The rise in prevalence of type 2 diabetes reflects a primary medical challenge of the 21st century. The mechanisms underlying glucose homeostasis in general, and glucose-stimulated insulin secretion (GSIS) in particular, are not fully understood. In health, a rise in blood glucose triggers a biphasic pattern of insulin response, consisting of a rapid (<10 min) first phase and a less prominent but sustained second phase (1). The precise mechanisms mediating the early phase of the insulin response are unclear. Nevertheless, impaired first-phase GSIS is a major pathological hallmark of the early stages of type 2 diabetes (2), suggesting that this may be an important determinant of the transition to diabetes in at-risk subjects.

Pancreatic β-cells can directly sense changes in blood glucose and alter insulin release as appropriate. In addition, the pancreas has rich autonomic innervations, and a number of experimental approaches have demonstrated that neural inputs may modulate insulin release (3,4) via muscarinic receptors or α-adrenergic signaling (5,6). A preponderance of the neural inputs that influence pancreatic β-cell activity emanate from the hypothalamus (5); however, the specific nature of hypothalamic pathways regulating insulin secretion is less clear.

In particular, hypothalamic melanocortin signaling (7–9) and inflammation (10) have been implicated in the control of insulin release. Additionally, there is increasing evidence of a role for hypothalamic nutrient-sensing pathways in the control of other facets of peripheral glucose metabolism, in particular for hepatic glucose production (11,12). It is therefore plausible that similar mechanisms might allow central facilitation of insulin secretion in response to a rise in blood glucose.

In order to control peripheral glucose metabolism, the brain must first rapidly and accurately detect changes in glucose availability. The hypothalamus contains glucose-sensing neurons, although the mechanisms used to sense glucose are not fully defined. However, some reports suggest that these hypothalamic neurons may sense products of glucose metabolism such as cellular ATP levels (11,13). To be metabolized, glucose must first be phosphorylated by hexokinases (HKs). The specialized low affinity HK isozyme glucokinase (GK), thought to be central in pancreatic glucose sensing, may also play a key role in hypothalamic glucose sensing (14). Accordingly, here we investigated the effects of acute activation of hypothalamic glucose sensing (by brain glucose infusion) or inhibition (using the competitive GK inhibitors glucosamine [GSN] or mannoheptulose [MHI]) on insulin secretion and glucose handling during intravenous glucose tolerance tests (IVGTT) in rats.

RESEARCH DESIGN AND METHODS

Animals. Healthy adult male Sprague Dawley rats ~250–350 g were used throughout. For each study, the cohorts were matched for weight and randomized into treatment groups. Procedures were approved in advance by both a local university and a national ethical review process (U.K. Home Office License held under the Animals [Scientific Procedures] Act). Chemicals were from Sigma-Aldrich (Gillingham, U.K.) unless otherwise stated.

