

Localization of Glucokinase Gene Expression in the Rat Brain

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The brain contains a subpopulation of glucosensing neurons that alter their firing rate in response to elevated glucose concentrations. In pancreatic β -cells, glucokinase (GK), the rate-limiting enzyme in glycolysis, mediates glucose-induced insulin release by regulating intracellular ATP production. A similar role for GK is proposed to underlie neuronal glucosensing. Via *in situ* hybridization, GK mRNA was localized to hypothalamic areas that are thought to contain relatively large populations of glucosensing neurons (the arcuate, ventromedial, dorsomedial, and paraventricular nuclei and the lateral area). GK also was found in brain areas without known glucosensing neurons (the lateral habenula, the bed nucleus stria terminalis, the inferior olive, the retrochiasmatic and medial preoptic areas, and the thalamic posterior paraventricular, interpeduncular, oculomotor, and anterior olfactory nuclei). Conversely, GK message was not found in the nucleus tractus solitarius, which contains glucosensing neurons, or in ependymal cells lining the third ventricle, where others have described its presence. In the arcuate nucleus, >75% of neuropeptide Y-positive neurons also expressed GK, and most GK⁺ neurons also expressed KIR6.2 (the pore-forming subunit of the ATP-sensitive K⁺ channel). The anatomic distribution of GK mRNA was confirmed in micropunch samples of hypothalamus via reverse transcription-polymerase chain reaction (RT-PCR). Nucleotide sequencing of the recovered PCR product indicated identity with nucleotides 1092-1411 (within exon 9 and 10) of hepatic and β -cell GK. The specific anatomic localization of GK mRNA in hypothalamic areas known to contain glucosensing neurons and the coexpression of KIR6.2 and NPY in GK⁺ neurons support a role for GK as a primary determinant of glucosensing in neuropeptide neurons that integrate multiple signals relating to peripheral energy metabolism. *Diabetes* 49:693-700, 2000

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ARC, arcuate nucleus; GK, glucokinase; HKI, hexokinase I; K_{ATP} channel, ATP-sensitive inward rectifier K⁺ channel; LHA, lateral hypothalamic area; NPY, neuropeptide Y; PBS, phosphate-buffered saline; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; RT-PCR, reverse transcription-polymerase chain reaction; SSC, sodium chloride-sodium citrate; VMH, ventromedial hypothalamus; VMN, ventromedial nucleus.

Mammalian feeding behavior and general energy homeostasis appear to be regulated by circulating levels of nutrients (glucose) and peptides (e.g., leptin, insulin). Sensors to detect levels of these factors have been found to reside within specific nuclei of the hypothalamus (1-8), where central regulation of energy homeostasis is believed to be coordinated. For example, large changes in blood glucose are correlated with centrally mediated responses such as thermogenesis through activation of the sympathetic nervous system. These changes are monitored by the brain (9-11), and such responses are altered in obesity-prone animals (11-13). Moreover, lesions of the ventromedial hypothalamus (VMH) prevent the hypoglycemic activation of the sympathetic response (14). Thus, available data indicate that glucose detection by hypothalamic neurons may play an important role in regulating energy homeostasis.

Glucosensing neurons are among the best characterized of such metabolic sensors. Unlike most neurons, they use glucose as a signaling molecule to alter their firing rate in response to changes in ambient glucose levels (1,2,7,15,16). Glucosensing neurons constitute 10-40% of the resident neuronal populations of the VMH and lateral hypothalamic area (LHA) (1,2,15,17). In addition, glucosensing neurons have been discovered in other brain areas including the amygdala (18), locus ceruleus (19), and nucleus tractus solitarius (20). Two distinct types of neuronal responses to changes in glucose concentrations have been observed, leading to characterization of the neurons as either glucose-responsive or glucose-sensitive. Glucose-responsive neurons are defined as those that increase their firing rates when ambient brain glucose levels rise; glucose-sensitive neurons decrease their firing rates when glucose levels increase (1,2,7,15,16).

Most attention has been focused on the hypothalamus because of its important role in energy homeostasis (7,8,21). Whereas areas such as the VMH contain neurons that are clearly inhibited by pathological lowering and stimulated by raising of glucose (14,22,23), both the VMH and LHA also contain neurons that are inhibited as brain glucose increases over a physiologic range of 1.5-4 mmol/l (15,17). Of the 2 types of glucosensing neurons, the glucose-responsive neurons have been best characterized physiologically because of their similarity to the glucosensing pancreatic β -cell (7,24). Alternatively, the islet α -cell may be the endocrine equivalent of the glucose-sensitive neuron, since glucagon secretion is decreased in response to elevated glucose (25).

The pancreatic β -cell, in turn, is the most extensively studied model of cellular glucose sensing (24-26). The enzyme

glucokinase (GK; hexokinase IV) (27) appears to play a central role in the ability of β -cells to sense changes in blood glucose (24,28,29). β -Cells are activated by increases in blood glucose over a wide range (5–20 mmol/l). GK has a relatively low affinity for glucose ($K_m \sim 10$ mmol/l [30,31]), which provides the β -cell with the ability to increase the rate of glucose phosphorylation in proportion to increases in blood glucose over the physiologic range (26,28). In β -cells isolated from rat pancreas, the absolute sensitivity of individual cells to glucose appears to be correlated with the level of GK expression (26), and overexpression of GK enhances secretion of insulin in response to elevated glucose (29). Moreover, modulation of GK activity in insulin-secreting cell lines modulates the rate of glucose utilization and the sensitivity of these cells to glucose (30). A similar role for GK in glucose sensing by human β -cells also has been demonstrated (31). Thus, it is clear that the presence of GK is required for normal sensing of glucose by β -cells, and the relative level of GK expression may set overall sensitivity or set point for activation by glucose (26).

To explain the coupling between changes in glucose phosphorylation rate and β -cell activation, it is proposed that ATP produced by GK-regulated glycolysis inactivates the ATP-sensitive inward rectifier K^+ channel (K_{ATP} channel), leading to depolarization of the cell limiting membrane, Ca^{2+} influx, and insulin release (24,32). The pore-forming subunit of the specific K_{ATP} channel expressed in β -cells is the KIR6.2, which, together with a sulfonylurea receptor, confers activity to the channel (32). In the brain, this same channel appears to modulate neuronal firing in select glucose-responsive neurons (7,33). Moreover, GK has been shown to be expressed in neuronal centers that exhibit responses to elevated glucose (34–36). Brain glucose concentrations change over a narrow range (1–4 mmol/l) during normal transitions in blood glucose (15); therefore the resolution of the glucose-sensing mechanism in these neurons must be high or, alternatively, these neurons may use a fundamentally different mechanism to sense changes in glucose.

