Original Article

Characterization of Glucosensing Neuron Subpopulations in the Arcuate Nucleus

Integration in Neuropeptide Y and Pro-Opio Melanocortin Networks?

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Four types of responses to glucose changes have been described in the arcuate nucleus (ARC): excitation or inhibition by low glucose concentrations <5 mmol/l (glucose-excited and -inhibited neurons) and by high glucose concentrations >5 mmol/l (high glucose–excited and –inhibited neurons). However, the ability of the same ARC neuron to detect low and high glucose concentrations has never been investigated. Moreover, the mechanism involved in mediating glucose sensitivity in glucose-inhibited neurons and the neurotransmitter identity (neuropeptide Y [NPY] or pro-opio melanocortin [POMC]) of glucosensing neurons has remained controversial. Using patch-clamp recordings on acute mouse brain slices, successive extracellular glucose changes greater than and less than 5 mmol/l show that glucose-excited, high glucose–excited, glucose-inhibited, and high glucose–inhibited neurons are different glucosensing cell subpopulations. Glucose-inhibited neurons directly detect decreased glucose via closure of a chloride channel. Using transgenic NPY–green fluorescent protein (GFP) and POMC-GFP mice, we show that 40% of NPY neurons are glucose-inhibited neurons. In contrast, <5% of POMC neurons responded to changes in extracellular glucose >5 mmol/l. In vivo results confirm the lack of glucose sensitivity of POMC neurons. Taken together, hypo- and hyperglycemia are detected by distinct populations of glucosensing neurons, and POMC and NPY neurons are not solely responsible for ARC glucosensing. *Diabetes* 56:1219–1227, 2007

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ARC, arcuate nucleus; CFTR, cystic fibrosis transmembrane regulator; GFP, green fluorescent protein; K_{ATP} channels, ATP-sensitive K^+ channels; NPY, neuropeptide Y; POMC, pro-opio melanocortin; PPAR, peroxisome proliferator–activated receptor; TTX, tetrodotoxin; VMN, ventromedian nucleus.

The hypothalamus integrates hormonal (e.g., insulin and leptin) and metabolic (e.g., glucose) signals of energy homeostasis and initiates adaptive responses. The arcuate nucleus (ARC) is crucial for this control (1–3). The ARC is located just above the median eminence, where the blood-brain barrier is "leaky" (4) and thus integrates peripheral signals of body energy status into changes in neuronal activity (5–9). The ARC possesses neurons expressing neuropeptide Y (NPY) and α-melanocyte-stimulating hormone (cleavage product of pro-opio melanocortin [POMC]) (2). Hypothalamic NPY pathways favor anabolic processes and increase food intake, whereas POMC neurons do the reverse. NPY and POMC neurons project to other hypothalamic areas as well as the spinal cord sympathetic preganglionic neurons. Moreover, they express insulin and leptin receptors (10,11).

Glucose plays a key role in the control of energy homeostasis (12). Hypoglycemia is sensed by the brain, which then initiates the counter-regulatory response (13). Hypoglycemia stimulates and hyperglycemia inhibits food intake (14–16). Glucosensing neurons have been identified in many hypothalamic nuclei, including the ARC and ventromedian nucleus (VMN) (6,7,9,17–19). Glucose-excited neurons increase and glucose-inhibited (GI) neurons decrease their electrical activity as extracellular glucose concentration increases from 0.1 to 5 mmol/l (9,19). The ATP-sensitive K^+ channel (K_{ATP} channel) mediates the response to glucose in ARC and VMN glucose-excited neurons, whereas an unidentified chloride channel mediates the glucose response in VMN glucose-inhibited neurons (19). The mechanism by which ARC glucose-inhibited neurons sense glucose remains unclear. ARC glucosensing neurons also exist that respond to changes in glucose concentration >5 mmol/l (6). These neurons were defined as high glucose–excited and –inhibited neurons. Interestingly, high glucose–excited neurons used a K_{ATP} channel–independent mechanism to sense glucose.

Thus, ARC glucosensing neurons sense changes in extracellular glucose greater than or less than 5 mmol/l (Table 1). The first aim of this study was to determine whether glucose-excited and high glucose–excited neurons or glucose-inhibited and high glucose–inhibited neurons were the same or different populations of neurons.

TABLE 1
ARC glucosensing neurons (6,9)

Glucosensing neurons	Glucose responses: Glucose sensitivity range <5 mmol/l	Glucose responses: Glucose sensitivity range >5 mmol/l	Mechanism involved
Glucose change tested	Decrease (2.5 → 0.1 mmol/l)	Increase (5 → 20 mmol/l)	
GE	Inhibition	?	K_{ATP}
GI	Excitation	?	?
HGE	?	Excitation	Nonselective cationic conductance
HGI	?	Inhibition	?

GE, glucose excited; GI, glucose inhibited; HGE, high glucose excited; HGI, high glucose inhibited. ?, unknown.

The latter has been suggested previously (6,9). Furthermore, we wished to determine whether ARC glucosensing neurons are of the NPY or POMC neuron phenotype. ARC neurons that increased their intracellular calcium levels as glucose levels decrease from 10 to 0 mmol/l were immunopositive for NPY (20). However, this glucose step does not differentiate between glucose-inhibited and high glucose–inhibited neurons. POMC neurons have been shown to be excited as glucose increased from 5 to 10 mmol/l (21). However, ARC glucose-excited neurons are not immunopositive for POMC (9). To address this controversy, we used transgenic NPY– or POMC–green fluorescent protein (GFP) mice, which specifically express the GFP in NPY or POMC neurons, to investigate their glucose sensitivity.

RESEARCH DESIGN AND METHODS

All experiments were carried out in nonfasted mice in accordance with European Community and U.S. guidelines. Electrophysiological recordings were performed on hypothalamic slices from 21- to 35-day-old C57Bl/6 mice. In vivo experiments used 7- to 8-week-old male C57BL/6J mice.

Some experiments used homozygous transgenic NPY-GFP or POMC-GFP mice provided by Dr. J.M. Friedman (Rockefeller University, New York, NY) (22). These two lines of transgenic mice were generated by using bacterial artificial chromosome expressing either sapphire GFP under the transcriptional control of the NPY genomic sequence (NPY-GFP mice) or topaz GFP under the transcriptional control of POMC genomic sequence (POMC-GFP mice) (22).

