ERK1/2 mediates glucose-regulated *POMC* gene expression in hypothalamic neurons

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Abstract

Hypothalamic glucose-sensing neurons regulate the expression of genes encoding feeding-related neuropeptides *POMC*, *AgRP*, and *NPY* – the key components governing metabolic homeostasis. AMP-activated protein kinase (AMPK) is postulated to be the molecular mediator relaying glucose signals to regulate the expression of these neuropeptides. Whether other signaling mediator(s) plays a role is not clear. In this study, we investigated the role of ERK1/2 using primary hypothalamic neurons as the model system. The primary neurons were differentiated from hypothalamic progenitor cells. The differentiated neurons possessed the characteristic neuronal cell morphology and expressed neuronal post-mitotic markers as well as leptin-regulated orexigenic *POMC* and anorexigenic *AgRP/NPY* genes. Treatment of cells with glucose dose-dependently increased *POMC* and decreased *AgRP/NPY* expression with a concurrent suppression of AMPK phosphorylation. In addition, glucose treatment dose-dependently increased the ERK1/2 phosphorylation. Blockade of ERK1/2 activity with its specific inhibitor PD98059 partially (approximately 50%) abolished glucose-induced *POMC* expression, but had little effect on *AgRP/NPY* expression. Conversely, blockade of AMPK activity with its specific inhibitor produced a partial (approximately 50%) reversion of low-glucose-suppressed *POMC* expression, but almost completely blunted the low-glucose-induced *AgRP/NPY* expression. The results indicate that ERK1/2 mediated *POMC* but not *AgRP/NPY* expression. Confirming the *in vitro* findings, i.c.v. administration of PD98059 in rats similarly attenuated glucose-induced *POMC* expression in the hypothalamus, but again had little effect on *AgRP/NPY* expression. The results are indicative of a novel role of ERK1/2 in glucose-regulated *POMC* expression and offer new mechanistic insights into hypothalamic glucose sensing.

Key Words

- ▶ ERK1/2
- ▶ AMPK
- ▶ POMC
- ▶ AgRP
- ▶ NPY
- ▶ glucose sensing

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Introduction

The body's nutritional state is sensed by the brain, whereby feed-back signals are generated to maintain metabolic homeostasis (Blouet & Schwartz 2010, Jordan *et al.* 2010). The hypothalamus, particularly the arcuate nucleus (ARC) where feeding-related neuropeptides

POMC and *AgRP/NPY* are expressed (Cone 2005), is central to the convergence of nutrient signals (e.g., glucose) and impaired hypothalamic nutrient sensing contributes to the onset of obesity and insulin resistance (Blouet & Schwartz 2010, Jordan *et al.* 2010).

Results from previous studies indicate that infusion of glucose into the hypothalamus leads to decreased food intake (Kurata *et al.* 1986), suggesting that one function of hypothalamic glucose sensing is appetite control. Consistent with this observation, systemic or i.c.v. administration of glucose stimulates POMC and inhibits AgRP/NPY expression (Chang *et al.* 2005, Wolfgang *et al.* 2007, Cha *et al.* 2008). Glucose-regulated AgRP or POMC expression has also been demonstrated in transformed cell lines (Lee *et al.* 2005, Cai *et al.* 2007, Cheng *et al.* 2008). Because the glucose-regulated gene expression can be blunted by the non-metabolizable glucose analogue 2-deoxyglucose *in vivo* as well as in cell lines (Lee *et al.* 2005, Wolfgang *et al.* 2007, Cha *et al.* 2008, Cheng *et al.* 2008), the results indicate that intracellular glucose metabolism is required for the expression of these genes.

