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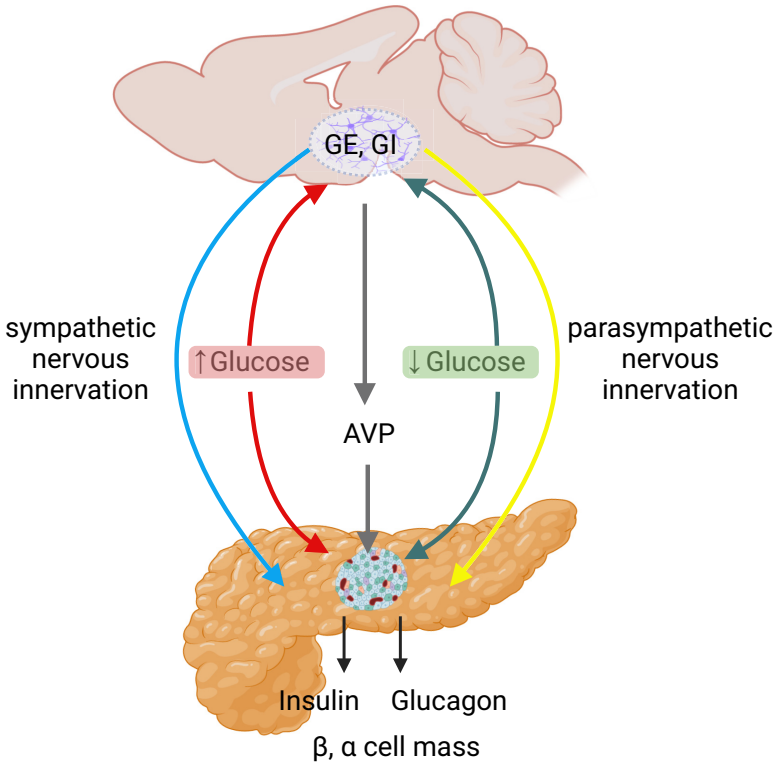

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KEY WORDS

autonomic nervous system; glucagon; glucose sensing; hypothalamus; insulin

CLINICAL HIGHLIGHTS

A major difficulty associated with the insulin treatment of patients with type 1 or type 2 diabetes is the risk of hypoglycemia, with initial hypoglycemic episodes increasing the risk of developing subsequent ones of greater severity. Hypoglycemia sensing by the central nervous system (CNS) leads to a coordinated counterregulatory hormonal response (CRR) initiated by a rapid stimulation of glucagon secretion and inhibition of insulin release. Recent studies have identified specific glucose sensing neurons in the brain stem and hypothalamus and, through the use of current genetic technologies, have characterized the circuits that connect these neurons to the endocrine pancreas to control hormone secretion. In addition, new investigations have characterized so far unknown hypothalamic mechanisms that adapt the response to hypoglycemia according to the prevailing metabolic and inflammatory states. These studies will pave the way for a more complete understanding of the defects in CRR in patients with diabetes treated with insulin and for the development of prevention or therapeutic strategies.

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NEURONAL GLUCOSE SENSING MECHANISMS AND CIRCUITS IN THE CONTROL OF INSULIN AND GLUCAGON SECRETION

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Abstract

Glucose homeostasis is mainly under the control of the pancreatic islet hormones insulin and glucagon, which, respectively, stimulate glucose uptake and utilization by liver, fat, and muscle and glucose production by the liver. The balance between the secretions of these hormones is under the control of blood glucose concentrations. Indeed, pancreatic islet β-cells and α-cells can sense variations in glycemia and respond by an appropriate secretory response. However, the secretory activity of these cells is also under multiple additional metabolic, hormonal, and neuronal signals that combine to ensure the perfect control of glycemia over a lifetime. The central nervous system (CNS), which has an almost absolute requirement for glucose as a source of metabolic energy and thus a vital interest in ensuring that glycemic levels never fall below ∼5 mM, is equipped with populations of neurons responsive to changes in glucose concentrations. These neurons control pancreatic islet cell secretion activity in multiple ways: through both branches of the autonomic nervous system, through the hypothalamic-pituitary-adrenal axis, and by secreting vasopressin (AVP) in the blood at the level of the posterior pituitary. Here, we present the autonomic innervation of the pancreatic islets; the mechanisms of neuron activation by a rise or a fall in glucose concentration; how current viral tracing, chemogenetic, and optogenetic techniques allow integration of specific glucose sensing neurons in defined neuronal circuits that control endocrine pancreas function; and, finally, how genetic screens in mice can untangle the diversity of the hypothalamic mechanisms controlling the response to hypoglycemia.

autonomic nervous system; glucagon; glucose sensing; hypothalamus; insulin

CLINICAL HIGHLIGHTS

A major difficulty associated with the insulin treatment of patients with type 1 or type 2 diabetes is the risk of hypoglycemia, with initial hypoglycemic episodes increasing the risk of developing subsequent ones of greater severity. Hypoglycemia sensing by the central nervous system (CNS) leads to a coordinated counterregulatory hormonal response (CRR) initiated by a rapid stimulation of glucagon secretion and inhibition of insulin release. Recent studies have identified specific glucose sensing neurons in the brain stem and hypothalamus and, through the use of current genetic technologies, have characterized the circuits that connect these neurons to the endocrine pancreas to control hormone secretion. In addition, new investigations have characterized so far unknown hypothalamic mechanisms that adapt the response to hypoglycemia according to the prevailing metabolic and inflammatory states. These studies will pave the way for a more complete understanding of the defects in CRR in patients with diabetes treated with insulin and for the development of prevention or therapeutic strategies.