In vitro experiments

Brain slice preparation. As previously described (6), after cervical dislocation, three 300-μm coronal slices per brain were cut in an ice-cold oxygenated (95% O_2-5% CO_2) saline solution containing 200 mmol/l sucrose, 28 mmol/l $NaHCO_3$, 2.5 mmol/l KCl, 7 mmol/l $MgCl_2$, 1.25 mmol/l NaH_2PO_4, 0.5 mmol/l $CaCl_2$, 1 mmol/l L-ascorbate, 3 mmol/l sodium pyruvate, and 8 mmol/l D-glucose (pH 7.4). Slices were then incubated at room temperature in oxygenated extracellular medium containing 118 mmol/l NaCl, 3 mmol/l KCl, 1 mmol/l $MgCl_2$, 25 mmol/l $NaHCO_3$, 1.2 mmol/l NaH_2PO_4, 1.5 mmol/l $CaCl_2$, 5 mmol/l HEPES, 5 mmol/l D-glucose, and 15 mmol/l sucrose (osmolarity adjusted at 300–310 mOsmol/l, pH 7.4) for a 1-h recovery period.

Patch-clamp recordings. Slices were transferred into a recording chamber on the stage of an upright microscope, immobilized by a nylon grid, and perfused at 2–3 ml/min with the extracellular medium described above. ARC neurons were visualized using a ×60 water immersion objective (Nikon-France/Leica-USA) under IR-DIC illumination and an infrared video camera (Hamamatsu Photonics). Topaz or sapphire GFP-positive neurons were visualized using epifluorescence and specific filter sets (AHF Analysentechnik).

Borosilicate pipettes (5–7 MΩ; GC150F-10; Phymep) were filled with an internal solution containing 155 mmol/l K-gluconate, 0.1 mmol/l EGTA, 1 mmol/l $CaCl_2$, 10 mmol/l HEPES, 5 mmol/l KCl, 10 mmol/l KOH, 5 mmol/l Mg-ATP, and 0.4 mmol/l Na-GTP (pH and osmolarity adjusted at 7.3 and 300 mOsmol/l, respectively). Recordings were made using an Axopatch 1D amplifier (Axon Instruments), digitized using the Digidata 1320A interface, and acquired using pClamp 9.2 software (Axon Instruments). The amplifier filter was set at 5 kHz. Pipettes and cell capacitances were fully compensated. Junction potential was calculated using pClamp 9.2 and corrected off-line.

Drugs and glucose application. Glucosensing neurons were identified by changes in extracellular glucose as indicated in the figures. For some studies, medium containing 20 or 0 mmol/l D-glucose (osmolarity adjusted for each solution with sucrose at 300–310 mOsmol/l, pH 7.4) was applied to 5 mmol/l glucose.

D-Glucose was purchased from Sigma. Tetrodotoxin (TTX; Latoxan) and gemfibrozil (Sigma) were prepared as concentrated stock solutions and stored at −80°C. Extracellular medium containing different glucose concentration and drugs diluted in these oxygenated extracellular mediums were delivered in the recording chamber at 3 ml/min by a multibarrel gravity-feed system (ALA Scientific Instruments, Sega Electronique) positioned at ~3 mm from the ARC. Using this application system, glucose concentration changes in the recording chamber were performed from 5 to 0.5 mmol/l or from 5 to 15 mmol/l. For a set of experiments (Fig. 4), a different system was used, and extracellular glucose concentration was decreased from 2.5 to 0.1 mmol/l.

Data analysis and representation. Recordings were analyzed using pClamp 9.2 and Clampfit 9.2 softwares (Axon Instruments) and plotted with Origin 5.0 (Microcal Software). Data were shown as means ± SE and differences between groups were analyzed by paired or unpaired Student's t test, with $P < 0.05$ taken as significant.

In vivo experiments

Surgery and injections. POMC-GFP mice were anesthetized by an i.p. injection of ketamine-xylazine (100 and 10 mg/kg body wt, respectively). A Silastic catheter (VWR) was implanted into the left internal carotid artery with the proximal end directed toward the brain. The catheter was filled with a 0.9% NaCl solution. The distal end of the catheter was put under the skin to the back of the neck where it was plugged. All mice were allowed to recover from surgical procedures for 1 week before experiment. Animals that did not fully recover their presurgery body weight were systematically excluded from the study.

On the morning of experiment (between 10:00 A.M. and 12:00 P.M.), freely moving mice were injected for 60 s through the catheter with isotonic NaCl-containing glucose solution (50 mg/kg body wt, 60 μl/min).

c-Fos and GFP immunohistochemistry. Two hours after glucose injection, mice were anesthetized by pentobarbital injection into the catheter. A rapid fall down of the animal allowed us to control the correct location and the nonobstruction of the catheter. Then, mice were intracardialy perfused with 3.6% formaldehyde/0.2% picric acid solution. Brains were removed, postfixed at 4°C overnight, sunk in 30% sucrose for 3 days, and frozen at −40°C in isopentane. The hypothalamus was cut into 30-μm serial sections on a cryostat (Leica), and free-floating sections were collected in PBS (pH 7.4).

After quenching peroxidase activity (0.1% H_2O_2, 30 min), sections were incubated in 3% normal goat serum (Sigma)/PBS/0.3% Triton X-100 (Sigma) for 6 h at room temperature and then in the rabbit antiserum against Fos protein (1/10,000; Ab-5 antibody; Oncogene) overnight at 4°C. After washing, sections were incubated in biotinylated goat anti-rabbit IgG (1/2,000; The Jackson Laboratories, Bar Harbor, ME) for 45 min at room temperature and finally incubated in streptavidin-peroxidase conjugate (1/2,000; The Jackson Laboratories) for 1 h at room temperature. Peroxidase activity was revealed using a mixture of diaminobenzidine and nickel ammonium sulfate chloride as described by Shu et al. (23).

After c-fos revelation, free-floating sections were incubated in rabbit polyclonal antibody against GFP (1/1,000; Molecular Probes) overnight at 4°C. Sections were then incubated for 1 h at room temperature in streptavidin-peroxidase conjugate (1/1,000; The Jackson Laboratories). Peroxidase activity was revealed by diaminobenzidine. Fos labeling was thus detected by a black precipitate in the nuclei when GFP-expressing cells presented a brown cytoplasm.

At the end of immunohistochemistry, sections were mounted on silanized slides, dehydrated in alcohol, cleared in Bioclear (MicroStain [D-limonene] and Micron), and examined under a transmitted-light microscope (Leica).

Quantification and statistical analysis. Fos-immunopositive cells were counted using a computerized image analysis (Visilog 6.2 software; Noesis).