The presence of GK in the purported glucose-responsive centers of the hypothalamus has been evaluated. Jetton et al. (35) expressed a GK promoter-driven human growth hormone transgene in mice to identify tissues in which the GK promoter was active. Promoter activity was observed in β -cells of the pancreas, specific segments of the alimentary canal, and several brain regions. In the hypothalamus, activity was observed in regions surrounding the third ventricle, including the arcuate nucleus (ARC) and the ventromedial nucleus (VMN), indicating that GK is normally expressed in these regions. Several hypothalamic areas also showed immunoreactivity for GK antibody, although it was unclear within which specific regions GK was expressed or if expression was in neurons or glia. In situ hybridization to identify GK mRNA showed low levels of GK mRNA expression in the basomedial hypothalamus and ependymal layers of the third ventricle within this region of the hypothalamus. Because of the low signal, expression could not be localized to specific regions. More recent studies of Navarro et al. (36) and Yang et al. (34) also identified GK message within the hypothalamus, but again the exact regions of expression could not be ascertained. Although these previous studies (34,36) indicate that GK promoter is active in the hypothalamus, and that both message and protein are expressed, a comprehensive analysis of the regional distribution of GK expression has not

been performed. This issue is of great importance, since GK has emerged as a central component for glucosensing by the β -cell (24,26,32) and may well serve the same function in both glucose-responsive (7) and glucose-sensitive (15) neurons in the brain. For this reason, we postulated that GK would be highly localized to those brain areas in which glucosensing neurons reside. In the current studies, we used an analysis of GK mRNA expression in sequential sections of entire rat brain by in situ hybridization. In addition, reverse transcription-polymerase chain reaction (RT-PCR) was used to confirm the localized expression in micropunches from specific hypothalamic nuclei and to sequence the code for the expressed GK.

RESEARCH DESIGN AND METHODS

Animals. For the in situ hybridization studies, 8 male Sprague-Dawley rats weighing 300–400 g were used. For the RT-PCR studies, 5 female 300-g Sprague-Dawley rats were used. Animals were kept on a 12 h:12 h light-dark schedule at 22–23°C with food and water provided ad libitum. Rats were anesthetized by CO_2 and decapitated, and their brains were removed quickly for further study.

In situ hybridization. Probes were generated from a 1.4-kb fragment of the mRNA sequence for rat GK (nucleotides 1–1422). This fragment was subcloned into the *Bam*HI site of a pBS2SK+ vector. Because this portion of the GK gene also contains a small area of partial homology to rat hexokinase I (HKI), a truncated probe that shared no homology with HKI (nucleotides 963–1422) was used in a separate set of experiments to confirm the identity of GK. For prepro-neuropeptide Y (NPY), a 511-bp fragment was subcloned into the *Eco*RI site of a bluescript SK(+) vector (36). Riboprobes labeled with ^{35}S , digoxigenin, or fluorescein were generated using standard techniques (37,38). Probes were hydrolyzed for 20 min at 60°C in 0.06 mol/l Na_2CO_3 and 0.04 mol/l $NaHCO_3$, and probe fragments <20 nucleotides were removed using microspin G-50 columns. Cryostat sections of rat brain were cut at 10 μ m, fixed in paraformaldehyde, and dehydrated. Hybridization was performed as previously described (38). Briefly, sections were treated with glycine, followed by pronase. They were then acetylated, dehydrated, and prehybridized at 60°C. Slides were incubated with the hybridization mix containing 40,000 dpm/ μ l [^{35}S]-labeled sense or antisense GK probe (for single labeling) or 0.5 ng/ μ l digoxigenin-labeled GK probe plus 0.5 ng/ μ l fluorescein-labeled NPY probe (for double labeling). After overnight hybridization at 60°C, sections were treated with β -mercaptoethanol followed by RNase. After a low-stringency rinse, slides received a high-stringency wash (0.1 \times sodium chloride-sodium citrate [SSC] [NaCl, 150 mmol/l, citric acid, 15 mmol/l; pH to 7.4] at 60°C). Preliminary results showed that high-stringency washing was essential for specificity of the GK probe. Washing with 0.25 \times SSC at 55°C resulted in extensive nonspecific background activity with both sense and antisense probes, whereas washing with 0.1 \times SSC at 60°C eliminated most of the background activity while retaining specific hybridization of the antisense probe. After hybridization, single-label brain sections were dehydrated, and the slides were apposed to Kodak (Rochester, NY) Biomax MR film for 2–4 weeks.

NPY and GK double-labeled slides were blocked with 2% goat serum, and hybridization of the fluorescein-labeled NPY probe was visualized using a peroxidase-conjugated anti-fluorescein antibody, followed by a renaissance green fluorescence in situ hybridization kit (New England Nuclear, Boston, MA). Next, hybridization of the digoxigenin-labeled GK probe was visualized using alkaline phosphatase-conjugated anti-digoxigenin, followed by a nitroblue tetrazolium/4-bromo-4-chloro-indolyl phosphate chromagen reaction. KIR6.2 and GK double-labeled slides used hybridization of a digoxigenin-labeled KIR6.2 probe that was recognized as described above. After this hybridization, ^{35}S -labeled GK probe was demonstrated by dipping slides in Ilford K5D autoradiographic emulsion (Ilford, Paramus, NJ) and exposing for 3 weeks.

Isolation of tissue from adult rat hypothalamus. Dissection blades, needle punches, and all dissection instruments were sterilized and maintained in saline supplemented with 4 mmol/l vanadyl ribonucleoside complex (Gibco BRL, Gaithersburg, MD) to inhibit RNase activity (39). Brains were placed in a Heffner slicer, and 1- to 2-mm coronal slices were obtained from the region posterior to the bifurcation of the optic tract. The slices were placed in phosphate-buffered saline (PBS) supplemented with 4 mmol/l vanadyl ribonucleoside. Area-specific tissues were obtained from the slices under $\times 20$ magnification using a blunt polished 16-gauge needle (1.5 mm inner diameter). Tissue cores were collected from specific hypothalamic nuclei including the ARC, VMN, paraventricular nucleus (PVN), and LHA. Cortex samples were taken from within the same slice. Care was taken to avoid sampling from the ependymal layer around the third ventricle. Vanadyl ribonucleoside complex was removed from the tissue before RNA extraction by rinsing the tissue cores in PBS. Region-specific tissue from 5 rats

(~40–60 mg total) were pooled in 1 ml Trizol (Gibco BRL). Tissue isolations were performed in duplicate to verify reproducibility of results.

