Original Article

Lowering glucose level elevates $[Ca^{2+}]_i$ in hypothalamic arcuate nucleus NPY neurons through P/Q-type Ca^{2+} channel activation and GSK3 β inhibition

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Aim: To identify the mechanisms underlying the elevation of intracellular Ca^{2+} level ($[Ca^{2+}]_i$) induced by lowering extracellular glucose in rat hypothalamic arcuate nucleus NPY neurons.

Methods: Primary cultures of hypothalamic arcuate nucleus (ARC) neurons were prepared from Sprague-Dawley rats. NPY neurons were identified with immunocytochemical method. $[Ca^{2+}]_i$ was measured using fura-2 AM. Ca^{2+} current was recorded using whole-cell patch clamp recording. AMPK and GSK3 β levels were measured using Western blot assay.

Results: Lowering glucose level in the medium (from 10 to 1 mmol/L) induced a transient elevation of $[Ca^{2+}]_i$ in ARC neurons, but not in hippocampal and cortical neurons. The low-glucose induced elevation of $[Ca^{2+}]_i$ in ARC neurons depended on extracellular Ca^{2+} , and was blocked by P/Q-type Ca^{2+} channel blocker ω -agatoxin TK (100 nmol/L), but not by L-type Ca^{2+} channel blocker nifedipine (10 μ mol/L) or N-type Ca^{2+} channel blocker ω -conotoxin GVIA (300 nmol/L). Lowering glucose level increased the peak amplitude of high voltage-activated Ca^{2+} current in ARC neurons. The low-glucose induced elevation of $[Ca^{2+}]_i$ in ARC neurons was blocked by the AMPK inhibitor compound C (20 μ mol/L), and enhanced by the GSK3 β inhibitor LiCl (10 mmol/L). Moreover, lowering glucose level induced the phosphorylation of AMPK and GSK3 β , which was inhibited by compound C (20 μ mol/L).

Conclusion: Lowering glucose level enhances the activity of P/Q type Ca^{2+} channels and elevates $[Ca^{2+}]_i$ level in hypothalamic arcuate nucleus neurons via inhibition of GSK3 β .

Keywords: hypothalamic arcuate nucleus; NPY neurons; glucose; calcium imaging; P/Q type Ca^{2+} channel; GSK3 β ; AMPK; whole-cell patch clamp

Acta Pharmacologica Sinica (2012) 33: 594–605; doi: 10.1038/aps.2012.17; published online 16 Apr 2012

Introduction

The prevalence of obesity, an important risk factor for the development of type 2 diabetes, is increasing dramatically, and the number of obese individuals worldwide is now projected to reach as many as 1.1 billion by 2030^[1,2]. Currently, however, the behavioral and physiological cues leading to imbalanced food intake and energy expenditure remains poorly defined.

Hypothalamic neuropeptide Y and agouti-related peptide (NPY/AgRP) neurons are critical in food intake and energy homeostasis^[3–5], especially in glucose homeostasis^[6] and it has

been reported that a decrease in extracellular glucose concentration increases the intracellular calcium signals in neurons isolated from arcuate nucleus (ARC) of rat, and 94% of them are immunoreactive for NPY^[7]. Thus ARC-NPY neurons are closely related to obesity and diabetes^[5,8]. Glucose sensing in hypothalamic NPY/AgRP neurons needs the activation of AMP-activated protein kinase (AMPK)^[9–12]. AMPK is activated when the AMP-to-ATP ratio increases^[9,13]. The knock-out of mice AMPK α 2 induces the responses to changes in extracellular glucose levels in NPY neurons^[13]. The change in action potential can be stimulated by AMPK activator AICAR and blocked by AMPK inhibitor compound C^[14–16]. Decreased glucose activates AMPK and elevates $[Ca^{2+}]_i$ in NPY neurons^[15]. The similar process is also observed by administration of many metabolic hormones, such as leptin, insulin and

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Received 2011-12-05 Accepted 2012-02-08

ghrelin to ARC NPY neurons^[17]. Our previous study showed that leptin, which inhibits AMPK in central nervous system^[12], can decrease the peak amplitude of high voltage-activated calcium currents in the isolated neurons from ARC^[18]. Therefore, the activation of AMPK is crucial in the change in excitability of ARC-NPY neurons. However, the possible role of AMPK activation in the regulation of ion channels in NPY neurons remains to be elucidated.

Calcium ions are probably the most widely used intracellular messengers, and voltage-gated calcium channels (VGCCs) play key role in regulating intracellular calcium concentration during depolarization^[19]. Being a state of energy deficit, decrease in extracellular glucose levels induces membrane depolarization and increases the firing rate of action potential in NPY neurons^[13]. But the underlying mechanism of glucose decrease induced intracellular elevation is still uncertain^[7]. Furthermore, the inhibition of AMPK-mediated GSK3 β inhibition has been found both in hepatic and cortical neuronal cells^[20–22]. In our previous study, it was found that GSK3 β phosphorylated P/Q-type calcium channel in hippocampal neurons^[23]. It is possible that AMPK may be involved in the depolarization due to calcium influx through VGCCs. However, little is known about the subtype of VGCC which contributes to the calcium elevation in ARC-NPY neurons.