1. INTRODUCTION

Glucose homeostasis is classically described as resulting from the equilibrium between glucose absorption, glucose utilization, and endogenous glucose production, a balance that is under the control of the pancreatic islet hormones insulin and glucagon (1, 2). Insulin stimulates glucose utilization by liver, fat, and muscle and inhibits hepatic glucose production. On the other hand, glucagon is secreted by pancreatic α-cells and stimulates glucose release from the liver to prevent hypoglycemia development. The equilibrium between insulin secretion and glucagon secretion as well as the action of these hormones on peripheral tissues need to be perfectly controlled to maintain normoglycemia over a lifetime to prevent development of diabetic hyperglycemia. Currently, >92% of the world population is free

of diabetes (3) despite the increase in life expectancy and the metabolic challenges associated with reduced physical activity and overnutrition inherent to modern lifestyle. What does ensure the robustness of this system? A key aspect is the functional plasticity of the islet β-cells and α-cells, which ensures not only the efficient minute-to-minute control of insulin and glucagon secretion in response to glucose variations but also a compensatory change in their total number to adapt to the sensitivity of target tissues to the action of insulin or glucagon (4–6). This plasticity is controlled by multiple signals such as metabolites, hormones, and cytokines produced by liver, fat, and muscle and by neuronal inputs from the autonomic nervous system (ANS). Among these signals, glucose itself has a major role in modulating pancreatic endocrine cell secretion and proliferation cell autonomously and cell nonautonomously. β-Cells and α-cells have intrinsic glucose sensing properties that trigger insulin and glucagon secretion when extracellular glucose concentrations increase above or fall below the euglycemic level, but glucose has also many indirect ways of controlling hormone secretion. For example, in the oral cavity, glucose activates taste cells, which trigger a vagal reflex that induces insulin secretion, the so-called cephalic phase of insulin release (CPIR) (7); in the duodenum, glucose triggers the secretion of the gluco-incretin hormones gastric inhibitory protein (GIP) and glucagon-like protein (GLP)-1, which potentiate glucose-stimulated insulin secretion (GSIS), protect β-cells from apoptosis, and increase their mass and function (8–11); in the liver, glucose regulates the expression of many genes (12) and, through the production of bile acids, controls a liver-β-cell axis that increases the capacity of these cells to respond to glucose (13); in the peripheral and central nervous systems, specific glucose-responsive neurons are activated by a rise [glucose-excited (GE) neurons] or a fall [glucose-inhibited (GI) neurons] in blood glucose levels (14, 15) and control the activity of the endocrine pancreas through the regulation of both branches of the autonomic nervous system (15, 16). Importantly, autonomic nervous innervation of the endocrine pancreas appears during the developmental period and is required for the establishment of normal adult pancreatic islet mass and function (17, 18). Thus, although endocrine cells from the pancreatic islets can respond directly to changes in glucose concentrations, the communication with other glucose sensing cells, located at different anatomical sites, is essential for islet development and to maintain glucose homeostasis over a lifetime.

The present review focuses on the role of nervous glucose sensing in the regulation of insulin and glucagon secretion. We present a description of the autonomic innervation of the pancreas; discuss how neurons sense glucose; and illustrate how recent viral tracing, chemogenetic, and optogenetic techniques, as well as genetic screens in mice, allow identification of the neuronal network that controls pancreatic hormone secretion. Collectively, these data illustrate the importance of nervous glucose sensing in the control of the endocrine pancreas as a means to preserve glucose homeostasis. They highlight the important role of hypoglycemia sensing by the central and peripheral nervous systems to suppress insulin and stimulate glucagon secretion to restore euglycemia and preserve continuous glucose supply to the brain. Because defective glucose sensing may cause life-threatening hypoglycemia in patients with diabetes (19), development of this area of research may lead to novel preventive and therapeutic interventions to manage this frequent and dangerous condition.

2. AUTONOMIC INNERVATION OF THE PANCREAS

2.1. *Sympathetic and Parasympathetic Innervation*

The pancreas is richly innervated by efferent projections from the sympathetic and parasympathetic branches of the autonomic nervous system (ANS), which ensure connections between the brain and the pancreas (**FIGURE 1**). It also receives neuronal input from the enteric nervous system. Afferent vagal and spinal sensory fibers originating from the hepatoportal vein area also convey information to the pancreas via vago-vagal or spino-vagal reflexes, as discussed further below (see **FIGURE 8**). Here, only a short description of this innervation is presented, as more emphasis will be placed on the central mechanisms that regulate the activity of the ANS. Detailed descriptions of the pancreas innervation by the ANS can be found in excellent recent reviews (20–22).

Vagal afferent nerves originate from the dorsal motor nucleus of the vagus (DMNX), a nucleus of the brain stem dorsal vagal complex (DVC), which also comprises the nucleus of the tractus solitarius (NTS) and the area postrema (AP) (**FIGURE 1**, **FIGURE 2**). These vagal neurons are cholinergic and project to intrapancreatic ganglia from which postganglionic neurons innervate the exocrine part of the pancreas and the pancreatic islets. These postganglionic neurons are cholinergic and can be identified by immunostaining of the vesicular acetylcholine transporter (vAChT). They also express neuropeptides such as vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), or gastrin-releasing peptide (GRP) (23). In mice, these postganglionic neurons contact α, β, δ, and PP cells. In humans, although it was suggested early on that there was no parasympathetic innervation of

