

Characterization of glucosensing neuron subpopulations in the arcuate nucleus: Integration in NPY and POMC networks?

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Running title: Arcuate glucosensing neuron subpopulations

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ABSTRACT

Four types of responses to glucose changes have been described in the arcuate nucleus (ARC): excitation or inhibition by low glucose concentrations below 5 mM (glucose-excited (GE) and -inhibited (GI) neurons) and by high glucose concentrations above 5 mM (high-glucose-excited (HGE) and -inhibited (HGI) 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 GI neurons, and the neurotransmitter identity (NPY or POMC) of glucosensing neurons remained controversial. Using patch-clamp recordings on acute mouse brain slices, successive extracellular glucose changes above and below 5 mM show that GE, HGE, GI and HGI neurons are different glucosensing cell subpopulations. GI neurons directly detect decreased glucose *via* closure of a chloride channel. Using transgenic NPY-GFP and POMC-GFP mice, we show that 40% of NPY neurons are GI neurons. In contrast, less than 5% of POMC neurons responded to changes in extracellular glucose above 5 mM. *In vivo* results confirm the lack of glucose sensitivity of POMC neurons. Taken together, hypo- and hyperglycaemia are detected by distinct populations of glucosensing neurons and that POMC and NPY neurons are not solely responsible for ARC glucosensing.

ABBREVIATIONS

ARC, arcuate nucleus; CFTR, cystic fibrosis transmembrane regulator; HGE neurons, high glucose-excited neurons; HGI neurons, high glucose-inhibited neurons; GE neurons, glucose-excited neurons; GI neurons, glucose-inhibited neurons; K_{ATP} channels, ATP-sensitive potassium channels; ME, median eminence; NPY, neuropeptide Y; POMC, pro-opio melanocortin; RT, room temperature; VMH, ventromedial hypothalamus; VMN, ventromedian nucleus.

INTRODUCTION

The hypothalamus integrates hormonal (e.g., insulin, 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 (ME), 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 (α -MSH; cleavage product of pro-opio melanocortin (POMC)) (2). Hypothalamic NPY pathways favor anabolic processes, and increase food intake, while 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). Hypoglycaemia is sensed by the brain, which then initiates the counter-regulatory response (13). Hypoglycaemia stimulates, and hyperglycaemia 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 (GE) neurons increase, and Glucose-inhibited (GI) neurons decrease their electrical activity as extracellular glucose concentration increases from 0.1 to 5 mM. (9,19). The ATP-dependent potassium channel (K_{ATP}) mediates the response to glucose in ARC and VMN GE neurons, while an unidentified chloride channel mediates the glucose response in VMN GI neurons (19). The mechanism by which ARC GI neurons sense glucose remains unclear. ARC glucosensing neurons also exist that respond to changes in glucose concentration above 5 mM (6). These neurons were defined as high glucose-excited (HGE) and high glucose-inhibited

(HGI) neurons. Interestingly, HGE neurons used a K_{ATP} channel-independent mechanism to sense glucose.

Thus, ARC glucosensing neurons sense changes in extracellular glucose below or above 5 mM (Table 1). The first aim of this study was to determine whether GE and HGE neurons, or GI and HGI neurons, were the same or different populations of neurons. 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 which increased their intracellular calcium levels as glucose levels decrease from 10 to 0 mM were immunopositive for NPY (20). However, this glucose step does not differentiate between GI and HGI-neurons. POMC neurons have been shown to be excited as glucose increased from 5 to 10 mM. (21). However, ARC GE neurons are not immunopositive for POMC (9). To address this controversy, we used transgenic NPY or POMC-GFP mice, which specifically express the green fluorescent protein (GFP) in NPY or POMC neurons, to investigate their glucose sensitivity.

MATERIALS AND METHODS

Animals

All experiments were carried out in non-fasted mice in accordance with European Community and United States guidelines. Electrophysiological recordings were performed on hypothalamic slices from 21- to 35-day old C57Bl/6 mice. *In vivo* experiments used 7-8 weeks old male C57BL/6J mice.

