological range; however, it is thought that the majority of glucose-sensing neurons use GLUT3 as their major glucose transporter (Kang *et al.* 2004). We have demonstrated the expression of components of the same system in our N-43/5 POMC-expressing hypothalamic cells, including glucose transporters 1–4, GK, the subunits of the K_{ATP} channel, and the subunits of the voltage-dependent calcium channels.

The activity of NPY and POMC cells is oppositely regulated by body energy status, and may directly respond to altered glucose availability, as glucose is the main metabolic fuel of the brain. To investigate the role of hypothalamic neurons in glucose homeostasis, it is important to define physiologic levels for extracellular glucose in the brain. As a rule, brain glucose levels are approximately 10–30% of that in the plasma (Routh 2002, de Vries *et al.* 2003). Thus, plasma glucose levels of 5–8 mM correspond to brain glucose levels of about 1–2.5 mM, which is considered to be euglycemic (Silver & Erecinska 1998). During insulin-induced hypoglycemia (plasma glucose 2–3 mM), brain glucose levels were approximately 0.5 mM. However, refeeding (15–17 mM plasma glucose levels) resulted in brain glucose levels of 4.5 mM

(Silver & Erecinska 1994). Brain glucose levels reach a 5 mM threshold with plasma hyperglycemia (Routh 2002). However, if the neurons are in or near a region lacking a blood–brain barrier, such as in the cells surrounding the third ventricle (including cells from the arcuate nucleus), brain glucose levels may approximate plasma glucose levels. Neurons in these regions may be normally exposed to higher levels of extracellular glucose in some pathological circumstances, but unlikely to be exposed to extracellular glucose levels above 5 mM under physiologic conditions (Routh 2002).

Emerging evidence indicates that AMPK may be a general regulator of energy homeostasis. AMPK activity in the hypothalamus is increased by fasting and normalizes upon refeeding in mice, which may correlate to changing levels of glucose and insulin (Minokoshi *et al.* 2004). Hypothalamic AMPK activity is acutely affected by the availability of specific nutrients, and using whole hypothalamic extracts or from specific regions of the brain, it has been shown that glucose directly regulates AMPK activity (Andersson *et al.* 2004, Kim *et al.* 2004, Minokoshi *et al.* 2004). Specifically, i.c.v. administration of glucose decreases hypothalamic AMPK activity (Minokoshi *et al.* 2004). Leptin and insulin produce a similar decrease in overall AMPK activity, while ghrelin, an orexigenic gut peptide, appears to increase it (Andersson *et al.* 2004, Minokoshi *et al.* 2004). Leptin has been shown to specifically affect neurons from the arcuate and paraventricular nuclei of the hypothalamus, indicating that the melanocortin neurons may be involved in this process. As such, the melanocortin receptor agonist MT II decreases, whereas AgRP increases phospho-AMPK levels (Minokoshi *et al.* 2004). Other known modulators of AMPK activity include C57, a fatty acid synthase (FAS) inhibitor, systemic hypoglycemia, streptozotocin-induced diabetes mellitus, and α -lipoic acid (Carling 2005). Therefore, we can speculate from this information that overall AMPK activity is decreased by anorexigenic factors and stimulated by orexigenic factors. A recent study using metformin, an anti-diabetic drug linked to weight loss, demonstrates that low glucose (1 mM) causes an increase in AMPK activity, resulting in a decrease in NPY gene expression. However, POMC gene expression was inhibited at low glucose, but was not linked to a change in AMPK activity in primary rat hypothalamic neurons (Chau-Van *et al.* 2006). In our N-43/5 cell model, we found that AMPK activity was decreased (at higher levels of glucose), resulting in an increase in POMC gene expression. Two cell lines that express AgRP were previously used to study AMPK activity, a neuroblastoma line N1E-115 and a gonadotropin-releasing hormone (GnRH) secreting cell model GT1-7 (Lee *et al.* 2005). In these cells, they found that increasing glucose to 25 mM decreased AMPK activity. Interestingly, AgRP expression decreased with increasing glucose concentrations. The mouse N1E-115 neuroblastoma cells, derived from a spontaneous tumor in a region of the spinal cord, are not likely representative of arcuate AgRP neurons due to the fact that they are not from the hypothalamus or central nervous system (CNS) and do not

co-express NPY, and the GT1-7 cells express GnRH and are a well-defined model of the GnRH neuron, which does not typically co-express AgRP or NPY. Therefore, it would be optimal to use cell models derived from the hypothalamus that express appropriate cell markers related to energy homeostasis. Our studies with the N-43/5 POMC neurons indicate that the levels of glucose available to the cell may affect AMPK responses to peripheral signals, particularly leptin and insulin.

While we have focused on a specific cell type from the hypothalamus, it is important to recognize that AMPK also has substantial effects on peripheral tissues, such as the maintenance of glucose homeostasis (Kahn *et al.* 2005). Thus, it has been suggested that AMPK activators may be used for the treatment of diabetes mellitus, as it stimulates glucose uptake in skeletal muscle and inhibits liver glucose synthesis (Musi & Goodyear 2002). The findings from the hypothalamus may, however, counteract these positive effects by increasing food intake. Therefore, it would be in our best interest to understand the specific cell types that are using AMPK to achieve changes in neuropeptide secretion, in order to specifically target therapeutic intervention to the appropriate cell and tissue. Our findings suggest that individual neuronal cell types, such as the POMC neuron, may indeed respond to peripheral signals differentially, and that the AMPK response may depend on glucose levels as well. The complexity of the hypothalamic architecture and the number of neuronal cell types involved in the regulation of energy homeostasis make these studies a daunting task. However, we suggest that this is a necessary exercise in order to understand the specific changes at the level of neuropeptide synthesis and secretion, the ultimate downstream effectors of overall changes in glucose concentrations and AMPK activity.

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