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Arcuate Na⁺,K⁺-ATPase senses systemic energy states and regulates feeding behavior through glucose-inhibited neurons

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Kurita H, Xu KY, Maejima Y, Nakata M, Dezaki K, Santoso P, Yang Y, Arai T, Gantulga D, Muroya S, Lefor AK, Kakei M, Watanabe E, Yada T. Arcuate Na⁺,K⁺-ATPase senses systemic energy states and regulates feeding behavior through glucose-inhibited neurons. Am J Physiol Endocrinol Metab 309: E320–E333, 2015. — Feeding is regulated by perception in the hypothalamus, particularly the first-order arcuate nucleus (ARC) neurons, of the body’s energy state. However, the cellular device for converting energy states to the activity of critical neurons in ARC is less defined. We here show that Na⁺,K⁺-ATPase (NKA) in ARC senses energy states to regulate feeding. Fasting-induced systemic ghrelin rise and glucose lowering reduced ATP-hydrolyzing activity of NKA and its substrate ATP level, respectively, preferentially in ARC. Lowering glucose concentration (LG), which mimics fasting, decreased intracellular NAD(P)H and increased Na⁺ concentration in single ARC neurons that subsequently exhibited [Ca²⁺]i responses to LG, showing that they were glucose-inhibited (GI) neurons. Third ventricular injection of the NKA inhibitor ouabain induced c-Fos expression in agouti-related protein (AgRP) neurons in ARC and evoked neuropeptide Y (NPY)-dependent feeding. When injected focally into ARC, ouabain stimulated feeding and mRNA expressions for NPY and AgRP. Ouabain increased [Ca²⁺], in single NPY/AgRP neurons with greater amplitude than in proopiomelanocortin neurons in ARC. Conversely, the specific NKA activator SSA412 suppressed fasting-induced feeding and LG-induced [Ca²⁺]i increases in ARC GI neurons. NPY/AgRP neurons highly expressed NKAα3, whose knockdown impaired feeding behavior. These results demonstrate that fasting, via ghrelin rise and LG, suppresses NKA enzyme/pump activity in ARC and thereby promotes the activation of GI neurons and NPY/AgRP-dependent feeding. This study identifies ARC NKA as a hypothalamic sensor and converter of metabolic states to key neuronal activity and feeding behaviour, providing a new target to treat hyperphagic obesity and diabetes.

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Arcuate nucleus; Na⁺,K⁺-ATPase; α3-isofrom; neuropeptide Y; agouti-related protein; feeding behavior; SSA412; ouabain; fasting; ghrelin; glucose; ATP; glucose-inhibited neuron

THE FIRST-ORDER NEURONS in the arcuate nucleus (ARC) of the hypothalamus perceive the nutrients and hormones that reflect the systemic energy state and thereby control feeding behavior (2, 10, 32, 34). These neurons are likely equipped with a device that senses these metabolic signals and couples them to neuronal activation. Candidate molecules have been proposed, which include Na⁺,K⁺-ATPase (NKA) (30), ATP-sensitive K⁺ channel (37), long-chain fatty acyl-CoA (LC-CoA) (21, 23), and AMP-activated protein kinase (AMPK) (21, 27). Oomura et al. first proposed NKA as the molecule that converts lowering of the extracellular glucose level to the neuronal excitability in the glucose-sensitive or glucose-inhibited (GI) neurons in the lateral hypothalamic area (LHA) (29, 30). GI neurons were later also found in the ventromedial hypothalamus (VMH) and ARC (9, 28, 31). NKA is regulated by the level of ATP concentration, which reflects the energy state. One operation of NKA molecule hydrolyzes one ATP molecule for pumping three Na⁺ out of and two K⁺ into the cell, exhibiting the enzyme and pump (enzyme/pump) activity. This reaction hyperpolarizes the plasma membrane (39, 42). Hence, suppression of the electrogenic NKA is capable of depolarizing the membrane potential to increase neuronal excitability. However, the role of NKA in regulation of feeding remains unclear.

This study aimed to clarify the role of NKA in ARC in sensing energy states and regulating feeding. We found that fasting, partly via systemic ghrelin rise and glucose lowering, reduced ouabain-sensitive ATP-hydrolyzing activity of NKA and its substrate ATP level preferentially in ARC. Lowering glucose concentration (LG) decreased NAD(P)H level and increased cytosolic Na⁺ concentration ([Na⁺]i) in GI neurons of ARC. Intra-ARC injection of the NKA inhibitor ouabain induced feeding, and ouabain increased cytosolic calcium concentration ([Ca²⁺]i) in ARC neurons expressing neuropeptide Y (NPY) and agouti-related protein (AgRP). Inversely, intra-ARC injection of the specific NKA activator SSA412 (42)
suppressed feeding, and SSA412 inhibited LG-induced [Ca^{2+}]; increases in GI neurons. ARC AgRP neurons highly expressed NKA α3 isoform, whose knockdown with shRNA impaired feeding behavior.

