

# Glucose Sensing During Hypoglycemia: Lessons From the Lab

Shortly after the introduction of insulin in the management of type 1 diabetes, clinicians became aware of the potential for insulin therapy to induce iatrogenic hypoglycemia. Hypoglycemia remains the major adverse effect of insulin therapy and has emerged as a significant limitation to achieving near-normal glucose control, which is required to reduce the risk of microvascular complications. The average individual with type 1 diabetes experiences around two episodes of symptomatic hypoglycemia per week. In the recent U.K. Hypoglycemia Study (1), the incidence of severe hypoglycemia (requiring external assistance) was 110 episodes per 100 patient-years in subjects with duration of diabetes <5 years and 320 episodes per 100 patient-years in subjects with duration of diabetes >15 years. Thus, despite the introduction of insulin analogues and improved delivery systems, hypoglycemia remains a major concern for individuals with type 1 and long-duration type 2 diabetes as well as their caregivers.

When glucose levels fall, a sequence of counterregulatory responses is triggered, which mainly involves the suppression of endogenous insulin secretion and the release of counterregulatory hormones that act rapidly to promote endogenous glucose production and limit peripheral glucose utilization. As glucose levels fall further, subjective awareness of hypoglycemia results in behavioral changes that ordinarily lead an individual to seek food. In healthy individuals, this homeostatic mechanism works well and hypoglycemia is rare, but for individuals with type 1 diabetes, these compensatory systems are disrupted at every level. First, for most individuals insulin delivery to the systemic circulation following a subcutaneous injection will continue despite the development of hypoglycemia. Second, almost all individuals with type 1 diabetes will over time develop defects in the hormonal counterregulatory defense against hypoglycemia. Within 5 years of diagnosis of type 1 diabetes, hypoglycemia fails to stimulate release of the major counterregulatory hormone glucagon (2). As a result, individuals with type 1 diabe-

tes are particularly dependent on the sympathoadrenal (primarily epinephrine and norepinephrine) response to low blood glucose. However, within 10 years of diagnosis, the majority of patients develop additional impairments in sympathoadrenal and other neurohormonal responses against hypoglycemia (2). In addition, symptom awareness becomes impaired in individuals with type 1 diabetes. This combination of disrupted physiological and behavioral responses to hypoglycemia markedly increases the risk of suffering severe hypoglycemia in patients with type 1 diabetes.