Some experiments used homozygous transgenic NPY-GFP or POMC-GFP mice provided by Dr JM Friedman (22). These two lines of transgenic mice were generated by using bacterial artificial chromosome (BAC) expressing either sapphire green fluorescent protein (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 μ m coronal slices per brain were cut in an ice-cold oxygenated (95% O₂ - 5% CO₂) saline solution containing (in mM): 200 sucrose, 28 NaHCO₃, 2.5 KCl, 7 MgCl₂, 1.25 NaH₂PO₄, 0.5 CaCl₂, 1 L-Ascorbate, 3 Na-Pyruvate, 8 D-glucose (pH 7.4). Slices were then incubated at room temperature (RT), in oxygenated extracellular medium containing (in mM): 118 NaCl, 3 KCl, 1 MgCl₂, 25 NaHCO₃, 1.2 NaH₂PO₄, 1.5 CaCl₂, 5 Hepes, 5 D-glucose, 15 sucrose (osmolarity adjusted at 300-310 mOsmol/l, pH 7.4) for 1 hour 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 X60 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 AG).

Borosilicate pipettes (5-7 M Ω ; GC150F-10, Phymep) were filled with an internal solution containing (in mM): 155 K-gluconate, 0.1 EGTA, 1 CaCl₂, 10 Hepes, 5 KCl, 10 KOH, 5 Mg-ATP, 0.4 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 mM or 0 mM D-glucose (osmolarity adjusted for each solution with sucrose at 300-310 mOsmol/l, pH 7.4) was applied to 5 mM glucose.

D-glucose was purchased from Sigma. Tetrodotoxin (TTX; Latoxan) and gemfibrozil (Sigma) were prepared as concentrated stock solution 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, Segre Electronique) positioned approximately at 3 mm from the ARC. **Using this application system, glucose concentration changes in the recording chamber were performed from 5 to 0.5 mM or from 5 to 15 mM.** For a set of experiments (Figure 4), a different system was used and extracellular glucose

concentration was decreased from 2.5 to 0.1 mM.

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 mean \pm SEM and differences between groups were analyzed by paired or unpaired Student's *t*-test, with $p < 0.05$ taken as significant (NS = not significant).

In vivo experiments

Surgery and injections

POMC-GFP mice were anaesthetized by an i.p. injection of ketamine-xylazine (100 and 10 mg/kg b.w., respectively). A Silastic[®] catheter (VWR) was implanted into the left internal carotid artery with the proximal end directed towards 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 which did not fully recover their pre-surgery body weight were systematically excluded from the study.

On the morning of experiment (between 10:00h and 12:00h), freely moving mice were injected, for 60 s, through the catheter, with isotonic NaCl-containing glucose solution (50 mg/kg b.w., 60 μ l/min).

c-Fos and *GFP* immunohistochemistry

Two hours after glucose injection, mice were anaesthetized by pentobarbital injection into the catheter. A rapid fall down of the animal allowed us to control the correct location and the non-obstruction of the catheter. Then, mice were intracardially 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- μ m serial sections on a cryostat (Leica,) and free-floating sections were collected in phosphate-buffered saline (PBS) (pH 7.4).

After quenching peroxidase activity (0.1% H₂O₂, 30 min), sections were incubated in 3% normal goat serum (Sigma)/PBS/0.3% triton X100 (Sigma) for 6 h at RT, and then in the rabbit antiserum against Fos protein (1/10000; Ab-5 antibody, Oncogene) overnight at 4 °C. After washing, sections were incubated in biotinylated goat anti-rabbit IgG (1: 2000, Jackson ImmunoResearch Laboratories) for 45 min at RT and finally incubated in streptavidin-peroxidase conjugated (1/2000, Jackson Laboratories) for 1 h at RT. Peroxidase activity was revealed using a mixture of diaminobenzidine and nickel ammonium sulphate chloride as described by Shu et al. (23).

Following *c-fos* revelation, free-floating sections were incubated in rabbit polyclonal antibody against GFP (1/1000; Molecular Probes) overnight at 4 °C. Sections were then incubated for 1 h at RT in streptavidin-peroxidase conjugated (1/1000; Jackson Laboratories). Peroxidase activity was revealed by diaminobenzidine. Fos labelling 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), Micron] and examined under a transmitted-light microscope (Leica).