AMP-activated protein kinase (AMPK) is a cellular energy sensor, activated under states of low cellular energy such as glucose deprivation (Kahn *et al.* 2005, Hardie *et al.* 2006). AMPK is also postulated to mediate hypothalamic glucose sensing. For instance, i.c.v. injection of glucose suppresses hypothalamic AMPK activity (Minokoshi *et al.* 2004, Cha *et al.* 2008); virally mediated expression of activated or dominant-negative mutants of AMPK in the hypothalamus modulates AgRP/NPY expression in the fasted or fed state (Minokoshi *et al.* 2004); expression of dominant-negative mutants of AMPK in cell lines also attenuates low-glucose-induced AgRP expression (Lee *et al.* 2005). In agreement with the regulatory role of AMPK in orexigenic neuropeptide expression, transgenic mice with AgRP-neuron-specific AMPK inactivation display decreased feeding and a lean phenotype (Claret *et al.* 2007). However, evidence supporting the role of AMPK in POMC expression is perplexing. For instance, mice with POMC-neuron-specific deletion of AMPK (POMC α 2KO), which would be predicted to have enhanced *Pomc* expression and thus a lean phenotype, are hyperphagic and obese (Claret *et al.* 2007); virally mediated expression of dominant-negative mutants of AMPK in the hypothalamus also produces a paradoxical suppression of POMC expression in the fed state (Minokoshi *et al.* 2004). Furthermore, using pharmacological inhibitors it has been demonstrated that AMPK mediates the glucose-inhibited electrical activity of hypothalamic NPY-expressing neurons, but its role in the activity of glucose-excited neurons (e.g., POMC neurons) is unclear (Mountjoy *et al.* 2007). However, POMC neurons from *Pomc* α 2KO mice show defective electrophysiology (Claret *et al.* 2007). It appears that the molecular mechanism governing glucose sensing in POMC-expressing neurons is complex and that other signaling mediator(s) might be involved.

Pancreatic islet cells are prototypic glucose-sensing cells (Schuit *et al.* 2001, Mountjoy & Rutter 2007). In β -cells, glucose not only induces insulin secretion but also insulin expression (Lawrence *et al.* 2008), the latter event is mediated by ERK1/2 (Khoo *et al.* 2003). Hypothalamic ERK1/2 has been implicated in energy homeostasis. Fasting activates ERK1/2 in the ARC and paraventricular nucleus in mice (Morikawa *et al.* 2004, Ueyama *et al.* 2004) and the activation is reversed by refeeding (Ueyama *et al.* 2004). Hypothalamic ERK1/2 has also been shown to mediate the anorectic and thermogenic effect of leptin (Rahmouni *et al.* 2009) and the leptin-induced glucose utilization (Toda *et al.* 2013).

To address whether ERK1/2 plays a role in hypothalamic glucose sensing, we generated primary hypothalamic neurons via differentiation of rat hypothalamic progenitor cells. The cultured cells exhibited neuronal cell morphology and expressed the neuron-specific cellular marker β III tubulin (β III) and post-mitotic marker NeuN, confirming the neuronal nature and maturation state. Using these primary neurons, we investigated the contribution of ERK1/2 to glucose-regulated POMC and AgRP/NPY expression.

Materials and methods

Animals

Sprague–Dawley (SD) rats were purchased from the Animal Center of Shantou University Medical College. The animals were bred and housed under standard conditions with food and water available and the animals allowed to eat and drink *ad libitum*. All experimental procedures were conducted in accordance with the guidelines published in the Ministry of Science and Technology of China for Care and Use of Laboratory Animals and approved by the Animal Care and Welfare Committee of Shantou University Medical College.

Hypothalamic cell isolation and primary cell culture

Timed-pregnant SD rats on day 18 of gestation (E18) were killed under anesthesia. Fetal brains were collected and hypothalami dissected as described previously (Bergonzelli *et al.* 2001). Hypothalamic cells were isolated according to a published method (Sousa-Ferreira *et al.* 2011) with modifications. Fetal hypothalami were immediately placed into Hank's Balanced Salt Solution (HBSS; Macgene, Beijing, China), washed with HBSS, cut into 5–10 fragments, and digested in Neurobasal medium