Total RNA isolation and RT-PCR. Tissue samples were incubated in Trizol for 20 min at room temperature and homogenized through a 1-ml pipette tip. Total RNA was extracted using phenol/chloroform, precipitated from the aqueous layer with isopropanol, and resuspended in diethyl pyrocarbonate-treated RNase free water. Total RNA was also collected from a central region of the cerebral cortex and from a rat insulinoma cell line (BG 40/110; P26) that overexpresses the rat islet form of GK (39). Total RNA (5 µg) was digested with RNase-free DNase for 60 min at 37°C to remove genomic DNA contamination. RNA was phenol/chloroform-extracted and precipitated overnight at -70°C in ethanol and 3 mol/l sodium acetate, pH 5.2. Single-stranded cDNA was synthesized from 2 µg RNA using the following reaction: 1 µg random primer, 200 ng oligo d(T) primer, 50 U RNase inhibitor, 50 U reverse transcriptase Moloney murine leukemia virus, and 1 mmol/l dNTP in 5× first-strand buffer containing 0.8 mmol/l dithiothreitol at 37°C for 1 h (40). The 5× first-strand buffer contained 250 mmol/l Tris HCl, pH 8.3; 375 mmol/l KCl, and 15 mmol/l MgCl₂. PCR was performed using sequence-specific primers for rat GK (5'-GTGGTCTTTGAGACCCGTT-3' and 5'-TTCGATGAAGGTGATTCGCA-3') corresponding to bases 1071–1091 and 1411–1391 (GenBank accession number X53598), respectively, of rat liver GK (41) and for rat cyclophilin (5'-GGGGAGA AAGGATTGGCTA-3' and 5'-ACATGCTTCCATCCAGCC-3') corresponding to bases 165–185 and 422–404 (GenBank accession number M19533), respectively (42). Each 50 µl of PCR reaction contained 100 ng cDNA template, 100 pmol sense and antisense primer, and 0.4 mmol/l dntps in 10× Taq buffer with 15 mmol/l MgCl₂ (Promega, Madison, WI), catalyzed with 2.5 U Taq polymerase. The 10× Taq buffer (Promega) contained 100 mmol/l Tris HCl, pH 8–3, 500 mmol/l KCl, 1% Triton X-100, and 15 mmol/l MgCl₂. cDNA from the GK-expressing cell line was used as a positive control, and 1.0 µg total DNase-treated RNA was used as a negative control reaction. All PCR reactions were run on a Perkin-Elmer GeneAmp model 2400 (Perkin-Elmer Biosystems, Foster City, CA) (16) as follows: 2 min at 94°C, 1 min at 94°C, 2 min at 50°C, and 1.5 min at 72°C (30 cycles) with a final 10 min at 72°C before storage at 4°C overnight. PCR products were analyzed by elec-

trophoresis on 2% agarose gels (Stratagene, La Jolla, CA), stained with ethidium bromide, visualized, and photographed (exposure 400 ms) on a Bio-Rad ultraviolet illuminator (Bio-Rad Laboratories, Hercules, CA). Images were acquired using Quantity One software (Bio-Rad Laboratories).

Purification, identification, and determination of relative abundance of PCR products. PCR products were excised from the agarose gel and extracted using the QiaQuick kit (Qiagen, Valencia, CA). PCR product (30 ng) was cycle-sequenced using fluorescent dideoxy chain termination (Arizona Research Labs, Division of Biotechnology, Tucson, AZ). Sequence data was obtained using 10 pmol of both reverse and forward PCR primers, and the compiled nucleotide sequence was entered as a BLAST query using the National Center for Biotechnology Information program software (National Institutes of Health, Bethesda, MD). To determine the relative abundance of the GK mRNA transcript, 8 µl PCR product was combined for sample-matched GK and cyclophilin reactions into the same lanes of a 2% agarose gel and run for 2 h at 80 V. Gels were stained in freshly prepared ethidium bromide and photographed. Images of each gel were then analyzed for intensity of ethidium labeling using Scion version 5.0.1 (Scion, Frederick, MD). To determine the total intensity within equal areas, a selected region was used that was held constant for measurement of background and signal of each GK and cyclophilin band. To correct for background noise, the signal from an area immediately above each band was subtracted. The corrected intensity for each GK band was standardized to the corrected intensity for the cyclophilin band in the same lane (43). Sample volumes were selected to provide similar loading of cyclophilin in all lanes and ensure that cyclophilin signal was within the linear range of the detection system.

RESULTS

Anatomic distribution of GK mRNA by in situ hybridization. According to film autoradiography (Figs. 1 and 2; Table 1), hybridization of the antisense GK probe was most extensive in the hypothalamus. The specificity of this

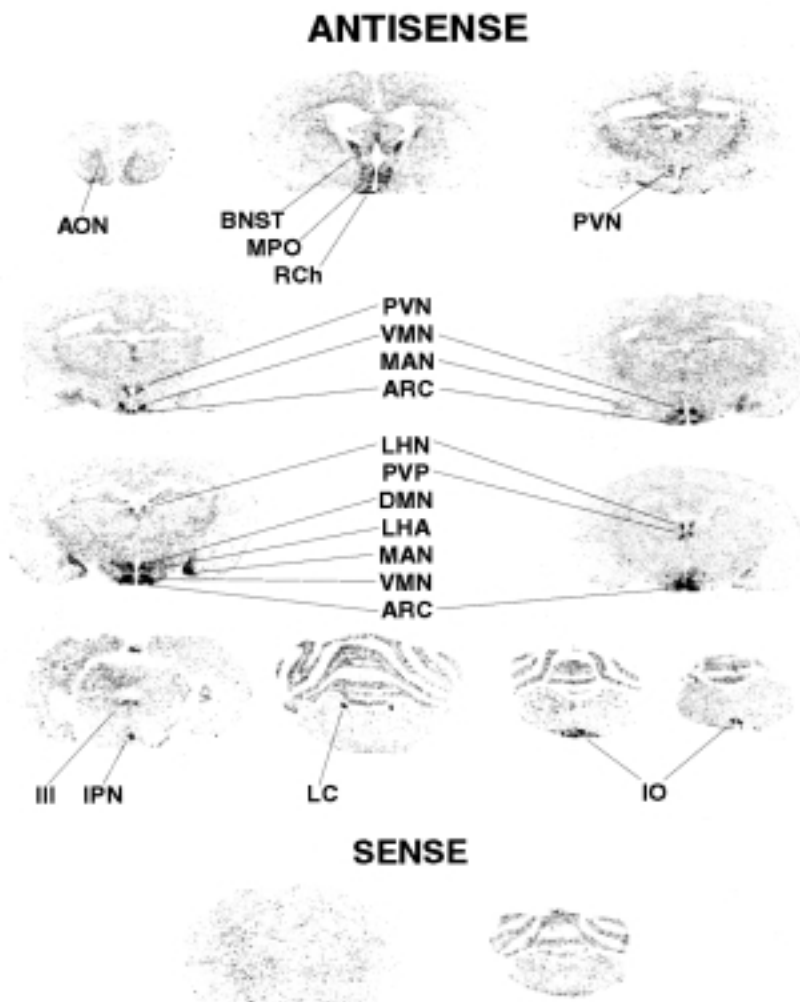


FIG. 1. Montage of in situ hybridization of radiolabeled sense and antisense GK probes in film autoradiographs of a series of coronal sections through the rat brain. The antisense hybridization is shown in the 11 sections at the top of the figure, which run from rostral (starting at the rostral pole of the cortex, top left) to caudal (ending at the caudal pole of the cerebellum, bottom right). Hybridization of the sense probe is shown in sections that pass through the hypothalamus at the level of the mid-hypothalamus (bottom left) and through the hindbrain at the level of the lower medulla (bottom right). Note that although hybridization to the molecular layer of the cerebellum was seen (bottom right antisense sections), similar hybridization occurred with the sense probe, indicating that this hybridization was non-specific. AON, anterior olfactory nucleus; BNST, bed nucleus stria terminalis; DMN, dorsomedial nucleus; III, oculomotor nucleus; IO, inferior olive; IPN, interpeduncular nucleus; LC, locus coeruleus; LHN, lateral habenular nucleus; MAN, medial amygdalar nucleus; MPO, medial preoptic area; PVP, thalamic posterior paraventricular nucleus; RCh, retrochiasmatic area.