MATERIALS AND METHODS

Materials. NKA activator SSA412 is a polyclonal antibody generated against the extracellular \textsuperscript{89}DVDSYGQWQTYEQR\textsuperscript{91} region of rat α-subunit of NKA and is further purified through an affinity column directed against the same synthetic peptide (42). SSA412 increases the ATP-hydrolyzing activity of NKA and consequently modulates intracellular Ca^{2+} transients (42). Western blots show that SSA412 recognizes the α-subunit of three isoforms of NKA. Oubain was from Wako Pure Chemical Industries (Osaka, Japan). Ghrelin was from Peptide Institute (Osaka, Japan). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. Adult male Sprague-Dawley (SD) rats were purchased from Japan SLC (Hamamatsu, Japan) and were maintained on a 12:12-h light-dark cycle and given conventional food (CE-2; CLEA Japan, Tokyo, Japan) and water ad libitum. The animal protocols for this study were approved by the Animal Care and Use Committee of Jichi Medical University.

Measurements of NKA’s ATP-hydrolyzing activity and ATP content in the hypothalamus and in vitro slices. For in vivo assays, bilateral portions of ARC, LHA, and VMH were dissected from rats aged 8 wk under ad libitum-fed or 24-h-fasted conditions. For in vitro assays, coronal brain slices containing ARC were prepared from rats aged 5 wk by using a microslicer. Bilateral ARC portions were dissected from slices, incubated in HEPES-buffered Krebs–Ringer bicarbonate buffer (pH 7.5) solution containing 0.5, 2.8, or 8.3 mM glucose for 10 min at 37°C. The ATPase assay was performed by the method of Kyte (20), with modifications as previously reported (42). The ATP-hydrolyzing activity of NKA was defined as the ouabain-sensitive hydrolysis of Mg^{2+}-ATP (3 mM) in the presence of both Na^{+} (100 mM) and K^{+} (20 mM) with or without ouabain (2 mM). Briefly, the incubation mixture containing samples in a final volume of 0.2 ml was maintained at 37°C, and reaction was initiated by adding Mg^{2+}-ATP and was stopped after 30 min by adding 0.75 ml of quench solution and 0.02 ml of developer. The color was allowed to develop for 30 min at room temperature. The free phosphate was then determined by reaction with molybdate, giving a product measured by spectrophotometer at 700 nm. For ATP content assay, samples were homogenized in 10 mM HEPES buffer (pH 7.5) and incubated with 100 μM of lysis buffer (Toyo B-net, Tokyo, Japan). The luminescence of each sample was measured using Luminoimage Analyzer LAS-1000 (Fuji Film, Tokyo, Japan) and normalized to the DNA content extracted from each homogenized solution.

Western blotting. Bilateral portions of ARC were dissected from ad libitum-fed or 24-h-fasted rats and lysed. The 5 μg of proteins were subjected to 10% SDS-PAGE and transferred to nitrocellulose filters. NKA-α1, -α2, and -α3 isoforms were detected with specific antibodies (Table 1). Immunoreactive proteins were detected with HRP-conjugated secondary antibody and the ECL system (Amersham, Arlington Heights, IL). The luminescence of each sample was calculated by Luminoimage Analyzer. Protein levels were normalized to those of β-actin from the same samples. β-Actin was detected with goat anti-β-actin polyclonal antibody (C-11) (sc-1615; Santa Cruz Biotechnology, Santa Cruz, CA).

Measurements of plasma ghrelin and blood glucose concentrations. Blood was sampled from the tails of 10-wk-old rats under ad libitum-fed and 24-h-fasted conditions in the same animals. Blood glucose and plasma ghrelin (acylated ghrelin) concentrations were measured using a GlucoCard DIA meter (Arkray, Kyoto, Japan) and enzyme-linked immunosorbent assay (ELISA) kits (Mitsubishi Kagaku Iatron, Tokyo, Japan), respectively, as described previously (14).

Table 1. Identities and conditions of primary and secondary antibodies used for double immunostaining of NKA α-isosfoms and AgRP

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
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<tr>
<td>NKA</td>
<td>AgRP</td>
</tr>
<tr>
<td>*Mouse anti-NKA-α monoclonal antibody</td>
<td>Goat anti-AgRP polyclonal antibody</td>
</tr>
<tr>
<td>C486.4; Santa Cruz Biotechnology, Santa Cruz, CA</td>
<td>GT15023, Neuramics Antibodies, Northfield, MN</td>
</tr>
<tr>
<td>*Goat anti-NKA-α2 polyclonal antibody</td>
<td>**Rabbit anti-AgRP polyclonal antibody</td>
</tr>
<tr>
<td>sc-16049; Santa Cruz Biotechnology,</td>
<td>H-003-53, Phoenix Pharmaceuticals, Burlingame, CA</td>
</tr>
<tr>
<td>*Mouse anti-NKA-α3 monoclonal antibody</td>
<td>Goat anti-AgRP polyclonal antibody</td>
</tr>
<tr>
<td>MA3-915; Thermo scientific, Rockford, IL</td>
<td>GT15023, Neuramics Antibodies</td>
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<th>NKA</th>
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<tr>
<td>*Mouse anti-AgRP antibody</td>
<td>Anti-AgRP antibody</td>
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<tr>
<td>Molecular Probes, Carlsbad, CA</td>
<td>Molecular Probes, Carlsbad, CA</td>
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<tr>
<td>*Goat anti-NKA-α2 polyclonal antibody</td>
<td>Goat anti-AgRP polyclonal antibody</td>
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<td>Molecular Probes</td>
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<tr>
<td>*Goat anti-NKA-α3 polyclonal antibody</td>
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Intra-ARC and third ventricle injections and measurements of food intake. For intra-ARC injections, a guide cannula was inserted with the tip located at 2.8 mm caudal, 0.3 mm lateral to the bregma and 8.8 mm below the skull (38) in 9- to 10-wk-old rats. Oubain (250 pmol/0.5 μl) was injected during the light phase (10:00) in ad libitum-fed animals, immediately followed by feeding experiments, in which food intake for 1 and 2 h was measured. After the feeding experiment, bilateral portions of ARC were immediately collected from hypothalamic slices for mRNA measurement. SSA412 (1.1 μg/0.5 μl) was injected after 24 h of fasting and 30 min prior to the start of feeding experiments, in which food intake for 1 and 2 h was also measured. For third ventricular injections, a guide cannula was inserted with the tip located at 2.5 mm caudal to the bregma in the midline and 8 mm below the surface of the skull, and ouabain (0.5 nmol/5 μl) and NPY Y1 receptor antagonist BIBP-3226 (30 nmol/5 μl) were injected for feeding experiments.

