Lowering glucose level elevates $[\text{Ca}^{2+}]_{i}$ in hypothalamic arcuate nucleus NPY neurons through P/Q-type Ca$^{2+}$ channel activation and GSK3β inhibition

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Aim: To identify the mechanisms underlying the elevation of intracellular Ca$^{2+}$ level ($[\text{Ca}^{2+}]_{i}$) 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. $[\text{Ca}^{2+}]_{i}$ was measured using fura-2 AM. Ca$^{2+}$ current was recorded using whole-cell patch clamp recording. AMPK and GSK3β 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 $[\text{Ca}^{2+}]_{i}$ in ARC neurons, but not in hippocampal and cortical neurons. The low-glucose induced elevation of $[\text{Ca}^{2+}]_{i}$ 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 µmol/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 $[\text{Ca}^{2+}]_{i}$ in ARC neurons was blocked by the AMPK inhibitor compound C (20 µmol/L), and enhanced by the GSK3β inhibitor LiCl (10 mmol/L). Moreover, lowering glucose level induced the phosphorylation of AMPK and GSK3β, which was inhibited by compound C (20 µmol/L).

Conclusion: Lowering glucose level enhances the activity of P/Q type Ca$^{2+}$ channels and elevates $[\text{Ca}^{2+}]_{i}$ level in hypothalamic arcuate nucleus neurons via inhibition of GSK3β.

Keywords: hypothalamic arcuate nucleus; NPY neurons; glucose; calcium imaging; P/Q type Ca$^{2+}$ channel; GSK3β; AMPK; whole-cell patch clamp
ghrelin to ARC NPY neurons\textsuperscript{[17]}. Our previous study showed that leptin, which inhibits AMPK in central nervous system\textsuperscript{[12]}, can decrease the peak amplitude of high voltage-activated calcium currents in the isolated neurons from ARC\textsuperscript{[19]}. 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\textsuperscript{[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\textsuperscript{[13]}. But the underlying mechanism of glucose decrease induced intracellular elevation is still uncertain\textsuperscript{[7]}. Furthermore, the inhibition of AMPK-mediated GSK3β inhibition has been found both in hepatic and cortical neuronal cells\textsuperscript{[20–22]}. In our previous study, it was found that GSK3β phosphorylated P/Q-type calcium channel in hippocampal neurons\textsuperscript{[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\textsuperscript{2+}], elevation and the signaling pathway, we provide evidence that AMPK activation increases glucose decrease-induced [Ca\textsuperscript{2+}], response in ARC-NPY neurons via regulation of P/Q-type calcium channel by GSK3β 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\textsuperscript{[24]}. Briefly, Sprague-Dawley rats, 2–4 d postnatal, were humanely killed by decapitation. After decapitation, brain slices containing 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 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 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 μg/mL for 1–2 h) and kept at 37°C in 5% CO\textsubscript{2} 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 μg/mL penicillin, and 10 μg/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\textsuperscript{[25]}. ARC neurons were fixed with 4% paraformaldehyde in 0.1 mol/L PBS overnight immediately after calcium imaging. They were pretreated with H\textsubscript{2}O\textsubscript{2} in methanol for 1 h. Nonspecific binding sites were then blocked with 10% goat serum. Cells were subsequently incubated with biotinated 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 antibody was then replaced by the corresponding nonspecific IgG and processed in parallel.