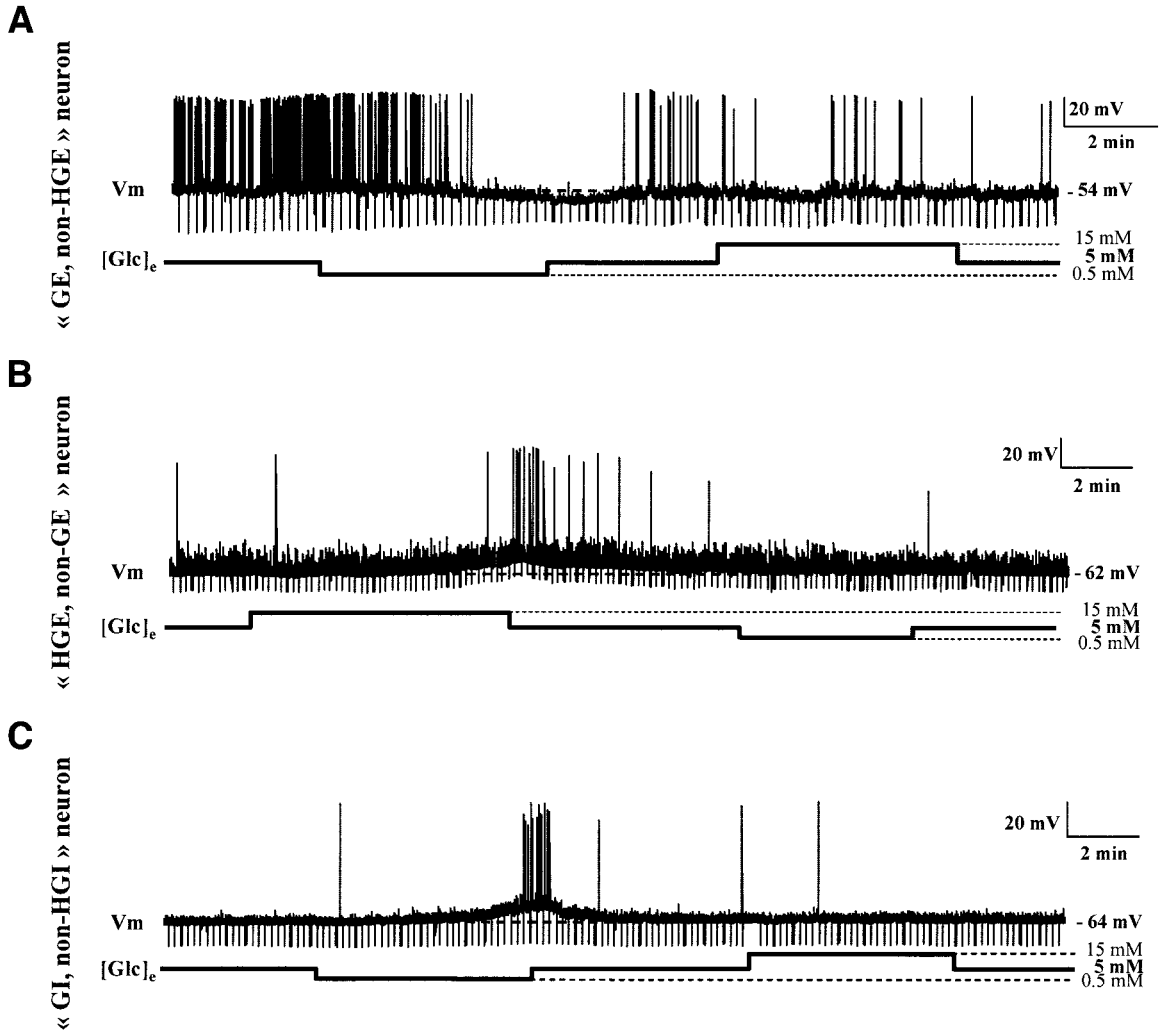

FIG. 1. Whole-cell current-clamp recordings of ARC glucosensing neurons as extracellular glucose concentration is altered. In this and all subsequent figures, downward deflections represent membrane voltage responses to hyperpolarizing current injection. Resting membrane potential is indicated by the dotted line and noted on the *right*. Glucose changes are schematically displayed below each recording. *A*: Decreased glucose inhibited this glucose-excited neuron; however, increased glucose had no effect. *B*: Increased glucose excited this high glucose–excited neuron; however, decreased glucose had no effect. *C*: Decreased glucose excited this glucose-inhibited neuron; however, increased glucose had no effect.

The results are expressed as the sum of single- or double-labeled cells counted bilaterally on six sections per ARC and are given as means ± SE for several independent experiments. The mean values from glucose-injected versus NaCl-injected mice were compared using unpaired Student's t test, with $P < 0.05$ taken as significant.

RESULTS

Presence of four distinct glucosensing neurons subpopulations in the ARC. Neurons in the median and anterior ARC were recorded under current-clamp whole-cell mode in acute brain slices (Bregma coordinates between −1.46 and −2.18 mm in adult mice [24]). Each ARC neuron was tested in a random order for a response to glucose changes <5 mmol/l, from 5 to 0.5 mmol/l, and >5 mmol/l, from 5 to 15 mmol/l. All recorded glucosensing neurons were sensitive to changes in glucose either greater than or less than 5 mmol/l but not in both glucose ranges. Thus, glucose-excited neurons never responded to an increase in glucose level >5 mmol/l ($n = 3$; Fig. 1*A*), nor did high glucose–excited neurons respond to a decrease in glucose <5 mmol/l ($n = 5$; Fig. 1*B*). Similarly, glucose-inhibited and high glucose–inhibited neurons were only sensitive to changes in glucose greater than or less than 5 mmol/l, respectively ($n = 13$; Fig. 1*C*). Thus, there are four distinct subpopulations of glucosensing neurons in the ARC.

Characterization of ARC glucose-inhibited neurons. To determine whether glucose acted directly on glucose-inhibited neurons or via presynaptic input, TTX was used to block action potentials and thus synaptic transmission. The excitatory effect of decreased glucose from 5 to 0.5 mmol/l persisted in the presence of TTX in all recorded glucose-inhibited neurons (five of five; Fig. 2*A*). Depolarization amplitude was similar with and without TTX (0.5 mmol/l glucose, 6.4 ± 1.6 mV vs. 0.5 mmol/l glucose + TTX, 4.9 ± 0.8 mV; $n = 5$; $P > 0.05$; Fig. 2*B*). These results suggest that ARC glucose-inhibited neurons directly detect changes in glucose concentration <5 mmol/l.