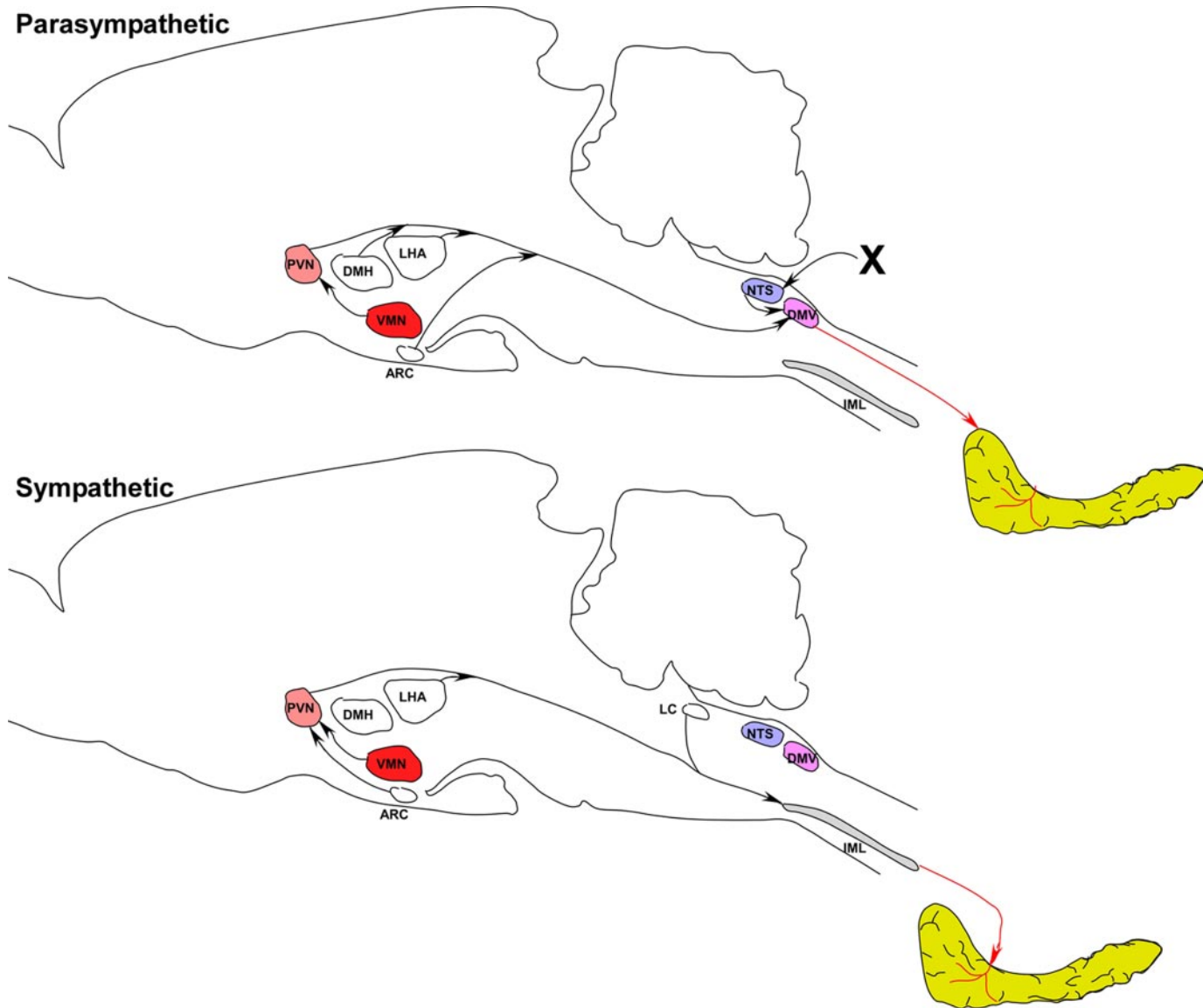
To understand why glucose counterregulation is impaired in diabetes requires a greater knowledge of where and how hypoglycemia is sensed, how hypoglycemia-sensitive cells trigger a counterregulatory response, and how the presence of diabetes influences these mechanisms. Research in this area has in recent years focused on animal and more basic models, although the groundwork for this was laid by a series of elegant human studies in the 1980s and 1990s. In this review, I will examine the literature, largely based on rodent studies, that is beginning to shed some light on the mechanisms that underpin hypoglycemia detection. This review will focus on the role of the ventromedial hypothalamus (VMH) (Fig. 1), a key central glucose-sensing region involved in the detection of hypoglycemia, to illustrate the advances that have been made in this field of research.

## The hypoglycemia sensors

Under most physiological conditions glucose is the primary fuel for the brain. The brain accounts for more than half the body's glucose use and because fuel stores such as glycogen are limited, it is very dependent on a continuous supply from the circulation. This probably explains why the glucose sensors thought to be dominant during hypoglycemia are found in the brain. Neurons shown to be regulated by glucose have been found in a number of brain areas that share certain common features (3). Within the brain they localize to regions adjacent to the III or IV ventricle or to the circumventricular organs

(these are regions of the brain where the blood-brain barrier is leaky or absent). This potentially allows glucose sensing neurons direct sampling and, hence, monitoring of glucose levels in the blood, brain, and cerebrospinal fluid. This is important because the presence of the blood-brain barrier ensures that brain glucose levels are only ~10–30% of the levels seen in the blood (4). Thus, glucose-sensing neurons are able to integrate changes in glucose within each of these different regions.