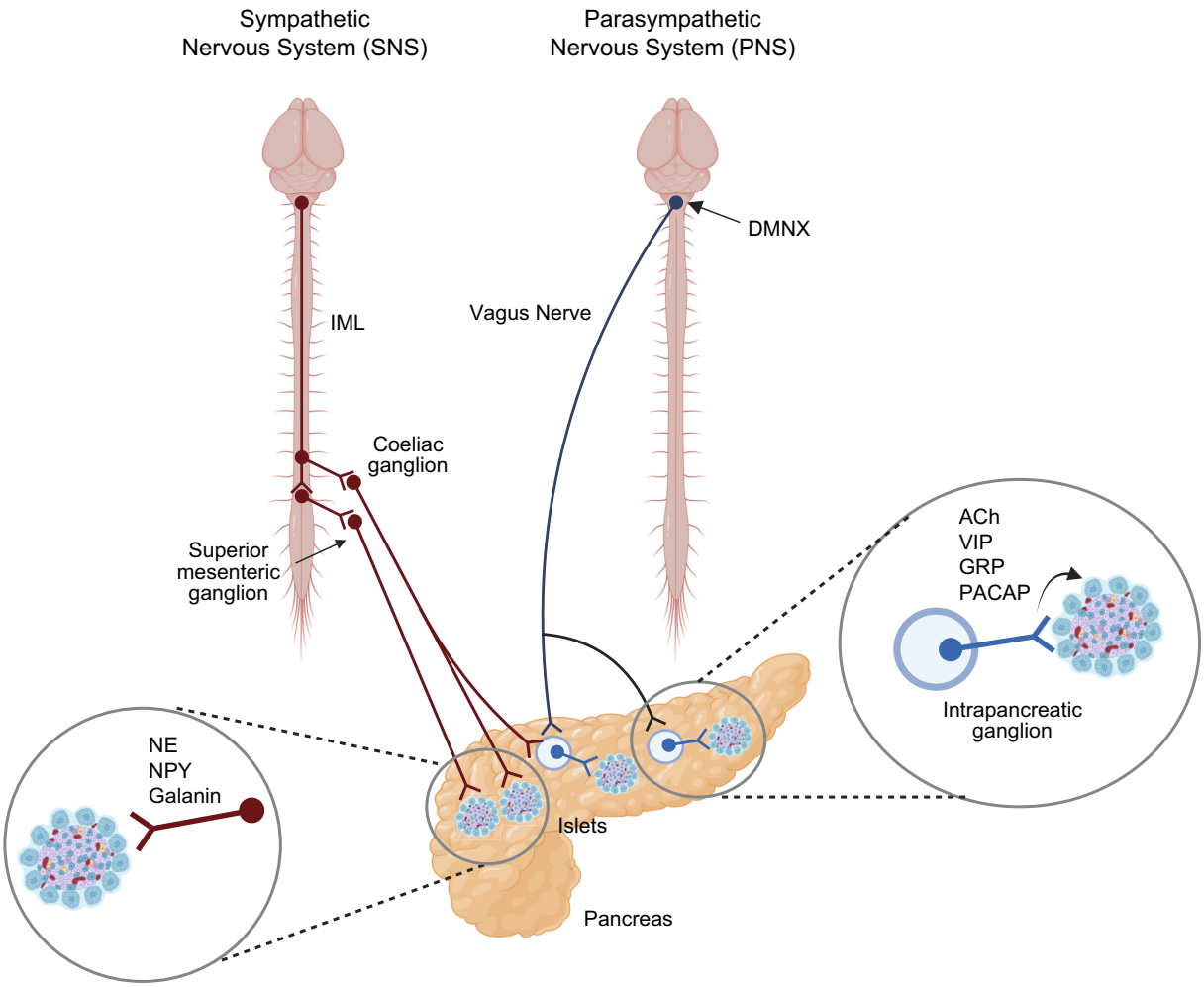


FIGURE 1. Schematic representation of the autonomous nervous system innervation of the endocrine pancreas. The sympathetic nerve extends through the intermediolateral column (IML) of the spinal cord and sends branches to the celiac and superior mesenteric ganglia. Postganglionic nerves project directly to the pancreatic islets and use norepinephrine (NE) as principal neurotransmitter, but neuropeptide Y (NPY) and galanin are also present in some nerve terminals. In the parasympathetic nervous system, the vagal nerve neurons have their cell bodies in the dorsal motor nucleus of the vagus (DMNX), which is part of the dorsal vagal complex that includes the area postrema (AP) and the nucleus tractus solitarii (NTS). The cholinergic vagal neurons send their projections to the intrapancreatic ganglia, and postganglionic neurons reach the islets. These neurons use acetylcholine (ACh) as main neurotransmitter but some also express vasoactive intestinal polypeptide VIP, pituitary adenylate cyclase-activating polypeptide (PACAP), and gastrin-releasing peptide (GRP). Figure generated with BioRender.com, with permission.

the islet endocrine cells (24), recent reports from optically cleared tissue and three-dimensional (3-D) imaging have established that vagal nerves are present within the islets making contact, in particular, with α-cells (25, 26).

The sympathetic nerve that innervates the pancreas is issued from neurons located in the intermediolateral cell column (IML) of the spinal cord, which send projections to the celiac and superior mesenteric ganglia (**FIGURE 1**) (27). The postganglionic neurons then reach the pancreas, where some contact vagal ganglia and others send terminals to islet α- and β-cells and the islet vasculature (25). These postganglionic neurons are catecholaminergic and can be identified by tyrosine hydroxylase (TH) immunostaining. In addition to norepinephrine, they can also carry neuropeptides such as neuropeptide Y and galanin (23).

Functionally, the vagal nerve stimulates insulin secretion by activating the β-cell's muscarinic acetylcholine receptor, m3AchR (23, 28). However, the effect of acetylcholine on insulin secretion requires glycemia to be above the euglycemic level. Importantly, vagal activity is also stimulated by hypoglycemia, and acetylcholine binding to the α-cell m3AchR increases glucagon secretion. Thus, the effects of vagal nerve activity on insulin or glucagon secretion are largely determined by the prevalent glycemic levels.

Norepinephrine secreted by intraislet sympathetic nerves also has a dual action on insulin and glucagon secretion. Pancreatic β-cells express the α2-adrenergic receptor (α2-AR), which when activated inhibits insulin secretion, whereas α-cells express the β2-adrenergic receptor, which stimulates glucagon secretion (29).