Surgical preparation. Under inhaled anaesthetic, rats underwent stereotaxic insertion of a guide cannula into the base of the third ventricle (coordinates from bregma: 2.2 mm posterior, 0.9 mm lateral, 8.4 mm below skull surface angled at 5° to vertical toward the midline) and placement of jugular vein catheter as previously described (15). Peri- and postoperative injectable analgesia and antibiotic were provided routinely, and only animals that had regained preoperative body weight with no signs of infection or illness were studied 1 week later.
Effect of intracerebroventricular infusion of α-glucose versus urea on glucose handling during IVGTT. Chronically catheterized intracerebroventricular (ICV) and intravenous (IV) rats prepared, acclimatized, and fasted overnight as above. Rats received ICV infusion of GSN or MH (300 nmol/min) or vehicle (aECF). 90 min after the start of ICV infusion. After 90 min, rats were rapidly euthanized and their brains removed; plasma samples were collected before and after ICV infusion. 13,000 rpm for 20 min at 4°C. Supernatants were collected and stored at −80°C before being analyzed. HK and GK activity was assayed spectrophotometrically (340 nm, room temperature, Beckmann DU-64 spectrophotometer) by coupling glucose phosphorylation to a reporter assay, which oxidizes glucose-6-phosphate to 6-phosphogluco-6-lactone with simultaneous reduction of NAD+ to NADH, as described previously (18). The reaction mixture in 1 mL final volume contained 20 mM HEPES, 150 mM KCl, 5 mM MgCl2, and 1 mM EDTA [pH 7.4], supplemented with 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μM leupeptin HCl) and centrifuged at 13,000 rpm for 20 min at 4°C. Supernatants were collected and stored at −80°C for further analysis. HK and GK activity was assayed spectrophotometrically (340 nm, room temperature, Beckmann DU-64 spectrophotometer) by coupling glucose phosphorylation to a reporter assay, which oxidizes glucose-6-phosphate to 6-phosphogluco-6-lactone with simultaneous reduction of NAD+ to NADH, as described previously (18). The reaction mixture in 1 mL final volume contained 20 mM HEPES (pH 7.1), 25 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 1 mM Na+ NAD+, 1 mM ATP, 10 units glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides, 1 mM 3-O-methyl-N-acetyl GSN (Axxora LLC, San Diego, CA, U.K.), 100 μM L-threohydroxypalmitic protein extract, and glucose in concentrations of 0.5 mM, 1 mM, and 20 mM. In all assays for GK activity, 3-O-methyl N-acetyl GSN was incorporated to inhibit N-acetyl GSN kinase (19), except inhibition studies with HK and GK. GK activity was calculated by subtracting glucose phosphorylation at 0.5 mM (hypothalamic) or 1 mM (liver) glucose from that measured at 20 mM glucose. Data were analyzed by fitting sigmoidal curves to the dose-response studies by nonlinear least square minimization method.

Determination of extent of GSN distribution following ICV infusion. Nonfasted catheterized rats under 90 min ICV infusion of 150 nmol/min GSN or aECF. Plasma samples were collected before and after ICV infusion. After 90 min, rats were rapidly euthanized and their brains removed; the hypothalamus, brain stem, and cortex were dissected rapidly and frozen in liquid nitrogen. Samples were stored at -80°C before being analyzed. Brain samples were homogenized in ice-cold lysis buffer, centrifuged at 13,000 rpm for 20 min at 4°C, and supernatants were collected for further analysis.

GSN concentration in plasma and brain homogenate was assayed by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS/MS). Analysis was performed using an Alliance 2995 separation module (Waters, Milford, MA) coupled to an ion trap mass spectrometer (Quattro LC; Micromass UK Ltd., Manchester, U.K.). Sample preparation was optimized by a slight modification of a previously published method for direct determination of GSN in plasma (20). Briefly, 45 μL of sample was mixed with 5 μL of O-[U-13C] GSN as the internal standard; then, 25 μL of trichloroacetic acid (200 g/L) were added to achieve protein precipitation. 3 μL of the supernatant was injected into the HPLC-ESI-MS/MS system.

A polymer-based amino column (5 μm, 2.0 mm i.d. × 150 mm), supplied by Showa Denko K.K. (Kanagawa, Japan), and mobile phase of 80:20 (volume for volume) acetonitrile-10 mM ammonium acetate (pH = 7.5) at 0.3 mL/min flow rate were used. MS/MS detection, using ESI source in positive ionization, was performed in a triple quadrupole monitoring mode, selecting the charge/mass ratio transition 180→72 for GSN and charge/mass ratio 181→73 for internal standard. The limit of quantification of the method was 0.28 μmol/L for both rat plasma and brain homogenate. Matrix-matched standards were used for calibration, obtaining good linearity up to 56 μmol/L (R² = 0.9997). For calculating absolute concentrations, the density of brain tissue was assumed to be 1.04 g/mL (21).

RESULTS

ICV infusion of α-glucose improved glucose handling during IVGTT. To investigate this, glucose was infused into the third ventricle (ICV) of adult male rats for 30 min followed by an IVGTT (0.35 g/kg) (Fig. 1A and B). ICV infusion of glucose did not change systemic plasma glucose during the 30 min infusion period (−30 to 0 min). However, ICV glucose-infused rats displayed improved glucose handling during the first 10 min of the IVGTT, with a significantly lower (P < 0.05) area under the excursion curve (AUC0-10) relative to control urea-infused animals (Fig. 1C and D).