The VMH was shown to play a significant role in the detection of insulin-induced hypoglycemia in a series of *in vivo* rodent studies in the 1990s. Borg et al. (5) were able to demonstrate that chemical destruction of the VMH in a rodent model with ibotenic acid caused ~75% reduction in the hormonal counterregulatory response to acute hypoglycemia. Subsequently, they showed that the local perfusion, using microdialysis in awake unrestrained rats, of the VMH with 2-deoxyglucose (a nonmetabolizable form of glucose that effectively causes local hypoglycemia) stimulated a classic systemic counterregulatory response (6). Finally, and perhaps most convincingly, they demonstrated again with microdialysis that local perfusion of the VMH with glucose, maintaining glucose levels in this select brain region during systemic insulin-induced hypoglycemia, markedly suppressed the hormonal counterregulatory response (7). The addition of tracer to the perfusate (6), as well as the lesion studies (5), confirmed that the intervention in these studies was local to the VMH. In addition, a number of electrophysiological studies from hypothalamic slice preparations have shown that the VMH contains neurons that respond directly to low glucose (8), as do dissociated VMH neurons (9). Finally, transgenic mice that lack the vesicular glutamate transporter VGLUT2 selectively in steroidogenic factor-1 (SF1) neurons, which in rodents are only expressed in the VMH, show markedly impaired counterregulatory hormonal responses to acute insulin-induced hypoglycemia (10). Taken together these studies all point toward the VMH playing



**Figure 1**—Neural pathways linking the VMH via sympathetic and parasympathetic neurons to the pancreas. Retrograde neuronal tracer studies using pseudorabies virus microinjected into the pancreas of the rats established the VMH as a third-order neuron reaching the parasympathetic and sympathetic innervation of the pancreas via the paraventricular hypothalamic nucleus (PVN) and intermediolateral (IML) nucleus as well as the dorsal motor nucleus of the vagus (DMV), respectively. The dorsomedial hypothalamus (DMH) and lateral hypothalamus (LHA) also feed into this pathway as well as the nucleus tractus solitarius (NTS) in the hindbrain, each of which plays a role on glucose sensing. The nucleus tractus solitarius also is the likely point of integration of inputs from peripheral sensors via vagal afferents (X). Reprinted with permission from Buijs et al. (11).

an important role in the mechanism by which the brain detects a falling glucose and triggers a counterregulatory hormone response.

For normal glucagon and epinephrine counterregulation to occur, neural pathways must exist between the glucose-sensing regions of the brain such as the VMH and the pancreatic  $\alpha$ -cell and adrenal medulla, respectively. These pathways have not yet been fully elucidated. However, using pseudorabies virus as a retrograde tracer, Buijs et al. (11) were able to characterize sympathetic and parasympathetic inputs to the pancreas.

In this neuroanatomical study, the VMH was identified as a third-order neuron (the last of three neurons in a pathway that ultimately leads to the discharge of a neurotransmitter into the synaptic cleft) innervating the pancreas by way of other nuclei in the hypothalamus and thalamus and, subsequently, the dorsal motor nucleus of the vagus (parasympathetic) and intermediolateral cell column (sympathetic) (Fig. 1). These studies provide a potential neural pathway through which VMH glucose-sensing neurons could initiate glucose counterregulation during hypoglycemia, although why, given the

major role that the VMH appears to play in coordinating the counterregulatory response in vivo, this should be an indirect one is not clear. It should also be pointed out that if the VMH is not a primary sensing region but rather an important relay station for other brain regions, then this pathway may be even more indirect.