Measurements of c-fos expression. Measurements of c-Fos expression were performed as previously reported (25). Briefly, in 2 h after ouabain injection, rats were transcardially perfused and coronal sections processed. Anti-c-Fos antiserum Ab-5 (dilution 1:40,000; Calbiochem, San Diego, CA), anti-AgRP antiserum (1:100; Neuramics Antibodies, Northfield, MN), and anti-propiomelanocortin (POMC) antiserum (1:5,000, provided by Dr. Tanaka) were used for immunohistochemistry. This anti-POMC antiserum was produced by Dr. Shigeyasu Tanaka (40) and shown to be highly specific to POMC (25). The number of c-Fos-positive cells per section in ARC was counted.
Measurements of $[\text{Ca}^{2+}]$, and immunocytochemistry in single neurons of ARC. Measurements of $[\text{Ca}^{2+}]$, in single neurons and subsequent immunocytochemical staining followed previous reports (18, 25, 28). In brief, single neurons were isolated from ARC of rats aged 5–7 wk by treatment with papain. The cells were loaded with 2 µM fura 2-acetoxyethyl ester (AM) (Dojin Chemical, Kumamoto, Japan). They were excited at 340 and 380 nm, respective emission signals at 510 nm (F340 and F380) were detected, and ratio (F340/F380) images were produced by Aquacosmos (Hamamatsu Photonics, Hamamatsu, Japan). After $[\text{Ca}^{2+}]$, measurements, neurons were immunocytochemically stained with rabbit antisera against AgRP (1: 200; Phoenix Pharmaceuticals, Burlingame, CA), NPY (1:5,000; ImmunoStar, Hudson, WI), and POMC (1:1,000) (40).

Measurements of NAD(P)H level in single neurons of ARC. Auto-fluorescence of NAD(P)H in single neurons isolated from ARC was measured with excitation at 360 nm and emission at 470 nm, as previously reported (43). Thereafter, the cells were loaded with fura 2-AM, and $[\text{Ca}^{2+}]$, was measured in the same neurons to examine the responses to LG.

Measurements of $[\text{Na}^{+}]$, in single neurons of ARC. $[\text{Na}^{+}]$, was measured as previously reported (16), with slight modifications. Briefly, the cells were incubated with $[\text{Na}^{+}]$ indicator SBFI-AM (2 µM; Molecular Probes, Carlsbad, CA) and Pluronic F-127 (0.0125%; Molecular Probes) for 2 h at 34°C. The procedures for ratiometric SBFI microfluorometry followed those for fura 2. After measurement of $[\text{Na}^{+}]$, $[\text{Ca}^{2+}]$, was measured using fluo-4 with distinct excitation (490 nm) and emission wavelengths (520 nm) from SBFI. The cells were incubated with 2 µM fluo-4-AM (Molecular Probes) for 1 h at room temperature.

Immunohistochemistry of NKA α-isofoms and AgRP. Coronal sections of hypothalamus containing ARC were prepared from SD rats aged 6 wk and were double-immunostained using antibodies against NKA-α1, α2, or α3 and against AgRP (Table 1) according to previously described methods (25). Fluorescence images were acquired with an Olympus FV1000 confocal microscope (Tokyo, Japan).

Measurement of mRNA. mRNAs were measured by real-time PCR as previously reported (13). Primers were as follows (forward and reverse): NPY, 5'-TTGCCCAGATACATCTCCGCTC-3' and 5'-AATGGAAGGCTTCACGCTC-3'; AgRP, 5'-GTTGGCTAGATCCACAGAACCG-3' and 5'-CCAAGCAGAGTCTGGCAG-3'; POMC, 5'-CCCTCATAAGCTGTTAGCCTG-3' and 5'-AAGGCTCTTGCTACATCCGGTT-3'; GAPDH, 5'-GGCCACTGTCAGCAGACGAA-3' and 5'-ATGCGTGTTGAAACGAGTA-3'; NKA-α1, 5'-TGCATTGACTTGGGACGCTAC-3' and 5'-TTACAGGCTTCA-TAGGGCAAG-3'; NKA-α2, 5'-CACCACCTGCAACCCACAGTT-3' and 5'-ATGTCCCGAAGTCCAGGATG-3'; NKA-α3, 5'-ACCGAAGTCCCGAAGTCCAGGATG-3' and 5'-AAGGCTCTTGCTACATCCGGTT-3'.