To identify the glucose-regulated conductance in ARC glucose-inhibited neurons, input resistance was measured by recording the membrane potential change in response to a constant hyperpolarizing current injection. Depolarization of glucose-inhibited neurons correlated with a significant increase of input resistance of 64% (5 mmol/l, 703 ± 138 MΩ vs. 0.5 mmol/l, 1,098 ± 235 MΩ; $n = 6$; $P <$

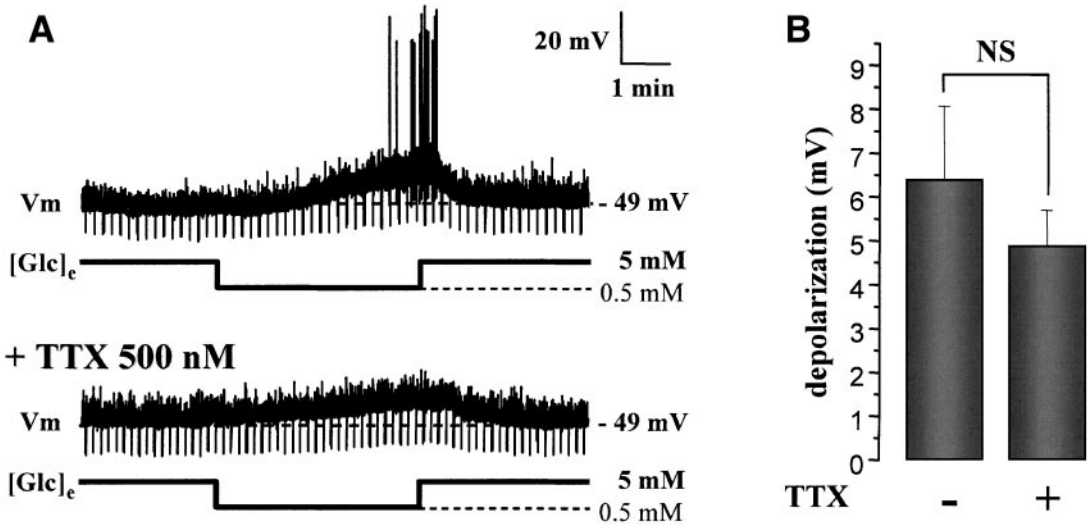

FIG. 2. ***A*****: Consecutive whole-cell current-clamp recordings in an ARC glucose-inhibited neuron. Decreased glucose excited this neuron in the presence (*bottom trace*) and absence (*top trace*) of 500 nmol/l TTX.** ***B*****: The presence of 500 nmol/l TTX did not alter glucose-inhibited neurons membrane potential; $n = 5$; NS, $P > 0.05$.**

0.05; Fig. 3*A*), indicating decreased membrane conductance. Voltage-current relationships were extrapolated from increasing hyperpolarizing current injections (−5 to −50 pA) to identify the ionic conductance involved. The glucose-sensitive current reversed at −80.4 ± 1.8 mV (n = 10; Fig. 3*B*), which is close to the theoretical chloride equilibrium potential (E_{Cl}) in our studies (−74 mV). Voltage-clamp recordings applying depolarizing voltage ramps (from −130 to −50 mV) confirmed this result (n = 4; Fig. 3*C*). To confirm involvement of a chloride conductance, we changed the chloride concentration in the internal medium to switch E_{Cl} to −57 mV. Under this condition, the reversal potential of the glucose response of glucose-inhibited neurons is −63 ± 8.1 mV (n = 3). Thus, these results suggest that decreased glucose <5 mmol/l depolarized ARC glucose-inhibited neurons through decreasing chloride conductance, as in VMN glucose-inhibited neurons (19).

The clofibric acid analog gemfibrozil is specific for the cardiac cystic fibrosis transmembrane regulator (CFTR) (25). In 0.1 mmol/l glucose, gemfibrozil (200 μmol/l) decreases input resistance of glucose-inhibited neurons from 1,166 ± 201 to 999 ± 187 MΩ (n = 8; $P < 0.05$), indicating an increased conductance. Moreover, the gemfibrozil-induced conductance reversed at −71.5 ± 1.4 mV (n = 3) (theoretical E_{Cl} calculated at −57 mV). This confirms that gemfibrozil opens a chloride channel in hypothalamic glucose-inhibited neurons. Here, gemfibrozil (200 μmol/l) reversed the effects of decreased glucose on action potential frequency and membrane conductance in ARC glucose-inhibited neurons (n = 4; Fig. 4*A*) and VMN glucose-inhibited neurons (n = 3; Fig. 4*B*). This suggests that gemfibrozil opened the CFTR channel on glucose-inhibited neurons. This effect was irreversible. After gemfibrozil treatment, glucose-inhibited neurons were no longer able to detect changes in glucose. These data suggest that the CFTR channel mediates glucose sensing in both ARC and VMN glucose-inhibited neurons.

Glucose sensitivity of NPY and POMC neurons. NPY-GFP transgenic mice were used to test the glucose sensitivity of ARC NPY neurons (Fig. 5*A*) (22). Forty percent (21 of 52) ARC NPY-GFP neurons were depolarized by glucose decrease <5 mmol/l; thus, they are glucose-inhibited neurons (Fig. 5*B*). ARC NPY-GFP neurons were never high glucose–inhibited neurons, as indicated by a lack of hyperpolarization when glucose increases >5 mmol/l (Fig. 4). Moreover, glucose-inhibited–like responses of NPY neurons persist in 500 nmol/l TTX, showing that NPY neurons directly detect glucose concentration decrease from 5 to 0.5 mmol/l (data not shown). In the presence of 5 mmol/l extracellular glucose, some ARC NPY-GFP neurons are silent, whereas some are spontaneously active. Most of the NPY-GFP ARC neurons that show a glucose-inhibited–like response are silent at a glucose concentration of 5 mmol/l. Finally, recordings of non–NPY-GFP neurons show that <5% of these cells are glucose-inhibited neurons (1 of 22). Thus, most glucose-inhibited neurons use NPY as a neurotransmitter.

As for NPY-GFP mice, detection of GFP fluorescence in brain slices of POMC-GFP mice allows us to specifically record POMC neurons. Their glucose sensitivity was then studied in response to glucose changes less than and greater than 5 mmol/l. The electrical activity of 95% (38 of 40) of these POMC-GFP neurons did not change in response to changes greater than or less than 5 mmol/l (Fig. 6*A*). In contrast, glucosensing neurons were found among non–POMC-GFP–recorded ARC cells (data not shown). These results strongly suggest that ARC POMC-GFP neurons are not glucosensing neurons.