Quantification and statistical analysis

Fos immuno-positive cells were counted using a computerized image analysis (Visilog 6.2 software, Noesis). The results are expressed as the sum of single or double labeled cells counted bilaterally on six sections per ARC and are given as mean \pm SEM for several independent experiments. The mean values from glucose- vs NaCl-injected mice were compared using unpaired

Student's *t*-test, with $p < 0.05$ taken as significant (NS = not 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 below 5 mM, from 5 to 0.5 mM and above 5 mM, from 5 to 15 mM. All recorded glucosensing neurons were sensitive to changes in glucose either above or below 5 mM, but not both glucose ranges. Thus, GE neurons never responded to an increase in glucose level above 5 mM ($n = 3$; Figure 1A), nor did HGE neurons respond to a decrease in glucose below 5 mM ($n = 5$; Figure 1B). Similarly, GI and HGI neurons were only sensitive to changes in glucose below and above 5 mM, respectively ($n = 13$; Figure 1C). Thus there are four distinct subpopulations of glucosensing neurons in the ARC.

Characterization of ARC GI neurons

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

To identify the glucose regulated conductance in ARC GI neurons, input resistance was measured by recording the membrane potential change in response to a

constant hyperpolarizing current injection. Depolarization of GI neurons correlated with a significant increase of input resistance of 64 % (5 mM: 703 ± 138 M Ω *vs* 0.5 mM: 1098 ± 235 M Ω ; $n = 6$; $p < 0.05$; Figure 3A), indicating decreased membrane conductance. Voltage-current relationships were extrapolated from increasing hyperpolarizing current injections (-5 to -50 pA) in order to identify the ionic conductance involved. The glucose sensitive current reversed at -80.4 ± 1.8 mV ($n = 10$; Figure 3B) which is close to the theoretical E_{Cl} in our studies (-74 mV). Voltage-clamp recordings applying depolarizing voltage ramps (from -130 to -50 mV) confirmed this result ($n = 4$; Figure 3C). **In order to confirm involvement of a chloride conductance, we changed the chloride concentration in the internal medium in order to switch E_{Cl} to -57 mV. Under this condition, the reversal potential of the glucose response of GI neurons is -63 ± 8.1 mV ($n=3$).** Thus, these results suggest that decreased glucose below 5 mM depolarized ARC GI neurons through decreasing chloride conductance, as in VMN GI neurons (19).

The clofibric acid analogue, gemfibrozil is specific for the cardiac cystic fibrosis transmembrane regulator (CFTR) (25). **In 0.1 mM glucose, gemfibrozil (200 μ M) decreases input resistance of GI neurons from 1166 ± 201 to 999 ± 187 M Ω ($n = 8$; $p < 0.05$), 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 chloride channel in hypothalamic GI neurons.** Here, gemfibrozil (200 μ M) reversed the effects of decreased glucose on action potential frequency and membrane conductance in ARC GI neurons ($n = 4$; Figure 4A) as well as VMN GI neurons ($n = 3$; Figure 4B). This suggests that gemfibrozil opened the CFTR channel on GI neurons. This effect was irreversible. Following gemfibrozil treatment, GI 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 GI neurons.