RESULTS

Fasting suppresses NKA’s ATP-hydrolyzing activity and substrate ATP level in ARC. We examined whether the NKA enzyme activity in ARC is physiologically controlled under fed vs. fasted conditions. We measured the NKA’s ATP-hydrolyzing activity and substrate ATP content, the two factors that determine the final NKA enzyme activity in living cells. In 24-h-fasted rats compared with ad libitum-fed rats, the ouabain-sensitive ATP-hydrolyzing activity of NKA decreased in ARC [100.0 ± 9.7% for fed (N = 11) vs. 54.0 ± 3.0% for fasted (N = 12), P < 0.05], LHA [100.0 ± 6.9% for fed (N = 11) vs. 57.8 ± 18.4% for fasted (N = 12), P < 0.05], and VMH [100.0 ± 6.0% for fed (N = 11) vs. 74.6 ± 2.8% for fasted (N = 12), P < 0.05], and the magnitude of reduction was greater in ARC than in LHA and VMH (Fig. 1A). However, the expressions of the catalytic NKA-α1, α2, and α3 proteins in ARC were unchanged (Fig. 1B). These results indicate that the ATP-hydrolyzing enzyme activity but not the expression of NKA molecule was suppressed. In rats fasted 24 h, the ATP content markedly decreased in ARC [100.0 ± 26.7% for fed (N = 5) vs. 32.6 ± 6.0% for fasted (N = 5), P < 0.05], whereas it only tended to decrease in LHA [100.0 ± 25.5% for fed (N = 5) vs. 62.2 ± 23.2% for fasted (N = 5)] and VMH [100.0 ± 19.4% for fed (N = 5) vs. 56.7 ± 18.6% for fasted (N = 5); Fig. 1C]. Furthermore, the ATP content in ARC of individual fed or fasted rats correlated significantly with the blood glucose concentration of the corresponding animal, indicating that ARC ATP content reflects the systemic energy state (Fig. 1D). These results demonstrate that the systemic energy state influences the final NKA enzyme/pump activity in ARC by regulating both the ATP-hydrolyzing activity and substrate ATP level.

Fasting-induced plasma ghrelin rise and glucose fall reduce NKA’s ATP-hydrolyzing activity and substrate ATP level, respectively, in ARC. We next investigated the messengers that link systemic energy states to NKA enzyme activity in ARC. Under 24-h-fasted conditions compared with fed conditions, plasma level of ghrelin, the candidate hunger hormone considered to be a physiological meal initiator (2, 12, 33), was increased [36.2 ± 8.7 fmol/ml for fed (N = 6) vs. 68.7 ± 9.4 fmol/ml for fasted (N = 6), P < 0.05], and blood glucose level was decreased [95.0 ± 5.0 mg/dl for fed (N = 6) vs. 60.2 ± 2.8 mg/dl for fasted (N = 6), P < 0.01] (Fig. 1, E and F). In isolated ARC slices, administration of ghrelin significantly decreased the ATP-hydrolyzing activity of NKA [100.0 ± 18.7% for vehicle (n = 6) vs. 47.0 ± 13.1% for ghrelin (n = 6), P < 0.05; Fig. 1G], whereas ATP content was unaltered (Fig. 1H). Conversely, ATP content measured in ARC slices was significantly decreased by incubation in 2.8 mM glucose (2.8G) compared with 8.3G [100.0 ± 14.0% for 8.3G (n = 8) vs. 56.1 ± 9.7% for 2.8G (n = 8), P < 0.05] and in 0.5G compared with 2.8G [100.9 ± 10.4% for 2.8G (n = 10) vs.
Fig. 1. Fasting, fasting-induced plasma ghrelin rise, and glucose fall reduce Na\(^+\)-K\(^+\)-ATPase (NKA)’s ATP-hydrolyzing activity and ATP level in the arcuate nucleus (ARC). Throughout the paper and figure legends, \(N\) signifies number of rats examined; \(n\) signifies number of cells, slices, and sections examined. 

**A:** ATP-hydrolyzing activity of NKA in ARC, lateral hypothalamic area (LHA), and ventromedial hypothalamus (VMH) was significantly decreased in 24-h-fasted rats (\(N = 12\)) compared with ad libitum-fed rats (\(N = 11\)). NKA activity specified by ouabain sensitivity was expressed as means ± SE. 

**B:** protein levels of NKA-α isoforms α1, α2, and α3 in ARC were not changed between fed and 24-h-fasted rats (\(N = 3\)). 

**C:** ATP contents in ARC, assayed by luminescence and expressed as means ± SE, were significantly decreased in 24-h-fasted rats (\(N = 5\)) compared with ad libitum-fed rats (\(N = 5\)). 

**D:** ATP contents in ARC were significantly correlated with arterial blood glucose concentrations (\(N = 10\), \(P = 0.01\)). Each point represents individual animal. 

**E:** plasma ghrelin concentrations under fed (\(N = 6\)) and 24-h-fasted (\(N = 6\)) conditions. 

**F:** Blood glucose concentrations under fed (\(N = 6\)) and 24-h-fasted (\(N = 6\)) conditions. 

**G** and **H:** administration of 10^{-8} M ghrelin suppressed ATP-hydrolyzing NKA activity (G, \(n = 6\) for vehicle and ghrelin) without altering ATP content (H, \(n = 6\) for vehicle and ghrelin) in ARC slices. Glucose concentration was 5.6 mM. 

**I:** ATP-hydrolyzing NKA activity in ARC slices was not altered by incubation in 2.8 mM glucose (2.8G; \(n = 7\)) compared with 8.3G (\(n = 7\)) and in 0.5G (\(n = 5\)) compared with 2.8G (\(n = 5\)). 