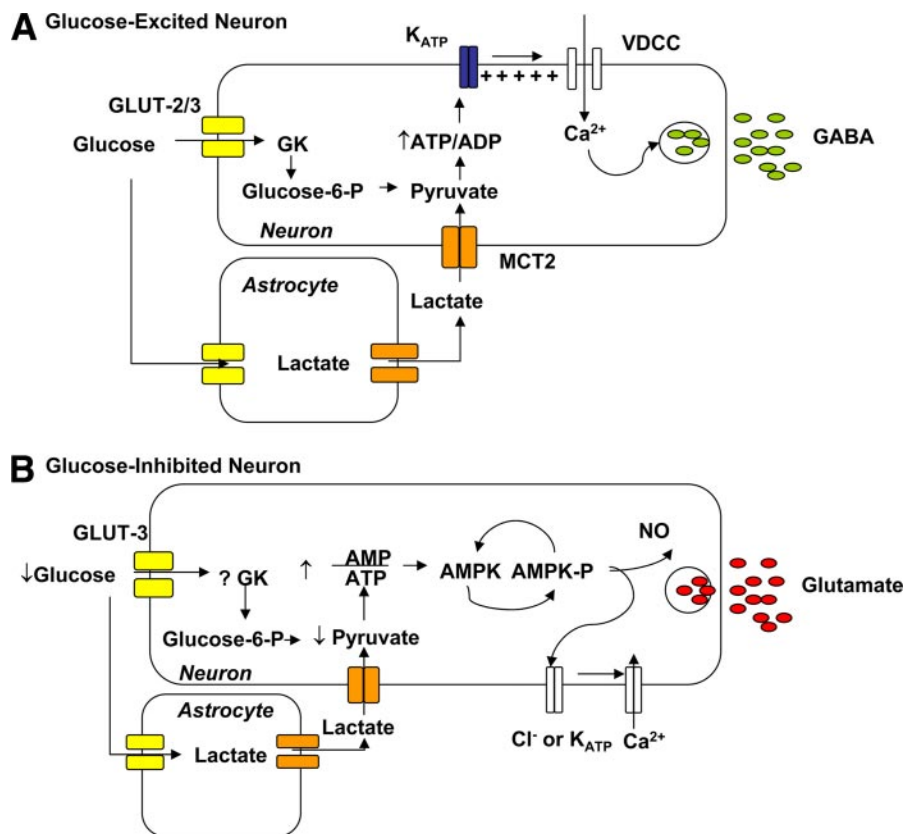
Glucose-sensing neurons are not unique to the brain and have also been identified in the intestine, hepatoportal vein, and carotid body as well as the classic glucose sensor the pancreatic  $\beta$ -cell (3). The hepato-portal glucose sensor in particular appears to play a significant

role when glucose levels fall slowly (12) and links to central glucose sensors via capsaicin-sensitive sensory neurons (13). It is interesting to speculate whether both central and peripheral sensors may form a neural network of glucose-sensing cells designed to monitor and maintain glucose homeostasis. Certainly, evidence of presynaptic regulation of VMH glucose-sensing neurons by glucose-sensing neurons in other brain regions has been demonstrated (14). Moreover, and particularly in the brain, glucose-sensing neurons are generally located in areas involved in the control of the autonomic nervous system, neuroendocrine function, nutrient metabolism, and energy homeostasis. What this effectively means is that brain glucose-sensing neurons are able to receive inputs from blood nutrient levels, a variety of endocrine hormones, as well as from numerous peripheral and central sensory systems.

### Mechanisms of glucose sensing

The defining feature of a glucose-sensing neuron is that it can use glucose not simply as a fuel but as a signaling molecule that regulates its activity. Such specialized neurons were first demonstrated by Oomura et al. (15) in 1969. These neurons are glucose sensing in so far as glucose is the major metabolic substrate for the brain, but the fact that these neurons can use other fuels such as lactate produced either by astrocytes (16) or delivered locally (17) to alter their function suggests that it is more likely glucose oxidation-derived intracellular ATP that determines the activity of these neurons. This is intriguing because neuronal levels of ATP are generally thought to be well maintained, which means that there would need to be subcellular compartmentalization of the glucose-sensing apparatus to provide sensing capability. Such compartmentalization has been shown within pancreatic  $\beta$ -cells (18).