(Gibco) containing 2 mg/ml papain (Sigma) and 20 U/ml DNAase I (Biotechnology Center, Guwahati, India) for 30 min at 37 °C. The tissue fragments were then mechanically dissociated with a sterile Pasteur pipette by gently pipetting them up and down for ten times. After sitting for 2 min, the supernatant was aspirated and residual tissue fragments were washed with Neurobasal medium twice by pipetting up and down as before. The pooled supernatants were centrifuged at 500 *g* for 2 min and the cell pellets resuspended in Neurobasal medium. Viable hypothalamic cells were counted using Trypan Blue staining and plated into poly-D-lysine (Sigma)-coated six-well culture plates at density of 2×10^6 cells per well or 2×10^5 cells per well containing coverslips. The cells were incubated at 37 °C with 5% CO₂ in the differentiation medium containing Neurobasal, 500 mM L-glutamine, 2% B27 supplement, and 2% penicillin–streptomycin (all from Gibco) to allow for neuronal differentiation. Neurobasal medium contained 25 mM glucose throughout the study unless specified otherwise.

Immunocytochemistry

DIV10 cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.3% Triton X-100 (Sigma). The cells were then blocked with 3% BSA (Sigma) for 1 h, followed by incubation overnight at 4 °C using a blocking buffer containing each of the following primary antibodies: rabbit anti-NeuN (1:5000, Cell Signaling Technology, Beverly, MA, USA), and mouse anti-βIII tubulin (1:200, Millipore). Following washes with PBS, the cells were incubated for 1 h at room temperature with secondary antibodies of goat anti-mouse Alexa-Fluor 594 or goat anti-rabbit Alexa-Fluor 488 (1:200, ZS-Bio, China). The cells were then stained with the nuclear dye DAPI (Vectashield, Burlingame, CA, USA) for 5 min. Coverslips were mounted on microscope slides and fluorescence images captured using a fluorescence microscope (LSM510 Meta (Zeiss, Jena, Germany)). The percentage of antibody-positive cells was calculated using the number of double-positive cells divided by the number of DAPI-positive cells in four independent microscopic fields (approximately 100 cells) per coverslip. At least three independent cell culture preparations were analyzed for each treatment.

Pharmacological treatment *in vitro*

E18 progenitor cells were cultured in the differentiation medium for 10 days (DIV10) and subjected to pharmacological treatments as specified in the Results section.

Rat leptin was obtained from Biovondor (Brno, Czech Republic). Glucose, compound C (CC), and PD098059 were purchased from Sigma. CC and PD098059 were dissolved in DMSO as 10 mM stocks, such that the final DMSO concentration used in cell culture was 0.2%.

Intracerebroventricular (i.c.v.) injection

SD rats (8–10 weeks of age) provided with standard rodent chow and allowed to feed *ad libitum* were food-deprived overnight and randomized into treatment and control group ($n=4$ /group). The rats were then anesthetized and stereotactically given i.c.v. injections of 10 μmol glucose in 2 μl saline. PD98059 (2.5 or 5 μg in 2 μl DMSO) was given i.c.v. 30 min before glucose administration. The control group was given equivalent volumes of the vehicles. After 1 h of glucose administration, rats were killed and ARC regions of hypothalami were collected for RNA and protein extraction.

Quantitative PCR

Hypothalami from E18, and postnatal days 0 and 28 (P0 and P28) were homogenized on ice in HBSS and cultured hypothalamic cells were washed with PBS on ice before RNA extraction. Total cellular RNAs were extracted using TRIzol (Life Technologies) according to the manufacturer's instructions and RNA purity was confirmed by the 260/280 absorption ratio (>1.8). cDNAs were prepared from 1 μg total RNAs using PrimeScript RT with gDNA eraser (Takara, Tokyo, Japan) according to the manufacturer's instructions. Quantitative PCRs (qPCRs) were performed using SYBR Premix Ex TaqII (Takara). The primers used were as follows (5'–3'): β-actin (forward: ACCCTGAAGTACCCCATTTG; reverse: TACGACCAGAGGCATACAG), *Npy* (forward: GACATGGCCAGATACTACTCCG; reverse: AAGGGTCTTCAAGCCTTGTCT), *Agrp* (forward: GCA-GAGGTGCTAGATCCACAGAA; reverse: AGGACTCGTGCAGCCTTACAC; Chang *et al.* 2004), *Pomc* (forward: AGTGCCAGGACCTCACCACG; reverse: AAGCGGTCC-CAGCGGAAG; Martins *et al.* 2010), *LepR* (forward: ACAGCCAAACAAAAGCACCATT; reverse: TCAGCAGCCTC-TCTTACGATTG). Reactions were run in the 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Each sample was run in triplicate. Quantification of relative mRNA levels was performed using the change in threshold cycles (ΔCt) method with β-actin as the internal reference with the 7500 Software v2.0.6 (Applied Biosystems).