In the present study, using calcium imaging to determine the source of glucose decrease induced $[Ca^{2+}]_i$ elevation and the signaling pathway, we provide evidence that AMPK activation increases glucose decrease-induced $[Ca^{2+}]_i$ response in ARC-NPY neurons via regulation of P/Q-type calcium channel by GSK3 β inhibition.

Materials and methods

Chemicals

DMEM/F12, Neurobasal and B27 supplement were obtained from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Fura-2 AM was obtained from biotium (Hayard, CA). Other general agents were available commercially. All the drugs were prepared as stock solutions. AICAR, compound C and Fura-2 AM were dissolved in dimethylsulfoxide (DMSO). These stock solutions were diluted to the final concentrations with the HEPES-buffered solution before application. The final concentration of DMSO or ethanol did not exceed 0.1%. No detectable effects of the vehicles were found in our experiments.

Preparation of single neurons from ARC

The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Huazhong University of Science and Technology. Primary cultures of ARC neurons were prepared as previously described with some modifications^[24]. Briefly, Sprague-Dawley rats, 2–4 d postnatal, were humanely killed by decapitation. After decapitation, brain slices con-

taining the entire ARC were prepared, and entire ARC was excised from the left and right sides. The dissected tissues were removed and transferred to PBS-buffered solution containing (in mmol/L) 135 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, pH 7.3 and finely chopped. The tissues were then treated with 0.125% trypsin in PBS-balanced salt solution for 20 min at 37°C and gently triturated using flame-polished Pasteur pipettes. Cell suspension was centrifuged for 7 min at 1000×g. Then, the cell pellets were resuspended in the Dulbecco's modified Eagle's medium (DMEM) and F-12 supplement (1:1) with 10% fetal bovine serum before plating onto glass-bottomed dishes (MatTek) coated with poly-L-lysine (20 μ g/mL for 1–2 h) and kept at 37°C in 5% CO₂ incubator. After overnight incubation in DMEM, the medium was changed to neurobasal medium (Gibco) containing 15 mmol/L glucose supplemented with 2% B27, 2 mmol/L glutamine, 10 μ g/mL penicillin, and 10 μ g/mL streptomycin. The ARC neurons were fed with fresh medium every 48 h. Microscopically, glial cells were not apparent in ARC neurons employing this protocol. The neurons were maintained for 7–10 d in primary culture until used for calcium imaging.

Immunocytochemical identification of single ARC neurons

The immunocytochemical identification of ARC neurons was prepared as previously reported with slight modifications^[25]. ARC neurons were fixed with 4% paraformaldehyde in 0.1 mol/L PBS overnight immediately after calcium imaging. They were pretreated with H₂O₂ in methanol for 1 h. Nonspecific binding sites were then blocked with 10% goat serum in 0.1 mol/L PBS for 1 h at room temperature. Cells were incubated overnight at 4°C with primary antiserum to NPY diluted 1:1000 in PBS containing 1.5% normal goat serum. Cells were subsequently incubated with biotinylated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The secondary antibody was then rinsed, and the sections were labeled with avidin-peroxidase complex reagent (ABC kit; Vector) for 1 h. The sections were developed with 3,3'-diaminobenzidine (DAB). In control sections, the primary antibodies were replaced by the corresponding nonspecific IgG and processed in parallel.

Ca²⁺ measurements

For $[Ca^{2+}]_i$ measurements, cultured ARC, hippocampus and cortical neurons were rinsed three times with HEPES-buffered solution containing (in mmol/L) 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES (pH 7.3). Then, cells were incubated with 2 μ mol/L Fura-2 AM for 30 min at 37°C and subsequent washed three times with HEPES to remove the excess extracellular Fura-2 AM. Coverslips of every group were mounted on a recording chamber after they were pretreated with corresponding tool agent (MK801, D-APV, CNQX, Nifedipine, ω -conotoxin GVIA, ω -agatoxin TK, Compound C, LiCl) of each group at final concentration for five minutes. Then, experiments were performed at room temperature. At the beginning of experiments, coverslips were perfused with HEPES-buffered solution with 10 mmol/L

glucose using a peristaltic pump at a rate of 3 mL/min till baseline was stable. After that, low glucose HEPES-buffered solution containing corresponding tool agent (the blocker of AMPA, NMDA, VGSC, and VGCCs) was administrated $[Ca^{2+}]_i$ changes were measured by a Ratio Vision digital fluorescence microscopy system (TILL Photonics GmbH, Germany). Fura-2 AM loaded cells were illuminated at 340 nm for 150 ms and 380 nm for 50 ms at 1 s intervals using a TILL Polychrome monochromator. Fura-2 fluorescence emission was imaged at 510 nm by an intensified cooled charge coupled device (TILL Photonics GmbH). F_{340}/F_{380} fluorescence ratios were generated by TILL VISION 4.0 software. The contours of a single neuron were used to define a region of interest (ROI) from which the mean fluorescence was measured. Paired F_{340}/F_{380} fluorescence ratio images of ROI were acquired every second. The intracellular free calcium concentration was presented as the ratio of the fluorescence signals obtained (340/380 nm). All experiments were repeated three times using different batches of cells.