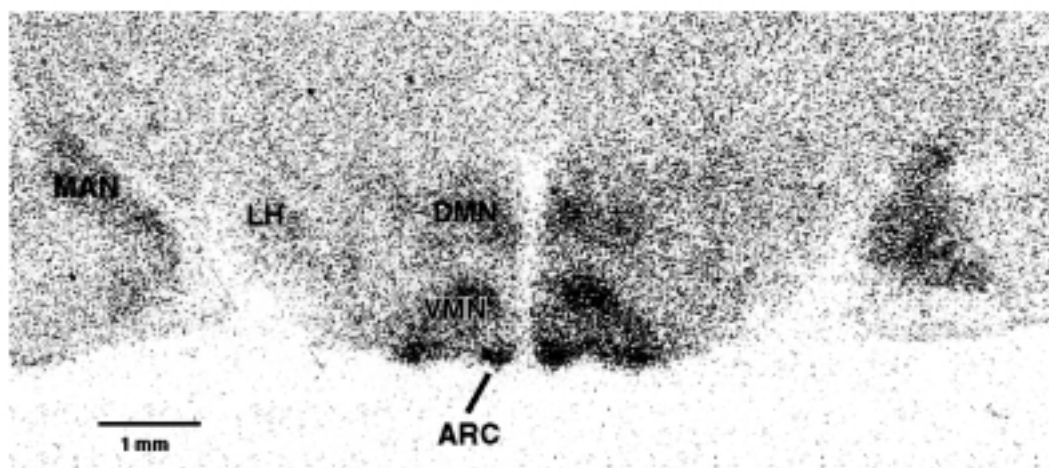


FIG. 2. High-power film autoradiograph of the in situ hybridization of the antisense GK probe in a coronal section through the hypothalamus and amygdala. DMN, dorsomedial nucleus; LH, lateral hypothalamus; MAN, medial amygdalar nucleus.

hybridization was demonstrated by the low level of nonspecific labeling using the sense probe (Fig. 1). In general, the relative abundance of brain GK mRNA appeared to be low. Exposure times on film ran 3–4 weeks as opposed to 3 days for comparable methods used to detect NPY mRNA (37). Given this low level of expression, heaviest labeling was seen in the ARC and VMN, especially the dorsomedial subdivision. Lesser expression was observed in the dorsomedial nucleus, retrochiasmatic area, and PVN, where the majority of labeling was observed in the caudal portion. There was also diffuse, low-level GK expression in the LHA. GK mRNA was expressed outside the hypothalamus as well. In the forebrain, high levels of expression were present in the medial amygdalar nucleus. Low to moderate levels of GK expression were observed in the anterior olfactory nucleus, bed nucleus of the stria terminalis, posterior paraventricular thalamic nucleus, lateral habenula, and interpeduncular nuclei. In the hindbrain, GK mRNA was expressed selectively in the oculomotor nucleus, locus ceruleus, and inferior olive. In situ hybridization with the truncated GK probe, which shared no homology with rat HKI, gave the same qualitative results as the full-length probe, although with somewhat lower levels of expression than those found with the full-length probe (data not shown). Thus, the full-length probe does not hybridize with the constitutively expressed HKI, and therefore, both probes are selective for GK transcripts.

Double-label in situ hybridization studies were carried out to assess GK, KIR6.2, and NPY mRNA expression at the cellular level. KIR6.2 was expressed throughout the hypothalamus, as previously described (44). Based on qualitative analysis of GK autoradiogram overlays with KIR6.2 micrographs, the majority of GK⁺ neurons in the ARC coexpressed KIR6.2 mRNA (Fig. 3), although this was only a fraction of all neurons that express KIR6.2. Double-label studies also showed that >75% of NPY neurons in the ARC coexpressed GK mRNA (Fig. 4). Importantly, within these same sections, no GK mRNA was observed in the ependymal cells lining the walls of the adjacent third ventricle (Fig. 2).

RT-PCR identification of GK in specific regions of the hypothalamus. The in situ hybridization studies showed the highest expression of GK in the ventrobasilar hypothal-

amus. RT-PCR was performed on total RNA extracted from micropunches of the hypothalamus to identify the presence or absence of GK mRNA transcripts in selected hypothalamic regions (Fig. 5). Using the GK primers, a single cDNA fragment of ~340 nucleotides was obtained. Cyclophilin primers produced a single band of ~259 nucleotides. GK was found in ARC, VMH, PVN, and LHA; however, the amount of product varied in the different regions. When expressed as a percentage of the constitutive cyclophilin signal in each lane, the ARC and LHA exhibited the highest signals, whereas GK mRNA was expressed at the lowest levels in the PVN (Fig. 6). **Nucleotide sequence of GK from arcuate tissue samples.** The PCR product from the ARC was purified from the agarose gel and sequenced using the PCR primers. Nucleotide sequence revealed 100% identity with the rat liver and β -cell sequence in this region (nucleotides 1092–1411 of

TABLE 1
Brain areas expressing GK mRNA by in situ hybridization

Area	Relative density
Anterior olfactory nucleus	+
Medial preoptic area	++
Bed nucleus stria terminalis	++
Hypothalamus	
Retrochiasmatic area	++
PVN	+
VMN	++++
Dorsomedial nucleus	++
ARC	+++
LHA	+
Amygdala, medial nucleus	+++
Thalamus	
PVN, posterior	++
Lateral habenular nucleus	++
Interpeduncular nucleus	++
Oculomotor nucleus	++
Locus coeruleus	++
Inferior olive	++

Relative density is an estimate in which + is the least and ++++ the greatest expression in areas where labeling was seen in Fig. 1.

