Original Article

Glucokinase Is a Critical Regulator of Ventromedial Hypothalamic Neuronal Glucosensing

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To test the hypothesis that glucokinase is a critical regulator of neuronal glucosensing, glucokinase activity was increased, using a glucokinase activator drug, or decreased, using RNA interference combined with calcium imaging in freshly dissociated ventromedial hypothalamic nucleus (VMN) neurons or primary ventromedial hypothalamus (VMH; VMN plus arcuate nucleus) cultures. To assess the validity of our approach, we first showed that glucose-induced (0.5–2.5 mmol/l) changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) oscillations, using fura-2 and changes in membrane potential (using a membrane potential–sensitive dye), were highly correlated in both glucose-excited and -inhibited neurons. Also, glucose-excited neurons increased (half-maximal effective concentration [EC_{50}] = 0.54 mmol/l) and glucose-inhibited neurons decreased (half-maximal inhibitory concentration [IC_{50}] = 1.12 mmol/l) $[Ca^{2+}]_i$ oscillations to incremental changes in glucose from 0.3 to 5 mmol/l. In untreated primary VMH neuronal cultures, the expression of glucokinase mRNA and the number of demonstrable glucosensing neurons fell spontaneously by half over 12–96 h without loss of viable neurons. Transfection of neurons with small interfering glucokinase RNA did not affect survival but did reduce glucokinase mRNA by 90% in association with loss of all demonstrable glucose-excited neurons and a 99% reduction in glucose-inhibited neurons. A pharmacological glucokinase activator produced a dose-related increase in $[Ca^{2+}]_i$ oscillations in glucose-excited neurons (EC_{50} = 0.98 mmol/l) and a decrease in glucose-inhibited neurons (IC_{50} = 0.025 μmol/l) held at 0.5 mmol/l glucose. Together, these data support a critical role for glucokinase in neuronal glucosensing. *Diabetes* 55:412–420, 2006

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AUC, area under curve; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; EC_{50}, half-maximal effective concentration; FLIPR, fluorometric imaging plate reader; IC_{50}, half-maximal inhibitory concentration; RNAi, RNA interference; siRNA, small interfering RNA; VMH, ventromedial hypothalamus; VMN, ventromedial hypothalamic nucleus.

Unlike most neurons in the brain that utilize glucose to fuel their metabolic needs (1), a select group of neurons use glucose as a signaling molecule to alter their firing rate as a means of glucosensing (2,3). Glucose-excited neurons increase, whereas glucose-inhibited neurons decrease, their firing rate as ambient glucose levels rise (2–7). During situations of low glucose availability, glucose-inhibited neurons are activated and glucose-excited neurons inactivated (4–8). The ventromedial hypothalamus (VMH) area contains both the ventromedial hypothalamic nucleus (VMN) and arcuate nucleus. Both contain glucosensing neurons that respond to differing levels of glucose and are linked to pathways involved in the regulation of glucose homeostasis (3–11) and the counterregulatory responses to hypoglycemia (12–19). Our work (6,7) and that of others (8) strongly support a role for glucokinase (hexokinase IV) as a key regulator of neuronal glucosensing, which is similar to its purported role in pancreatic β-cell glucosensing (20,21). We previously demonstrated that inhibition of glucokinase activity reduced glucose-excited and increased glucose-inhibited neuronal activity at 2.5 mmol/l glucose, the concentration at which they are normally active and inactive, respectively (6,7).

Recurrent hypoglycemia is common in patients with type 1 diabetes, especially in children (22–25). This leads to hypoglycemia-associated autonomic failure, in which counterregulatory responses to subsequent bouts of hypoglycemia are severely blunted (26–28). Our previous studies suggested that the development of hypoglycemia-associated autonomic failure might be associated with changes in the ability of VMH neurons to sense and respond to glucose (6,28). Furthermore, its development is associated with upregulation of glucokinase mRNA in the VMH (6,28,29). This upregulation might be a compensatory response that makes glucosensing neurons more sensitive to glucose by shifting their concentration-response to the left. If so, this could underlie the development of hypoglycemia-associated autonomic failure because it would require lower levels of glucose to be reached before counterregulatory responses were initiated. This would predict that increasing glucokinase activity would produce a leftward shift in glucose sensitivity in VMH glucosensing neurons such as it does in pancreatic β-cells, using a drug that increases glucokinase activity (30). It would also predict that reducing glucokinase activity would inhibit the response to glucose. Here, we tested these hypotheses in dissociated VMN and cultured VMH neurons, using calcium imaging combined with a glucokinase activator