**Ca\textsuperscript{2+} measurements**

For [Ca\textsuperscript{2+}], measurements, cultured ARC, hippocampus and cortical neurons were rinsed three times with HEPES-buffered solution containing (in mmol/L) 140 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 glucose and 10 HEPES (pH 7.3). Then, cells were incubated with 2 μmol/L Fura-2 AM for 30 min at 37°C and subsequent washed three times with HEPES to remove the 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 of DMSO or ethanol did not exceed 0.1%. No detectable effects of the vehicles were found in our experiments.
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\textsuperscript{2+}], changes were measured by a Ratio Vision digital fluorescence microscope 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 \( \beta \)-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: anti-phospho-AMPK\( \alpha \) (Thr172; 1:1000; CST, USA), anti-AMPK\( \alpha \) (1:1000; CST, USA). Monoclonal antibody (mAb) against total GSK-3\( \beta \) (1:1000 for Western blotting, Abcam, Cambridge, UK), polyclonal antibody (pAb) against phosphorylated GSK-3\( \beta \) at Ser9 (1:1000 for Western blotting, Abcam, Cambridge, UK), anti-\( \beta \)-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 \( \beta \)-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\textsuperscript{[26, 27]} and as what described in our previous reports with minor modification\textsuperscript{[18, 29]}. The bath solution for recording high-voltage activated calcium current (Ik,V)\textsuperscript{re} contained (in mmol/L): Choline-Cl 110, MgCl\textsubscript{2} 2, CaCl\textsubscript{2} 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\( \Omega \) when filled with the following solution (in mmol/L): CsF 64, CsCl 64, CaCl\textsubscript{2} 0.1, MgCl\textsubscript{2} 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, Southburo, 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 \([\text{Ca}^{2+}]\), 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 \( (R_{\text{peak}}-R_{\text{basal}}) \) divided by \( R_{\text{basal}} \). 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\textsuperscript{[18, 29]} and our previous work\textsuperscript{[18, 29]} (2008 and 2010). 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\textsuperscript{[25]} (2004) and our previous work\textsuperscript{[18, 29]} (2008 and 2010).

**Decreased extracellular glucose elevates \([\text{Ca}^{2+}]\), in ARC-NPY neurons**

We first tested the effect of glucose decrease on intracellular \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) (Figure 2A) in 25% control neurons (37 of 146, Figure 2E). In contrast, this elevation can 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 \([\text{Ca}^{2+}]\), response is extracellular calcium-dependent but not receptor-operated calcium channels (ROCC) dependent

We firstly carried out the \( \text{Ca}^{2+} \)-free external solution to investigate the involvement of extracellular \( \text{Ca}^{2+} \) in this [\( \text{Ca}^{2+} \)], elevation. Removal of extracellular \( \text{Ca}^{2+} \) suppressed the [\( \text{Ca}^{2+} \)], to basal
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\%±3.6\% in MK801 (10 μmol/L)-treated group (n=16, Figure 3C), 39.2\%±7.6\% in D-APV (20 μmol/L)-treated group (n=9, Figure 3D) and 28.7\%±1.6% in CNQX (50 μmol/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\(^{2+}\)] 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 reduced [Ca\(^{2+}\)] increase could not be observed when TTX (5 μmol/L) existed (Figure 4A, 4B, and 4C). Thus, this glucose decrease induced [Ca\(^{2+}\)] 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 \(I_{\text{HVA}}\) 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. \(I_{\text{HVA}}\) 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 \(I_{\text{HVA}}\) amplitude in NPY neurons.

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**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 ×40; B original magnification ×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 and cortical neurons. Mean±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.
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 $[Ca^{2+}]_i$ response in ARC-NPY neurons

To identify which kind of VGCC is involved in the glucose decrease-induced $[Ca^{2+}]_i$ elevation, specific calcium channel blockers were used as described before [27, 30–32]. Mibebradil (5 μmol/L), the blocker of T-type calcium channel, could not block the glucose decrease-induced $[Ca^{2+}]_i$ elevation. The amplitude of $[Ca^{2+}]_i$ elevation was 39.0%±5.8% ($n=8$, Figure 5B). Neither L-type calcium channel blocker nifedipine (10 μmol/L) nor N-type calcium channel blocker ω-conotoxin GVIA (300 nmol/L) inhibited the glucose decrease-induced $[Ca^{2+}]_i$ elevation. The basal level was increased by 31.8%±6.3% ($n=6$) and 26.1%±2.9% ($n=17$), respectively (Figure 5C and 5D).

AMPK activation contributes to $[Ca^{2+}]_i$ 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 AMPKα-subunit phosphorylation in primary ARC neurons to 142%±5.9% ($n=3$, $P<0.05$) and 118%±1.2% ($n=3$, $P<0.05$ 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 μmol/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 $[Ca^{2+}]_i$ elevation induced by decreased glucose in ARC-NPY neurons. As shown in Figure 6D and
6E compound C (20 μmol/L) largely depressed [Ca\(^{2+}\)]\(_i\) elevation from 28.7%±1.6% (\(n=37\), Figure 6C) to 16.9%±2.5% (\(n=14\), \(P<0.05\) vs control). It indicates that AMPK activation is essential in glucose decrease induced [Ca\(^{2+}\)]\(_i\) increase.