Western blotting analysis

Proteins were extracted from cultured cells using RIPA buffer (CellSignalingTechnology) containing 1 mM NaF and 1 mM PMSF (Solarbio, Beijing, China). Protein concentrations were determined using the BCA assay (Thermo Scientific, Waltham, MA, USA). Equivalent amounts of protein samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman, Dusseldorf, Germany). The membranes were incubated with 5% BSA in TBST at room temperature to block nonspecific binding and then incubated with primary antibodies of rabbit anti-phospho-AMPK α (Thr172) or rabbit anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1000, Cell Signaling Technology), or mouse anti- β -actin (1:2000, ZS-Bio) overnight at 4 °C. Subsequently, the membranes were incubated with secondary antibodies (IRDye 680RD-conjugated goat anti-rabbit or IRDye 800CW-conjugated goat anti-mouse (1:10 000, LI-COR, USA)) for 1 h at room temperature. The band

density was visualized, scanned, and analyzed using the Odyssey Two-Color Infrared Imaging System (LI-COR) and quantified using the Quantity OneSystem (Bio-Rad).

Statistical analysis

Student's *t*-test was used throughout the study. Data were expressed as the mean $\pm$ S.E.M. Differences with *P* < 0.05 were considered to be statistically significant.

Results

Characterization of cultured hypothalamic neurons

Primary hypothalamic neurons were generated by differentiating rat hypothalamic progenitor cells collected on embryonic day 18 (E18). Upon *ex vivo* differentiation, we saw a time-dependent increase in the expression of *Agrp*, *Npy*, *Pomc*, and leptin receptor (*LepR*) genes, peaking at around 10 days (DIV10) in culture (Fig. 1A) and the expression levels

Figure 1

Characteristics of differentiated hypothalamic neurons. (A) Hypothalamic progenitor cells were grown in differentiation medium for 0, 7, and 10 days and mRNAs were isolated for the analyses of expression of the indicated genes expression by qPCR. (B) Hypothalamic mRNAs isolated from E18, P0, and P28 rats were analyzed identically to those examined in A. (C) DIV10 neurons were cultured in Neurobasal medium containing 5 mM glucose

overnight before being treated with 20 or 40 nM leptin for 1 h. Cellular mRNAs were isolated for gene expression analysis. (D) DIV10 neurons were stained with antibodies against β III and NeuN and double-stained with the nuclear dye DAPI. All experiments were independently carried out at least three times and the results are representative. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

declined thereafter (results not shown). DIV10 cells were therefore chosen for further characterization. A 3.7- to 5.3-fold increase in the expression of the genes was found on DIV10 compared with the levels on day 0 (DIV0) in culture (Fig. 1A). As for comparison, rat hypothalamic mRNAs from developmental stages of E18, postnatal day 0 (P0), and 28 (P28) were analyzed. A 1.3- to 4.5- and 11- to 18-fold increase was found at P0 and P28, respectively, compared with those at E18 (Fig. 1B). As DIV0 cells (freshly isolated from E18) and E18 hypothalami were similar with respect to gene expression (results not shown), DIV10 cells appeared to have expression levels approximating to those of neonatal rats.