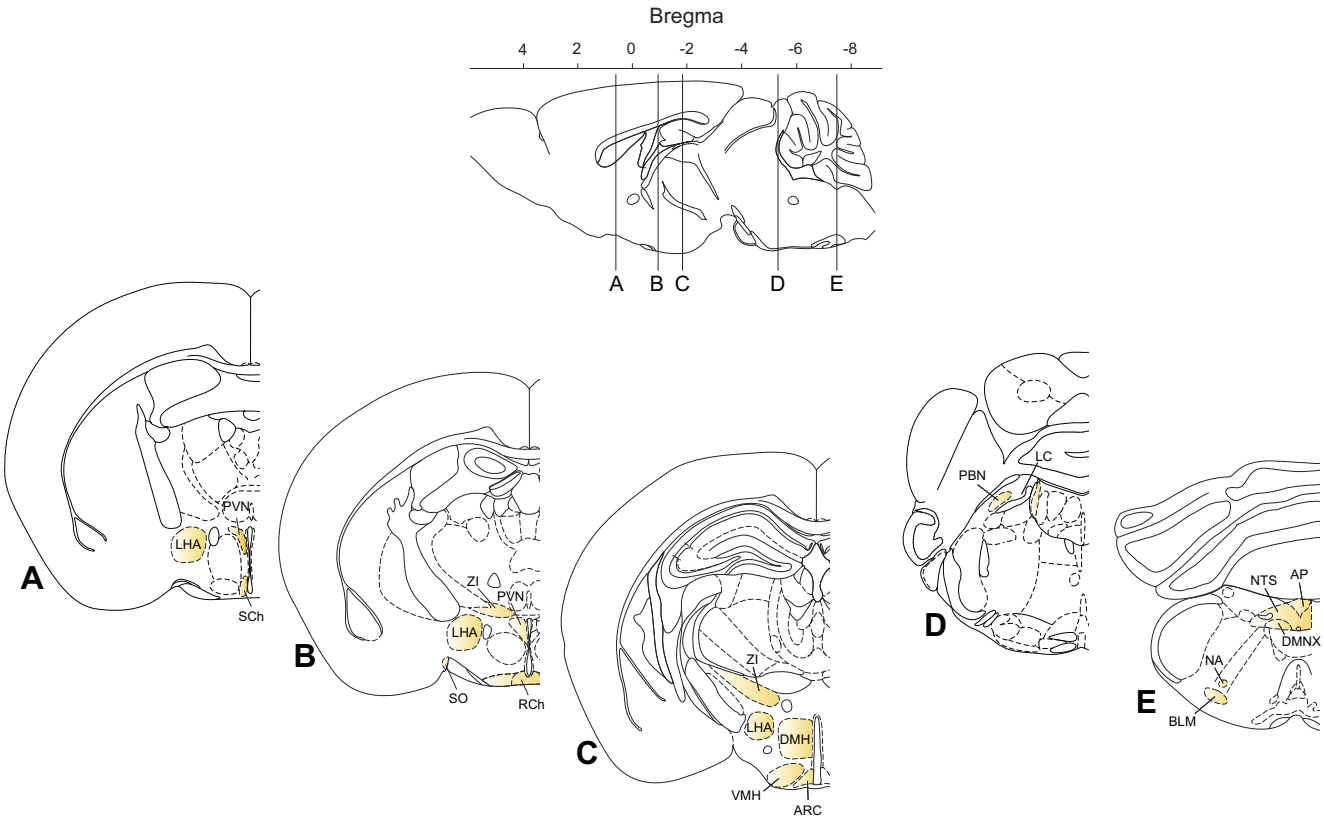


FIGURE 2. Schematic localization of the brain stem and hypothalamic nuclei containing glucose sensing neurons. *A*: sagittal section of a mouse brain with indication of the bregma levels at which the coronal sections in *B* are prepared. *B*: coronal sections with indications of the nuclei where glucose-excited (GE) and glucose-inhibited (GI) neurons are present (colored regions). *Sections A*, *B*, and *C* are from the hypothalamus and *sections D* and *E* from the brain stem. Amyg, amygdala; AP, area postrema; ARC, arcuate nucleus of the hypothalamus; BLM, basolateral medulla; DMH, dorsomedial hypothalamus; DMNX, dorsal motor nucleus of the vagus; LC, locus coeruleus; LHA, lateral hypothalamus; NA, nucleus ambigus; NTS, nucleus tractus solitarii; PBN, parabrachial nucleus; PVN, paraventricular nucleus of the hypothalamus; RCH, retrochiasmatic nucleus; SCh, suprachiasmatic nucleus; SO, supraoptic nucleus; VMH, ventromedial nucleus of the hypothalamus; ZI, zona incerta.

2.2. *Pancreas Innervation during Development and Diseases*

Innervation of the pancreas by the vagal nerve develops during midgestation in mice, a process that is under negative regulation by leptin (17). Parasympathetic innervation of the islets requires expression in developing nerve fibers of GRFA2, the receptor for the neurotrophic factor neurturin. Mice lacking this receptor display marked defects in neuroglucopenia-stimulated insulin, glucagon, and pancreatic polypeptide secretion (30).

Sympathetic innervation of pancreatic islets develops during prenatal life but fully matures around weaning (31, 32). This innervation depends on the production of neurotrophic factors, such as nerve growth factor (NGF), by the islets (33–35). Conversely, sympathetic innervation is required for the development of the normal islet architecture. In mice, sympathetic denervation, or inactivation of the NGF receptor TrkA that leads to defective sympathetic innervation, disrupts the normal structure of the islets, with α-cells being present in the core of the islets; this also leads to defect in insulin secretion capacity and the development of glucose intolerance (18). The fact that islet autonomic innervation is guided by neurotrophic factors secreted by islets is further evidenced by the observation that islets transplanted in the liver (36), under the kidney capsule (37), or in the back chamber of the eye (38) become progressively innervated by autonomic nerves. In the eye, transplanted islets are innervated by vagal nerves and are responsive to topical application of the AChR antagonist atropine (38). In the mouse liver, however, only sympathetic innervation is observed (36).