Western blot analysis

The protocol of Western blot refers to our previous articles. Cultures were treated as described in results; 30 mg of intracellular proteins were loaded in each lane for comparison with β -actin as loading control. Following SDS-PAGE, the proteins were transferred to polyvinylidene difluoride membrane. Immunodetection was performed overnight at 4°C using rabbit polyclonal antibodies against each protein below: anti-phospho-AMPK α (Thr172; 1:1000; CST, USA), anti-AMPK α (1:1000; CST, USA). Monoclonal antibody (mAb) against total GSK-3 β (1:1000 for Western blotting, Abcam, Cambridge, UK), polyclonal antibody (pAb) against phosphorylated GSK-3 β at Ser9 (1:1000 for Western blotting, Abcam, Cambridge, UK), anti- β -actin (1:5000; Santa Cruz, CA, USA). Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit 1:10 000; Jackson ImmunoResearch) for 1 h at room temperature before signals were visualized using the SuperSignal West Pico ECL kit (Thermo Scientific, Rockford, IL, USA). Quantification was performed using Scion Image software (Fredrick, MD, USA). Results are presented as percentage of control after normalization to β -actin. The effect of experimental manipulations on AMPK phosphorylation was determined using one-way ANOVA.

Whole-cell patch-clamp recording

The procedures for whole-cell patch-clamp recording were according to previous research^[26, 27] and as what described in our previous reports with minor modification^[18, 28]. The bath solution for recording high-voltage activated calcium current (I_{HVA}) contained (in mmol/L): Choline-Cl 110, MgCl₂ 2, CaCl₂ 10, TEA-Cl 20, HEPES 10, glucose 10, and the pH was adjusted to 7.4 with Tris base. Glass pipettes were used with a resistance of about 2–4 M Ω when filled with the following solution (in mmol/L): CsF 64, CsCl 64, CaCl₂ 0.1, MgCl₂ 2, EGTA 10.0, HEPES 10.0, Tris-ATP 5.0, and the pH was adjusted to 7.2 with Tris base. After establishing a whole-cell configuration, the

adjustment of capacitance compensation and series resistance compensation were done before recording. The current signals were acquired at a sampling rate of 10 kHz and filtered at 3 kHz. Whole-cell patch-clamp recordings were carried out using an EPC-10 amplifier (HEKA, Lambrecht, Germany) driven by Pulse/PulseFit software (HEKA, Southboro, Germany). Drug actions were measured only after steady-state conditions were reached, which were judged by the amplitudes and time courses of currents remaining constant. All the recordings were made at room temperature (20–22°C). All experiments were repeated three times using different batches of cells and at least three to four dishes with cells were used for recording in different batches of cells.

Statistical analysis

The amplitude of $[Ca^{2+}]_i$ transient represents the difference between baseline concentration and the transient peak response to the stimulation. This amplitude is expressed in the percentage (%) change of F_{340}/F_{380} and calculated by $(R_{peak} - R_{basal})$ divided by R_{basal} . Statistical significance between the multiple groups was determined using one-way ANOVA followed by *post hoc* comparisons (SPSS 11.5 software). Data are presented as mean $\pm$ SEM. Differences at the $P < 0.05$ level were considered statistically significant.

Results

Morphological features of ARC-NPY neurons

The cells were fixed for immunocytochemical identification of NPY neurons immediately after the calcium imaging records were finished with the method as described before^[18, 29]. The NPY neurons are typically small and medium neurons with triangular or spindle-shaped perikaryons. Most of them have one to three slender, poorly ramified primary dendrites (Figure 1), which are consistent with the observations by van den^[25] (2004) and our previous work^[18, 29] (2008 and 2010).

Decreased extracellular glucose elevates $[Ca^{2+}]_i$ in ARC-NPY neurons

We first tested the effect of glucose decrease on intracellular Ca^{2+} mobilization in ARC-NPY neurons. Primary cultured rat ARC NPY neurons were loaded with Fura-2 AM and monitored by fluorescence imaging with excitation wavelengths of 340 and 380 nm. We found that a decrease in glucose concentration from 10 mmol/L to 1 mmol/L induced a transient increase in intracellular Ca^{2+} (Figure 2A) in 25% control neurons (37 of 146, Figure 2E). In contrast, this elevation can not be observed in cultured cortical and hippocampal neurons (Figure 2B and Figure 2C). Thus, our following research focused on these positive-reaction ARC-NPY neurons.

The decreased glucose-induced $[Ca^{2+}]_i$ response is extracellular calcium-dependent but not receptor-operated calcium channels (ROCC) dependent

We firstly applied the Ca^{2+} -free external solution to investigate the involvement of extracellular Ca^{2+} in this $[Ca^{2+}]_i$ elevation. Removal of extracellular Ca^{2+} suppressed the $[Ca^{2+}]_i$ to basal

Figure 1. The morphological features of NPY neurons in cultured ARC neurons. A and B are the immunocytochemical identification of morphological features of NPY neurons in cultured ARC neurons. C is morphological features in calcium imaging. The arrows point at a typical NPY neuron. A, C original magnification $\times 40$; B original magnification $\times 100$.