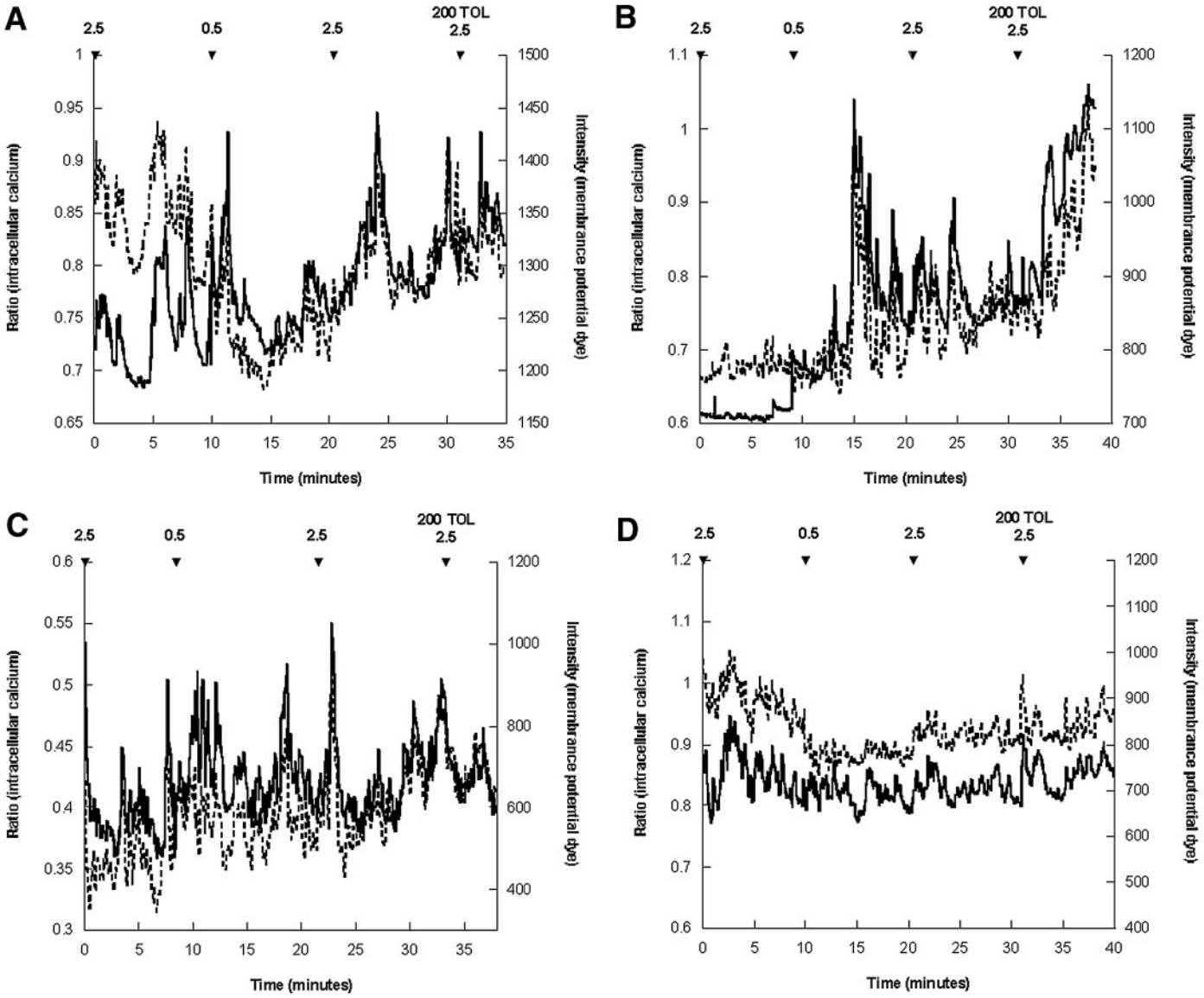

FIG. 1. Effect of glucose and tolbutamide (TOL) on membrane potential (dotted line) and $[Ca^{2+}]_i$ (solid line) in VMN neurons. *A*: Lowering glucose from 2.5 to 0.5 mmol/l comparably suppressed, whereas raising glucose from 0.5 to 2.5 mmol/l or the addition of 200 μmol/l tolbutamide comparably increased, $[Ca^{2+}]_i$ oscillations (ratio) and membrane potential fluorescence (intensity) in this glucose-excited neuron. *B*: This glucose-inhibited neuron had comparable changes in $[Ca^{2+}]_i$ oscillations and membrane potential fluorescence to altered glucose levels and the addition of 200 μmol/l tolbutamide. Spontaneously active (*C*) and inactive (*D*) nonglucosensing neurons had comparable spontaneous oscillations of $[Ca^{2+}]_i$ and membrane potential fluorescence, despite their nonresponsiveness to glucose and 200 μmol/l tolbutamide.

drug to increase glucokinase activity and small interfering RNA (siRNA) to glucokinase mRNA to decrease glucokinase activity.

RESEARCH DESIGN AND METHODS

All work was approved by the institutional animal care and use committee of the New Jersey Medical School. Male outbred 4- to 5-week-old Sprague-Dawley rats (Charles River Labs) were housed on a 12-h light/dark schedule (lights on at 0700) and kept at 22–23°C. Food (Purina rat chow no. 5001) and water were available ad libitum.

Single neuron measurements of glucose-induced intracellular Ca^{2+} concentration fluctuations. Freshly prepared single hypothalamic VMN neurons were prepared as described previously (7). Briefly, the VMN was punched, and single cells were papain digested (2 mg/ml, 30 min, 37°C) and mechanically triturated. Cells were plated onto coverslips and allowed to adhere for 60 min before loading with the Ca^{2+} fluorophore fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) for 20 min in Hanks' balanced salt solution buffer (135 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, and 10 mmol/l HEPES, pH 7.4) containing 0.25% (wt/vol) fatty acid–free BSA and 2.5 mmol/l glucose at 37°C. After washing twice with Hanks' balanced salt solution buffer to remove fura-2, cells were transferred to a microscope chamber held at 37°C. Fura-2 fluorescent images were acquired every 5 s by alternating excitation at 340 and 380 nm, and emissions (420–600 nm) were collected using a cooled, charge-coupled device camera. Fura-2 calibration parameters were determined in vitro (31). The values for R_{min}/R_{max} and constant β were calculated from measurements with fura-2–free acid in solution in the microscope chamber. The dissociation constant (K_d) for fura-2·Ca^{2+} complex was taken as 224 nmol/l (31). Neurons were classified as glucose-excited, glucose-inhibited, and nonglucosensing, as previously described (7).

Simultaneous assessment of membrane potential and intracellular Ca^{2+} concentration fluctuations. The lipophilic, anionic, bis-oxonol dye in the fluorometric imaging plate reader (FLIPR) membrane potential assay kit (Molecular Devices, Sunnyvale, CA) and fura-2 was used in dissociated VMN neurons to assess simultaneous fluorescent imaging of glucose-induced changes in membrane potential and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) flux. The distribution across the plasma membrane of FLIPR dye is dependent on the membrane potential of the cell (32). Depolarization causes dye entry, where it binds to intracellular proteins and lipids, producing increased fluorescence signal. Hyperpolarization causes the dye to exit the cell, which decreases fluorescence (32). The VMN neurons were attached to coverslips,