The inhibition effect of AMPK activation on GSK3β mimics the glucose decrease induced [Ca\(^{2+}\)]\(_i\) elevation in primary cultured ARC-NPY neurons

AMPK activation inhibits the activity of GSK3β 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 μmol/L), an AMPK activator for 2 min, the phosphorylation of GSK3β was significantly increased (\(n=3\), \(P<0.01\) vs control); and this effect could be blocked by compound C (20 μmol/L) (\(n=3\), \(P<0.01\) vs AICAR group, Figure 7A and 7B). Our previous study has shown that GSK3β 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 GSK3β phosphorylation in primary ARC neurons by 1, 2, and 5 min treatment (Figure 7C). Similar to the result of AMPK phosphorylation, p-GSK3β was significantly increased by 77.4%±8.9% and 67.4%±2.5% 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 μmol/L) could depress the increased p-GSK3β induced by glucose decrease (\(n=3\), \(P<0.05\) vs control).

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) [Ca\(^{2+}\)]\(_i\) elevation of ARC-NPY neurons was inhibited by TTX (5 μmol/L). (C) Histogram summarizes the effect of TTX on the amplitude of glucose decrease-induced [Ca\(^{2+}\)]\(_i\) elevation in ARC-NPY neuron. Mean±SEM. \(^{1}\)P<0.01 vs control. (D) Representative traces of \(I_{\text{HVA}}\) under control, glucose decrease and washout. \(I_{\text{HVA}}\) were evoked by the protocol shown on the top. (E) Time-course curve of \(I_{\text{HVA}}\) before, during and after 1 mmol/L glucose, corresponding to the neuron showed in Figure 4D. (F) Representative traces of \(I_{\text{LVA}}\) under control, glucose decrease and washout. \(I_{\text{LVA}}\) were evoked by the protocol shown on the top. (G) Time-course curve of \(I_{\text{LVA}}\) 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±SEM. \(I_{\text{HVA}}\), \(n=15\); \(I_{\text{LVA}}\), \(n=10\), \(^{b}\)P<0.05 vs control).
mmol/L glucose, Figure 7C and 7D). Thus we can find that GSK3β is inhibited 2 min and 5 min after glucose decrease; furthermore AMPK activation is required in the inhibition of GSK3β in this process.

Considering that glucose decrease induced GSK3β inhibition, we therefore investigated whether AMPK activation contributed to GSK3β inhibition in glucose decrease-induced [Ca^{2+}]_i change in ARC-NPY neurons. Lithium chloride can often be used to inhibit GSK3β activity. In our experiment lithium (10 mmol/L) obviously increased [Ca^{2+}]_i from 28.7%±1.6% (n=37, Figure 7E) to 45.3%±14.6% (n=8, Figure 7F). It suggests that AMPK activation contributes to GSK3β inhibition in glucose decrease-induced [Ca^{2+}]_i, change in ARC-NPY neurons (Figure 7G).

**Discussion**

In this study, it was demonstrated that P/Q-type calcium channel contributed to glucose decrease-induced [Ca^{2+}]_i elevation via an AMPK-dependent manner, and GSK3β inhibition facilitated P/Q-type calcium channel activity. Decreased glucose concentration activated cellular energy sensor AMPK, and, in turn, phosphorylated GSK3β. Thus, phosphorylated GSK3β alleviated its inhibition on P/Q-type calcium channel and induced [Ca^{2+}]_i, elevation in ARC-NPY neurons.