**J:** ATP content measured in ARC slices was significantly decreased by incubation in 2.8G (\(n = 8\)) compared with 8.3G (\(n = 8\)) and in 0.5G (\(n = 10\)) compared with 2.8G (\(n = 10\)). *\(P < 0.05\), **\(P < 0.01\).
74.5 ± 3.8% for 0.5G (n = 10), P < 0.05] (Fig. 1J), whereas ATP-hydrolyzing activity of NKA was not different between 8.3G and 2.8G and between 2.8G and 0.5G (Fig. 1I). These results suggest that fasting downregulates NKA enzyme/pump activity in ARC via two modes (see Fig. 8): increased plasma ghrelin reduces ATP-hydrolyzing activity of NKA, and lowered blood glucose reduces its substrate ATP level.

Lowering glucose reduces intracellular NAD(P)H level and increases Na⁺ concentration in ARC GI neurons. To assess whether the fasting- and low blood glucose-induced reduction of ATP level or energy state in ARC slices also takes place at the neuronal level, we measured the autofluorescence of NAD(P)H, a molecule that couples glucose metabolism to intracellular energy production in islet β-cells and hypothalamic neurons (1, 15). We found that LG from 8.3G to 2.8G reduced NAD(P)H level in a single ARC neuron, whereas a subsequent shift from 2.8G to 8.3G increased it (Fig. 2A, left). This neuron, after being loaded with fura 2-AM, subsequently responded to the same LG protocol with increases in [Ca²⁺], (Fig. 2A, right; n = 5), showing that this neuron was a GI neuron. Average NAD(P)H autofluorescence at 2.8G was significantly smaller than that at 8.3G in GI neurons (n = 5, Fig. 2B). These results indicate that LG decreases the intracellular energy state and increases neuronal activity in GI neurons of ARC. Since more than 90% of the ARC GI neurons are IR to NPY (28), the observed responses to LG may take place, at least partly, in NPY/AgRP neurons.

NKA activity was assessed by measuring [Na⁺], with SBFI. LG from 8.3G to 2.8G increased [Na⁺], in a single ARC neuron (Fig. 2C, left). This neuron, after being loaded with Fluo-4, subsequently responded to the same LG protocol with [Ca²⁺] increases (Fig. 2C, right), showing that this neuron was a GI neuron. By contrast, LG from 8.3G to 2.8G little altered [Na⁺], (Fig. 2D, left) in the ARC non-GI neurons that did not respond to LG with [Ca²⁺] increases (Fig. 2D, right). Average amplitude of [Na⁺] increases was significantly larger in GI than non-GI neurons (Fig. 2E). According to a calibration curve, LG from 8.3G to 2.8G increased [Na⁺], by 7.5 ± 1.1 mM in GI neurons, consistent with a previous report (35). These results showed that LG induces [Na⁺] increases and neuronal activation in ARC GI neurons.

Intra-ARC injection of NKA inhibitor promotes feeding and NPY mRNA expression in ARC. The results that fasting suppressed NKA enzyme activity, most markedly in ARC, prompted us to examine whether the energy state-sensing ARC NKA is related to feeding behavior. For this, we manipulated NKA in ARC by injecting its specific inhibitor and activator. Focal injection of the NKA inhibitor ouabain (250 pmol/0.5 μl) into ARC in ad libitum-fed rats during the light phase markedly increased food intake for 1 and 2 h (Fig. 3A). This condition also increased NPY, tended to increase AgRP, and tended to decrease POMC mRNA expressions in ARC (Fig. 3B). Thus, ouabain increased mRNA expression for orexigenic peptides and tended to decrease that for anorexigenic peptide. These changes could account for the feeding stimulation.

Third ventricular injection of NKA inhibitor induces feeding in an NPY-dependent manner and c-fos expression in AgRP neurons in ARC. Since fasting suppressed ATP-hydrolyzing activity of NKA not only in ARC but in LHA and VMH, and the ARC NPY/AgRP neurons project to various brain regions, the effect of intracerebroventricular injection of ouabain and NPY antagonist was examined. The third ventricular injection of the NKA inhibitor ouabain, compared with vehicle, increased food intake, and the ouabain-induced food intake was blocked by simultaneous third ventricular injection of NPY Y1 receptor antagonist BIBP-3226 (Fig. 3C). The third ventricular injection of ouabain also induced c-Fos expression in AgRP neurons in ARC (Fig. 3, D and E), and the c-Fos induction was much greater in AgRP (Fig. 3F) than in POMC neurons (Fig. 3G).

NKA inhibitor ouabain activates NPY/AgRP neurons more potently than POMC neurons. Administration of ouabain (100 μM) increased [Ca²⁺], in single ARC neurons that were subsequently shown to be immunoreactive (IR) to NPY (Fig. 4A), AgRP (Fig. 4B), and POMC (Fig. 4C). Ouabain evoked [Ca²⁺], responses in 15 of 22 (59.1%) NPY-IR neurons, 17 of 32 (53.1%) AgRP-IR neurons, and 13 of 36 (36.1%) POMC-IR neurons. These results indicate that ouabain activates NPY/AgRP neurons in ARC. The rest of [Ca²⁺], and immunohistochemical experiments were performed mainly for AgRP rather than NPY (neurons), since this peptide is expressed exclusively in ARC and is colocalized with NPY (10). Ouabain at a lower concentration of 1 μM induced solid increases in [Ca²⁺], in AgRP-IR neurons (Fig. 4D) but much smaller increases in [Ca²⁺], in POMC-IR neurons (Fig. 4E). The average amplitude of the [Ca²⁺], responses to ouabain at 1 and 100 μM were both much greater in AgRP-IR neurons than in POMC-IR neurons (Fig. 4F). These results taken together suggest that NKA suppression is linked to cellular activation in NPY/AgRP neurons more efficiently than in POMC neurons in ARC.