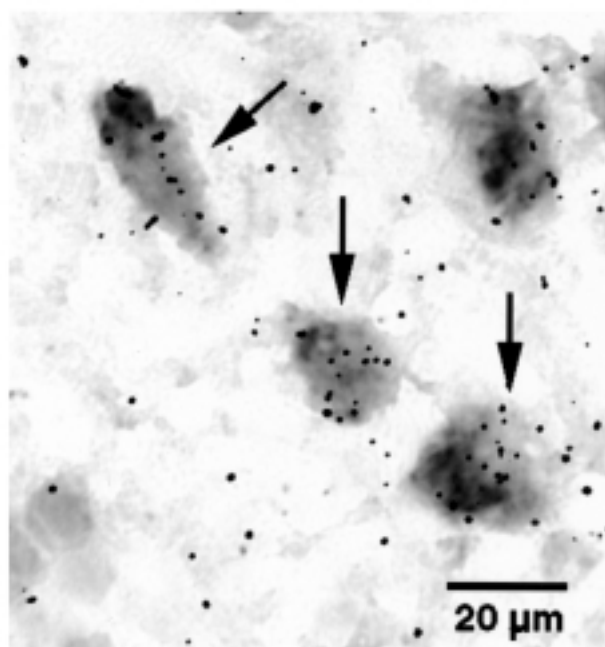


FIG. 3. Double-labeled photomicrograph of cells in the ventromedial hypothalamus. Double in situ hybridization techniques were used to examine the distribution of KIR6.2 mRNA (dark precipitate within cell profiles) and glucokinase mRNA (black autoradiographic grains overlaying cells). Arrows point to several cells that contain labels for both types of mRNA.

rat liver GK [41]). Genomic contamination of the samples would have resulted in a larger fragment than the observed 340 nucleotides because of the presence of an intron at this position. These results were confirmed by performing PCR on RNA samples that had not been DNase treated to remove contaminating genomic DNA. A PCR fragment of ~550 nucleotide pairs was observed in those samples (data not shown), suggesting that the gene transcribed to produce GK in the brain is the same as that used to express GK in the β -cell and liver with respect to the positions of exon 9 and 10.

DISCUSSION

These studies were undertaken to test the hypothesis that GK would be localized in brain areas containing relatively large populations of glucosensing neurons. The rationale was that GK appears to act as a gatekeeper for the glycolytic production of ATP and thereby cell activation (24,25). Binding of ATP inactivates (closes) the K_{ATP} channel, leading to membrane depolarization and increased firing rate (7,33). Such cells, by definition, are glucose responsive (7,16), using glucose as a signaling molecule to control the rate of cell firing. The current results show definitive evidence for the localization of GK gene expression in specific hypothalamic nuclei that are also known to contain glucose-responsive neurons (VMN, ARC, PVN, and LHA [2,15,16,45]). On the other hand, GK mRNA was not found in the nucleus tractus solitarius or substantia nigra, even though these areas appear to contain glucose-responsive neurons (20,46,47). Surprisingly, GK mRNA was expressed in several areas not known to contain glucosensing neurons. These include the bed nucleus of the stria terminalis, the medial preoptic area, inferior olive, and the dorsomedial hypothalamic, medial amygdalar, oculomotor, lateral habenular, interpeduncular, anterior olfactory, and posterior paraventricular thalamic nuclei. High-stringency conditions were used as a critical determinant to ensure the specificity of hybridization. Under such conditions, we were unable to confirm the presence of GK in the ependymal lining of the third ventricle, as was previously described (34,35). Finally, although it is likely that cells expressing GK mRNA also express GK protein, the current studies do not address this issue. In situ hybridization studies using a truncated form of the GK sequence showed results identical to those of the full-length probe. This truncated probe shares no homology with HKI, which is ubiquitously expressed in neurons and glia throughout the brain (48,49). This observation demonstrates the absence of hybridization between the full-length probe and HKI and verifies the specificity of both probes for GK.

RT-PCR identified the hypothalamic GK mRNA as one of the previously described rat GK isoforms. However, no differentiation could be made between the hepatic and β -cell

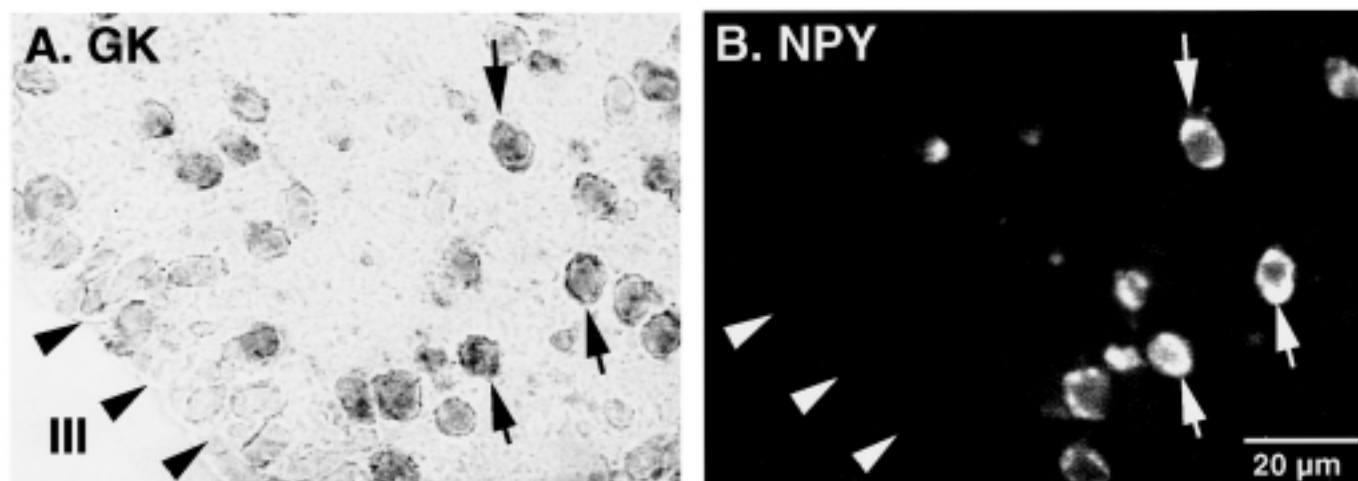


FIG. 4. Double-labeled in situ hybridization in a coronal section through the arcuate nucleus adjacent to the third ventricle (III). In situ hybridization of the antisense GK (A) and prepro-NPY (B) probes are shown. Arrows point to individual neurons that colocalize GK and NPY. Arrowheads point to ependymal cells lining the third ventricle that show no detectable hybridization with either probe.