The VMH, like other central glucose-sensing regions, has been shown to contain neurons whose activity is altered in response to changes of glucose within the physiological range. Two predominant subtypes of glucose-sensing neurons have been identified: namely, glucose-excited (GE) neurons whose activity increases as glucose levels rise and glucose-inhibited (GI) neurons whose activity decreases as glucose levels rise (8). The prevailing theory in the field at the moment is that these neurons use sensing mechanisms very similar to those found in the pancreatic  $\beta$ -



**Figure 2**—Hypothetical sensing mechanism of GE (A) and GI (B) neurons. Glucose enters the GE neuron through GLUT2 or GLUT3 and is phosphorylated by glucokinase, acting as the gatekeeper and regulating the production of cytosolic ATP in a subcellular compartment. The ATP closes  $K_{ATP}$  channels in the plasma membrane causing depolarization. In turn, this leads to  $Ca^{2+}$  influx through voltage-dependent calcium channels (VDCC), stimulating neurotransmitter release and/or increased action potential frequency. Lactate, produced locally by astrocytes or arriving systemically, enters the neuron via monocarboxylate transporter-2 (MCT2) and can then be metabolized to form ATP. In GI neurons, glucokinase may once again act as the gatekeeper. A falling glucose results in an increase in the AMP:ATP ratio activating AMPK and stimulates the formation of NO, which may diffuse out to adjacent glial cells or act as a neurotransmitter. In addition, AMPK may act on chloride (Cl) channels leading to neuronal depolarization and neurotransmitter release and/or increased action potential frequency.

and  $\alpha$ -cells (19). In this paradigm, the GE neuron is analogous to the pancreatic  $\beta$ -cell whereas the GI neuron behaves like the  $\alpha$ -cell (Fig. 2).

### The VMH $\beta$ -cell

Insulin secretion from the pancreatic  $\beta$ -cell follows membrane depolarization, which in turn is a response to a rise in glucose within the islet. Key steps in this process are thought to be 1) entry of glucose into the  $\beta$ -cell via the high-capacity GLUT (GLUT2) to allow rapid equilibration of extracellular and cytosolic glucose; 2) phosphorylation of glucose by the low-affinity hexokinase, glucokinase, linking changes in extracellular glucose with proportional changes in the cytosolic ATP:ADP ratio; and 3) closure of ATP-sensitive  $K^+$  channel ( $K_{ATP}$ ) in the plasma mem-

brane of metabolism that leads to depolarization and increased electrical activity. There is some support for this mechanism being present in VMH GE neurons. GLUT2, glucokinase, and the Kir6.2 subunit of the  $K_{ATP}$  channel are expressed in some but not all VMH GE neurons (9). Electrophysiological studies suggest glucokinase plays a regulatory role in VMH GE neuron sensing ability (20), whereas selective downregulation of glucokinase in primary VMH neuronal cultures led to the loss of all demonstrable glucose-sensing activity (21). In addition, in vivo microinjection of glucokinase activators or selective downregulation of VMH glucokinase modifies the counterregulatory response to acute hypoglycemia (22).

Similarly,  $K_{ATP}$  channels have been demonstrated throughout the brain, in-

cluding hypothalamic regions thought to be involved in glucose sensing (23). Using single-cell RT-PCR to analyze glucose-sensing neurons (identified electrophysiologically in a hypothalamic slice preparation), investigators have shown they express mRNA for Kir6.2 and sulfonylurea receptor-1 (SUR1), the two subunits that comprise the  $K_{ATP}$  channel in the pancreatic  $\beta$ -cell (9). In addition, electrophysiological studies in rat (20,24) and mouse (25) VMH have demonstrated that sulfonylureas (agents that block the  $K_{ATP}$  channel) can alter the response of GE neurons to changes in ambient glucose, and Kir6.2 knockout mice show impaired glucose counterregulation to systemic hypoglycemia (25). Finally, in vivo perfusion of VMH of rodents with glibenclamide (a  $K_{ATP}$  channel blocker) suppresses (26) whereas diazoxide (a  $K_{ATP}$  channel opener) amplifies (27) hormonal counterregulatory responses to acute hypoglycemia.