NKA activator SSA412 suppresses fasting-induced feeding behavior and LG-induced neuronal activation. We found that fasting decreases NKA activity in ARC. If this change is related to the fasting-evoked appetite, the food intake following fasting would be suppressed by activation of NKA. When the specific NKA activator SSA412 (1.1 μg/0.5 μl) was focally injected into ARC, food intake at 1 and 2 h of refeeding following 24-h fasting was significantly attenuated (Fig. 5A). Furthermore, whether the decreased NKA activity mediates the LG-induced activation of GI neurons was investigated. In control experiments, LG from 8.3G to 2.8G induced [Ca²⁺], increases twice, first immediately after the treatment with control nonimmune IgG (Fig. 5B, left) and subsequently following 70 min of incubation in HKRB (Fig. 5B, right). The majority of these GI neurons were IR to NPY (Fig. 5C). In test experiments, soon after the treatment with SSA412, LG from 8.3G to 2.8G failed to significantly increase [Ca²⁺], in an ARC neuron (Fig. 5D, left). This neuron, after incubation in HKRB to washout the influence of the antibody, responded to the same LG protocol with [Ca²⁺], increases (Fig. 5D, right), showing the recovery of the response to LG. Thus, [Ca²⁺], responses to LG in GI neurons were significantly inhibited by SSA412 compared with that with control IgG and that after washout incubation (Fig. 5E).

NKA-α isoforms are present in ARC and localized abundantly in NPY/AgRP neurons. The finding that ouabain induced much greater increases in [Ca²⁺], in NPY/AgRP neurons than in POMC neurons prompted us to study the molecular identity of NKA in ARC NPY/AgRP neurons. We examined the colocalization of the catalytic NKA-α isoforms α1, α2, and α3 with AgRP in ARC. As shown in Fig. 6, in the first, third, and fifth lanes, double immunohistochemistry revealed that the
neuron-preferential isoforms $\alpha_1$ and $\alpha_3$ proteins (8) were abundantly located in ARC, confirming a previous report (3). The glial cell-preferential isoform $\alpha_2$ (8) was also observed. In an expanded scale, the three NKA-$\alpha$ isoforms were located in AgRP-IR neurons in ARC (Fig. 6, 2nd, 4th, and 6th lanes). The immunoreactivities for NKA-$\alpha_3$ and $\alpha_1$ were frequently and intensely colocalized with the AgRP immunoreactivity (Fig. 6, 6th lane), indicative of high-level expression of $\alpha_3$ and $\alpha_1$ in ARC AgRP neurons.

ARC-selective knockdown of NKA-$\alpha_3$ impairs feeding behavior without altering the number and function of AgRP neurons. To further assess the role of these $\alpha$-subunit isoforms, shRNAs for NKA-$\alpha_1$, $\alpha_2$, and $\alpha_3$ were focally injected into ARC, which resulted in reduction of the mRNA expressions of

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**Fig. 2.** Lowering glucose concentration decreases intracellular NAD(P)H level and increases $[\text{Na}^+]_i$, in glucose-inhibited (GI) neurons. A: NAD(P)H autofluorescence decreased with 2.8G and increased with 8.3G in a single neuron isolated from ARC. Fluorescence intensity was normalized to that at the beginning (F/F0) (left). The same neuron responded to lowering glucose concentration (LG) from 8.3G to 2.8G, indicating that it was a GI neuron (right). This neuron also responded to $10^{-4}$ M glutamate with $[\text{Ca}^{2+}]_i$ increases. These results showed that LG decreases intracellular energy state and induces neuronal activation in ARC GI neurons. Results are representative of 5 neurons ($n = 5$). B: average NAD(P)H autofluorescence at 2.8G was significantly smaller than at 8.3G ($n = 5$). C: LG from 8.3G to 2.8G increased $[\text{Na}^+]_i$ (left) and subsequently increased $[\text{Ca}^{2+}]_i$ (right) in the same neuron. Fluo-4 fluorescence intensity was normalized to that at the beginning (F/F0) ($n = 13$). These results showed that LG increases $[\text{Na}^+]_i$, and induces neuronal activation in ARC GI neurons. D: LG from 8.3G to 2.8G did not alter $[\text{Na}^+]_i$ in a non-GI neuron (left), determined by a lack of subsequent $[\text{Ca}^{2+}]_i$ response to LG (right) ($n = 8$). E: $[\text{Na}^+]_i$ increases during exposure to 2.8G were significantly greater in GI ($n = 13$) than in non-GI neurons ($n = 8$). **$P < 0.01$.**
NKA-α1, -α2, and -α3 to the levels ~30% (Fig. 7A). In rats injected with shRNA for α3, food intake during the 2-h refeeding period following 16 h of fasting was significantly reduced, whereas that during the 1-h refeeding period tended to decrease and 24-h food intake was not changed (Fig. 7B). Treatment with shRNAs for NKA-α1 and -α2 tended to decrease food intake during 1- and 2-h refeeding periods but not statistically significantly. The treatment with NKA-α3 shRNA...
also significantly decreased NPY mRNA expression in ARC at 2 h of refeeding following 16 h of fasting (Fig. 7C). In the rats treated with NKA–α3 shRNA, the number of AgRP-IR neurons in ARC were not altered (Fig. 7, D and E), and the [Ca^{2+}]i responses to glutamate in ARC neurons were not altered (Fig. 7, F and G) compared with control rats treated with scramble shRNA. These results indicate that the treatment with NKA–α3 shRNA little affected the survival and functionality of ARC AgRP neurons. These results taken together suggest that NKA–α3 is implicated in the fasting-induced feeding and NPY expression in ARC.