In diabetic NOD mice, a model of type 1 diabetes, the density of intraislet parasympathetic and vagal fibers is increased and positively correlates with the glycemia (39). In humans, the density of islet sympathetic innervation is increased up to threefold in type 2 diabetes (40). The increased nerve fiber density may be due to the infiltration of the pancreas parenchyma by adipocytes, which provide neurogenic signals (26). This increased sympathetic tone can contribute to the hyperglucagonemia present in both forms of diabetes. An interesting observation was made by Groop's team, which reported

that islets of Goto-Kakizaki rats, a model of type 2 diabetes, display abnormally elevated expression of the α2-AR. They showed that reducing this receptor's expression or its intracellular signaling restored normal glucose-stimulated insulin secretion. They further found, in humans, a genetic variant in the *ADRA2A* gene (encoding the α2-AR) that leads to increased receptor expression in β-cells, diminished insulin secretion, and higher risk of developing type 2 diabetes (41). This important human study shows that autonomic nervous inputs into pancreatic β-cells play a critical role in the long-term maintenance of glucose homeostasis.

2.3. Enteric Nervous System

In addition to sympathetic and parasympathetic innervation, the endocrine pancreas receives nerve fibers from the enteric nervous system (42–44). Some of these nerves are equipped with proteins required for glucose sensing, such as the ATP-sensitive K^+ (K_{ATP}) channel, and may provide information related to gut nutrient absorption (43). These nerves and their role in controlling pancreatic endocrine secretion have been little described so far.

3. CENTRAL GLUCOSE SENSING MECHANISMS THAT CONTROL AUTONOMOUS NERVOUS ACTIVITY

How is the autonomic nervous tone to the endocrine pancreas controlled by the CNS? This is an important question that, if fully described at the molecular, cellular, and neuronal circuit levels, can lead to a better understanding of the pathogenesis of obesity and diabetes as well as of the defective counterregulatory response to hypoglycemia that often develops in insulin-treated patients with type 1 or type 2 diabetes. **FIGURE 2** presents a schematic description of the hypothalamic and brain stem nuclei where glucose sensing neurons are located and that are discussed in the present review.

Anatomical description of the vagal and sympathetic neuronal connections between CNS nuclei and the pancreas has been established by following the retrograde transport of recombinant pseudorabies viruses injected in the pancreas (45, 46). First-order vagal neurons are located in the DMNX; second-order neurons are in the adjacent NTS and in several hypothalamic nuclei, including the arcuate (ARH), paraventricular (PVN), lateral (LH), and dorsomedial (DMH) nuclei, as well as in the zona incerta (ZI). Third-order neurons are found in the ventromedial hypothalamus (VMN) and the suprachiasmatic nucleus (SCN).

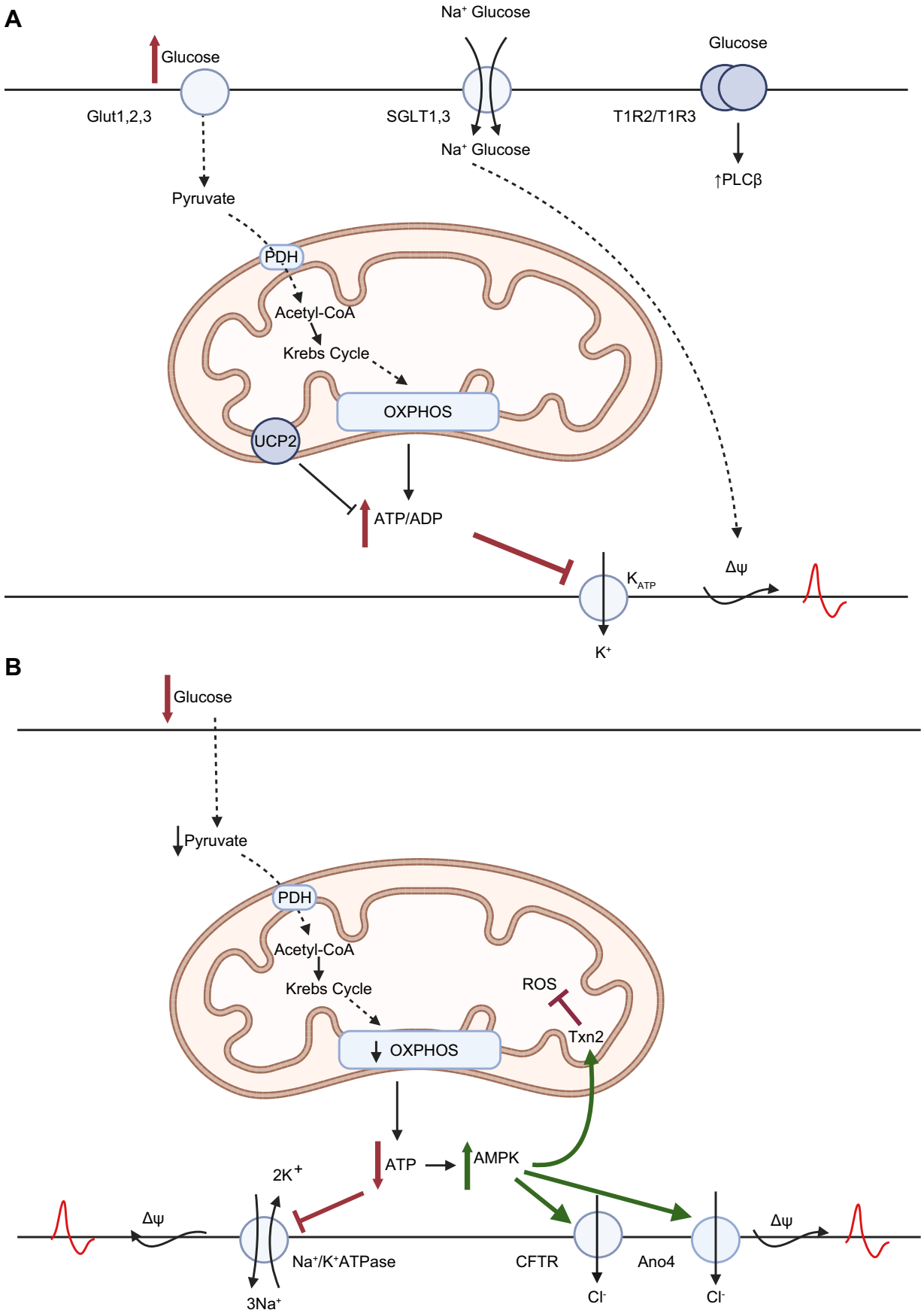
The first-order neurons of the pancreas-projecting sympathetic neurons are in the intermediolateral column of the spinal cord; second-order neurons are found in the A5 region of the basolateral medulla and the locus coeruleus (LC) as well as in the hypothalamic LH, PVN, ZI, and retrochiasmatic area (RCA); third-order neurons are present in ARH, VMN, DMH, and SCN.