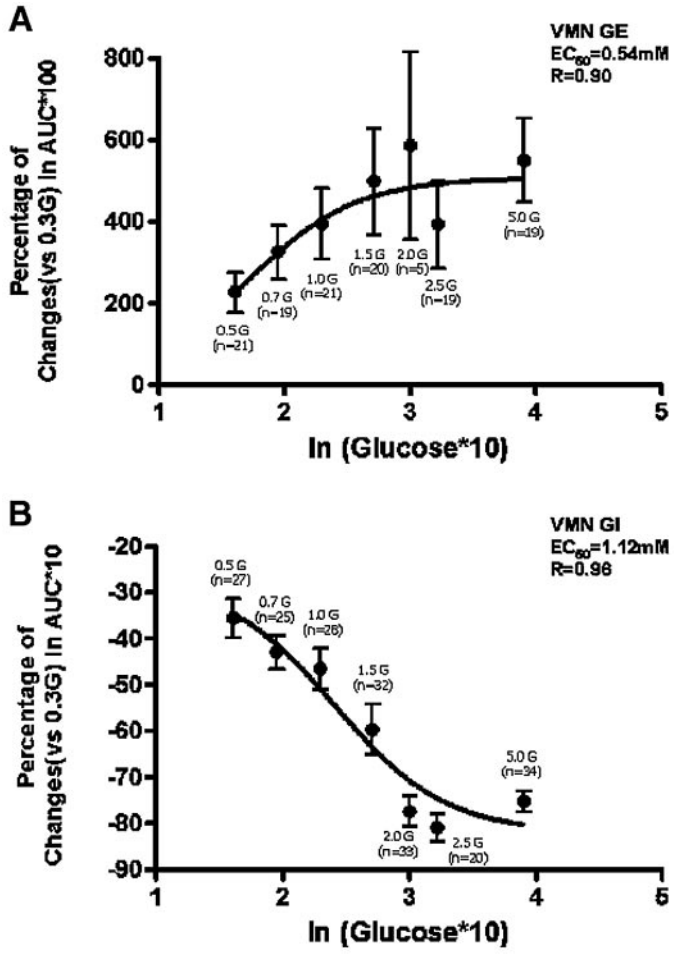

FIG. 2. Glucose concentration-response relationship for VMN glucosensing neurons at different concentrations between 0.3 and 5 mmol/l glucose. Data points were derived by calculating the difference between $[Ca^{2+}]_i$ AUC at each glucose (G) concentration. Each AUC was then normalized as a percent of the average maximal change in AUC between 0.3 and 5 mmol/l glucose. *A*: Glucose concentration-response for the activation of glucose-excited (GE) neurons. *B*: Glucose concentration-response for the inhibition of glucose-inhibited (GI) neurons. Data are the means ± SE of percent of changes in $[Ca^{2+}]_i$ flux. *n*, number of neurons assessed per data point.

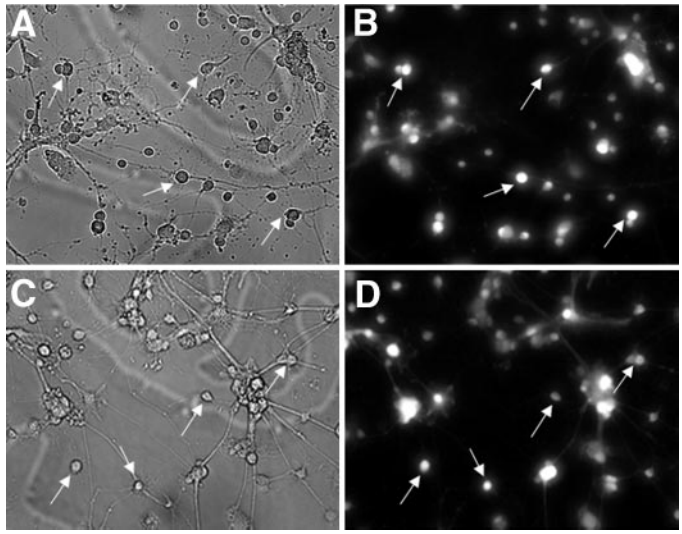

FIG. 3. Effect of glucokinase siRNA on neuron viability in cultured VMH neurons. Neurons were untreated (*A* and *B*) or transfected with glucokinase siRNA (*C* and *D*) after 24 h in culture. After 72 h (96 h in culture), they were counted using phase contrast microscopy (*A* and *C*) and also tested for viability by their uptake of fura-2 acetoxymethyl (*B* and *D*). Arrows mark individual and clustered neurons visualized by both phase (*A* and *C*) and fluorescence microscopy (*B* and *D*).

loaded with 2.5 μmol/l fura-2 acetoxymethyl for 20 min, washed twice, and transferred to the microscope chamber in 2.5 mmol/l glucose Hanks' balanced salt solution with 0.1% FLIPR membrane potential dye for 30 min. Membrane potential and fura-2 fluorescent images were collected at 5-s intervals. Excitations were set to 548 nm for membrane potential and 340–380 nm for Ca^{2+}. Emissions were set to 420–600 nm.

Primary culture of VMH neurons. The protocol followed was adapted from our previously published methods (7,9), based on the original description by Hentges et al. (33). We used VMH rather than just VMN neurons to increase the number of neurons available for culture. Brain slices containing VMH sections were digested for 30 min with papain (2 mg/ml) in Hibernate media (Brainbit) containing 2.5 mmol/l glucose, 1 mmol/l L-lactate, 0.23 mmol/l L-pyruvate, 2% (vol/vol) B-27, 0.5 mmol/l L-glutamine, and 100 units/ml penicillin/streptomycin, and the slices were rinsed and triturated. The cell suspension was centrifuged and the pellet resuspended with 1–1.5 ml Neurobasal-A media (Invitrogen, Carlsbad, CA) containing 2.5 mmol/l glucose, 1 mmol/l L-lactate, 0.23 mmol/l L-pyruvate, 2% B27, 0.5 mmol/l L-glutamine, and 100 units/ml penicillin/streptomycin. Cells were plated on coverslips in Neurobasal-A media containing different concentrations of glucose to investigate the effect of culture on glucokinase mRNA.