Our results confirm those obtained by Wang et al. (9) for glucose changes <5 mmol/l but are not consistent with Ibrahim et al. (21) for glucose changes >5 mmol/l. To confirm our data for glucose changes >5 mmol/l, we investigated the glucose sensitivity of POMC neurons in an in vivo model of brain hyperglycemia (26). POMC-GFP mice were implanted with an intracarotid catheter and injected with a bolus of glucose (50 mg/kg body wt, 60 μl/min, 60 s) corresponding to the maximal glucose concentration that does not change peripheral blood glucose level (n = 11; P = 0.98). We then looked for the presence of c-Fos protein as a marker of stimulus-specific cellular activation. Fos labeling was detected as a black precipitate in cell nuclei, which strongly contrasted with the background color. To visualize the POMC neurons, we performed immunohistochemistry against GFP, which colors the cytoplasm brown (Fig. 6*C*). The glucose sensitivity of

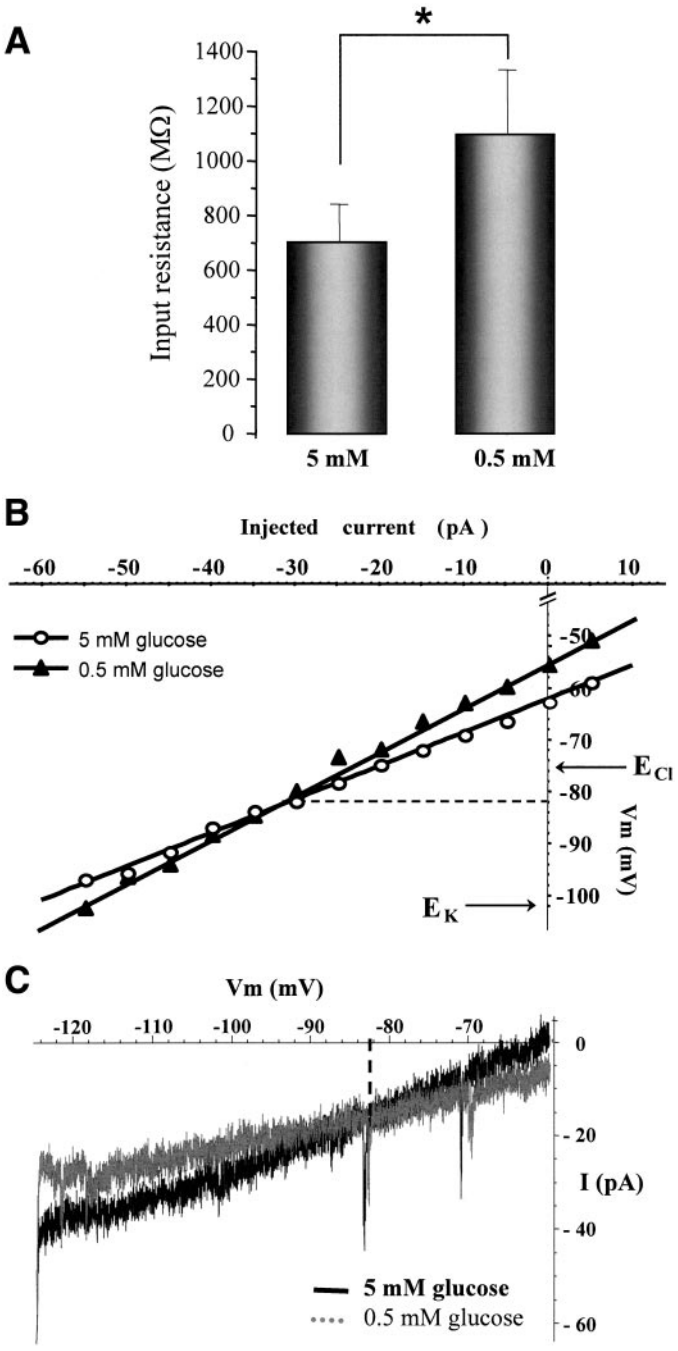

FIG. 3. *A*: The input resistance of glucose-inhibited neurons was greater in 0.5 vs. 5 mmol/l glucose. *$P < 0.0005$. *B*: Voltage-current curve extrapolated from membrane potential changes recorded in response to increasing hyperpolarizing current injection, in 5 mmol/l (○) and 0.5 mmol/l (▲) glucose. The reversal potential was approximately −82 mV for this glucose-inhibited neuron (dotted line). The theoretical equilibrium potentials for Cl^- and K^+ are indicated on the *y*-axis. *C*: Representative current-voltage relationship obtained by applying slow voltage ramps, in 5 mmol/l (black line) and 0.5 mmol/l (gray line) glucose. The reversal potential was approximately −84 mV for this glucose-inhibited neuron (dotted line).

POMC neurons was investigated by quantifying the number of double-labeled cells.

After intracarotid injection of glucose, a significant increase ($P < 0.05$) in the number of c-Fos–positive nuclei is observed in ARC compared with mice given an intracarotid injection of vehicle (glucose, 1,010 ± 141 vs. NaCl, 456 ± 80; $n = 4$; Fig. 6*B*). However, the number of c-Fos–immunopositive POMC neurons is not increased by the glucose injection (glucose, 7 ± 1 vs. NaCl, 9 ± 2; $n = 4$; $P > 0.05$; Fig. 6*B*). These results show that *1*) cells activated by an increase in brain glucose concentration are present in ARC but are not POMC neurons, and *2*) POMC neurons are not glucose sensitive.

DISCUSSION

We show that four distinct subpopulations of glucosensing neurons exist in the ARC. Wang et al. (9) showed previously that some ARC neurons were excited (glucose-excited neurons) or inhibited (glucose-inhibited neurons) in response to glucose changes <5 mmol/l. The same year, we showed that some ARC neurons were excited (high glucose–excited neurons) or inhibited (high glucose–inhibited neurons) by glucose changes >5 mmol/l (6). By evaluating ARC neurons under both glucose ranges, we show here that the glucose-excited and high glucose–excited and, respectively, glucose-inhibited and high glucose–inhibited are distinct glucosensing neurons (Fig. 7). These data are consistent with the data showing that the action potential frequency of glucose-excited neurons is not significantly different in 5 and 10 mmol/l glucose. Moreover, glucose-excited neurons sense glucose through the K_{ATP} channel, whereas high glucose–excited neurons use a K_{ATP}-independent mechanism (6,9).