Figure 2. Decreased extracellular glucose-induced $[Ca^{2+}]_i$ elevation is hypothalamic specific effect. (A) An acute decrease in extracellular glucose concentration from 10 to 1 mmol/L induced an elevation of $[Ca^{2+}]_i$ in ARC-NPY neurons. (B) The similar $[Ca^{2+}]_i$ change could not be observed in hippocampal neurons. (C) The similar $[Ca^{2+}]_i$ change could not be observed in cortical neurons. (D) Histogram summarizes the effect of glucose decrease on arcuate nucleus-neuropeptide Y (ARC-NPY) neurons hippocampal neurons and cortical neurons. Mean $\pm$ SEM. F value is 53,676, $^*P < 0.01$ vs control. (E) 25.2% (37/146) ARC-NPY neurons responded to the decreased glucose concentration. PRN, positive response neurons.

levels (Figure 3B), indicating an extracellular calcium-dependent mechanism.

To determine whether receptor-operated calcium channels (ROCC) are involved in decreased glucose concentration-induced $[Ca^{2+}]_i$ response, we used NMDA receptor blocker MK801, D-APV and specific AMPA receptor blocker CNQX. After pretreated with one of the three blockers for 5 min, the ARC-NPY neurons were perfused with HEPES-buffered low glucose (1 mmol/L) in the presence of blockers. The basal level of $[Ca^{2+}]_i$ was elevated by $32.8\% \pm 3.6\%$ in MK801 (10 $\mu\text{mol/L}$)-treated group ($n=16$, Figure 3C), $39.2\% \pm 7.6\%$ in D-APV (20 $\mu\text{mol/L}$)-treated group ($n=9$, Figure 3D) and $28.7\% \pm 1.6\%$ in CNQX (50 $\mu\text{mol/L}$)-treated group ($n=29$, Figure 3E), respectively. There are no significant difference between control group and those groups (Figure 3F), suggesting that neither NMDA receptor nor AMPA receptor mediates the reduction of glucose concentration-induced $[Ca^{2+}]_i$ increase in ARC-NPY neurons.

The amplitude of HVA current, not LVA current, is augmented by glucose decrease

Next we want to know whether action potential is involved in glucose decrease induced calcium influx. We firstly used voltage-gated sodium channel blocker tetrodotoxin (TTX) to identify this possibility. We found that glucose decrease induced $[Ca^{2+}]_i$ increase could not be observed when TTX (5 $\mu\text{mol/L}$) existed (Figure 4A, 4B, and 4C). Thus, this glucose decrease induced $[Ca^{2+}]_i$ increase is probably related to the change of membrane potential. Therefore, we used patch clamp to identify the effect of glucose decrease on VGCC currents.

In the whole-cell patch-clamp recording for I_{HVA} , the cells were stepped from the holding potential of -80 mV to -40 mV (50 ms), and then depolarized to +10 mV (200 ms) after briefly hyperpolarized to -45 mV for 10 ms. I_{HVA} was activated by the second depolarization. The protocol was applied about every 40 s. Extracellular glucose decrease from 10 mmol/L to 1 mmol/L significantly augmented I_{HVA} amplitude in NPY

Figure 3. The $[\text{Ca}^{2+}]_i$ elevation in ARC-NPY neurons is extracellular calcium-dependent and is not via ROCC. (A) Representative experiment showing glucose decrease induced $[\text{Ca}^{2+}]_i$ elevation in ARC-NPY neurons. (B) $[\text{Ca}^{2+}]_i$ elevation of ARC-NPY neurons was inhibited in a Ca^{2+} -free external solution. (C) $[\text{Ca}^{2+}]_i$ elevation in ARC-NPY neurons could not be blocked by NMDA receptor blocker MK801 (10 $\mu\text{mol/L}$). (D) $[\text{Ca}^{2+}]_i$ elevation in ARC-NPY neurons could not be blocked by NMDA receptor blocker D-APV (20 $\mu\text{mol/L}$). (E) $[\text{Ca}^{2+}]_i$ elevation in ARC-NPY neurons could not be blocked by AMPA receptor blocker CNQX (50 $\mu\text{mol/L}$). (F) Histogram summarizes the effect of external calcium, MK801, D-APV, and CNQX on the amplitude of glucose decrease-induced $[\text{Ca}^{2+}]_i$ elevation in ARC-NPY neuron. Mean $\pm$ SEM. F value is 15.006, $^*P<0.01$ vs control.

neurons (Figure 4D, 4E, and 4H). The amplitude was about 1.44 ± 0.20 times ($n=15$, $P<0.05$) compared to control groups.

In the whole-cell patch-clamp recording for I_{LVA} , the cell membrane voltage was held at -90 mV, and whole cell currents were recorded in response to step pulses at different test potentials. I_{LVA} were elicited by voltage steps to -20 mV from a holding potential of -90 mV, and the amplitude of the peak current was measured for analysis. Extracellular glucose decrease from 10 mmol/L to 1 mmol/L did not significantly alter I_{LVA} amplitude in NPY neurons (Figure 4F, 4G, and 4H).