Assessment of neuronal survival over time in culture and after the RNA interference (RNAi) procedure (see below) was carried out by plating neurons on coverslips with fiducial markings and counting them over 24–96 h in culture, using phase-contrast microscopy. Comparable counts were made over 96 h in cultures treated with glucokinase or nonsense siRNA 24 h after plating. Viability was tested at 96 h by uptake of fura-2 acetoxymethyl. Counting of cells was performed on a Bioquant image analysis system, using digitized images at each time period.

Design and transfection of glucokinase siRNA. Glucokinase siRNA was designed according to criteria described previously (34). Glucokinase siRNA was 21 nucleotides long and contained symmetric 3′ overhangs of two uridines that targeted the DNA sequence GGTACGACTTGTGCTGCTTAA (pancreatic glucokinase, GenBank accession no. M25807, 888-908). Nonsense siRNA that contained a sequence that did not correspond to any known gene (Qiagen, Valencia, CA) was used as a control. The RNAi procedure was carried out in isolated VMH neurons cultured overnight. They were transfected with glucokinase or nonsense siRNA and compared with untreated cultures. Briefly, 0.33 μg siRNA stock solution was diluted in a final volume of 100 μl of complete culture medium and incubated for 15 min with 3 μl RNAiFect transfection reagent (Qiagen). Fresh medium was added, and the cells were incubated at 37°C, 5% CO_2. All transfections were performed in at least triplicate. At 24, 48, and 96 h postplating, glucosensing ability of cultured neurons was determined by calcium imaging.

Assessment of cultured neuron glucokinase mRNA expression. Cultured neuron mRNA was quantified with real-time quantitative RT-PCR, as previously described (35). The mRNA was extracted from cells using an RNAqueous-96 automated kit (Ambion, Austin, TX) and reverse transcribed with superscript III (Invitrogen) with oligo-dT priming and treated with RNase H. The resultant cDNA was analyzed on a Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using TAQman MGB primer/probe sets targeting pancreatic glucokinase (GenBank accession no. NM 012565, forward 241–262 CGAGGAGGCCAGTGTAAAGATG, reverse 309–288 TCTC CGACTTCTGAGCCTTCTG, probe 281–266 AACGCACGTAGGTGGG), cyclophilin (GenBank accession no. M19533, forward 253–272 AATGGCACT GGTGGCAAGTC, reverse 330–310 GCCAGGACCTGTATGCTTCAG, probe 275–290 TCTACGGAGAGAAATT), and hexokinase I (GenBank accession no. NM 012734, forward 264–282 CAGCCTCCGTCAAGATGCT, reverse 347–326 CGAGATCCAGGGCAATGAAATC, probe 290–306 TTCGTCCGGTCCATTCC). Samples were compared against serially diluted reference standards prepared from pooled aliquots of cDNA from each sample. Data were expressed as the ratio of the standardized amount of the gene of interest (glucokinase or hexokinase I) to the standardized amount of cyclophilin.

Glucose concentration–dependent responses of VMN neurons. Dissociated VMN neurons were first classified by their $[Ca^{2+}]_i$ flux changes to a decrease in extracellular glucose (from 2.5 to 0.3 mmol/l) as glucose excited if they had a >20% decrease, as glucose inhibited if they had a >59% increase, and as nonglucosensing if they had no change in $[Ca^{2+}]_i$ oscillations (7). Then, neurons were exposed to seven incremental changes (0.5, 0.7, 1.0, 1.5, 2.0, 2.5, and 5 mmol/l) of extracellular glucose concentrations. Because of limited viability during testing, any given set of neurons was evaluated at only 2–3 concentrations of glucose. Changes in $[Ca^{2+}]_i$ oscillations from one condition to another were quantified as the area under curve (AUC) (7) and normalized

TABLE 1
Cultured VMH neurons that were untreated or transfected with glucokinase or nonsense siRNA after 24 h in culture

Cell dish	A (24 h postplating [baseline])	B (24 h post-siRNA)	B/A (%)	C (72 h post-siRNA)	C/A (%)	D (72 h post-siRNA fura-2)	D/C (%)
Culture time (h)	24	48	—	96		96	—
Untreated (*n*)	56	50	89	72	129	69	96
Nonsense siRNA (*n*)	98	70	75	108	117	100	93
Glucokinase siRNA (*n*)	60	63	102	94	156	87	93

Neurons were counted after 24, 48, and 96 h under phase contrast and at 96 h with fluorescence microscopy after loading cells with fura-2 at 96-h culture.

by determining the percentage of responses to a given concentration of glucose relative to the response at 0.3 mmol/l.

Dose-responsiveness of VMN glucosensing neurons to a glucokinase activator. Dissociated VMN neurons were classified by their glucosensing response to increasing glucose from 0.5 to 2.5 mmol/l, using calcium imaging. Preliminary studies were carried out in different concentrations of DMSO (0.005–5%). Only the 0.005% concentration had no effect on spontaneous $[Ca^{2+}]_i$ oscillations, and this concentration was used as the vehicle for all subsequent studies. After determining their glucosensing responses, neurons were held at 0.5 mmol/l glucose and exposed to incremental concentrations (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 5, and 10 mmol/l) of a glucokinase activator (Compound A; Merck Research Labs) dissolved in 0.005% DMSO. This compound is an analog of a previously published glucokinase activator (36) and acts to both increase the V_{max} and decrease the K_m of glucokinase for glucose. $[Ca^{2+}]_i$ oscillations in 0.5 mmol/l glucose and in the presence of glucokinase activator were quantified using AUC.

Statistics. Data were analyzed using the χ^2 test for nonparametric statistics. Significant intergroup differences were defined at the $P = 0.05$ level. In this study, we integrated AUC of $[Ca^{2+}]_i$ oscillations using Origin software (OriginLab) and followed the criteria for glucose-excited and -inhibited neurons as previously described (7). The half-maximal effective concentration [EC_{50}] of glucose-excited neurons and the half-maximal inhibitory concentration [IC_{50}] of glucose-inhibited neurons for glucose and in response to the glucokinase activator were determined by nonlinear regression analysis (sigmoidal dose-response curve fit; GraphPad Prism).