Despite being exposed to equivalent or lower circulating plasma glucose levels prior to and during IVGTT respectively, plasma insulin levels in ICV glucose-infused rats were significantly higher than controls, seemingly starting to rise even before the delivery of an external glucose load (Fig. 1E). Insulin secretion between the start of the brain infusion and the 10 min time point of the IVGTT (40 min total), which was used as the cutoff between early and late-phase insulin secretion, was significantly higher in ICV glucose-infused rats (Fig. 1F). These data suggest that elevation of hypothalamic glucose leads to a centrally driven insulin secretory response.

ICV infusion of GSN impaired glucose handling and insulin secretion during IVGTT. As a possible mechanism underlying this observation, we examined the role of hypothalamic GK-dependent sensing pathways in insulin secretion in response to a systemic glucose load. The competitive inhibitor of glucose phosphorylation, GSN, has previously been shown to inhibit hepatic GK (22). To confirm that GSN inhibits hypothalamic GK, we first examined the effect of GSN on GK activity in hepatic and hypothalamic protein preparations ex vivo. GK activity was detectable in hepatic and hypothalamic protein preparations from healthy Sprague Dawley rats ex vivo (Fig. 2A and B). GSN dose-dependently inhibited rat liver GK activity, with a half-maximal inhibitory concentration (IC50) of 4.2 mM in the presence of 20 mM glucose (Fig. 2C). GSN also dose-dependently inhibited hypothalamic GK activity with an IC50 of 5.0 mM in the presence of 20 mM glucose (Fig. 2D).

To determine the appropriate infusion regimen to be used in subsequent studies and the sites and concentrations of GSN achieved, the extent of GSN distribution following ICV infusion (150 nmol/min) was measured using HPLC-ESI-MS/MS method. ICV GSN resulted in a marked rise in hypothalamic GSN to levels of approximately 1 mM, a modest rise in brain stem GSN levels (~15% of hypothalamic levels), but did not alter cerebral cortex levels (Table 1). Importantly, although our sensitive assay was able to detect a small statistically significant change in plasma values, levels of GSN in the blood stream remained
at low micromolar levels, three orders of magnitude below the levels required to inhibit GK.

Next, we examined whether hypothalamic levels of GSN attained by 90 min ICV infusion (1 mM) would be sufficient to inhibit hypothalamic GK activity. We aimed to measure hypothalamic GK activity in the presence of 1 mM GSN in vitro at a glucose concentration that is likely to simulate ambient hypothalamic glucose levels during the IVGTT. A concentration of 3 mM glucose was selected for in vitro assays based on peak plasma glucose levels observed during IVGTT (17 mM, Fig. 1) and on the fact that accepted approximations of brain extracellular glucose concentrations are roughly 20% of that of plasma glucose (23,24). GK represented ~5% of total hypothalamic glucose phosphorylation activity at 3 mM glucose and was selectively inhibited by 1 mM GSN (reduced to 48.1 ± 7.3% and total glucose phosphorylating activities was 97 ± 1% of control, n = 3). These kinetic studies suggested that ICV GSN in vivo, even at the highest dose used, is unlikely to result in major spillage of GSN outside the brain and that levels achieved might be sufficient to inhibit, at least in part, hypothalamic GK activity.

We next investigated the effects of 90 min ICV infusion of GSN at 75 and 150 nmol/min on plasma glucose and insulin responses during IVGTT (0.5 g/kg—a higher dose than that used in ICV-glucose studies above) (Fig. 3A). ICV GSN significantly and dose-dependent impaired glucose handling during IVGTT (Fig. 3B and C). Despite the higher plasma glucose in the ICV GSN rats, insulin responses
were reduced, particularly during the first few minutes of the glucose challenge, with peak and AUC\textsubscript{0-10} insulin being significantly lower in ICV 150 nmol/min GSN rats compared with controls. As might be expected, ICV 75 nmol/min GSN showed a pattern intermediate between the other two groups. Insulinogenic index was significantly lower in both ICV 150 nmol/min and 75 nmol/min GSN rats relative to controls (Fig. 3D–F). Taken together, these data show that third ventricle infusion of a low dose of a GK inhibitor, GSN, predominantly distributed into the surrounding hypothalamus, impaired insulin secretion and glucose tolerance during the first few minutes of an IVGTT in rats. Moreover, these findings suggest a role for hypothalamic GK mediated glucose sensing in the central regulation of GSIS.