Brain extracellular glucose concentration is ~30% that of plasma glucose, ranging from 0.1 to 4.5 mmol/l for hypo- to hyperglycemic states, with a steady-state value ~2.5 mmol/l (27–29). However, glucose levels seen by ARC neurons may be higher because of its proximity to the median eminence where the blood-brain barrier is "leaky" (4). That is, if dendrites from ARC neurons project to the median eminence, they would be exposed to levels of glucose seen in the plasma. Thus, ARC neurons may be exposed to changes in glucose less than (like hypoglycemia or fasted status) or greater than 5 mmol/l (like hyperglycemia or postprandial status) (Fig. 7). Our data showing the presence of ARC glucosensing neurons that are specific for changes in glucose less than (glucose-excited and glucose-inhibited neurons) and greater than 5 mmol/l (high glucose–excited and high glucose–inhibited neurons) support this hypothesis. These data suggest that the ARC may be able to sense and integrate changes in both brain and plasma glucose. This is consistent with the role of the ARC as an integrator of peripheral and central signals of energy homeostasis.

Several studies demonstrated involvement of K_{ATP} channels in the response of glucose-excited neurons to glucose (9,17). A K_{ATP} channel–independent mechanism, involving changes in a cationic nonselective conductance, mediates the glucose response in high glucose–excited neurons (6). However, the mechanism mediating the response of glucose-inhibited neurons to glucose remains unclear (rev. in 30). Inhibition of the Na^+-K^+ ATPase pump has been suggested (31). More recently, glucose has been shown to activate a chloride channel in VMN glucose-inhibited neurons (19). Our data suggest that glucose also increases conductance in ARC glucose-inhibited neurons. Therefore, these data are not in agreement with the Na^+-K^+ pump involvement because a modification of its activity cannot change input resistance. Furthermore, I-V curves performed in current- and voltage-clamp mode suggest that like VMN glucose-inhibited neurons (19), ARC glucose-inhibited neurons use a glucose-sensitive chloride conductance to sense glucose.

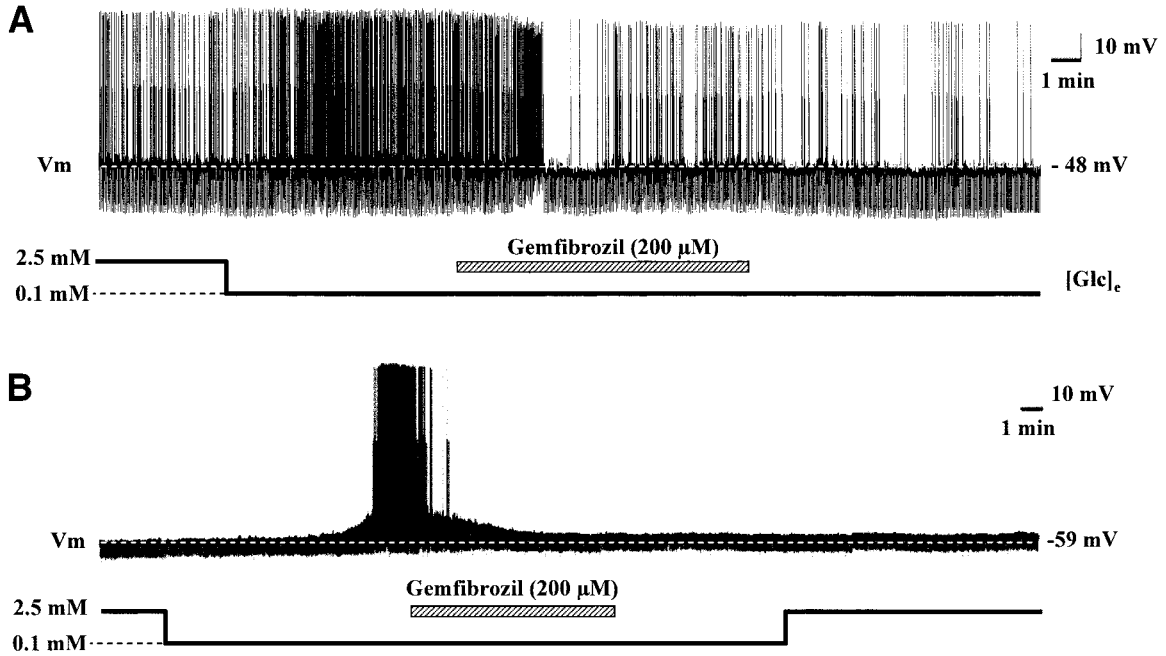

FIG. 4. Whole-cell current-clamp recordings in glucose-inhibited neurons. *A*: In this ARC glucose-inhibited neuron, gemfibrozil (200 μmol/l) reversed the excitatory effect of decreased glucose. This did not reverse upon washout. *B*: The effects of gemfibrozil were similar in this VMN glucose-inhibited neuron. After gemfibrozil washout, this neuron did not respond to changes in glucose.

One candidate for the ion channel involved in the glucose-sensitive chloride conductance is the CFTR (32). The CFTR belongs to the ATP binding cassette (ABC) family, like the K_{ATP} channel. An increase in ATP opens the channel, causing chloride influx and hyperpolarization (33). The CFTR channel gene, mRNA, and protein are expressed in the human and rat brain and in hypothalamic cell lines (32–34). Here, we show that the clofibric acid analog gemfibrozil, a nonselective CFTR inhibitor (25,35), reverses the effects of low glucose in ARC and VMN glucose-inhibited neurons and blocks their ability to detect subsequent changes in glucose. These data support the hypothesis that the CFTR channel could mediate glucose sensing in ARC and VMN glucose-inhibited neurons. However, these data need to be interpreted with caution. Although gemfibrozil is specific for the cardiac CFTR channel, it has never been tested in brain (25). In the heart, gemfibrozil decreases the CFTR channel current, which is opposite to our results in the brain. However, individual enantiomers of clofibric acid can either increase or decrease chloride conductances in skeletal muscle (36). Thus, it is conceivable that gemfibrozil increases the CFTR channel conductance in glucose-inhibited neurons. More importantly, the effect of gemfibrozil was irreversible and blocked the ability of glucose-inhibited neurons to detect glucose. Gemfibrozil also inhibits long-chain acyl-CoA hydrolases in the liver. However, this does not occur in brain (37). Lastly, gemfibrozil is an agonist of the peroxisome proliferator–activated receptor (PPAR)-α (38). However, our data showing that gemfibrozil increases a chloride conductance in glucose-inhibited neurons within several minutes is more consistent with a direct action on an ion channel rather than effects mediated via PPARα. Direct effects of gemfibrozil on ion channels have been reported previously (25,39). Nevertheless, further in vitro and in vivo studies need to be performed to confirm the involvement of a CFTR channel in ARC glucose-inhibited neurons and in brain glucose sensing.