Blockade of P/Q-type calcium channel, not T-type, L-type, and N-type calcium channel, inhibits the decreased glucose-induced $[\text{Ca}^{2+}]_i$ response in ARC-NPY neurons

To identify which kind of VGCC is involved in the glucose decrease-induced $[\text{Ca}^{2+}]_i$ elevation, specific calcium channel blockers were used as described before^[27, 30-32]. Mibefradil (5 $\mu\text{mol/L}$), the blocker of T-type calcium channel, could not block the glucose decrease-induced $[\text{Ca}^{2+}]_i$ elevation. The amplitude of $[\text{Ca}^{2+}]_i$ elevation was $39.0\%\pm5.8\%$ ($n=8$, Figure 5B). Neither L-type calcium channel blocker nifedipine (10 $\mu\text{mol/L}$) nor N-type calcium channel blocker ω -conotoxin GVIA (300 nmol/L) inhibited the glucose decrease-induced $[\text{Ca}^{2+}]_i$ elevation. The basal level was increased by $31.8\%\pm6.3\%$ ($n=6$) and $26.1\%\pm2.9\%$ ($n=17$), respectively (Figure 5C and 5D). On the contrast, P/Q-type calcium channel blocker ω -agatoxin

TK (100 nmol/L) completely depressed $[\text{Ca}^{2+}]_i$ elevation in ARC-NPY neurons ($n=8$, Figure 5E, $P<0.01$ vs control). There is no significant difference between control group and those groups except ω -agatoxin TK group (Figure 5F). Thus, glucose decrease-induced $[\text{Ca}^{2+}]_i$ influx is via P/Q-type calcium channel.

AMPK activation contributes to $[\text{Ca}^{2+}]_i$ increase induced by decreased glucose in ARC-NPY neurons

To identify whether AMPK is activated in glucose decrease, the time dependent AMPK activation is tested 1, 2, and 5 min after glucose reduction in NPY neurons. By Western blot analysis, we found that decreasing glucose concentration from 10 to 1 mmol/L remarkably increased AMPK α -subunit phosphorylation in primary ARC neurons to $142\%\pm5.9\%$ ($n=3$, $P<0.05$) and $118\%\pm1.2\%$ ($n=3$, $P<0.05$ vs 10 mmol/L glucose) by 2 min and 5 min glucose decrease; and this increased phosphorylation could be abolished by pretreatment with Compound C (20 $\mu\text{mol/L}$), the AMPK inhibitor ($n=3$, $P<0.05$ vs 1 mmol/L glucose). However there was no significant difference between each group by 1 min treatment (Figure 6A, 6B, $n=3$). It suggests that AMPK is activated 2 min and 5 min after glucose decrease.

Using calcium imaging, we next determined whether AMPK activation mediated the $[\text{Ca}^{2+}]_i$ elevation induced by decreased glucose in ARC-NPY neurons. As shown in Figure 6D and

Figure 4. The amplitude of HVA current, not LVA current, is augmented by glucose decrease from 10 mmol/L to 1 mmol/L in ARC-NPY neurons. (A, B) $[\text{Ca}^{2+}]_i$ elevation of ARC-NPY neurons was inhibited by TTX (5 $\mu\text{mol/L}$). (C) Histogram summarizes the effect of TTX on the amplitude of glucose decrease-induced $[\text{Ca}^{2+}]_i$ elevation in ARC-NPY neuron. Mean $\pm$ SEM. $^{\circ}P<0.01$ vs control. (D) Representative traces of I_{HVA} under control, glucose decrease and washout. I_{HVA} were evoked by the protocol shown on the top. (E) Time-course curve of I_{HVA} before, during and after 1 mmol/L glucose, corresponding to the neuron showed in Figure 4D. (F) Representative traces of I_{LVA} under control, glucose decrease and washout. I_{LVA} were evoked by the protocol shown on the top. (G) Time-course curve of I_{LVA} before, during and after 1 mmol/L glucose, corresponding to the neuron showed in Figure 4F. (H) Histogram summarizes the amplitude change of currents induced by glucose decrease. (Mean $\pm$ SEM. I_{HVA} , $n=15$; I_{LVA} , $n=10$, $^bP<0.05$ vs control).

6E compound C (20 $\mu\text{mol/L}$) largely depressed $[\text{Ca}^{2+}]_i$ elevation from $28.7\%\pm1.6\%$ ($n=37$, Figure 6C) to $16.9\%\pm2.5\%$ ($n=14$, $P<0.05$ vs control). It indicates that AMPK activation is essential in glucose decrease induced $[\text{Ca}^{2+}]_i$ increase.