Glucose sensitivity of NPY and POMC neurons

NPY-GFP transgenic mice were used to test the glucose sensitivity of ARC NPY neurons (Figure 5A) (22). Forty percent (21/52) ARC **NPY-GFP neurons** were depolarized by glucose decrease below 5 mM, thus they are GI neurons (Figure 5B). ARC **NPY-GFP neurons** were never HGI neurons as indicated by a lack of hyperpolarization when glucose increases above 5 mM (Figure 4). **Moreover, GI-like responses of NPY neurons persist in 500 nM of TTX showing that NPY neurons directly detect glucose concentration decrease from 5 to 0.5 mM (data not shown).** In the presence of 5 mM extracellular glucose, some ARC **NPY-GFP neurons** are silent while some are spontaneously active. Most of the **NPY-GFP ARC neurons** which show a GI-like response are silent at 5 mM glucose concentration. Finally, recordings of **non-NPY-GFP neurons** show that less than 5 % of these cells are GI neurons (1/22). Thus, most GI neurons use NPY as 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 below and above 5 mM. The electrical activity of 95 % (38/40) of these **POMC-GFP neurons** did not change in response to changes above or below 5 mM (Figure 6A). 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 below 5 mM but are not consistent with Ibrahim et al. (21) for glucose changes above 5 mM. In order to confirm our data for glucose changes above 5 mM, 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 b.w., 60 μ l/min, 60 s) corresponding to the maximal glucose concentration which 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 (Figure 6C). The glucose-sensitivity of 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 to mice given an intracarotid injection of vehicle (Glucose: 1010 ± 141 vs NaCl: 456 ± 80 ; $n = 4$; Figure 6B). However, the number of c-Fos immunopositive POMC neurons is not increased by the glucose injection (Glucose: 7 ± 1 vs NaCl: 9 ± 2 ; $n = 4$; $p > 0.05$; Figure 6B). These results show that i) cells activated by an increase in brain glucose concentration are present in ARC but are not POMC neurons and ii) POMC neurons are not glucose-sensitive.

DISCUSSION

We show that four distinct subpopulations of glucosensing neurons exist in the arcuate nucleus (ARC). Wang et al. showed previously that some ARC neurons were excited (GE neurons) or inhibited (GI neurons) in response to glucose changes below 5 mM (9). The same year, we showed that some ARC neurons were excited (HGE neurons) or inhibited (HGI neurons) by glucose changes above 5 mM (6). By evaluating ARC neurons under both glucose ranges, we show here that the

GE and HGE, and respectively GI and HGI, are distinct glucosensing neurons (Figure 7). These data are consistent with the data showing that the action potential frequency of GE neurons is not significantly different in 5 and 10 mM glucose. Moreover, GE neurons sense glucose through *via* the K_{ATP} channel while HGE neurons use a K_{ATP} -independent mechanism (6,9).

Brain extracellular glucose concentration is approximately 30% that of plasma glucose, ranging from 0.1 to 4.5 mM for hypo- to hyperglycaemic states, steady state value around 2.5 mM (27-29). However, glucose levels seen by ARC neurons may be higher due to 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 below (like hypoglycaemia or fasted status) or above 5 mM (like hyperglycaemia or postprandial status) (Figure 7). Our data showing the presence of ARC glucosensing neurons which are specific for changes in glucose below (GE and GI neurons) and above 5 mM (HGE and HGI neurons) supports 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 GE neurons to glucose (9,17). A K_{ATP} channel-independent mechanism, involving changes in a cationic non-selective conductance mediates the glucose response in HGE neurons (6). However, the mechanism mediating the response of GI neurons to glucose remains unclear (for review (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 GI neurons (19). Our data suggest that glucose also increases conductance in ARC GI

neurons. Therefore, these data are not in agreement with the Na^+K^+ pump involvement since a modification of its activity can not change input resistance. Furthermore, I-V curves performed in current- and voltage-clamp mode, suggest that, like VMN GI neurons (19), ARC GI neurons use a glucose sensitive chloride conductance to sense 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, as well as in hypothalamic cell lines (32-34). Here, we show that the clofibric acid analog, gemfibrozil, a non-selective CFTR inhibitor (25,35), reverses the effects of low glucose in ARC and VMN GI 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 GI neurons.** However, these data need to be interpreted with caution. While gemfibrozil is specific for the cardiac CFTR channel, it has never been tested in brain (25). In heart, gemfibrozil decreases the CFTR channel current which is opposite to our results in 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 GI neurons. More importantly, the effect of gemfibrozil was irreversible and blocked the ability of GI neurons to detect glucose. Gemfibrozil also inhibits long-chain acyl-CoA hydrolases in the liver. However, this does not occur in brain (37). Last, gemfibrozil is an agonist of the peroxisomal proliferators-activator receptor-(PPAR) $_{\alpha}$ (38). However, our data showing that gemfibrozil increases a chloride conductance in GI neurons within several minutes is more consistent with a direct