RESULTS

Glucose-induced changes in membrane potential fluorescence and $[Ca^{2+}]_i$ responses. We made simultaneous recordings of oscillations in $[Ca^{2+}]_i$ and FLIPR membrane potential fluorescence in response to altering ambient glucose concentrations from 2.5 to 0.5 mmol/l and adding 200 μmol/l tolbutamide (Fig. 1). The changes in $[Ca^{2+}]_i$ oscillations to glucose and tolbutamide were consistent with our previous study (7). The changes in membrane potential fluorescence occurred slightly earlier than changes in $[Ca^{2+}]_i$ oscillations but were highly correlated in both glucose-excited and -inhibited neurons during changes in glucose concentrations and with spontaneous changes in nonglucosensing neurons (mean of $r = 0.81$, $P = 0.975$, $n = 104$).

TABLE 2
Freshly dissociated and 12- to 96-h cultured VMH neurons assessed for expression of hexokinase I and glucokinase mRNA relative to cyclophilin

Timing/ treatment	Change in VMH gene expression: Hexokinase I/cyclophilin	% fresh	Glucokinase/ cyclophilin	% fresh
Freshly isolated	0.88 ± 0.06	—	1.76 ± 0.2	—
12 h	0.86 ± 0.04	98	1.00 ± 0.09*	57
24 h	1.08 ± 0.04	123	1.16 ± 0.06*	66
48 h	1.14 ± 0.05	130	0.93 ± 0.11*	53
72 h	0.92 ± 0.05	105	0.84 ± 0.08*	48
96 h	0.80 ± 0.04	91	0.69 ± 0.11*	39

Data are the means ± SE. $n = 8$ cultures/time period. *$P \leq 0.05$ when values for neurons in culture were compared with those from freshly isolated neurons.

Glucose concentration responses of VMN glucosensing neurons. There was a nonlinear response of glucose-excited neuron $[Ca^{2+}]_i$ oscillations to incremental increases in extracellular glucose concentrations from 0.5 to 5.0 mmol/l, with a plateau at 2.0 mmol/l glucose and no further increase at 5.0 mmol/l (Fig. 2*A*). This yielded a calculated EC_{50} of 0.54 mmol/l ($r = 0.90$). In contrast, glucose-inhibited neurons decreased their $[Ca^{2+}]_i$ oscillations when extracellular glucose levels were increased from 0.5 to 5.0 mmol/l (Fig. 2*B*). This yielded a calculated IC_{50} of 1.12 mmol/l ($r = 0.96$), which was significantly higher than EC_{50} for glucose-excited neurons ($P = 0.001$).

Glucokinase siRNA attenuates glucosensing in primary VMH neuronal cultures. We first demonstrated that the RNAi procedure itself had no effect on neuronal survival over time in culture. Neurons in control untreated cultures had progressive outgrowth of processes over 96 h, and many formed clusters (Fig. 3*A*). All visible neurons took up fura-2 acetoxymethyl, demonstrating their viability (Fig. 3*B*). Transfection with glucokinase siRNA produced no reduction in the total number of surviving neurons over 96 h (Table 1), and there was, if anything, a small increase in the number of neurons in all groups at 96 h (Fig. 3*C* and *D*).

Relative to constitutively expressed cyclophilin, glucokinase mRNA expression in control neurons fell to 57% of freshly dissociated control neurons after only 12 h in culture, and expression was reduced to 39% of the freshly dissociated state by 96 h (Table 2). On the other hand, there was no appreciable change in absolute cyclophilin mRNA expression (data not shown) or in the expression of hexokinase I (Table 2) relative to cyclophilin over 96 h. The reduction in glucokinase mRNA in cultured neurons was not reversed by either longer times in culture (up to 17 days) or culturing neurons in 10–25 mmol/l glucose (data not shown). In parallel with the reduction in glucokinase mRNA, and without loss of the total number of neurons, the number of demonstrable glucosensing neurons fell to 38% for glucose-excited neurons and 50–63% for glucose-inhibited neurons after 24 h in culture, compared with the freshly dissociated state. This percentage did not change appreciably over 96 h (Table 3).

Relative to untreated neurons, there was an 85 ± 1% reduction of glucokinase mRNA expression in neurons transfected with glucokinase siRNA after 24 h. There was no change in glucokinase mRNA expression in cultures treated with nonsense siRNA or transfected with MAP2 (microtubule-associated protein-2) siRNAi. Their glucoki-

TABLE 3
Neurons transfected with glucokinase or nonsense siRNA after 24 h in culture and assessed for glucosensing

Timing/treatment		Change in glucosensing ability				
	N	Glucose excited	% fresh	Glucose inhibited	% fresh	Nonglucosensing
Freshly isolated	759	13 (97)	—	16 (122)	—	71 (540)
24 h cultures	831	5 (45)†	38	10 (87)†	63	84 (699)
48 h cultures	356	5 (18)†	38	8 (30)†	50	87 (308)
96 h cultures	114	5 (5)†	38	9 (10)†	56	87 (99)
24 h glucokinase siRNA	204	6 (12)†	46	10 (20)†	63	84 (172)
48 h glucokinase siRNA	158	4 (6)†	31	13 (20)†	81	84 (132)
96 h glucokinase siRNA	66	0*†	0	1.5 (1)*†	9	98 (65)
96 h nonsense siRNA	103	7 (7)†	54	7 (7)†	44	86 (89)

Data are *N*, % (*n*), and %. Neurons were assessed for glucosensing with Ca^{2+} imaging after 24, 48, and 96 h in culture. *N*, total number of neurons assessed at each time period; %, percent of neurons; *n*, number of neurons classified as glucose excited, glucose inhibited, and nonglucosensing at each time period. *$P \leq 0.05$ when the percentage of neurons in the 96-h glucokinase siRNA group was compared with all other groups. †$P \leq 0.05$ when the percentage of neurons in each glucosensing category was compared with that in freshly dissociated neurons by χ^2 test.

nase mRNA levels were 114 ± 28 and 110 ± 12% of untreated controls, respectively. Exposure to glucokinase siRNA had no appreciable effect on the number of demonstrable glucosensing neurons until 72 h after transfection (96 h in culture). At that time, no glucose-excited neurons were found, and there was a 98.5% reduction in the number of identified glucose-inhibited neurons (Table 3). On the other hand, there was no appreciable effect of nonsense siRNA on the percentage of glucose-excited neurons, although there appeared to be a slight reduction in the percent of glucose-inhibited neurons. Given that the siRNA procedure had no appreciable effect on neuronal survival or the expression of either cyclophilin or hexokinase I mRNA, it is most likely that the failure to demonstrate functional glucosensing neurons at 72 h after glucokinase siRNA transfection was because of a loss of glucosensing ability rather than their death.