The inhibition effect of AMPK activation on GSK3 β mimics the glucose decrease induced $[\text{Ca}^{2+}]_i$ elevation in primary cultured ARC-NPY neurons

AMPK activation inhibits the activity of GSK3 β in the liver and epithelial cells^[20, 33, 34], next we want to reveal whether this inhibition also exists in the hypothalamus. After cultured ARC-NPY neurons were treated with AICAR (200 $\mu\text{mol/L}$), an AMPK activator for 2 min, the phosphorylation of GSK3 β was significantly increased ($n=3$, $P<0.01$ vs control); and this effect

could be blocked by compound C (20 $\mu\text{mol/L}$) ($n=3$, $P<0.01$ vs AICAR group, Figure 7A and 7B). Our previous study has shown that GSK3 β can regulate P/Q-type calcium channel via phosphorylation^[23]. Therefore, we investigated whether decreased glucose concentration from 10 to 1 mmol/L remarkably increased GSK3 β phosphorylation in primary ARC neurons by 1, 2, and 5 min treatment (Figure 7C). Similar to the result of AMPK phosphorylation, p-GSK3 β was significantly increased by $77.4\%\pm8.9\%$ and $67.4\%\pm2.5\%$ by 2 min and 5 min after glucose decrease ($n=3$, $P<0.05$ vs 10 mmol/L glucose, Figure 7D). There was no significant difference after 1 min treatment (Figure 7D, $n=3$). Additionally, inhibition of AMPK by compound C (20 $\mu\text{mol/L}$) could depress the increased p-GSK3 β induced by glucose decrease ($n=3$, $P<0.05$ vs 1

Figure 5. The $[\text{Ca}^{2+}]_i$ elevation of ARC-NT neurons is mediated by P/Q-type calcium channel. (A) Representative trace of control group. (B) $[\text{Ca}^{2+}]_i$ elevation of ARC-NT neurons could not be blocked by selective T-type calcium channel blocker Mibefradil (5 $\mu\text{mol/L}$). (C) $[\text{Ca}^{2+}]_i$ elevation of ARC-NT neurons could not be blocked by selective L-type calcium channel blocker nifedipine (10 $\mu\text{mol/L}$). (D) $[\text{Ca}^{2+}]_i$ elevation of ARC-NT neurons could not be blocked by N-type calcium channel blocker ω -conotoxin GVIA (300 nmol/L). (E) Selective P/Q-type calcium channel blocker ω -agatoxin TK (100 nmol/L) completely blocked the $[\text{Ca}^{2+}]_i$ elevation of ARC-NT neurons. (F) Histogram summarizes the effect of VGCC blockers on the amplitude of glucose decrease-induced $[\text{Ca}^{2+}]_i$ elevation in ARC-NT neuron. Mean $\pm$ SEM. F value is 12.825. ^b $P < 0.05$ vs control.

mmol/L glucose, Figure 7C and 7D). Thus we can find that GSK3 β is inhibited 2 min and 5 min after glucose decrease; furthermore AMPK activation is required in the inhibition of GSK3 β in this process.

Considering that glucose decrease induced GSK3 β inhibition, we therefore investigated whether AMPK activation contributed to GSK3 β inhibition in glucose decrease-induced $[\text{Ca}^{2+}]_i$ change in ARC-NT neurons. Lithium chloride can often be used to inhibit GSK3 β activity. In our experiment lithium (10 mmol/L) obviously increased $[\text{Ca}^{2+}]_i$ from $28.7\% \pm 1.6\%$ ($n=37$, Figure 7E) to $45.3\% \pm 14.6\%$ ($n=8$, Figure 7F). It suggests that AMPK activation contributes to GSK3 β inhibition in glucose decrease-induced $[\text{Ca}^{2+}]_i$ change in ARC-NT neurons (Figure 7G).

Discussion

In this study, it was demonstrated that P/Q-type calcium channel contributed to glucose decrease-induced $[\text{Ca}^{2+}]_i$ elevation via an AMPK-dependent manner, and GSK3 β inhibition facilitated P/Q-type calcium channel activity. Decreased glucose concentration activated cellular energy sensor AMPK, and, in turn, phosphorylated GSK3 β . Thus, phosphorylated GSK3 β alleviated its inhibition on P/Q-type calcium channel and induced $[\text{Ca}^{2+}]_i$ elevation in ARC-NT neurons.

Using $[\text{Ca}^{2+}]_i$ as a marker of neuronal excitability, our data shows that $[\text{Ca}^{2+}]_i$ in hypothalamic NPY neurons increases

when extracellular glucose concentration decreases from 10 mmol/L to 1 mmol/L, and up to 25% of all ARC neurons were excited and induced $[\text{Ca}^{2+}]_i$ elevation by decreasing concentrations of glucose. In contrast, neither hippocampal nor cortical neurons responded to decreased glucose over the same concentration range and induce the increase of $[\text{Ca}^{2+}]_i$, which shows that the effect is specific in glucose sensing neurons in hypothalamus. This is concordant with the previous report that the activity of cortical neurons becomes silent when glucose is reduced from 5 mmol/L to 0 mmol/L^[35].