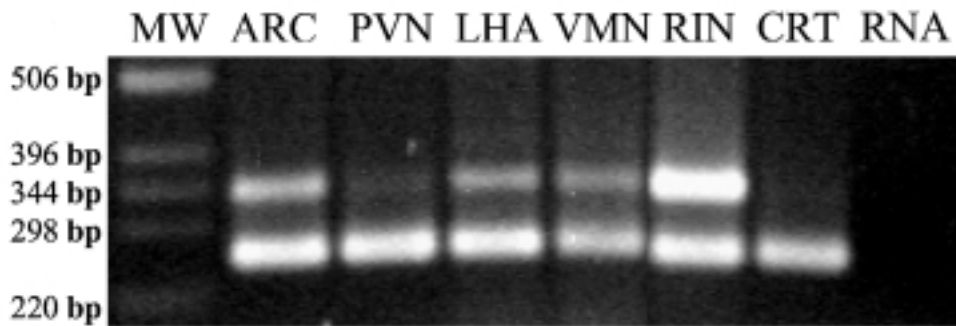


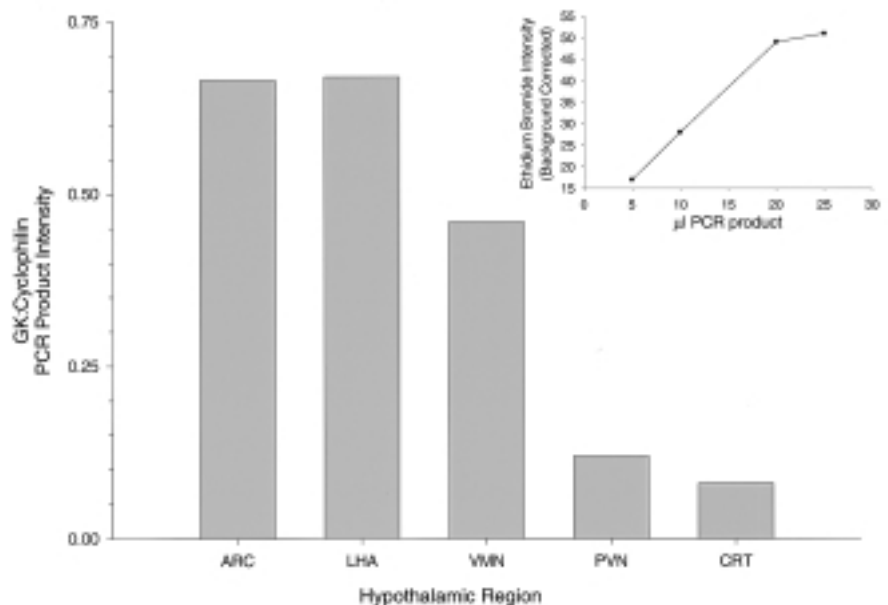
FIG. 5. PCR analysis of hypothalamic GK mRNA transcripts. PCR products were synthesized using 100 ng cDNA templates reverse transcribed from polyadenylated cytoplasmic RNA samples isolated from specific regions of the adult rat hypothalamus (primer sequences are given in RESEARCH DESIGN AND METHODS). Sample-matched GK (340 bp) and cyclophilin (259 bp) PCR products were run on a 2% agarose gel. Lane MW: 2 μ g 1-kb ladder (Gibco BRL); lane ARC: arcuate nucleus; lane PVN: paraventricular nucleus; lane LHA: lateral hypothalamic area; lane VMN: ventromedial nucleus; lane RIN: rat GK-expressing rat insulinoma cell line BG-40/110; lane CRT: cortex; lane RNA: 1 μ g hypothalamic total RNA (DNase treated) as control for genomic contamination.

forms of this enzyme, since the PCR primers were specifically designed to cover a region homologous to both. This issue may be of some importance, because only hepatic GK expression is reduced by fasting and reinstated by refeeding (50). Such responsiveness to the metabolic state of the animal plays an important regulatory role in the expression of both pro-opiomelanocortin (POMC) and NPY in the ARC. Fasting decreases POMC (51) and increases NPY expression in these neurons (37,52). Here we show that GK is colocalized with NPY in ARC neurons. Our unpublished studies have also found colocalization of GK in ARC POMC neurons. Obviously, these findings do not exclude expression of GK within glia. However, NPY neurons, but not astrocytes or oligodendroglia, also contain the pore-forming unit (KIR6.2) of the K_{ATP} channel (38). Moreover, expression of KIR6.2 in neurons expressing GK (Fig. 3) is consistent with these neurons playing dual sensory and regulatory functions. The high-affinity (3,53) and low-affinity (3) sulfonylurea receptors, which are an integral part of the K_{ATP} channel, are also present in the ARC, suggesting that neuropeptide neurons in the ARC, such as those expressing NPY, are capable of using glucose as a sig-

naling molecule to regulate cell firing. If the analogy to the β -cell is correct (26,32), then GK may be the critical regulator of the neuronal firing rate through its control of glycolytic production of ATP and thereby the activity of the K_{ATP} channel. With its relatively high K_m for glucose (30,31), GK would enable such neurons to sense glucose within the physiologic range seen by the brain.

Brain glucose levels vary from 0.16 to 4.5 mmol/l as plasma glucose levels move from 2.8 to 15.2 mmol/l (15,17). Such a narrow range of glucosensing capacity would be required if a neuron were to play an important role in the physiologic regulation of energy homeostasis and glucose metabolism. Such potential roles include modulation of food intake (54), sympathetic nervous system activity (11–13), and body weight (11), although in none of these functions has a definitive role for glucose been proven. Glucosensing neurons containing GK might be important to the counterregulatory response to hypoglycemia (14,22,23). All of these functions may actually represent an interplay between glucose-responsive and glucose-sensitive neurons (7). Studies in the pancreatic β -cell (and by inference glucose-responsive neurons) suggest a central role

FIG. 6. Relative expression of GK transcripts in specific regions of the adult rat hypothalamus. Semiquantitative analysis was performed by measuring the ethidium bromide signal intensity of each band from the scanned images of an agarose gel (as described in RESEARCH DESIGN AND METHODS). Band signal intensity for GK was normalized to that of cyclophilin within the same lane. Sample volume was held constant for each PCR product and hypothalamic region. Volumes were chosen to provide loading of each lane with similar levels of cyclophilin. Exposure duration and sample volume (5–15 μ l) were selected to ensure that signal intensity of all bands were in the linear range of the detection system (insert).



for GK in regulating K_{ATP} channel activity (24,26,32), but GK may also regulate the activity of the Na^+/K^+ ATP pump, which has been postulated to control the firing rate of glucose-sensitive neurons (1,15). Glucose-sensitive neurons, which decrease their firing rate as glucose levels rise, are highly localized in the LHA (1), where GK mRNA was localized by in situ hybridization and RT-PCR. The LHA is also the site of orexigenic neurons, which contain orexin (55), and melanin-concentrating neurons (56). This finding raises the potential for the regulation of another set of neuropeptide neurons involved in energy homeostasis by changes in brain glucose. But the ability to sense glucose is only one of several ways in which central neurons can monitor peripheral metabolism. In addition to containing the components of the K_{ATP} channel and GK, NPY (and POMC) neurons also contain leptin receptors (4,5). Both may also express insulin receptors, since these receptors are highly localized in the ARC (6). Thus, such neurons appear to be pluripotent with regard to their ability to sense and integrate signals relating to the metabolic status of the periphery. Given their connections with autonomic and hypophysial output pathways (10,57–60), ARC NPY and POMC neurons represent a class of neurons that is uniquely equipped to act as a central clearing station for such information.