Leptin regulates *POMC* and *AgRP/NPY* gene expression in an opposing manner (Barsh & Schwartz 2002, Varela & Horvath 2012). Consistently, DIV10 cells exhibited a dose-dependent decrease in *AgRP/Npy* and increase in *Pomc* expression upon leptin challenge (Fig. 1C), indicating that DIV10 cells are leptin-sensitive.

The neuronal homogeneity and maturity of DIV10 cells were examined using the neuron-specific cellular marker β III and post-mitotic marker NeuN. β III-positive cells exhibited an exclusive cytoplasmic staining with long cellular processes characteristic of neurons: 94% of cells were double-positive for β III and DAPI (Fig. 1D). NeuN-positive cells displayed an exclusive nuclear staining and 98% of cells were double-positive for NeuN and DAPI (Fig. 1D).

Glucose modulates the expression of feeding-related neuropeptides in DIV10 neurons

To assess the effect of glucose on the expression of feeding-related neuropeptides, DIV10 neurons were treated with increasing glucose concentrations of 1, 5, 10, 25 mM for 1 h. The *Pomc* mRNA levels were dose-dependently increased, with a 2.4-fold elevation with 25 mM glucose compared with the 1 mM glucose treatment (Fig. 2). Conversely, the *AgRP* and *Npy* expression was dose-dependently decreased, with a respective 65% and 60% reduction for treatment with 25 mM glucose compared with the 1 mM treatment (Fig. 2). The regulatory effect of glucose on the orexigenic and anorexigenic neuropeptide expression in DIV10 neurons agrees with the results reported previously (Chang *et al.* 2005, Lee *et al.* 2005, Cai *et al.* 2007, Wolfgang *et al.* 2007, Cha *et al.* 2008, Cheng *et al.* 2008).

Glucose activates ERK1/2 in DIV10 neurons

To investigate the role of ERK1/2 in glucose sensing, DIV10 neurons were treated with increasing concentrations of glucose for 1 h and the ERK1/2 phosphorylation

Figure 2

Glucose regulates the *Pomc* and *AgRP/Npy* gene expression in DIV10 neurons. DIV10 neurons were incubated in Neurobasal medium containing 1 mM glucose overnight before being treated with the indicated concentrations of glucose for 1 h. Cellular mRNAs isolated from each treatment were subjected to qPCR analyses. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with cells treated with 1 mM glucose.

was examined. A dose-dependent increase in phosphorylated ERK1/2 (pERK1/2) was seen, which was paralleled by a dose-dependent reduction in phosphorylated AMPK (pAMPK) (Fig. 3). The results indicate that glucose, in addition to inhibiting AMPK as expected, activates ERK1/2 in DIV10 neurons.

Contribution of ERK1/2 to *Pomc* expression in glucose sensing

To establish that ERK1/2 mediates the appetitive gene expression in glucose sensing, the MEK (upstream

Figure 3

Glucose inhibits AMPK and promotes ERK1/2 phosphorylation in DIV10 neurons. DIV10 neurons were incubated in Neurobasal medium containing 1 mM glucose overnight, followed by 1, 5, and 25 mM glucose (Glu) treatment for 1 h. The pAMPK and pERK1/2 levels were analyzed by western blotting and quantified by densitometry scan. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared with cells treated with 1 mM glucose.

kinase of ERK1/2)-specific inhibitor PD98059 was used to modulate ERK1/2 activity. The inhibitor dose-dependently blocked glucose-induced pERK1/2 levels (Fig. 4A). PD98059 at 10 μ M suppressed the pERK1/2 level by 71% (Fig. 4A) while it produced a 28% reduction in glucose-stimulated *Pomc* expression (Fig. 4B). PD98059 at 20 μ M, which depressed glucose-induced pERK1/2 levels by 125%, further blunted the *Pomc* expression by 53% (Fig. 4B). In contrast, PD98059 at 10 μ M had no effect on glucose-suppressed *Agrp/Npy* expression ($P > 0.05$, Fig. 4B). At 20 μ M, at which the pERK1/2 level was inhibited below basal level (125%), PD98059 only produced a modest attenuation of 15–17% (Fig. 4B). Because PD98059 is a highly specific ERK1/2 inhibitor with few known off-target effects even when used at 50 μ M (Alessi *et al.* 1995), the results indicate that ERK1/2 partially

participates in glucose-regulated *Pomc* expression, but its role in *Agrp/Npy* expression, if any, is minimal. The results also indicate the existence of additional regulatory pathway(s) for *Pomc* expression.