In addition, this $[\text{Ca}^{2+}]_i$ elevation can be abolished via a Ca^{2+} free extracellular fluid perfusion. This result suggests an extracellular calcium-dependent mechanism. Thus, concerning depolarization of ARC-NT neurons induced by glucose decrease^[16, 36], we wondered that whether action potential is associated with this $[\text{Ca}^{2+}]_i$ elevation. The administration of TTX verified the $[\text{Ca}^{2+}]_i$ elevation is related to the change of membrane potential. Therefore, we hypothesized VGCC as a source of glucose decrease-induced $[\text{Ca}^{2+}]_i$ elevation in NPY neuron. Using whole cell patch clamp, we found that when extracellular glucose concentration decreases from 10 mmol/L to 1 mmol/L, the amplitude of I_{HVA} , but not I_{LVA} , was significantly augmented in ARC NPY neurons. Some previous studies demonstrate that N-type calcium channel may be involved in the regulation of hormones on rat arcuate nucleus^[37, 38]; our present results showed that P/Q-type calcium channel con-

Figure 6. The effect of AMPK activation mediates the elevation of $[Ca^{2+}]_i$ induced by glucose decrease in ARC-NPY neurons. (A) Representative immunoblot of total AMPK, and p-AMPK α in primary cultured ARC-NPY neurons after 1, 2, and 5 min treatment. p-AMPK α increased after 2 min treatment and this effect could be inhibited by compound C (20 μ mol/L). This change could not be observed after 1 min treatment. (B) The difference of p-AMPK α between each group was not significant when neurons were treated for 1 min ($n=3$). There was significant difference between 1 mmol/L glucose group and other group after 2 min treatment, while the difference between control group and compound C group was not significant. Immunoblots of p-AMPK α were measured using image J. The n of each group is 3, F value of 1 min is 2.461; 2 min, 7.005; 5 min, 4.673. ^b $P<0.05$ vs 10 mmol/L glucose. ^e $P<0.05$ vs 1 mmol/L glucose. (C) Representative trace of control group. (D) The response of NPY neuron to glucose decrease was largely depressed by compound C (20 μ mol/L). (E) The amplitude of $[Ca^{2+}]_i$ elevation of control ($28.7\pm8.5\%$, $n=37$) is different from compound C ($16.9\pm9.3\%$, $n=14$), ^b $P<0.05$ vs control.

tributed to the response of glucose decrease-induced effect. Subsequently, the $[Ca^{2+}]_i$ elevation will influence the downstream signaling molecules, such as CaMKK2, and finally regulate food intake^[39].

It has been reported that AMPK activation can simulate the effect of glucose decrease in NPY neurons^[14]. Since extracellular glucose concentrations decrease, the rate of glucose uptake^[40] and metabolism^[41] will fall. Then, the resultant increase in the AMP to ATP ratio is expected to activate AMPK^[42], which will in turn promote ATP synthesis and restore the AMP to ATP ratio. AMPK is activated when cells undergo an energy stress and it has been hypothesized that glycogen synthesis would be shut down by energy consuming processes through activation of AMPK in skeletal muscle^[43]. The later study provides evidence for GSK-3 β inhibition by AMPK activator, AICAR^[44]. In addition, AMPK can inactivate downstream GSK3 β through Ser⁹ phosphorylation both in direct^[20] and indirect^[45] ways. p-AMPK is elevated and GSK3 β is also inhibited via phosphorylation in ARC-NPY neurons facing glucose decrease. Although previous researches have reported the inhibitory effect of p-AMPK on GSK3 β , it is

intriguing to observe this hypothalamic regulation of glucose decrease on $[Ca^{2+}]_i$ of ARC-NPY neurons through this signaling pathway, and reveal the potential involvement of the inhibition effect of p-AMPK on GSK3 in food intake and energy homeostasis.

Lithium is used to inhibit GSK-3 β activity^[46]. And calcium imaging showed that lithium could largely enhance $[Ca^{2+}]_i$ elevation in ARC-NPY neurons, which evidenced a GSK3 β -dependent mechanism. As illustrated above, this glucose decrease-dependent $[Ca^{2+}]_i$ elevation is probably related to P/Q-type calcium channels. While in recent report, GSK3 β can phosphorylate the intracellular loop-connecting domains of P/Q-type calcium channels, which leads to a decrease of $[Ca^{2+}]_i$ elevation through the P/Q-type voltage-dependent calcium channel^[23]. Therefore, it is reasonable to attribute $[Ca^{2+}]_i$ elevation to de-inactivation of P/Q-type calcium channel (Figure 8). Compare to the relatively slow time-course change of Western blot, $[Ca^{2+}]_i$ elevation happened very fast (approximately 20–40 s), according calcium imaging, after lowering the glucose concentration. Although it seems complex, the glucose decrease induced $[Ca^{2+}]_i$ elevation may be the directly initiated by the