These studies provide support for the  $\beta$ -cell model, but inconsistencies in the literature are apparent that mean this hypothesis is not yet established. For instance, extracellular levels of glucose in the brain are only about 10–30% of the levels found in blood. Microdialysis studies in rats (4,28) and human subjects (29) have shown that the extracellular fluid (ECF) glucose levels to which neurons are exposed are in the range of 1–2 mmol/l under basal conditions and fall markedly during acute hypoglycemia ( $\sim$ 0.5 mmol/l) (30). This means that during hypoglycemia, brain ECF glucose levels are well beneath the range in which glucokinase or GLUT2 usually act in a regulatory manner. In addition, using genetically targeted luciferases to monitor cytosolic ATP, Ainscow et al. (31) were unable to detect a rise in ATP during exposure of hypothalamic neurons to a high glucose, although they were able to see increased cytosolic ATP in  $\beta$ -cells, hypothalamic astroglia, and cerebellar neurons. Interestingly, transgenic mice that express GLUT2 only in astroglial cells in the central nervous system are rescued from the defect in counterregulation seen in whole body GLUT2<sup>-/-</sup> mice (32), raising the intriguing possibility that astroglial cells might function as detectors of extracellular glucose change. Tanycytes, which are specialized glial cells that line the dorsal lateral, ventral lateral, and floor of the third ventricle express GLUT2; glucokinase; and the  $K_{ATP}$  channel send long processes that terminate in the VMH (33).

Burdakov et al. (34) have proposed two further possibilities through which glucose might activate glucose-sensing neurons that do not involve the metabolism of glucose. In the first, glucose transport is coupled directly with the transmembrane movement of ions, such as those used by sodium-glucose cotransporters (SGLTs). In this way, an inward current is generated while glucose is transported into the neuron, and this leads to depolarization and increased excitability. Phloridizin, a nonselective inhibitor of SGLTs, inhibits glucose-induced excitation of VMH neurons (20). Alternatively, glucose might bind to an extracellular receptor that could alter electrical activity without transporting the glucose into the neuron (34). Recently, such a mechanism has been demonstrated in GI orexigenic neurons in the lateral hypothalamus (35).

Once a change in glucose is sensed by the GE neuron, it needs to then communicate that signal to a downstream neuron in the pathway that leads eventually to glucose counterregulation. In general, neural communication relies on the release of classic neurotransmitters such as GABA, neuropeptides, or unconventional transmitters such as nitric oxide (NO). No definitive transmitter has been identified for GE neurons; it is very possible that a number of transmitters and peptides are important, but most current data support a role for the inhibitory neurotransmitter GABA. GAD, the rate-limiting enzyme in GABA synthesis, is expressed in 56% of GE and 36% of GI neurons (9). In vivo antagonism of the VMH GABA receptor amplifies the counterregulatory hormone response to acute hypoglycemia (36), and VMH GABA release is regulated by  $K_{ATP}$  channel modulation (37). Finally, GABA tone in the VMH is increased following recurrent hypoglycemia (38).

### The VMH $\alpha$ -cell

GI neurons show a decrease in activity as glucose levels rise (8). It is perhaps easier to think of GI neurons as those glucose-sensing neurons that become more active when glucose levels fall, and as such they may use signaling mechanisms more relevant to the pancreatic  $\alpha$ -cell. Unfortunately, like the  $\alpha$ -cell, the signaling pathways used by the GI neuron are not well understood. This may reflect the fact that they appear to be few in number, comprising only 3–14% of neurons in the VMH nucleus (14,39). Although recent evidence suggests GI neurons may be more prev-

alent, when improved slice techniques are used in conjunction with novel methods for identifying GI neurons (e.g., membrane potential dyes) as many as  $\sim$ 30–40% of all neurons in the VMH are reported to be GI neurons (40).