**DISCUSSION**

We found that fasting suppresses NKA enzyme activity by decreasing its ATP-hydrolyzing activity and substrate ATP level in ARC. Furthermore, fasting induces ghrelin rise and glucose lowering in the plasma, and ghrelin suppresses NKA’s ATP-hydrolyzing activity and LG reduces its substrate ATP level in ARC slices. At the cellular level, LG decreases NAD(P)H level and increases [Na^{+}]i in ARC GI neurons. Intra-ARC injection of NKA inhibitor ouabain stimulates feeding, and administration of ouabain activates NPY/AgRP neurons preferentially to POMC neurons in ARC. Intracerebroventricular injection of ouabain induces NPY/AgRP-dependent feeding. Inversely, NKA activator SSA412 suppresses both fasting-induced feeding and LG-induced activation of ARC GI neurons. Thus, the correlation between the ARC neuronal activity and the feeding behavior was observed in two conditions, the NKA suppression with ouabain and the NKA activation with SSA412. NKA–α1 and -α3 are abundant in ARC Na,K pump controls glucose-inhibited neuron and feeding.
and intensely localized in NPY/AgRP neurons, and NKA-α3 knockdown attenuates fasting-induced feeding behavior, suggesting an important role for NKA-α3 and -α1 isoforms in linking energy state to neuronal activity and feeding behavior. These results demonstrate that fasting, via plasma ghrelin rise and glucose lowering, suppresses the final enzyme/pump activity of NKA including its α3 and α1 isoforms in ARC, which in turn activates ARC GI neurons to promote NPY/AgRP-dependent feeding behavior.

In ARC, fasting decreased NKA’s ATP-hydrolyzing activity approximately to the level of one-half and its substrate ATP content to one-third, suggesting that the final NKA enzyme/pump activity might markedly drop. The fasting-induced decreases in the energy state and NKA activity observed in ARC slices may take place at least partly in the neurons, since the ARC GI neurons exhibited NAD(P)H reduction and [Na⁺]i increase, possibly reflecting the NKA suppression, in response to LG. A greater reduction of NKA enzyme activity in ARC than in other areas is in accord with the concept that the ARC neurons are the first-order neurons that initially sense systemic signals (26, 34). We identified plasma ghrelin rise and glucose lowering as the key systemic signals that link fasting conditions to the NKA enzyme/pump activity in ARC. Hence, ARC neurons may have the ability to efficiently sense the increased ghrelin and decreased glucose. This process most likely takes place in ghrelin-responsive neurons and GI neurons in ARC, both of which are the substantial subpopulations of ARC neurons (18, 28). Importantly, over 80% of ghrelin-responsive (18) and more than 90% of GI neurons (28) are NPY neurons, and ghrelin receptor GHSR is expressed on more than 90% of NPY neurons in ARC (2, 41).

In this study, we found that the NKA suppression with ouabain induced greater increases in [Ca²⁺]i in NPY/AgRP neurons than in POMC neurons in ARC. In contrast, intra-ARC injection of ouabain increased NPY and AgRP mRNA expression but tended to decrease POMC mRNA expression in ARC.
An apparent discrepancy was observed in the ouabain effects comparing the [Ca^{2+}]_{i} increase in POMC neurons in single-cell experiments and the tendency of reduction of POMC mRNA in ARC in vivo. It is suggested that ouabain suppressed POMC mRNA expression by activating NPY/AgRP neurons and thereby driving their intra-ARC circuit to inhibit POMC neurons (11), and that this inhibitory effect could exceed the direct stimulatory effect on POMC neurons. Furthermore, central injection of ouabain evoked NPY-dependent feeding behavior. These data indicate a tight link of the suppression of NKA to the activation of the NPY/AgRP system. Although the mechanism underlying this link remains to be elucidated, it could be partly mediated by the NKA-α3 isoform. We found that the α3 isoform is expressed abundantly in ARC and intensely localized in NPY/AgRP neurons. These results suggest that NKA-α3 plays a pivotal role in the regulation of the NPY/AgRP neuron excitability and feeding.