Effect of activating glucokinase on neuronal glucosensing. To test the hypothesis that increasing glucokinase activity would make glucosensing neurons more sensitive to low glucose levels, dissociated VMN neurons were first characterized with Ca^{2+} imaging and then treated with either 0.005% DMSO vehicle or increasing concentrations of glucokinase activator (0.01–10 μmol/l) in 0.005% DMSO. There was no effect of DMSO on glucose-excited or -inhibited neuronal glucosensing. Of all VMN neurons tested, ~18% were glucose excited, and they were essentially inactive at 0.5 mmol/l glucose. Of these, 44–75% responded to glucokinase activator by increasing their $[Ca^{2+}]_i$ oscillations in the presence of increasing concentrations of glucokinase activator (Figs. 4*B* and 5*A*), with an EC_{50} of 0.98 μmol/l ($r = 0.97$). Of the 17% of VMN neurons classified as glucose inhibited, most were active at 0.5 mmol/l glucose. Of these, 26–47% responded to the glucokinase activator with decreased $[Ca^{2+}]_i$ oscillations, with an IC_{50} of 0.025 μmol/l ($r = 0.92$). There were no effects of glucokinase activator on nonglucosensing neurons at any concentration. Thus, glucokinase activator increased the sensitivity of both glucose-excited and -inhibited neurons to low glucose levels, and glucose-inhibited neurons were ~40-fold more sensitive to this drug than were glucose-excited neurons.

DISCUSSION

The current studies confirm prior ones supporting the hypothesis that glucokinase is a critical regulator of neuronal activity in VMH glucosensing neurons. Prior studies demonstrated the presence of glucokinase activity (37,38), mRNA (6–8,39,40), and immunoreactive protein (41) in selective brain areas and in specific glucosensing neurons. Furthermore, pharmacological inhibition of glucokinase activity reduces $[Ca^{2+}]_i$ oscillations in glucose-excited neurons and increases them in glucose-inhibited neurons (6,7,42), and it decreases the firing rate of glucose-excited neurons (43). On the other hand, glucokinase is probably not the only regulator of glucosensing because it is not found in all glucosensing neurons, and inhibition of glucokinase does not alter the response to glucose in all glucosensing neurons (7).

The current studies were undertaken to provide a more thorough understanding of the physiological properties of VMH glucosensing neurons and the role that glucokinase plays as a regulator of their activity. First, we demonstrated that $[Ca^{2+}]_i$ oscillations provide a reasonable surrogate for glucose-induced changes in membrane potential across a physiological range of glucose concentrations. Also, $[Ca^{2+}]_i$ oscillations in glucose-excited and -inhibited neurons responded to physiological changes in glucose in a concentration-dependent fashion similar to that shown for glucose-excited neurons, using a patch-clamp technique in VMN slice preparations (44). Finally, pharmacological activation and molecular inactivation of glucokinase markedly altered the ability of glucosensing neurons to respond to glucose. These findings have potentially important clinical implications for the treatment of diabetic patients who develop hypoglycemia-associated autonomic failure after repeated bouts of insulin-induced hypoglycemia. Glucosensing neurons in the VMH are clearly implicated in the counterregulatory responses to hypoglycemia (11,29,45–47), and a blunted counterregulatory response to glucoprivation is associated with increased VMH glucokinase mRNA expression (6,29), suggesting that drugs that alter hypothalamic glucokinase activity might affect the counterregulatory responses to hypoglycemia.

Interstitial brain levels of glucose, even during hypoglycemia, are generally 15–30% of blood levels across a wide range of values (48–50). Electrophysiological studies in slice preparations clearly demonstrate that both VMN (5) and arcuate (9) glucose-excited neurons exhibit a reproducible concentration-dependent response to glucose across this physiological range. An important unresolved issue is how neuronal glucokinase, which is identical to pancreatic glucokinase (39,40,51), can act as a regulator of neuronal glucosensing at brain levels that are only ~1–2%