Contribution of AMPK to *Pomc* expression in glucose sensing

Although glucose coregulates *Pomc* expression and AMPK activity *in vivo* (Minokoshi *et al.* 2004, Cha *et al.* 2008), no clear evidence yet indicates AMPK to be the mediator relaying glucose signals to *Pomc* expression. To address this question, the AMPK-specific inhibitor CC was employed to modulate its activity. CC dose-dependently inhibited low-glucose-induced elevation of AMPK phosphorylation, achieving a 100% inhibition ($P > 0.05$) at 20 μ M. At this inhibitor concentration, the low-glucose-suppressed *Pomc* expression was attenuated by 55% (Fig. 5B). In contrast, the low-glucose-induced *Agrp/Npy* expression was almost completely (approximately 90%) abolished (Fig. 5B). Although CC may have off-target effects, the effects influencing the results observed here might be minimal, because i) similar CC concentrations have been used in cultured hypothalamic neurons, which showed no apparent off-target effects (Mountjoy *et al.* 2007) and ii) CC modulated the *Pomc* and *Agrp/Npy* expression in manners (i.e., opposite directions) consistent with its specific effect. Thus, the results indicate that AMPK is the primary mediator of glucose-regulated *Agrp/Npy* expression, but only a partial contributor to *Pomc* expression, which supports the hypothesis that ERK is an additional mediator relaying glucose signals to anorexigenic neuropeptide expression.

Effects of hypothalamic ERK1/2 on glucose-regulated *Pomc* and *Agrp/Npy* expression in rats

To further validate the role of ERK1/2 observed *in vitro*, food-deprived rats were given i.c.v. injections of glucose in the presence or absence of PD98059 and hypothalamic ARC tissues were collected 1 h later for protein and gene analyses. Consistent with results described in previous reports (Cha *et al.* 2008, Minokoshi *et al.* 2004), glucose injection suppressed the hypothalamic pAMPK (Fig. 6A), upregulated the *Pomc* and downregulated *Agrp/Npy* expression (Fig. 6B). It also markedly raised hypothalamic pERK1/2 levels (Fig. 6A) in agreement with the results of the *in vitro* experiments (Fig. 3). Administration of PD98059 dose-dependently depressed glucose-induced ERK1/2 phosphorylation with no effect on

Figure 4

Effects of PD98059 on glucose-regulated *Pomc* and *Agrp/Npy* gene expression. DIV10 neurons were incubated in Neurobasal medium containing 1 mM glucose overnight, followed by pretreatment with either DMSO or 10 μM or 20 μM PD98059 (PD) in Neurobasal medium containing 1 mM glucose for 30 min before treatment with Neurobasal medium

containing 25 mM glucose in the presence or absence of PD98059 for 1 h. The pERK1/2 level was analyzed by western blottings (A) and gene expression was analyzed by qPCR (B). Experiments were repeated at least twice and the data were consistent between experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

pAMPK levels (Fig. 6A). PD98059 at 2.5 μg, at which 57% inhibition of pERK1/2 was achieved (Fig. 6A), blocked glucose-induced *Pomc* expression by 33% and had no effect on *Agrp/Npy* expression ($P > 0.05$, Fig. 6B). PD98059 at 5 μg, which gave a 123% inhibition of pERK1/2 levels, attenuated the *Pomc* expression by 57%, whereas the glucose-suppressed *Agrp/Npy* expression was only modestly (17–19%) reversed (Fig. 6B) despite an excess inhibition of the ERK1/2 activity. The i.c.v. dose of 5 μg PD98059 has been used in food intake studies, and did not exhibit apparent toxicities in rats (Rahmouni *et al.* 2009). Thus the *in vivo* results confirm that ERK1/2 is an indispensable mediator in glucose-regulated *Pomc* but not *Agrp/Npy* expression.