Fig. 6. NKA-α isoforms are present in ARC and localized abundantly in NPY/AgRP neurons. Double immunohistochemistry showed that NKA-α1 and -α3 were abundantly, and -α2 to a much lesser extent, located in ARC. In the area marked with dotted squares and expanded in the corresponding bottom panels, 3 α-isforms were localized in AgRP-IR neurons in ARC, as indicated by arrows (N = 4 for each isoform). Scale bars, 100 μm for low-power and 20 μm for high-power fields.
Fig. 7. ARC-selective knockdown of NKA-α3 impairs fasting-induced feeding behavior and NPY expression without altering the number and function of AgRP neurons. A: focal injection of shRNAs for NKA-α1, α2, and α3 into ARC significantly reduced target mRNA expression in the ARC [N = 3 for α1, 6 for α2, 6 for α3, and 3 for scramble (scr)]. B: cumulative food intake on refeeding for 1, 2, and 24 h after 16-h fasting in rats receiving intra-ARC shRNAs for NKA-α1, -α2, or -α3. Transfection with α3 shRNA significantly reduced food intake on refeeding for 2 h after 16-h fasting; N = 10 for scr, 7 for α1, 6 for α2, and 6 for α3. C: NPY mRNA expression in ARC on refeeding for 2 h after 16-h fasting was reduced in rats receiving intra-ARC shRNAs for α3; N = 8 for scr and 6 for α3. D and E: localization of AgRP-IR neurons in ARC was not different between scramble (N = 4) and NKA-α3 shRNA (N = 4) injected rats. Scale bars, 20 μm. F and G: [Ca^{2+}]_{i} responses to 1 μM glutamate in single ARC neurons were not different between scramble (N = 4) and NKA-α3 shRNA (N = 3) injected rats. *P < 0.05.
Our results are consistent with the well-established notion that NPY/AgRP neurons fire much more action potentials in the fasting state than other neurons (7). However, it is perplexing how inhibition of the NKA in NPY/AgRP neurons leads to a sustained higher action potential firing rate, while the proper function of NKA to maintain intracellular ion concentrations is required for sustaining action potential firing. The mechanism underlying this intriguing question remains to be elucidated. However, it is speculated that suppression of the NKA activity slightly changes the intracellular environment but is capable of triggering the voltage-dependent activation of the signal transduction cascade that can last to maintain neuronal activity. Alternatively, counterregulatory mechanisms might operate to maintain the intracellular ion environment.

We have shown that NKA suppression promotes feeding primarily via activation of NPY/AgRP neurons in ARC, while reduced POMC mRNA expression could additionally be implicated. The essential role of NPY/AgRP neurons in stimulating feeding has been well established: selective ablation of ARC AgRP neurons in adult mice results in immediate and severe reduction of food intake and body weight (17, 24), and selective activation of ARC AgRP neurons using optogenetics initiates orchestrated feeding behavior (4, 5). Taken together, the current finding of the NPY/AgRP dependence of the NKA suppression-induced food intake reinforces the relevance of the feeding regulation by NKA. However, these results do not exclude other possible mechanisms for fasting-induced feeding. It has previously been shown that fasting-induced NPY/AgRP neuron activation may be mediated through a presynaptic mechanism (36) or a postsynaptic NMDA receptor mechanism (22). The Kv2.1 channel is implicated in fasting-induced excitation of the ARC NPY/AgRP neurons and its inhibition by leptin (6). Ghrelin may elevate excitatory synaptic input on ARC NPY/AgRP neurons (44). This study, together with previous reports, suggests that under fasting conditions the NKA-mediated mechanism cooperates with other cellular and/or synaptic mechanisms to efficiently activate the ARC NPY/AgRP neurons to evoke feeding behavior.

In this study, tonic silencing of NKA-α3 with shRNA treatment suppressed food intake after fasting. This result is in apparent discrepancy with the acute orexigenic effect of the administration of the NKA inhibitor ouabain. Regarding the underlying mechanism, we speculate that knockdown of NKA-α3 decreases the number of NKA-α3 molecules, resulting in reduced amplitude of the integrated change of NKA enzyme/pump activity per ARC neuron in response to fasting. Consequently, the impact of the fasting-induced suppression of NKA activity on ARC neurons and feeding might be attenuated. Hence, the presence of NKA-α3 beyond a critical level might be required for ARC to fully respond to fasting with sufficient stimulation of the NPY/AgRP system and feeding behavior.

This has study provided data to support a novel role of the ARC NKA in the stimulation of feeding under fasting/hunger conditions (Fig. 8). A rise in plasma ghrelin suppresses NKA’s ATP-hydrolyzing activity, and lowering of blood glucose reduces its substrate ATP level in ARC including GI and possibly NPY/AgRP neurons. These two changes in combination effectively suppress the final enzyme/pump activity of NKA. It has been shown (8) that NKA-α1 is ubiquitously expressed and functions as the housekeeping NKA, and that NKA-α3 is less widely distributed and has different affinities to cations and ATP from other isoforms, indicative of its spare or additional functions. Hence, the abundant expression of α3 and α1 in ARC NPY/AgRP neurons, observed in the present study, might enable these neurons to respond to systemic and intracellular metabolic alteration with large amplitude of changes in the total NKA enzyme/pump activity, which might result in greater excitation of NPY/AgRP neurons. This is supported by the greater impact of ouabain on NPY/AgRP neurons than POMC neurons, as judged by [Ca2+]i increase and mRNA expression.

As an additional finding of this study, fasting suppressed NKA activity also in VMH and LHA, albeit to a lesser extent than in ARC. This result suggests that NKA might function as the energy sensor in VMH and LHA as well and that this sensing might also be implicated in feeding and other hunger.
associated metabolic regulation such as defense against hypoglycemia (9).

Until now, efforts to develop antiobesity medicines have largely focused on suppression of feeding by targeting the release, reuptake, and/or action of neurotransmitters, including serotonin and cannabinoïd but have failed to produce safe and effective drugs without adverse effects and rebound weight gain after discontinuation (19). A novel feeding-suppressing compound working through a distinct mode is still awaited. The action of the NKA activator bears important clinical implications for providing a novel tool to treat obesity, diabetes, and metabolic syndrome with excessive appetite.

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29. No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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