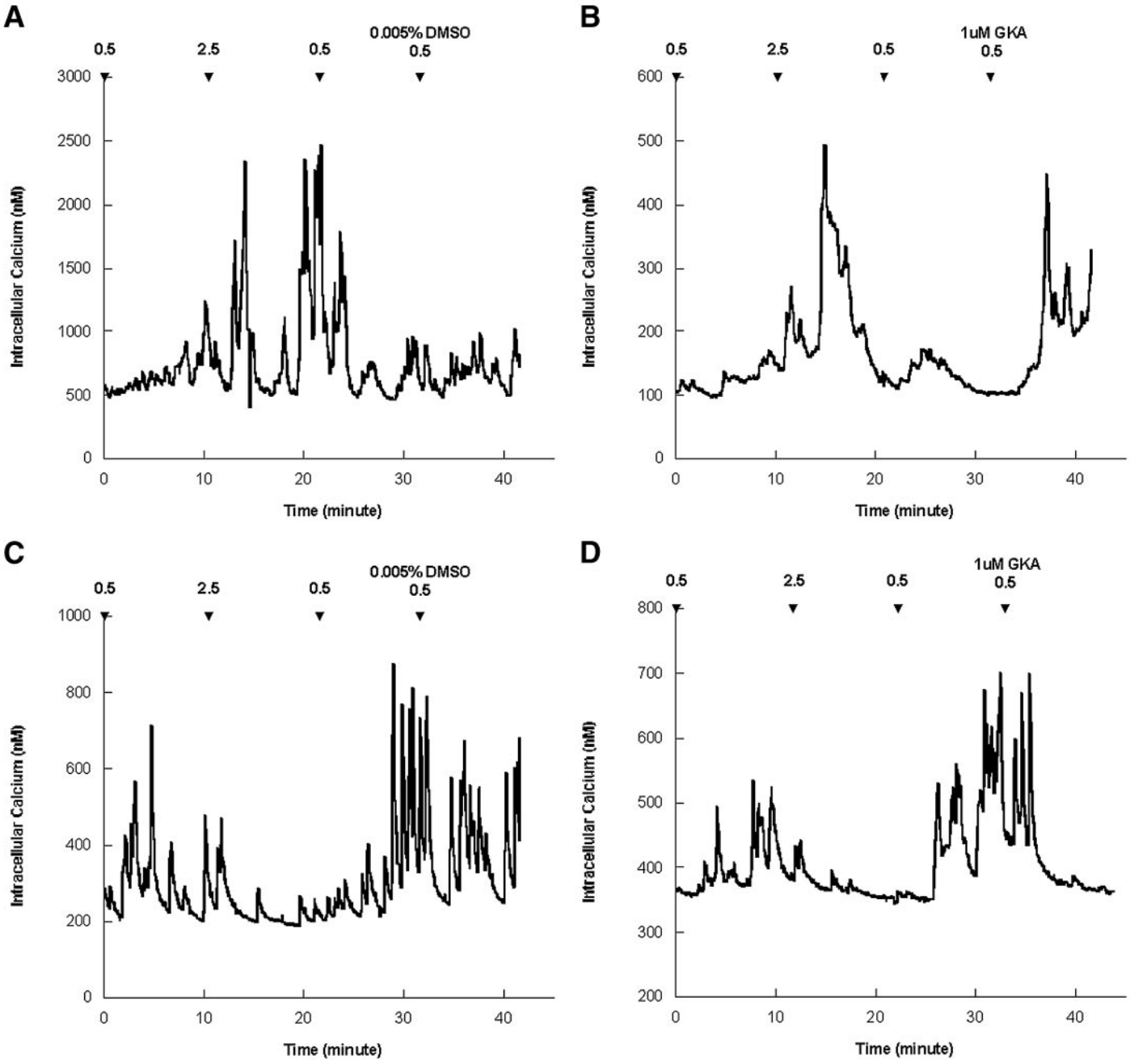

FIG. 4. Responses to a glucokinase activator (0.01–10 μmol/l) or 0.005% DMSO vehicle were assessed by changes in $[Ca^{2+}]_i$ oscillations at 0.5 mmol/l glucose in freshly dissociated VMN neurons after they were first characterized as being glucose excited or glucose inhibited by their responses to increasing glucose from 0.5 to 2.5 mmol/l glucose. *A*: A glucose-excited neuron unaffected by 0.005% DMSO at 0.5 mmol/l glucose. *B*: A glucose-excited neuron activated by 1 μmol/l glucokinase activator (GKA) at 0.5 mmol/l glucose. *C*: A glucose-inhibited neuron unaffected by 0.005% DMSO. *D*: A glucose-inhibited neuron that had robust $[Ca^{2+}]_i$ oscillations at 0.5 mmol/l was inactivated by 1 μmol/l glucokinase activator.

of its maximal velocity for glucose (52). One might question our results because we did not assess neuronal activity directly but instead measured glucose-induced changes in $[Ca^{2+}]_i$ oscillations. However, the fact that these oscillations are tightly linked to changes in membrane potential suggests that measures of $[Ca^{2+}]_i$ oscillations may provide a reasonable surrogate for glucose-induced changes in membrane potential. We used FLIPR membrane potential dye to assess membrane potential because it has the highest signal-to-background ratio and fastest response time of most membrane potential dyes (32). Also, its fluorescence intensity correlates highly with changes in membrane potential, as determined by patch-clamping (53). Finally, the fact that the EC_{50} for glucose in glucose-excited neurons using calcium imaging almost exactly matches that found using the patch-clamp technique in VMN neurons (5) strongly supports a close relationship between glucose-induced changes in $[Ca^{2+}]_i$ oscillations and neuronal activity. Thus, even though the issue of how glucokinase might perform a regulatory role when brain glucose levels are so low remains unresolved at present, our data strongly suggest that it nevertheless is a critical regulator of neuronal glucosensing.

An important new finding is that VMN glucose-excited

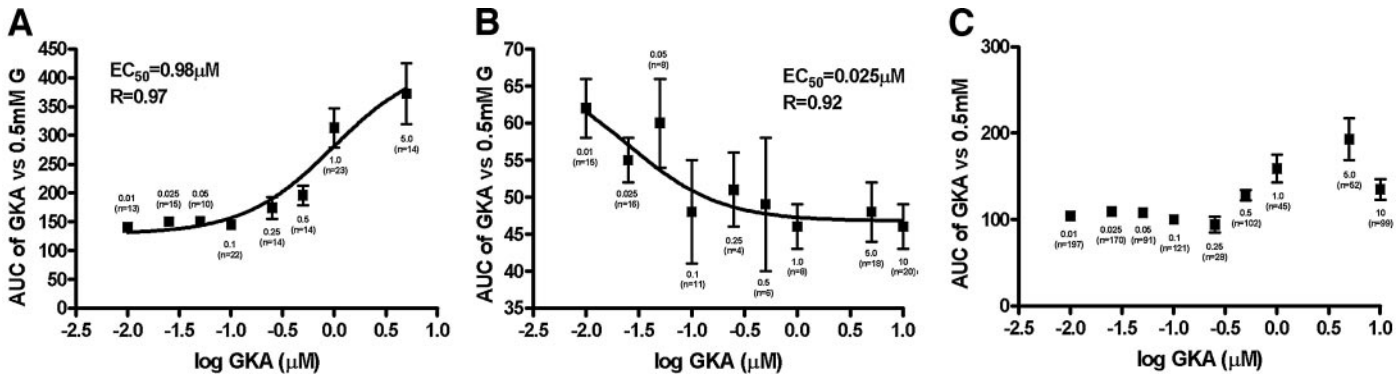

FIG. 5. Freshly dissociated VMN neurons were characterized for their glucosensing properties and then treated with glucokinase activator (GKA; 0.01–10 μmol/l) in the presence of 0.5 mmol/l glucose. Data points (means ± SE) were derived by calculating the difference of $[Ca^{2+}]_i$ AUC at 0.5 mmol/l from the AUC in the presence of a given dose of glucokinase activator. *A*: Glucokinase activator dose response for activation of glucose-excited neurons. *B*: Glucokinase activator dose response for inhibition of glucose-inhibited neurons. *C*: Glucokinase activator had no significant effect on nonglucosensing neurons. G, glucose.