Glucose sensing neurons have been identified in each of these brain stem and hypothalamic nuclei (15). Neurons located in nuclei not protected by a blood-brain barrier, such as the NTS and ARH, can respond to changes in blood glucose concentrations (47). The glucose sensing GE or GI neurons present in other nuclei detect changes in parenchymal glucose concentrations, which vary in parallel with, and are about a third of, the blood glucose concentrations (48). The characteristics of these neurons have been elucidated by recording their electrophysiological properties in live animals (47, 49) or by patch-clamp analysis performed on acute brain sections or isolated neurons with extracellular glucose concentrations varying between 0.5–1 mM and 5 mM (50–53); a few studies reported changes in electrical activity over the 5 mM to 10 mM glucose concentration range, defining high-glucose-excited (HGE) or high-glucose-inhibited (HGI) neurons (54, 55).

3.1. How Do GE Neurons Sense Glucose?

GE neurons are activated by a mechanism that requires glucose uptake, metabolism, and closure of the K_{ATP} channel to induce membrane depolarization and firing activity (49, 54, 56–58) (**FIGURE 3*A***). The presence of the K_{ATP} channel has been identified by its characteristic electrophysiological and pharmacological properties (49, 56), and its role has been confirmed by genetic approaches. For instance, in mice with whole body knockout of the Kir6.2 subunit of the K_{ATP} channel, glucose-responsive neurons are no longer detected in the VMN, and the mice show defective glucagon secretion in response to hypoglycemia (58). Also, specific overexpression in hypothalamic proopiomelanocortin (POMC) or melanin-concentrating hormone (MCH) neurons of a mutant of the Kir6.2 subunit, which suppresses the ATP sensitivity of the channel, blocks the response of these neurons to high glucose, leading to the development of glucose intolerance (59–61). In POMC and MCH neurons, the mitochondrial UCP2 uncoupling protein has been found to control ATP production and glucose responsiveness (59, 60).

In VMN neurons, UCP2 is also required to control the level of phosphorylation of Drp1, a protein that regulates mitochondria fission and activation of GE neurons by glucose (62). A requirement for adequate regulation of mitochondria dynamics for the glucose responsiveness



of POMC GE neurons has also been reported in mice with knockout of Mitofusin 2, a regulator of mitochondria fusion, or of Drp1 (63–65). These studies highlight the critical role of mitochondria and ATP production in the signaling pathway that activates GE neuron firing. An obligatory role for mitochondrion-produced reactive oxygen species (ROS) has also been reported for the response of GE neurons of the VMN and ARH to high glucose (10 mM), a response that can be suppressed by various antioxidants (66, 67).

However, how glucose metabolism is regulated is not fully established. Because of the similarity with the activation of pancreatic β-cells by glucose, notably the role of the K_{ATP} channel, it is widely assumed that glucokinase (Gck), which catalyzes the rate-limiting step in glucose-stimulated insulin secretion (68), also plays a critical role in both GE and GI neurons (50, 69, 70). However, the glucose responsiveness of GE and GI neurons is over a glucose concentration range, 0.5 mM to 5 mM, that is much lower than the K_m for glucose of glucokinase (~8 mM) and below the glucose concentration range that stimulates insulin secretion, ~3.4 mM to 20 mM (71, 72). In addition, these studies (50, 69, 70) mostly used glucokinase pharmacological inhibitors such as alloxan, glucosamine, or mannoheptulose or siRNA-mediated silencing of the glucokinase mRNA in dissociated neuronal cells, and nonspecific or off-target effects cannot be excluded. In contrast, targeted inactivation of *Gck* in Sf1-expressing neurons of the VMN, which represent a very large majority of the VMN neurons, had no impact on the glucose responsiveness of GE or GI neurons as detected by electrophysiological recordings on acute brain slices (73). Thus, although widely considered in the literature to be required for the function of the classically defined GE and GI neurons, *Gck* inactivation did not confirm the assumed role of this enzyme. Nevertheless, mice with inactivation of *Gck* in Sf1 neurons displayed impaired control by glucose of both branches of the autonomic nervous system, reduced hypoglycemia-induced glucagon secretion, and increased fat mass; these phenotypes were, however, only observed in female mice (73). The role of glucokinase in hypothalamic neurons, which appears to be sex dependent, still needs to be fully elucidated.