In conclusion, we have shown that GK mRNA is expressed in neurons in an anatomically specific pattern. Several brain areas that express high levels of GK mRNA also contain glucosensing neurons, which respond to changes in ambient glucose by changing their firing rates. We show that ARC NPY neurons specifically coexpress GK. Because GK should regulate the glycolytic production of ATP in such neurons (26,32), our results suggest that GK might be the critical characteristic that confers the ability to be glucose-responsive, in contrast to the many other neurons that contain components of the K_{ATP} channel but are not glucosensing (7,53). The presence of GK in brain areas in which glucosensing neurons have not been identified previously suggests that these neurons might also serve a glucosensing function. However, this remains to be shown. Finally, ARC neuropeptide neurons may represent a class of neurons that have evolved mechanisms for sensing and integrating a host of signals from the periphery and passing this information on to autonomic and hypophysial pathways critical to the regulation of energy homeostasis (61).

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REFERENCES

- Oomura Y, Ooyama H, Sugimori M, Nakamura T, Yamada Y: Glucose inhibition of the glucose-sensitive neurone in the rat lateral hypothalamus. *Nature* 247:284–286, 1974
- Oomura Y, Ono T, Ooyama H, Wayner MJ: Glucose and osmosensitive neurons of the rat hypothalamus. *Nature* 222:282–284, 1969
- Dunn-Meynell AA, Routh VH, McArdle JJ, Levin BE: Low affinity sulfonylurea binding sites reside on neuronal cell bodies in the brain. *Brain Res* 745:1–9, 1997
- Glaum SR, Hara M, Bindokas VP, Lee CC, Polonsky KS, Bell GI, Miller RJ: Leptin, the obese gene product, rapidly modulates synaptic transmission in the hypothalamus. *Mol Pharmacol* 50:230–235, 1996
- Spanswick D, Smith MA, Groppi VE, Logan SD, Ashford ML: Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature* 390:521–525, 1997
- Routh VH, McArdle JJ, Spanswick DC, Levin BE, Ashford MLJ: Insulin modulates the activity of glucose-responsive neurons in the ventromedial hypothalamic nucleus (VMN). *Soc Neurosci Abst* 23:577A, 1997
- Levin BE, Dunn-Meynell AA, Routh VH: Brain glucosensing and body energy homeostasis: role in obesity and diabetes. *Am J Physiol* 276:R1223–R1231, 1999
- Woods SC, Seeley RJ, Porte D Jr, Schwartz MW: Signals that regulate food intake and energy homeostasis. *Science* 280:1378–1387, 1998
- Levin BE: Intracarotid glucose-induced norepinephrine response and the development of diet-induced obesity. *Int J Obes* 16:451–457, 1992
- Dunn-Meynell AA, Govek E, Levin BE: Intracarotid glucose infusions selectively increase Fos-like immunoreactivity in paraventricular, ventromedial and dorsomedial nuclei neurons. *Brain Res* 748:100–106, 1997
- Levin BE, Govek EK, Dunn-Meynell AA: Reduced glucose-induced neuronal activation in the hypothalamus of diet-induced obese rats. *Brain Res* 808:317–319, 1998
- Levin BE, Sullivan AC: Glucose-induced norepinephrine levels and obesity resistance. *Am J Physiol* 253:R475–R481, 1987
- Levin BE: Glucose increases rat plasma norepinephrine levels by direct action on the brain. *Am J Physiol* 261:R1351–R1357, 1991
- Borg WP, During MJ, Sherwin RS, Borg MA, Brines ML, Shulman GI: Ventromedial hypothalamic lesions in rats suppress counterregulatory responses to hypoglycemia. *J Clin Invest* 93:1677–1682, 1994
- Silver IA, Erecinska M: Glucose-induced intracellular ion changes in sugar-sensitive hypothalamic neurons. *J Neurophysiol* 79:1733–1745, 1998
- Ono T, Nishino H, Fukada M, Sasaki K, Muramoto K, Oomura Y: Glucose-responsive neurons in rat hypothalamic tissue slices in vitro. *Brain Res* 232:494–499, 1982
- Silver IA, Erecinska M: Extracellular glucose concentrations in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals. *J Neurosci* 14:5068–5076, 1994
- Nakano Y, Oomura Y, Lenard L, Nishino H, Aou S, Yamamoto T, Aoyagi K: Feeding-related activity of glucose- and morphine-sensitive neurons in the monkey amygdala. *Brain Res* 399:167–172, 1986
- Finta EP, Harms L, Sevcik J, Fischer HD, Illes P: Effects of potassium channel openers and their antagonists on rat locus coeruleus neurones. *Brit J Pharmacol* 109:308–315, 1993
- Mizuno Y, Oomura Y: Glucose responding neurons in the nucleus tractus solitarius of the rat, in vitro study. *Brain Res* 307:109–116, 1984
- Levin BE, Routh VH: Role of the brain in energy balance and obesity. *Am J Physiol* 271:R491–R500, 1996
- Niimi M, Sato M, Tamaki M, Wada Y, Takahara J, Kawanishi K: Induction of Fos protein in the rat hypothalamus elicited by insulin-induced hypoglycemia. *Neurosci Res* 23:361–364, 1995
- Borg WP, Sherwin RS, During MJ, Borg MA, Shulman GI: Local ventromedial hypothalamic glucopenia triggers counterregulatory hormone release. *Diabetes* 44:180–184, 1995
- Newgard CB, McGarry JD: Metabolic coupling factors in pancreatic β -cell signal transduction. *Annu Rev Biochem* 64:689–719, 1995
- Heimberg H, De Vos A, Moens K, Quartier E, Bouwens L, Pipeleers D, Van Schftingen E, Madsen O, Schuit F: The glucose sensor protein glucokinase is expressed in glucagon-producing α -cells. *Proc Natl Acad Sci U S A* 93:7036–7041, 1996
- Heimberg H, De Vos A, Vandercammen A, Van Schftingen E, Pipeleers D, Schuit F: Heterogeneity in glucose sensitivity among pancreatic β -cells is correlated to differences in glucose phosphorylation rather than glucose transport. *EMBO J* 12:2873–2879, 1993
- Sweet IR, Li G, Najafi H, Berner D, Matschinsky FM: Effect of a glucokinase inhibitor on energy production and insulin release in pancreatic islets. *Am J Physiol* 271:E606–E625, 1996
- Matschinsky FM, Glaser B, Magnuson MA: Pancreatic beta-cell glucokinase: closing the gap between theoretical concepts and experimental realities (Review). *Diabetes* 47:307–315, 1998