Figure 7. The inhibition effect of AMPK activation on GSK3β mimics the glucose decrease induced $[Ca^{2+}]_i$ elevation in primary cultured ARC-NPY neurons. (A) Representative immunoblot of total GSK3β, p-GSK3β in primary cultured ARC-NPY neurons 2 min after treated with AICAR (200 μmol/L). (B) The statistic of pGSK3β after AMPK activation. The level of p-GSK3β could be promoted by direct activation of AMPK by AICAR; and this effect could be blocked by compound C (20 μmol/L). $n=3$, F value of 1 min group is 30.27; 2 min, 38.82; 5 min, 33.08. $^cP<0.01$ vs control, $^fP<0.01$ vs AICAR group. (C) Representative immunoblot of total GSK3β, p-GSK3β in primary cultured ARC-NPY neurons by 1, 2, and 5 min treatment separately. The level of p-GSK3β can be inhibited by compound C and can be promoted by glucose decrease after 2 min treatment. (D) The statistic of p-GSK3β at 1, 2, and 5 min time points. There is significant difference between the level of p-GSK3β of control group and other groups after 2 min treatment. Immunoblots of p-GSK3β and GSK3β were measured using image J. $^bP<0.05$ vs 10 mmol/L glucose. $^eP<0.05$ vs 1 mmol/L glucose. (E) Representative trace of control group. (F) The response of NPY neuron to glucose decrease was enhanced by lithium chloride. (G) The amplitude of $[Ca^{2+}]_i$ elevation of control ($28.7\% \pm 8.5\%$, $n=37$) is different from lithium ($45.3\% \pm 14.6\%$, $n=8$). $^bP<0.05$ vs control.

depolarization of neurons and the subsequent AMPK activation can be the potent promoter of P/Q-type calcium channel opening.

The increase of $[Ca^{2+}]_i$ reflects the excitation of neuron. Glucose-inhibited neurons are always excited when glucose concentration decrease to a certain level. In previous report, the *in vivo* glucose concentration to which hypothalamic glucose-inhibited neurons are exposed is still controversial. In early studies about hypothalamic glucose-sensing, the extracellular glucose levels in the brain were measured at different plasma

glucose concentrations. The brain glucose level varies from 1 to 2.5 mmol/L when the plasma glucose is altered from 5 to 8 mmol/L in normoglycemia^[47]. When plasma glucose level is at 15–17 mmol/L in hyperglycemia, brain concentrations are at 4.5 mmol/L^[48]. While all those researches focused on glucose-excited neuron to illustrate a mechanism of glucose-increase excitation. Therefore, in most hypothalamic neuron culture protocol, neurons were cultured in a relatively high glucose surroundings depending on different medium used in experiments^[15, 49]. Moreover, it has been argued that the hypo-

Figure 8. The summary diagrams of current studies and proposed model. Schematic of glucose decrease induced hypothalamic ARC-NPY neurons intracellular calcium elevation. The activation of AMPK inhibited GSK3 β , and subsequently alleviated the inhibition of GSK3 β on P/Q type calcium channel and lastly increased calcium influx through P/Q type calcium channel.

thalamal blood-brain barrier is deficient to allow hormones pass^[50], so that hypothalamus might be exposed to the levels closer to those measured in the plasma. Therefore, to avoid the disturbance of a dramatic change of extracellular fluid on neurons which are to be tested, we used a relatively acceptable glucose level of 10 mmol/L for incubation and the low limit of perfusion fluid is 1.0 mmol/L in the range as reported^[48, 51, 52].

To maintain the glucose concentration in a certain scale, the regulation of daily change of glucose concentration is important. Therefore, the regulation of ARC-NPY excitability is crucial, which underlies the mechanism of food intake regulation and energy homeostasis^[25, 53]. As a feature of central neurons, electrophysiology activity is an intrinsic character of hypothalamic ARC-NPY neurons. Therefore, their excitation is undoubtedly regulated by ion channel especially calcium channels. And calcium is important for ARC-NPY neurons' activity as a second messenger^[37]. Thus, we focused on calcium channels to illustrate their role in hypothalamic metabolism homeostasis. In our results, GSK3 β and P/Q-type calcium channel integrate energy sensing signaling to hypothalamic electrophysiology activity. Although this P/Q-type calcium channel-based mechanism contributes to the glucose-decrease dependent $[Ca^{2+}]_i$ increase, we still can't exclude the possibility of calcium-induced calcium release. Accordingly, it will be the next step for us to identify the intracellular mechanism.

Conclusion

In summary, we showed that P/Q-type calcium channel contributes to glucose-decrease induced $[Ca^{2+}]_i$ increase in hypothalamic ARC-NPY neurons. This process is dependent on the activation of AMPK, and its inhibitory effect on GSK3 β contributes to the attenuation of the blockade on P/Q-type calcium channels. These results directly link VGCCs to glucose sensing. Our work opens up the possibility of using

modulators of ion channels to control energy homeostasis and the conditions of aberrant food intake such as obesity and diabetes.

Acknowledgements

This work was supported by the grants from the National Basic Research Program of China (973 Program) (No 2007CB507404), the Chang Jiang Scholar Program of the Ministry of Education of China, the Key Project of NSFC (No 30930104) to Dr Jian-guo CHEN, and NSFC (No 30801390) to Dr Li-hong LONG. It was also supported by the Program for New Century Excellent Talents in Universities of China (NCET-08-0225) to Dr Fang WANG.

Author contribution

Yu CHEN and Li-hong LONG designed research; Yu CHEN, Jun ZHOU, Chao HUANG, and Jun-qi ZHANG performed research; Lan NI and You JIN contributed new reagents or analytic tools; Yu CHEN, Na XIE, and Zhuang-li HU analyzed data; Yu CHEN and Li-hong LONG wrote the paper; Fang WANG and Jian-guo CHEN critically revised the manuscript.

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