Additional unresolved questions related to the function of the GE neurons include the following: Why does glucose lead to closure of the K_{ATP} channel only in GE neurons when this channel is expressed in all neurons? Do GE neurons that that are protected or not by the blood-brain barrier sense glucose with the same or a different mechanism? In the ARH, which is at least in part outside the blood-brain barrier, neurons have been characterized that respond to increases in glucose concentrations from 5 mM to 20 mM (54, 55). These HGE neurons are activated in a K_{ATP} channel-independent manner but require the production of ROS and the activation of the TRPC3 nonselective cation channel (67). Other mechanisms associated with GE neuron activation include the detection of glucose by the T1R2/T1R3 sweet taste receptor or the uptake of glucose by the Na^+-glucose symporters Sglt1 and Sglt3 (66, 74) (**FIGURE 3*A***). There is obviously a need to better define the respective importance of each of these mechanisms in the activation of GE neurons, whether they are present in the same or distinct GE neurons, and whether these neurons are part of different neuronal circuits that control pancreatic endocrine cells.

3.2. *How Are GI Neurons Activated by Hypoglycemia?*

The mechanisms of activation of GI neurons by hypoglycemia are also diverse and depend on the modulation of either K^+ or Cl^- conductance to depolarize the plasma membrane in low-glucose conditions (49, 75–78) (**FIGURE 3*B***). One study also suggested that activation of orexin GI neurons of the LH depends on a cell surface glucose receptor (79), which has, however, not yet been characterized. Thus, activation of GI neurons is currently viewed as being triggered by a decrease in cellular glucose metabolism that lowers intracellular ATP levels, leading to activation of AMP-dependent protein kinase (AMPK) and reduced activity of the Na^+-K^+-ATPase.

FIGURE 3. Proposed modes of glucose signaling in glucose-excited (GE) and glucose-inhibited (GI) neurons. *A*: GE neurons. Glucose induces membrane depolarization through a glucose metabolism-dependent pathway, which requires glucose uptake by Glut1, Glut2, or Glut3, although whether specific transporters are required for glucose sensing by various GE neurons has not yet been tested. After glucose uptake, glucose phosphorylation by glucokinase (Gck) has been reported to be required, although gene knockout studies in mice showed that Gck was not required for GE neuron activation by glucose. Increased oxidative phosphorylation (OXPHOS)-dependent ATP production then leads to the inhibition of the ATP-sensitive K^+ (K_{ATP}) channel and membrane depolarization ($\Delta\psi$). K_{ATP} channel-independent GE neuron activation has been reported that required TRPC3 channel participation or the induction of membrane depolarization by Na^+ cotransport with glucose through the SGLT1 or SGLT3 cotransporter. Other reports provided evidence for the T1R2/T1R3 heterodimer sweet taste receptor, which is linked to intracellular phospholipase Cβ (PLCβ) signaling. PDH, pyruvate dehydrogenase. *B*: GI neurons. Decreases in extracellular glucose concentrations reduce the glycolytic flux, Krebs cycle activity, and OXPHOS-dependent ATP production. The decrease in intracellular ATP lowers the activity of the Na^+-K^+-ATPase, leading to membrane depolarization and neuronal firing. Reduced ATP production also leads to increased intracellular AMP levels that activate AMP-dependent protein kinase (AMPK), which can control the membrane potential by regulating the activity of Cl^- channels, such as the cystic fibrosis transmembrane regulator (CFTR) or, as demonstrated in gene knockout experiments, Ano4. AMPK also controls intracellular reactive oxygen species (ROS) levels, by triggering the expression of the mitochondria detoxifying enzyme Txn2. Figure generated with BioRender.com, with permission.

In GI neurons of the VMN, AMPK has been reported to phosphorylate and activate guanylate cyclase; the increased production of cGMP further stimulates AMPK activity, which regulates plasma membrane ion channels, possibly the cystic fibrosis transmembrane regulator (CFTR), a chloride channel (77). However, more recent studies have identified and provided genetic evidence that the chloride channel anoctamin 4 (*Ano4*) is required for hypoglycemia sensing by GI neurons of the VMN (78, 80).

The role of AMPK in activation of neurons by hypoglycemia has been shown with the GT1-7 hypothalamic GI cell line (81). In mice, this has been demonstrated by genetic inactivation of AMPK in Sf1 neurons of the VMN (82). Inactivation of either the α1 or α2 catalytic subunit of AMPK only partially reduced the number of GI neurons, whereas simultaneous inactivation of both subunits completely suppressed their presence. Of note, VMN GE neurons were not affected by these genetic modifications. Analysis of the transcriptome of the VMN neurons of double α1-α2-subunit-knockout mice revealed a strong decreased expression of *Txn2*, which encodes a mitochondrial reactive oxygen species (ROS) scavenging enzyme. Virus-directed overexpression of *Txn2* in the VMN of the double-knockout mice restored GI neuron activity. Thus, AMPK contributes to hypoglycemia sensing also by inducing the expression of a gene that limits the production of ROS. This is also supported by another study that showed that overexpressing Txn1, a cytoplasmic form of the enzyme, in the VMN of streptozotocin-induced diabetic rats preserves normal glucagon secretion (83). ROS are known to be increased upon hypoglycemia following the activation of various enzymes, such as NADPH oxidase, xanthine oxidase, or the Ca^{2+}-dependent phospholipase A_2, and their overproduction may have a deleterious impact on glucose sensing (84–86).

Another established mechanism by which a fall in intracellular ATP levels induces neuronal firing, possibly independently of a role of AMPK, is by reducing the activity of the Na^{+}-K^{+}-ATPase, leading to membrane depolarization and neuron activation. This mechanism has been observed in the rat LH and VMN, in experiments where blood and parenchymal glucose levels and firing activity were recorded simultaneously (49): after insulin injection, GI neuron activity progressively increases until the parenchymal glucose levels decrease to 1.0 to 0.5 mM; at lower glucose levels the neurons stop firing at all. In mouse AgRP neurons, evidence has been obtained for the crucial role of the Na^{+}-K^{+}-ATPase in triggering firing activity in response to hypoglycemia; this response can be replicated by application of the sodium pump inhibitor ouabain (87).