Current evidence favors a role for AMP-activated protein kinase (AMPK) in the sensing pathway used by GI neurons. AMPK has been described as an intracellular fuel gauge in that it is activated in response to a rise in the intracellular ratio of AMP:ATP and acts to switch off energy-consuming anabolic processes and switch on energy-producing catabolic processes (41). Canabal et al. (40) demonstrated that in VMH GI neurons exposed to 2.5 mmol/l glucose, AICAR (an activator of AMPK) mimicked the excitatory effect of low glucose (0.5 mmol/l) on action potential frequency. Both low glucose and AICAR were shown to mediate their effects, in part, through an increase in NO production in GI neurons. Conversely, increased NO production in response to low glucose was blocked by compound C, an inhibitor of AMPK (40). In a related series of studies, Mountjoy et al. (42) reported that activation of AMPK with AICAR or inhibition with compound C altered neuronal activity in GI but not GE neurons in an ex vivo hypothalamic cell culture system obtained from the medio-basal (including VMH and Arc) hypothalamus. In this study, AMPK had no effect on the  $K_{ATP}$  channel (42), but others have suggested AMPK may instead act on a chloride channel to depolarize the plasma membrane (43). In rodent studies, in vivo pharmacological activation of AMPK in the VMH during acute hypoglycemia amplifies the glucose counterregulatory response in normal Sprague-Dawley rats (44) and restores the hormonal counterregulatory response to hypoglycemia in rats with defective counterregulation (45). Conversely, downregulation of AMPK in the VMH using specific RNA interference suppresses the counterregulatory response to acute hypoglycemia (46). In addition, mice that selectively lack the  $\alpha$ -catalytic subunit of AMPK in proopiomelanocortin or agouti gene-related protein neurons (both neuronal types are located in the medio-basal hypothalamus and are involved in feeding behavior and energy homeostasis) lose the responsiveness of these neurons to changes in extracellular glucose (47).

As in the GE neuron, the mode of neurotransmission in GI neurons has yet to be

established. Local administration of norepinephrine to the hypothalamus increases circulating glucose and counterregulatory hormones, whereas norepinephrine receptor antagonists block the counterregulatory response to 2-deoxyglucose (DG) (48). Glucoprivation induced through 2-DG (49) or insulin (50) increases VMH norepinephrine levels, whereas local perfusion of the VMH with glucose during systemic hypoglycemia prevents the local increase in norepinephrine (51). Intriguingly, terminals from noradrenergic neurons form a shell around the VMH, implying that the detection of hypoglycemia by these neurons takes place at a site distant from the VMH. Brainstem norepinephrine cell groups, for instance, which respond to glucoprivation, project to the VMH and may ultimately form a neural circuit through which peripheral and hypothalamic glucose sensors integrate (52).

Additionally, a potential role for the excitatory transmitter glutamate recently has been revealed. Neurons in the VMH are largely glutaminergic and express high levels of the vesicular glutamate transporter VGLuT2. Mice lacking VGLuT2 selectively in steroidogenic factor-1 (a transcription factor unique to a population of VMH neurons) neurons show a markedly suppressed counterregulatory response to hyperinsulinemic hypoglycemia (10). These findings support a potential role for excitatory glutaminergic transmission in the output signal from VMH GI neurons. Finally, the unconventional transmitter NO may also play a role in GI neuron signaling (40).

### Recurrent hypoglycemia and glucose sensing

Studies in rodent models suggest that recurrent hypoglycemia results in altered glucose sensing within the brain. Recurrent hypoglycemia lowers the glucose level at which activation of glucose-inhibited neurons in the VMH is initiated (53). Moreover, *in vivo* activation of AMPK in the VMH of rats that have experienced prior recurrent hypoglycemia can restore hormonal counterregulatory responses to a subsequent hypoglycemic challenge (45). Similarly, opening of  $K_{ATP}$  channels in the VMH of recurrently hypoglycemic rats leads to the restoration of normal counterregulatory responses to subsequent hypoglycemia (27).

There are a number of potential explanations for altered glucose sensing in the VMH following recurrent hypoglycemia. One possibility is that glucose sensing neurons are seeing higher glucose

and/or ATP levels during a subsequent hypoglycemic challenge caused by an increased supply of fuel through altered glucose or alternate fuel transport. However, whereas these changes suggest an increased capacity to transport fuels across the blood-brain barrier and into glucose-sensing neurons, the data from human studies have been mixed with some indicating increased whole brain glucose, or acetate, uptake after chronic hypoglycemia and others showing no change (3). Alternatively, the VMH might obtain additional metabolic substrates from more local sources. Brain glycogen can act as a fuel reserve during acute hypoglycemia (54), and brain glycogen levels may actually increase in response to an acute episode of hypoglycemia (54). Glycogen supercompensation could provide an additional fuel reserve that leads to defective glucose sensing during a subsequent episode of hypoglycemia. However, brain glycogen levels appear to be very low and in rodents return to baseline levels within 24 h of a hypoglycemic episode (55).