29. Becker TC, Noel RJ, Johnson JH, Lynch RM, Hirose H, Tokuyama Y, Bell GI, Newgard CB: Differential effects of overexpressed glucokinase and hexokinase I in isolated islets. *J Biol Chem* 271:390-394, 1996
30. Wang H, Iynedjian P: Modulation of glucose responsiveness of insulinoma β -cells by graded overexpression of glucokinase. *Proc Natl Acad Sci U S A* 94:4372-4377, 1997
31. De Vos A, Heimberg H, Quartier E, Huypens P, Bouwens L, Pipeleers D, Schuit F: Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. *J Clin Invest* 96:2489-2495, 1995
32. Inagaki N, Gono T, Clement JP IV, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J: Reconstitution of I_{KATP} : an inward rectifier subunit plus sulphonylurea receptor. *Science* 270:1166-1170, 1995
33. Routh VH, McArdle JJ, Levin BE: Phosphorylation modulates the activity of the ATP-sensitive K^+ channel in the ventromedial hypothalamic nucleus. *Brain Res* 778:107-119, 1997
34. Yang X, Kow L, Funabashi T, Mobbs C: Hypothalamic glucose sensor: similarities to and differences from pancreatic β -cell mechanisms. *Diabetes* 48:1763-1772, 1999
35. Jetton TL, Liang Y, Pettepher CC, Zimmerman EC, Cox FG, Horvath K, Matschinsky FM, Magnuson MA: Analysis of upstream glucokinase promoter activity in transgenic mice and identification of glucokinase in rare neuroendocrine cells in the brain and gut. *J Biol Chem* 269:3641-3654, 1994
36. Navarro M, Rodriguez DF, Alvarez E, Chowen JA, Zueco JA, Gomez R, Eng J, Blazquez E: Colocalization of glucagon-like peptide-1 (GLP-1) receptors, glucose transporter GLUT-2, and glucokinase mRNAs in rat hypothalamic cells: evidence for a role of GLP-1 receptor agonists as an inhibitory signal for food and water intake. *J Neurochem* 67:1982-1991, 1996
37. Higuchi H, Yang HYT, Sabol SL: Rat neuropeptide Y precursor gene expression. *J Biol Chem* 263:6288-6295, 1988
38. Levin BE, Dunn-Meynell AA: Dysregulation of arcuate nucleus preneuropeptide Y mRNA in diet-induced obese rats. *Am J Physiol* 272:R1365-R1370, 1996
39. Berger SL, Birkenmeier CS: Inhibition of intractable nucleases with ribonucleoside-vanadyl complexes: isolation of messenger ribonucleic acids from resting lymphocytes. *Biochemistry* 18:5143-5149, 1979
40. Clark SC, Quaade C, Constandy H, Hansen P, Halban P, Ferber S, Newgard CB, Normington K: Novel insulinoma cell lines produced by iterative engineering of GLUT-2, glucokinase and human insulin expression. *Diabetes* 46:958-967, 1997
41. Sambrook J, Fritsch EF, Maniatis T, Eds.: *Molecular Cloning: A Laboratory Manual*. Cold Springs Harbor, NY, Cold Springs Harbor Laboratory Press, 1989
42. Wang MY, Koyama K, Shimabukuro M, Mangelsdorf D, Newgard CB, Unger RH: Overexpression of leptin receptors in pancreatic islets of Zucker diabetic fatty rats restores GLUT-2, glucokinase and glucose-stimulated insulin secretion. *Proc Natl Acad Sci U S A* 95:11921-11926, 1998
43. Danielson PE, Forss-Petter S, Brow MA, Calavetta L, Milner RJ, Sutcliffe JG: pIB15: a cDNA clone of the rat mRNA encoding cyclophilin. *DNA* 7:261-267, 1988
44. Dunn-Meynell AA, Rawson NE, Levin BE: Distribution and phenotype of neurons containing the ATP-sensitive K^+ channel in rat brain. *Brain Res* 814:41-54, 1998
45. Muller Y, Reitsetter R, Yool AJ: Regulation of Ca^{2+} -dependent K^+ channel expression in rat cerebellum during postnatal development. *J Neurosci* 18:16-25, 1998
46. Kow LM, Pfaff DW: Responses of hypothalamic paraventricular neurons *in vitro* to norepinephrine and other feeding-relevant agents. *Physiol Behav* 46:265-271, 1989
47. Roeper J, Ashcroft FM: Metabolic inhibition and low internal ATP activate K_{ATP} channels in rat dopaminergic substantia nigra neurones. *Pflügers Arch* 430:44-54, 1995
48. Illes P, Sevcik J, Finta EP, Frolich R, Nieber K, Norenberg W: Modulation of locus coeruleus neurons by extra- and intracellular adenosine 5'-triphosphate. *Brain Res Bull* 35:513-519, 1994
49. Garner JA, Linse KD, Polk RK: Type I brain hexokinase: axonal transport and membrane associations within central nervous system presynaptic terminals. *J Neurochem* 67:845-856, 1996
50. Lusk JA, Manthorpe CM, Kao-Jen J, Wilson JE: Predominance of the cytoplasmic form of brain hexokinase in cultured astrocytes. *J Neurochem* 34:1412-1420, 1980
51. Iynedjian PB, Pilot PR, Nospikel T, Milburn JL, Quaade C, Hughes S, Ucla C, Newgard CB: Differential expression and regulation of the glucokinase gene in liver and islets of Langerhans. *Proc Natl Acad Sci U S A* 86:7838-7842, 1989
52. Kim EM, Welch CC, Grace MK, Billington CJ, Levine AS: Chronic food restriction and acute food deprivation decrease mRNA levels of opioid peptides in arcuate nucleus. *Am J Physiol* 270:R1019-R1024, 1996
53. Brady LS, Smith MA, Gold PW, Herkenham M: Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. *Neuroendocrinology* 52:441-447, 1990
54. Karschin C, Ecke C, Ashcroft FM, Karschin A: Overlapping distribution of K_{ATP} channel-forming unit Kir6.2 subunit and the sulphonylurea receptor SUR1 in rodent brain. *FEBS Lett* 401:59-64, 1997
55. Campfield A, Brandon P, Smith F: On-line continuous measurement of blood glucose and meal pattern in free feeding rats: the role of glucose in meal initiation. *Brain Res Bull* 14:605-616, 1985
56. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JRS, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terrett JS, Eshourbagy NA, Bergsma DJ, Yanagisawa M: Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92:573-585, 1998
57. Ludwig DS, Mountjoy KG, Tatro JB, Gillette JA, Frederich RC, Flier JS, Maratos-Flier E: Melanin-concentrating hormone: a functional melanocortin antagonist in the hypothalamus. *Am J Physiol* 274:E627-E633, 1998
58. Baker RA, Herkenham M: Arcuate nucleus neurons that project to the hypothalamic paraventricular nucleus: neuropeptidergic identity and consequences of adrenalectomy on mRNA levels in the rat. *J Comp Neurol* 358:518-530, 1995
59. Sawchenko PE, Swanson LW: Immunohistochemical identification of neurons in the paraventricular nucleus of the hypothalamus that project to the medulla or to the spinal cord in the rat. *J Comp Neurol* 205:260-272, 1982
60. Jansen ASP, Wessendorf MW, Loewy AD: Transneuronal labeling of CNS neuropeptide and monoamine neurons after pseudorabies virus injections into stellate ganglion. *Brain Res* 683:1-24, 1995
61. Elias CF, Saper CB, Maratos-Flier E, Tritos NA, Lee C, Kelly J, Tatro JB, Hoffman GE, Ollmann MM, Barsh GS, Sakurai T, Yanagisawa M, Elmquist JK: Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. *J Comp Neurol* 402:442-459, 1998