Discussion

Hypothalamic neuroprogenitor cells have been used to study the molecular mechanisms of neuroendocrine hormones (Bergonzelli *et al.* 2001), leptin sensitivity in

developing hypothalamic neurons (Carlo *et al.* 2007), and hormonal effects on hypothalamic neural differentiation (Desai *et al.* 2011). The progenitor cells also express genes and proteins of feeding-related neuropeptides (Sousa-Ferreira *et al.* 2011). In this study, we established primary hypothalamic neurons from progenitor cells to study glucose sensing. Using hypothalamic cell models to study hypothalamic nutrient sensing could be particularly fitting because changes in extracellular nutrient levels directly trigger the cellular events (e.g., gene expression, electrical activity) without the mediation of neurotransmission. Nevertheless, it should be stressed that although DIV10 neurons are post-mitotic (i.e., expressing *NeuN*) and express leptin-regulated *POMC* and *AgRP/NPY* genes, which may make them more applicable than immortalized cell lines as a model system, they are not adult hypothalamic neurons (e.g., at minimum, expression levels of the few genes examined differ between cultured neurons and adult hypothalami). Besides, whether these neurons express other feeding-related neuropeptides

Figure 5

Effects of CC on glucose-regulated *Pomc* and *Agrp/Npy* gene expression. DIV10 neurons were pretreated with either DMSO or 10 or 20 μM CC in Neurobasal medium containing 25 mM glucose for 30 min before treatment with Neurobasal medium containing 1 mM glucose in the

presence or absence of CC for 1 h. Thereafter, the levels of pAMPK were analyzed by western blots (A) and gene expression analyzed by qPCR (B). Experiments were repeated at least twice and the results were consistent between experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

(e.g., MCH, orexin, and kisspeptin) or whether they represent a mixed population of hypothalamic neurons, as is the case *in vivo*, is not known. Nonetheless, because the results of *in vivo* experiments validate the findings obtained with the cultured neurons, the cell system may have utility for exploring the molecular details of hypothalamic nutrient sensing.

ARC is in close contact with the median eminence, a region characterized by a leaky blood–brain barrier (Banks 2006). Therefore, ARC neurons may be exposed to glucose concentrations close to those of the peripheral blood. In this study, cultured hypothalamic neurons detect a broad range of glucose levels (1–25 mM) from clinical hypoglycemia to hyperglycemia and in turn produce a dynamic change in neuropeptide expression. The finding appears to differ from those obtained in electrophysiological studies, where the responsiveness of hypothalamic neurons to glucose concentrations is narrower and less dynamic. For instance, *Pomc*-expressing neurons hyperpolarize at glucose concentrations lowered from 10 to 5 or 5 to 3, or 2 to 0.1 mM (Ibrahim *et al.* 2003, Claret *et al.* 2007, Parton *et al.* 2007) and

NPY-expressing neurons depolarize at glucose levels dropping from 2.5 to 0.1 mM or 15 to 1 mM (Fioramonti *et al.* 2007, Mountjoy *et al.* 2007). Whether the differential glucose sensitivity between the two glucose-sensing read-outs (gene expression and electrical activity) reflects differences in methodologies used or mechanisms requires future studies. It is conceivable though that the dynamic changes in appetitive gene expression in response to glucose signals would correlate well with the continuity in changes to physiological endpoints such as food intake and body weight.