It has also emerged that within the brain there exist mechanisms for regulating the magnitude of the neuroendocrine response to acute hypoglycemia. The corticotrophin-releasing hormone (CRH) family of ligands and receptors form an ancient and highly conserved means of regulating the neuroendocrine stress response. At key sites involved with autonomic activation within the brain, CRH acting through the CRH receptor (CRHr)-1 appears to lead to an amplification of the autonomic response to stress, whereas urocortin (part of the CRH family of peptides) acting through the CRHr2 receptor suppresses the autonomic response to stress (56). We recently were able to show that such a mechanism operated in the VMH with urocortin locally delivered to the VMH markedly suppressing the counterregulatory response to acute hypoglycemia (57), whereas CRH amplified the response (58). Thus, an alteration in the balance between those mechanisms that regulate the hypoglycemia stress response might contribute to the development of defective counterregulation.

If our model is correct, these studies suggest that the balance between GE and GI neuronal activity is altered by recurrent hypoglycemia. The important point here is that both the GE and GI neurons operate over a range of glucose values and therefore are not either on or off but rather the balance tips in favor of one or other population as the glucose levels fluctuate. We would anticipate that GE

neurons (acting to suppress counterregulation) are more likely to be active and/or GI neurons (acting to amplify counterregulation) less likely to be active following recurrent hypoglycemia. Consistent with this hypothesis, there is increased GABAergic inhibitory tone (38) (hypothetically GE mediated) and reduced AMPK activity (59) (hypothetically GI mediated) in the VMH following recurrent hypoglycemia, the net effect being that full glucose counterregulation is initiated at a lower glucose level. This gives individuals less time to react to, and seek treatment for, hypoglycemia.

**SUMMARY**— Within the body a number of highly specialized regions sense alterations in glucose levels and, in the case of hypoglycemia, this leads to the generation of a glucose counterregulatory defense response. The VMH, discussed in detail in this article, reflects only one of a number of brain regions thought to be important in the detection of hypoglycemia, and together these brain regions may form an integrated neural network coordinating physiological and behavioral responses to a hypoglycemic challenge. Glucose-sensing neurons, by virtue of specific sensing systems, directly or indirectly translate the rate or quantity of glucose oxidation into a neural signal that alters neuronal firing rates. These neurons appear to use signaling mechanisms that parallel those used by pancreatic  $\beta$ - and  $\alpha$ -cells. The GE neuron, more likely to operate under conditions of euglycemia or hyperglycemia, may use glucokinase as its key regulatory step and the  $K_{ATP}$  channel to then translate that signal into altered neuronal firing rates. In contrast, the GI neuron, active under hypoglycemic conditions, may be dependent on alterations in intracellular AMPK activity that, in turn, may act via NO to alter neuronal firing rates. Potentially, the counterbalance between GI and GE neuronal activity forms the most sensitive means of regulating and maintaining blood glucose within a narrow physiological range as well as ensuring an adequate supply of glucose to the brain. Recurrent exposure to hypoglycemia disturbs this relationship in a number of ways, which may include an increased capacity of glucose-sensing regions of the brain to use glucose and/or alternate fuels, as well as changes in both the mechanisms that sense glucose and those that fine-tune the hypoglycemic stress response, the net effect being

to reduce the glucose level at which counterregulation is initiated.

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Received 21 January 2009 and accepted 16 March 2009.

DOI: 10.2337/dc09-0123

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**Acknowledgments**— This research was supported by a research grant from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (69831) and the Juvenile Diabetes Research Foundation.

No potential conflicts of interest relevant to this article were reported.

The author acknowledges the postdoctoral fellows and technical staff who contributed greatly to the research that underpins this article.



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