**FIG. 2.** Ex vivo GK activity assays in protein preparations. A: Sigmoidal dependence of hepatic GK (n = 2) on glucose concentration. B: Glucose increases hypothalamic GK activity. C and D: GSN dose dependently inhibits hepatic (n = 3–5) and hypothalamic (n = 3) GK ex vivo. E: MH dose dependently inhibits hypothalamic GK ex vivo (n = 3), although with reduced potency as compared with GSN (IC\textsubscript{50} =12 mM vs. 5 mM, MH vs. GSN, respectively). **P < 0.01.
ICV infusion of MH impaired glucose handling and insulin secretion during IVGTT. A limitation of the study above is that GSN has other biological actions in addition to inhibiting glucose metabolism via GK, for instance acting through the hexosamine pathway (25). The study was repeated using MH, an alternative GK inhibitor that is structurally unrelated to GSN and does not act through the hexosamine pathway. Again, we first confirmed ex vivo that MH dose-dependently inhibited hypothalamic GK (Fig. 2E). Given the lower potency of MH for GK inhibition, a higher dose of MH was used for in vivo studies. In keeping with an effect mediated by GK inhibition, ICV 300 nmol/min MH-treated rats displayed impaired glucose handling with a significantly higher AUC\(_{0-10}\) glucose relative to control rats (Fig. 4A and B). Consistently, insulinogenic index was significantly lower in ICV 300 nmol/min MH rats relative to controls (Fig. 4C and D). These findings further support a role for hypothalamic GK-mediated glucose sensing in the regulation of GSIS in response to an IV glucose challenge.

DISCUSSION

Although there is increasing evidence that hypothalamic glucose sensing may contribute to the integrated control of aspects of whole body glucose homeostasis (26) such as hepatic glucose output (11, 12) and hypoglycemia counter-regulation (13), its role in the regulation of insulin secretion has been less clear. Here, we provide the first direct evidence that hypothalamic glucose sensors play a significant role in the control of insulin secretion, one of the most important systems in the maintenance of whole-body glucose homeostasis.

Specifically, we observed that activation of hypothalamic glucose sensing by ICV infusion of glucose improved glucose handling and insulin secretion during IVGTT. Furthermore, we demonstrated that pharmacological inhibition of hypothalamic glucose sensing by ICV infusion of GK and HK inhibitors, GSN and MH, significantly impaired glucose handling and first-phase insulin secretion during IVGTT. These data suggest a critical role for brain glucose sensing in the regulation of the first phase of pancreatic GSIS and in turn whole-body glucose tolerance. Such a role is consistent with emerging evidence that hypothalamic glucose sensors contribute to the integrated control of peripheral glucose homeostasis (26).

As in humans, glucose infusion in rats elicits a biphasic insulin response (27). In this study, we elected to use an IVGTT rather than oral challenge because it allowed investigation of GSIS without a confounding effect from incretins. Furthermore, IVGTT as a measure of insulin secretion has been used successfully in rats. Similar to the pattern seen in humans, both an early peak response during the first few minutes after a glucose load and a later sustained insulin release can be identified (28). Analogous to humans, the early phase of insulin release is suppressed in some rodent models of diabetes (29).