Using [Ca^{2+}]_i as a marker of neuronal excitability, our data shows that [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 [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 [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 [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-NPY induced by glucose decrease[16, 36] we wondered that whether action potential is associated with this [Ca^{2+}]_i elevation. The administration of TTX verified the [Ca^{2+}]_i elevation is related to the change of membrane potential. Therefore, we hypothesized VGCC as a source of glucose decrease-induced [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-
tributed to the response of glucose decrease-induced effect. Subsequently, the \([Ca^{2+}]i\) elevation will influence the downstream signaling molecules, such as CaMKII, 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\(^{45}\). The later study provides evidence for GSK-3\(\beta\) inhibition by AMPK activator, AICAR\(^{46}\). In addition, AMPK can inactivate downstream GSK3\(\beta\) through Ser\(^9\) phosphorylation both in direct\(^{29}\) and indirect\(^{45}\) ways. p-AMPK is elevated and GSK3\(\beta\) 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\(\beta\), 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\(\beta\) activity\(^{46}\). And calcium imaging showed that lithium could largely enhance \([Ca^{2+}]i\) elevation in ARC-NPY neurons, which evidenced a GSK3\(\beta\)-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\(\beta\) 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 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\(\alpha\) in primary cultured ARC-NPY neurons after 1, 2, and 5 min treatment. p-AMPK\(\alpha\) increased after 2 min treatment and this effect could be inhibited by compound C (20 \(\mu\)mol/L). This change could not be observed after 1 min treatment. (B) The difference of p-AMPK\(\alpha\) 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\(\alpha\) 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. *\(P<0.05\) vs 10 mmol/L glucose. \(b\ P<0.05\) 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 \(\mu\)mol/L). (E) The amplitude of \([Ca^{2+}]i\) elevation of control (28.7\%±8.5\%, n=37) is different from compound C (16.9\%±9.3\%, n=14), *\(P<0.05\) 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 \([\text{Ca}^{2+}]\), 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 7. The inhibition effect of AMPK activation on GSK3\(\beta\) mimics the glucose decrease induced \([\text{Ca}^{2+}]\) elevation in primary cultured ARC-NPY neurons. (A) Representative immunoblot of total GSK3\(\beta\), p-GSK3\(\beta\) in primary cultured ARC-NPY neurons 2 min after treated with AICAR (200 \(\mu\)mol/L). (B) The statistic of p-GSK3\(\beta\) after AMPK activation. The level of p-GSK3\(\beta\) could be promoted by direct activation of AMPK by AICAR; and this effect could be blocked by compound C (20 \(\mu\)mol/L). \(n=3\), F value of 1 min group is 30.27; 2 min, 38.82; 5 min, 33.08. \(^{b}P<0.01\) vs control, \(^{f}P<0.01\) vs AICAR group. (C) Representative immunoblot of total GSK3\(\beta\), p-GSK3\(\beta\) in primary cultured ARC-NPY neurons by 1, 2, and 5 min treatment separately. The level of p-GSK3\(\beta\) can be inhibited by compound C and can be promoted by glucose decrease after 2 min treatment. (D) The statistic of p-GSK3\(\beta\) at 1, 2, and 5 min time points. There is significant difference between the level of p-GSK3\(\beta\) of control group and other groups after 2 min treatment. Immunoblots of p-GSK3\(\beta\) and GSK3\(\beta\) were measured using image J. \(^{c}P<0.05\) vs 10 mmol/L glucose. \(^{e}P<0.05\) vs 1 mmol/L glucose. (E) Representative trace of NPY neuron to glucose decrease was enhanced by lithium chloride. (F) The amplitude of \([\text{Ca}^{2+}]\) elevation of control (28.7\(\pm\)8.5\%, \(n=37\)) is different from lithium (45.3\(\pm\)14.6\%, \(n=8\)). \(^{a}P<0.05\) vs control.
perfusion fluid is 1.0 mmol/L in the range as reported glucose level of 10 mmol/L for incubation and the low limit of neurons which are to be tested, we used a relatively acceptable the disturbance of a dramatic change of extracellular fluid on closer to those measured in the plasma. Therefore, to avoid regulation and energy homeostasis. As a feature of central crucial, which underlies the mechanism of food intake regu-
ation is undoubtedly regulated by ion channel especially
calcium channels. And calcium is important for ARC-NPY
of hypothalamic ARC-NPY neurons. Therefore, their exci-
tation is undoubtedly regulated by ion channel especially
calcium channels. And calcium is important for ARC-NPY
neurons’ activity as a second messenger. Thus, we focused
on calcium channels to illustrate their role in hypothalamic
metabolism homeostasis. In our results, GSK3β and P/Q-type
calcium channel integrate energy sensing signaling to hypo-
thalamic electrophysiology activity. Although this P/Q-type
calcium channel-based mechanism contributes to the glucose-
decrease dependent [Ca2+]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 mecha-
nism.

Conclusion
In summary, we showed that P/Q-type calcium channel
contributes to glucose-decrease induced [Ca2+]i increase in
hypothalamic ARC-NPY neurons. This process is dependent
on the activation of AMPK, and its inhibitory effect on GSK3β
contributes to the attenuation of the blockade on P/Q-type
calcium channels. These results directly link VGCCs to glu-
cose 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 dia-
betes.

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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
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