action on an ion channel rather than effects mediated via PPAR α . 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 GI 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 hypoglycaemia 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). This further supports a role for GE and GI 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 visa versa (2). Thus it is reasonable to hypothesize that GI and/or HGI neurons should express NPY and GE and/or HGE neurons should express POMC. Our data showing that: i) 40 % of NPY neurons are GI neurons; ii) only GI-response was found in NPY neurons; and iii) less than 5 % GI-responses were found in non-NPY neurons support this hypothesis. **Our results are consistent with study Muroya et al., showing that ARC neurons excited by 10 to 1 mM glucose decrease express NPY (20).**

The role of POMC neurons as glucose sensors is more controversial (for review (30)). ARC GE neurons were not immunopositive for POMC (9). However, 80 % of POMC neurons (10/12) were excited an increase in glucose from 5-10 mM (21). Thus, we hypothesized that HGE neurons may be of the POMC phenotype. Using POMC-GFP mice, our results confirm

that POMC neurons are not GE neurons. However, only 2 of 40 POMC neurons were also HGE neurons. This is a smaller percentage (5%) of occurrence of HGE neurons than we found in the ARC overall (6).

Double POMC/GFP immunohistochemistry shows that GFP fluorescent cells were indeed POMC neurons. To confirm that POMC neurons are not HGE 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 hyperglycaemia since peripheral blood glucose concentration is unchanged. The presence of activated cells in the ARC confirm the presence of hyperglycaemia-activated neurons i.e. HGE 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 GE and HE, as well as HGI, neurons remains unknown. One possible peptide candidate for the GE or HGE 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 (Figure 7). GE and GI neurons specifically detect changes in glucose levels below 5 mM, while HGE and HGI neurons sense changes above 5 mM. GI neurons may use the CFTR channel to take part in the control of energy homeostasis in response to decreased glycaemia *via* NPY release. Finally, we show that, converse to a classical idea, POMC neurons are not glucose sensitive.

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FIGURES LEGENDS

FIGURE 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 GE neuron; however increased glucose had no effect. **B:** Increased glucose excited this HGE neuron; however decreased glucose had no effect. **C:** Decreased glucose excited this GI neuron; however increased glucose had no effect.

FIGURE 2: **A:** Consecutive whole-cell current-clamp recordings in an ARC GI neuron. Decreased glucose excited this neuron in the presence (lower trace) and absence (upper trace) of 500 nM TTX. **B:** The presence of 500 nM TTX did not alter GI neurons membrane potential; $n = 5$; NS: $p > 0.05$.

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

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

FIGURE 5: **A:** A NPY-GFP neuron (white arrow) visualized with X40 objective under (a) infrared (b) fluorescent light. **B:** Whole-cell current-clamp recording of an ARC NPY neuron to altered glucose concentration.

FIGURE 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 (arrow head) following intracarotid injection of NaCl (0.9 %; left panel) or glucose (50 mg/kg; right panel) in POMC-GFP mice.

FIGURE 7: ARC glucosensing mechanisms

Decreased glucose concentration below 5 mM in ARC inhibits GE neurons by opening the K_{ATP} channel and excites NPY/GI neurons causing NPY release by closing a CFTR-like chloride channel. Increased glucose above 5 mM in ARC excites HGE neurons by closing a non-selective cation channel and inhibits HGI neurons.

Figures are available in a separate .pdf file.

TABLE 1: ARC glucosensing neurons (6,9).

Table 1

Glucosensing neurons	Glucose		responses	Mechanism involved
	sensitivity range :	<u>below</u> 5 mM	<u>above</u> 5 mM	
Glucose tested :	<u>change</u>	Decrease (2.5 → 0.1 mM)	Increase (5 → 20 mM)	
GE		Inhibition	?	K _{ATP}
GI		Excitation	?	?
HGE		?	Excitation	Non-selective cationic conductance
HGI		?	Inhibition	?