Although GSN and MH inhibit other HKs in addition to inhibiting GK, the conditions of our studies suggest that these effects are likely mediated via GK. Brain extracellular glucose concentration is ~20% of that of plasma glucose (23, 24). Plasma glucose levels during IVGTT studies attained peak values of approximately 17–20 mM (Figs. 1–3) and 20% of these values are roughly 3–4 mM. However, it is possible that glucose levels sensed by hypothalamic arcuate nucleus (ARC) neurons may be higher than this because of the proximity of the ARC to the median eminence, where the blood-brain barrier is thought to be leaky (30). Since GK is active in the hypothalamus at glucose concentrations ranging from about 3 to 20 mM (31), it is better suited for high capacity glucose phosphorylation necessary for glucose sensing in this range of glucose concentrations than other high affinity HKs (which are easily saturated at glucose concentrations less than 500 \(\mu\)M). Our in vitro studies demonstrating that 1 mM GSN selectively inhibits hypothalamic GK without interfering with other HKs at 3 mM glucose further suggest that ICV GSN’s effects during IVGTT are mediated via inhibition of hypothalamic GK activity.

It is important to note that our studies do not permit us to exclude a potential contribution from extrahypothalamic brain regions influenced by third ventricle infusion in mediating centrally driven GSIS responses. However, given that the relative levels of GSN achieved by our third ventricle infusion in hypothalami were approximately 5-fold higher than in the brain stem, the data suggest that the hypothalamus received the highest concentration of GSN. Furthermore, even though the data clearly demonstrate a direct effect of brain glucose sensors on pancreatic GSIS, the involvement of additional synergistic effects to improve glucose handling by hypothalamic efferents, for example, altering hepatic glucose output directly, cannot be excluded. To delineate further the specific role of the hypothalamus and specific hypothalamic subnuclei in the effects of GSIS, localized injections of glucose and inhibitors of GK should be used. In addition, many elegant genetic and pharmacogenetic tools are now available which will enable further refined probing of the discrete role of specific chemically defined neurons, such as those expressing the melanocortin neuropeptides in the ARC, in GSIS.

### Table 1

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<th>GSN concentrations in brain and plasma</th>
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<td>ICV 150 mol/min GSN</td>
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Data are mean ± SEM. GSN concentrations in brain areas \((n = 3–5)\) and plasma \((n = 11)\) following 90-min ICV 150 nmol/min GSN or aECF (vehicle). Chronically catheterized (jugular vein and third ventricle) adult male Sprague Dawley rats underwent 90-min ICV infusion of 150 nmol/min GSN or aECF, with blood sampling from ICV GSN rats at start and end of infusion. Following 90 min ICV infusions, animals were killed and brains collected for ex vivo GSN assays. ND, no data. *\(P < 0.05\) Mann-Whitney test for GSN vs. aECF and after vs. before ICV infusion.
Overall, our data support a role for hypothalamic glucose sensing in the integrated control of peripheral glucose homeostasis. In particular, our findings suggest a novel model for the regulation of GSIS. We identified for the first time that GK-dependent glucose phosphorylation in the hypothalamus may play a facilitatory role in the regulation of the first phase of insulin secretion in response to a systemic glucose load. GK activators have been highlighted as potential therapeutic candidates for type 2 diabetes (32), in particular focusing on effects mediated by activation of...
hepatic and pancreatic GK. Our data suggest that a further beneficial action might be targeting GK-mediated glucose sensing in the hypothalamus.

In conclusion, our findings delineate a novel central mechanism in the control of glucose-stimulated insulin release and suggest that this may offer a future therapeutic target for improving glycemic control in type 2 diabetes.

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No potential conflicts of interest relevant to this article were reported.
M.A.O., D.D.L., P.H., S.P.M., and M.L.E. conceived and designed the experiments; performed the experiments; analyzed the data; contributed reagents, materials, and analysis tools; and wrote the manuscript. J.S. conceived and designed the experiments; performed the experiments; contributed reagents, materials, and analysis tools; and wrote the manuscript. C.-Y.Y. conceived and designed the experiments, performed the experiments, and analyzed the data. L.K.H. conceived and designed the experiments; analyzed the data; contributed reagents, materials, and analysis tools; and wrote the manuscript. C.C. and A.R. performed the experiments, analyzed the data, and contributed reagents, materials, and analysis tools. M.L.E. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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