neurons are twofold more sensitive to glucose than glucose-inhibited neurons. The relatively low EC_{50} (0.54 mmol/l) suggests that glucose-excited neurons are most sensitive at the relatively low brain levels seen during hypoglycemia (48–50). On the other hand, the relatively high IC_{50} (1.12 mmol/l) for glucose-inhibited neurons suggests that they may be most important in mediating the changes in blood glucose seen both during diurnal fluctuations in glucose and after ingestion of nutrients (48). Glucose-excited neurons utilize ATP-sensitive K^+ channels as their transduction mechanism for glucosensing (5,8,54,55), whereas glucose-inhibited neurons appear to utilize a nonspecific Cl^- channel (5). Although some glucose-inhibited neurons can also respond to sulfonylureas (5), the fact that drugs that alter ATP-sensitive K^+ channel activity have their predominant effect on glucose-excited neurons suggests that they might provide potential therapeutic tools for differentially affecting the physiological responses to insulin-induced hypoglycemia. In fact, sulfonylureas infused directly into the VMH markedly blunt the counterregulatory response to hypoglycemia (47).

Regardless of the ion channels used as a final common pathway, a large proportion of glucose-excited and -inhibited neurons appear to utilize glucokinase as a regulator of glucosensing (6–8). Here, we confirmed this dependence on glucokinase by demonstrating that transfecting cultured VMH neurons with glucokinase siRNA almost completely abolished neuronal glucosensing and that pharmacological activation of glucokinase increased the sensitivity of glucosensing neurons to glucose. Interestingly, the expression of glucokinase, but not hexokinase I mRNA, fell by ~50% from 12 to 96 h in untreated VMH neurons in culture, and this reduction could not be prevented by longer time in culture or by raising the glucose concentration of the culture medium. This spontaneous reduction in glucokinase mRNA expression was paralleled by a comparable reduction in the percentage of demonstrable glucosensing neurons over the first 24 h in culture, even though there was no appreciable overall loss of neurons during this time. This suggests that neither glucokinase nor glucosensing per se are critical for neuronal survival in culture, whereas the ability to utilize glucose to maintain metabolic function via hexokinase I is. The reduction of both the expression of glucokinase mRNA and the number of glucosensing neurons in culture meant that detection of changes induced by glucokinase siRNA transfection would be more difficult to detect. Nevertheless, an 85% reduction in glucokinase mRNA with RNAi reduced the number of detectable glucose-excited neurons to 0 and glucose-inhibited neurons to 1.5% of the freshly dissociated state. Unlike the spontaneous reduction in glucokinase mRNA expression and the number of glucosensing neurons during the first 24 h in culture, the RNAi-induced reduction in the number of functional glucosensing neurons took 72 h to occur, even though glucokinase mRNA was reduced by 85% after only 12 h. This suggests that it may take 48–72 h for glucokinase enzymatic activity to fall after reduction of glucokinase mRNA by RNAi, whereas the rapid loss of apparent glucokinase activity after plating may represent inactivation caused by injury and initial repair. Unfortunately, this issue remains unresolved because we have been unable to assess either the amount of glucokinase protein or its activity in individual glucosensing neurons. Nevertheless, the marked fall in the number of detectable glucosensing neurons after glucokinase RNAi strongly suggests that the overwhelming majority of VMH glucosensing neurons in primary culture depend on glucokinase for glucosensing regulation, even though this may not be the case in freshly dissociated VMN neurons (6,7).

Our previous data suggest that the blunting of the counterregulatory responses to glucoprivation is associated with an increase in VMH glucokinase mRNA expression (6,29). Similarly, an increase in VMH glucokinase mRNA is also associated with reduced counterregulatory responses to hypoglycemia and abnormalities of glucosensing in rats genetically predisposed to develop diet-induced obesity (6,56–58). Here, we demonstrate that a compound that enhances glucokinase enzymatic activity can dose-dependently increase the sensitivity of both glucose-excited and glucose-inhibited neurons to low levels of glucose. The fact that glucose-inhibited neurons were almost 40-fold more responsive than glucose-excited neurons to this glucokinase activator may be because they were assessed at low glucose levels, where glucose-excited neurons are more sensitive to glucose-induced changes in their activity. On the other hand, this may well reflect differences in the amount of glucokinase protein and/or enzymatic activity expressed in glucose-excited versus glucose-inhibited neurons. Regardless of their differences in sensitivity, the important point is that the glucokinase activator enhanced glucose sensitivity in both glucose-excited and -inhibited neurons just as inhibitors of

glucokinase activity alter glucosensing in both glucose-excited and -inhibited neurons (6,7).

In conclusion, our current and previous data demonstrate that VMN neurons can respond to small incremental changes in ambient glucose levels that are well within those experienced in vivo. The current data also support previous studies demonstrating that VMN neurons express glucokinase mRNA and alter their activity at these same glucose levels in response to pharmacological agents that increase or decrease glucokinase enzyme activity. Finally, we demonstrate here that near complete knockdown of glucokinase mRNA, using glucokinase siRNA in primary hypothalamic neuronal cultures, almost totally ablates the ability of those neurons to sense glucose. Thus, our present and previous data lend very strong support for a critical role for glucokinase in neuronal glucosensing.

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