Interestingly, defects in both the K_{ATP} and CFTR channels are associated with altered glucose homeostasis and diabetes. For example, the counter-regulatory response and food intake were blunted in response to hypoglycemia in K_{ATP} channel–deficient mice (40). Moreover, a K_{ATP} channel mutation is responsible for persistent hyperinsulinemia-hypoglycemia in infancy in humans (41). Similarly, patients with cystic fibrosis often develop diabetes (42,43).

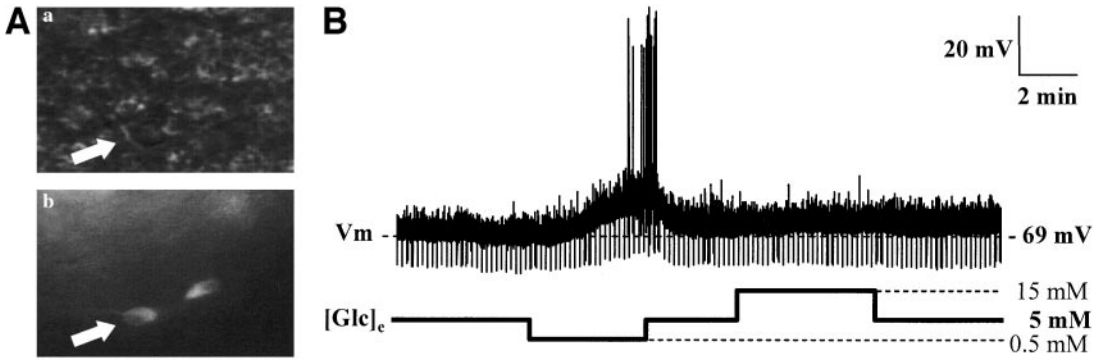

FIG. 5. *A*: A NPY-GFP neuron (white arrow) visualized with ×40 objective under (*a*) infrared and (*b*) fluorescent light. *B*: Whole-cell current-clamp recording of an ARC NPY neuron to altered glucose concentration.

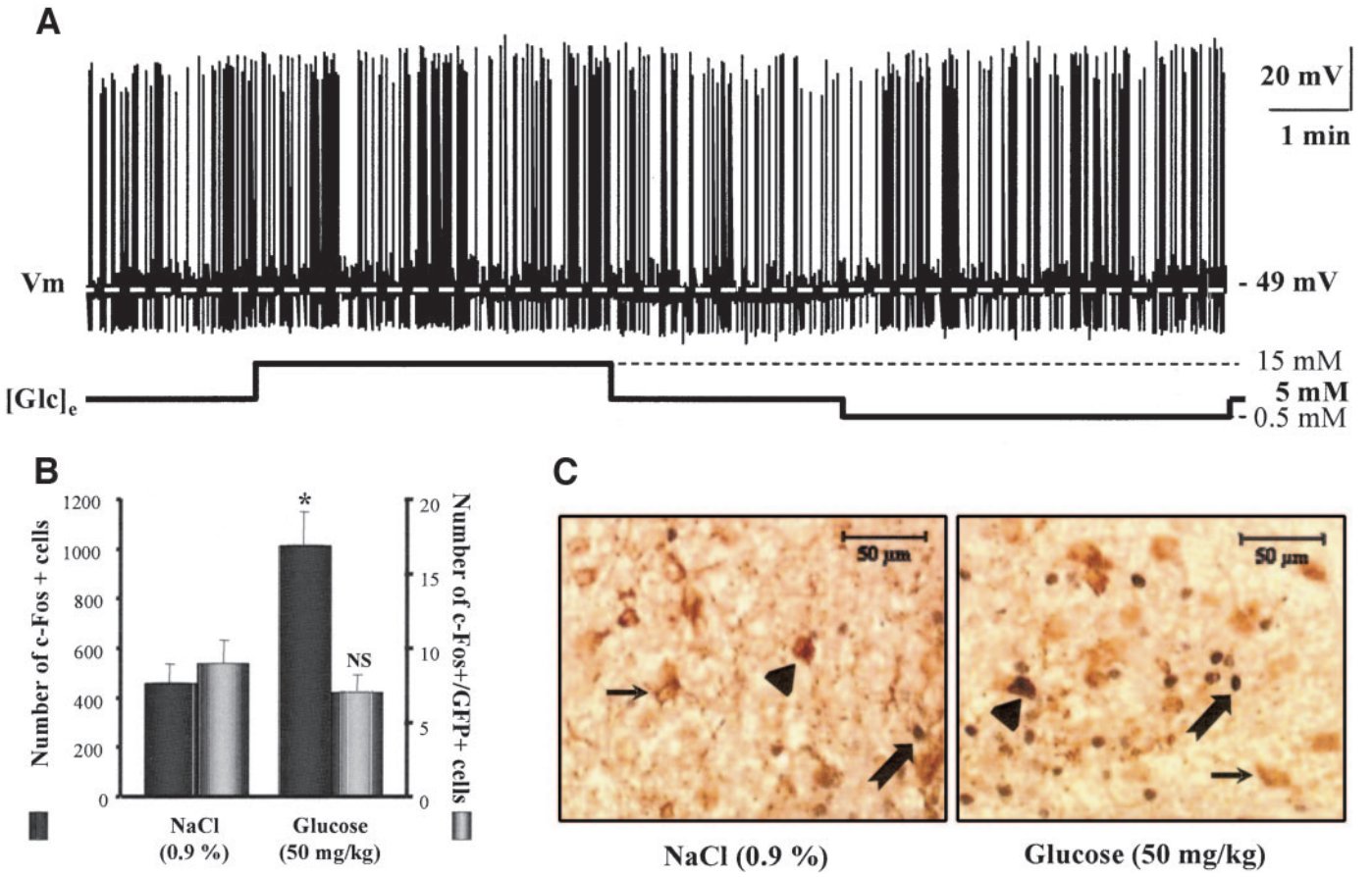

FIG. 6. ***A*****: Whole-cell current-clamp recording of an ARC POMC neuron to altered glucose concentration. Changes in glucose did not change this POMC neuron's activity.** ***B*****: The mean number of c-Fos–immunopositive nuclei (left** ***y*****-axis) and the number of GFP and c-Fos double immunopositive cells (right** ***y*****-axis) in ARC after NaCl or glucose intracarotid injection. *$P < 0.05$ vs. NaCl; NS, $P > 0.05$ vs. NaCl.** ***C*****: Illustration of GFP-immunopositive neurons (thin arrow), c-Fos–immunopositive nuclei (large arrow), and double-labeled cells (arrowhead) after intracarotid injection of NaCl (0.9%;** ***left panel*****) or glucose (50 mg/kg;** ***right panel*****) in POMC-GFP mice.**

This further supports a role for glucose-excited and glucose-inhibited neurons (and the K_{ATP} and CFTR channels) in the regulation of glucose homeostasis.