Altered intracellular ATP levels are thought to be the main trigger to initiate signaling cascades in hypothalamic nutrient sensing (Blouet & Schwartz 2010, Jordan *et al.* 2010). AMPK is activated by increased cellular AMP or decreased ATP levels (Kahn *et al.* 2005, Hardie *et al.* 2006). In β-cells, the glucose-induced ERK1/2 activation is also thought to be ATP-dependent, where glucose is taken up and metabolized to generate ATP, leading to a series of events including closure of the ATP-dependent potassium channel, activation of the voltage-dependent calcium channel, and the final release of calcium from intracellular

Figure 6

Effects of i.c.v. injection of PD98059 on glucose-induced ERK1/2 phosphorylation and feeding-related neuropeptide expression. Food-deprived rats were given i.c.v. injection of PD98059 (2.5 μg or 5 μg) or vehicle 30 min

before the i.c.v. administration of glucose (10 μmol). The rat hypothalami were removed 1-h post glucose treatment for western blotting (A) and qPCR analyses (B). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

stores; a rise in intracellular calcium activates ERK1/2 (Lawrence *et al.* 2008). It is likely that nutrient metabolism also drives glucose-induced ERK1/2 activation in hypothalamic neurons. The common metabolism-dependent mechanism would explain the finding that glucose regulates AMPK and ERK1/2 activities in opposing directions. However, besides ATP as the shared trigger to either activate or deactivate the two signaling molecules, the two signaling pathways do not seem to be in tandem but parallel, as an ERK1/2-specific inhibitor had no effect on AMPK phosphorylation (Fig. 6A) or vice versus (results not shown).

We used pharmacological tools to modulate ERK1/2 or AMPK activities and showed that both ERK1/2 and AMPK signaling pathways are required for glucose-regulated *POMC* expression. Because the inhibitors used do not show apparent toxic effects under the similar conditions used in other studies (Alessi *et al.* 1995, Mountjoy *et al.* 2007, Rahmouni *et al.* 2009) and the inhibitors behaved as expected in this study (i.e., either increasing or decreasing gene expression), it is unlikely that the results are artifacts. Our findings thus may provide

an explanation for earlier perplexing observations. For instance, *Pomc*2KO transgenic mice display a paradoxical phenotype of increased feeding and body weight (Claret *et al.* 2007). Here, we show that there are dual ERK1/2 and AMPK signaling pathways exerting opposite effects on *Pomc* expression. It is possible that a compensatory suppression of the ERK1/2 pathway leading to an over-inhibition of *Pomc* expression in *Pomc*2KO mice might have led to the discordant phenotypes, especially it has been suggested that hypothalamic circuits are biased towards weight gain (Schwartz *et al.* 2003, Morton *et al.* 2006). It is also attractive to speculate that ERK1/2 might participate in the glucose-regulated membrane potential change of POMC neurons. A clear role of AMPK in the electrical activity of basomedial hypothalamic glucose-excited (including *Pomc*-expressing) neurons (Mountjoy *et al.* 2007) could have been masked by the presence of ERK1/2 activities.

In this study, we showed the acute effect (1 h) of ERK1/2 on glucose-regulated *Pomc* expression. The phosphorylation of ERK1/2 and *Pomc* expression in DIV10 neurons also remained high after 6-h glucose treatment

(data not shown). However, whether ERK1/2 exerts a longer-term effect on *Pomc* expression remains to be examined. It is also not clear how ERK1/2 differentially mediates glucose-regulated orexigenic and anorexigenic neuropeptide expression. Because both *Pomc* and *Agrp/Npy* neurons express ERK1/2 (Morikawa *et al.* 2004, Rahmouni *et al.* 2009), it is possible that the difference may reside in their promoter regions. Two glucose-responsive elements (A and E boxes) in the insulin promoter drive the ERK1/2-mediated insulin expression in β -cells (Lawrence *et al.* 2008). Whether such DNA elements are present in the *Pomc* promoter (and cryptic/absent in the *Agrp/Npy* promoters) would be worth exploring.

In conclusion, using primary hypothalamic neurons and pharmacological tools we investigated the molecular mediators relaying glucose signals to the expression of feeding-related neuropeptides. Our data establish for the first time, to our knowledge, that AMPK mediates glucose-regulated *POMC* expression in manners as proposed. In addition, the results indicate ERK1/2 to be a novel co-mediator governing *POMC* expression. As the *in vitro* data is supported by the *in vivo* results, cultured hypothalamic neurons may have utility for studying hypothalamic nutrient sensing.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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