The peptide phenotype of hypothalamic glucosensing neurons remains unclear. Within the ARC, the NPY and POMC neurons are prime candidates. It is suggested that decreased glucose levels will stimulate NPY and inhibit POMC neurons and vice versa (2). Thus, it is reasonable to hypothesize that glucose-inhibited and/or high glucose–inhibited neurons should express NPY, and glucose-excited and/or high glucose–excited neurons should express POMC. Our data showing that *1*) 40% of NPY neurons are glucose-inhibited neurons; *2*) only glucose-inhibited response was found in NPY neurons; and *3*) <5% glucose-inhibited responses were found in non-NPY neurons support this hypothesis. Our results are consistent with the study by Muroya et al. (20), showing that ARC neurons excited by a glucose decrease from 10 to 1 mmol/l express NPY.

The role of POMC neurons as glucose sensors is more controversial (rev. in 30). ARC glucose-excited neurons were not immunopositive for POMC (9). However, 80% of POMC neurons (10 of 12) were excited by an increase in glucose from 5 to 10 mmol/l (21). Thus, we hypothesized that high glucose–excited neurons may be of the POMC phenotype. Using POMC-GFP mice, our results confirm that POMC neurons are not glucose-excited neurons. However, only 2 of 40 POMC neurons were also high glucose–excited neurons. This is a smaller percentage (5%) of occurrence of high glucose–excited neurons than we found in the ARC overall (6). Double POMC/GFP immunohistochemistry shows that GFP fluorescent cells were POMC neurons. To confirm that POMC neurons are not high glucose–excited neurons in vivo, we evaluated the presence of the neuronal activation marker c-Fos in the ARC after an intracarotid glucose injection shown to increase ARC c-Fos (26). This activation is induced by the cerebral hyperglycemia because peripheral blood glucose concentration is unchanged. The presence of activated cells in the ARC confirm the presence of hyperglycemia-activated neurons i.e., high glucose–excited neurons. However, the number of c-Fos–immunopositive POMC neurons did not increase after intracarotid glucose injection. Altogether, our in vitro and in vivo data strongly suggest that POMC neurons are not glucosensing neurons. Thus, the peptide phenotype of the glucose-excited and high glucose–excited neurons and of the high glucose–inhibited neurons remains unknown. One possible peptide candidate for the glucose-excited or high glucose–excited neurons is Neuromedin U, which is expressed in the ARC and inhibits food intake (44,45).

In conclusion, four distinct glucosensing neuron subpopulations exist in ARC (Fig. 7). Glucose-excited and glucose-inhibited neurons specifically detect changes in glucose levels <5 mmol/l, whereas high glucose–excited and high glucose–inhibited neurons sense changes >5

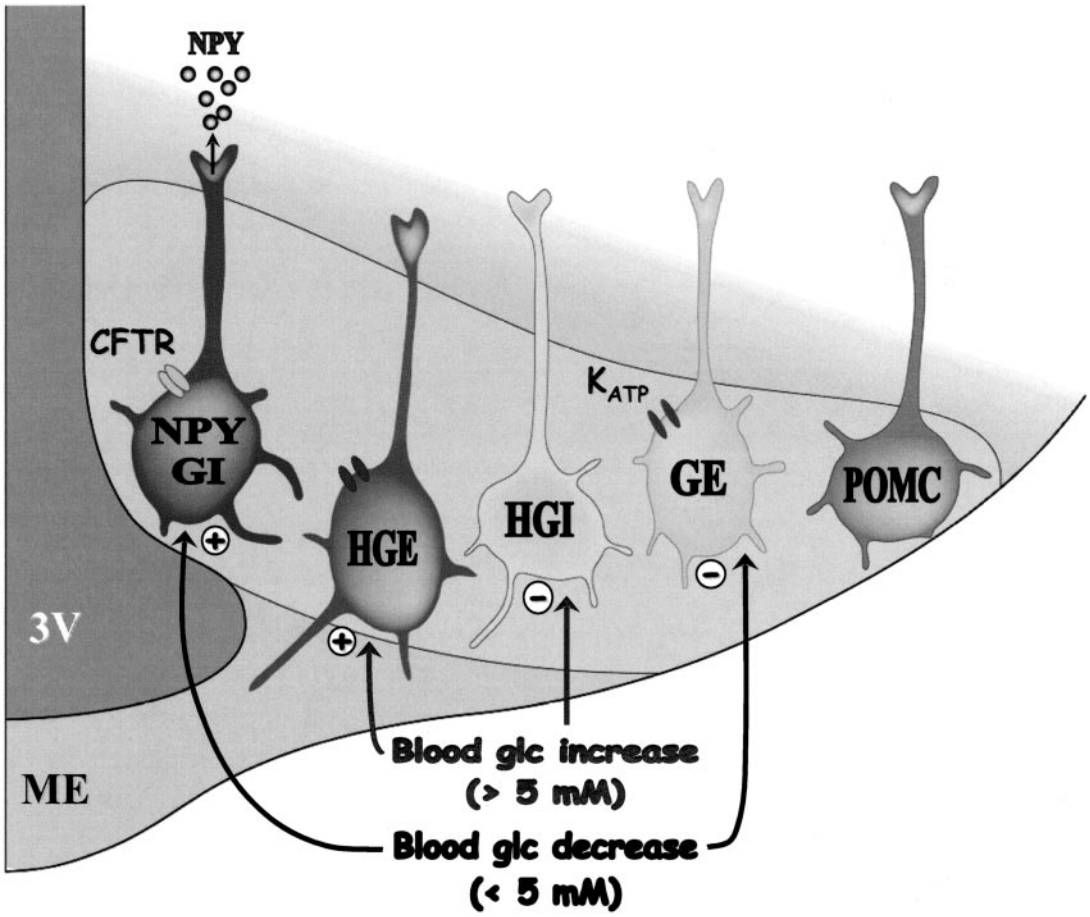

FIG. 7. ARC glucosensing mechanisms. Decreased glucose concentration <5 mmol/l in ARC inhibits glucose-excited neurons by opening the K_{ATP} channel and excites NPY/glucose-inhibited neurons, causing NPY release by closing a CFTR-like chloride channel. Increased glucose >5 mmol/l in ARC excites high glucose–excited neurons by closing a nonselective cation channel and inhibits high glucose–inhibited neurons.

mmol/l. Glucose-inhibited neurons may use the CFTR channel to take part in the control of energy homeostasis in response to decreased glycemia via NPY release. Finally, we show that, converse to a classical idea, POMC neurons are not glucose sensitive.

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