4. NEURONAL CONTROL OF INSULIN SECRETION

4.1. Cephalic Phase of Insulin Release

The cephalic phase of insulin release (CPIR) is triggered by stimuli conveyed by the senses of sight, smell, and taste (7). In the oral cavity, glucose is detected by taste cells, which transfer this information to the chorda tympani, the nerve that connects the tongue to the NTS (87). This signal is then transferred to the vagal nerve to stimulate insulin secretion, a response that can be blocked by vagotomy or by administration of the muscarinic antagonist atropine (88–90). How glucose is initially detected by taste cells to control CPIR is not fully characterized yet. Taste cells express the sweet taste receptor T1R2-T1R3 (91), which is activated by nonnutritive sweeteners and by glucose, and the glucose transporters Glut2, Glut5, and SGLT1 (92). In T1R3-knockout mice glucose still activates the CPIR (93, 94), whereas it is reduced by the glucose transporter inhibitors phloretin and phlorizin (95); this suggests that glucose transporters rather than the sweet taste receptors are involved in triggering CPIR.

In addition, CPIR can also be stimulated by the sight or smell of food. For instance, in a recent report Montaner et al. (96) showed that the odor of food, without direct contact with the food pellets, induced insulin release. They showed that this response required the production of GLP-1 by olfactory bulb glomerular layer cells and the activation of local GLP1-R-expressing cells; this was followed by vagal nerve activation and stimulation of insulin secretion. In a different setting, a CPIR was elicited in mice by a short-term (60 s) contact with food pellets. In this experiment, activation of the vagal nerve, and the consequent secretion of insulin, were found to depend on Il-1β secreted by microglial cells (97), suggesting that the CPIR can be modulated by the local inflammatory state. Together, these observations indicate that the vagal nerve innervating the β-cells can be activated by multiple pathways in anticipation of food ingestion. This anticipatory insulin secretion plays an important role to ensure normal postprandial glucose tolerance (98, 99).

4.2. Vagal Control of β-Cell Proliferation and Mass

Appropriate insulin secretion to maintain normoglycemia over a lifetime requires preservation of β-cell mass (100, 101) and function (102, 103). The role of the vagal nerve in controlling β-cell mass has been demonstrated, for instance, in rats with lesion of the ventromedial hypothalamus, which display higher vagal nerve activity, leading to increased insulin secretion, β-cell proliferation, and mass (104, 105). Also, intracerebroventricular injection of NPY, which is normally secreted by ARH NPY/AgRP

neurons, increases insulin secretion and triggers an obesity phenotype; the hyperinsulinemic response depends on vagal afferent signals (106). A more recent study showed that inactivation of *Glut2* selectively in neurons in mice suppresses the activation by glucose of the vagal nerve (107). This led to a lower rate of β-cell proliferation around the weaning period and a consequent ~30% reduction in adult β-cell mass. This defect, together with the loss of vagally controlled first-phase insulin secretion, induces a progressive development of glucose intolerance (107). This indicates that the direct effects of glucose on β-cell proliferation and GSIS are not sufficient to maintain glucose homeostasis in the absence of glucose-dependent vagal input (**FIGURE 4**).

A physiological increase in β-cell mass is also a compensatory mechanism to the development of insulin resistance. In a mouse model of liver-specific insulin resistance induced by activation of the hepatocyte Erk pathway, β-cell proliferation and mass are strongly increased (109). The signal linking liver to β-cells was shown to depend on splanchnic afferent signals that increase vagal nerve-dependent β-cell proliferation, an effect mediated by cholinergic and VIP/PACAP signaling and induction of the β-cell transcription factor FoxM1 (109–111).

4.3. *Neuronal Circuits Controlling Insulin Secretion*

With the use of green fluorescent protein (GFP)-expressing pseudorabies viruses in retrograde labeling techniques, the hypothalamic neurons connected to the pancreas and β-cells could be more precisely mapped. For instance, infection of mouse pancreatic β-cells led to the expression of a reporter GFP protein in hypothalamic PVN, VMN, ARH, and LH neurons (112). Adenovirus-directed overexpression of hexokinase 1 in these nuclei, which was hypothesized to alter their glucose sensing properties, induced glucose intolerance with reduced insulin secretion when the virus was injected in the ARC and VMN and increased glucagon secretion when injected in the LH. No discernible effect on glucose homeostasis was observed when hexokinase 1 was overexpressed in the PVN. These experiments confirmed that neurons within several hypothalamic nuclei control efferent circuits that regulate insulin and glucagon secretion. In addition, they show that modifying the glucose metabolism of these neurons can have a direct impact on glucose homeostasis.

With the development of genetically engineered mice in combinations of viral tracing, chemogenetic, and optogenetic techniques it is now possible to test the role of specific genes, expressed in defined brain cells, in the control of pancreatic hormone secretion (108). For instance, a conditional retrograde viral tracing study that used Ins1-Cre mice to selectively label the neurons that contact β-cells established the existence of a multisynaptic, sympathetic connection between β-cells and oxytocin neurons of the PVN (113) (**FIGURE 5**). These neurons are activated by glucoprivation, and their chemogenetic activation suppresses insulin secretion whereas their inhibition by expression of tetanus toxin, which blocks synaptic transmission, increases insulin secretion, and induces hypoglycemia. Similarly, chemogenetic activation of PACAP-expressing GI neurons of the VMN inhibits insulin secretion (114). This study indicates that the VMN has a tonic inhibitory role in insulin secretion, in line with the early report that lesion of this nucleus increases vagal activity and β-cell proliferation (104). Thus, the neuronal circuits that control β-cells characterized so far are recruited by hypoglycemia to inhibit insulin secretion; they are thus part of the global counterregulatory response to hypoglycemia.

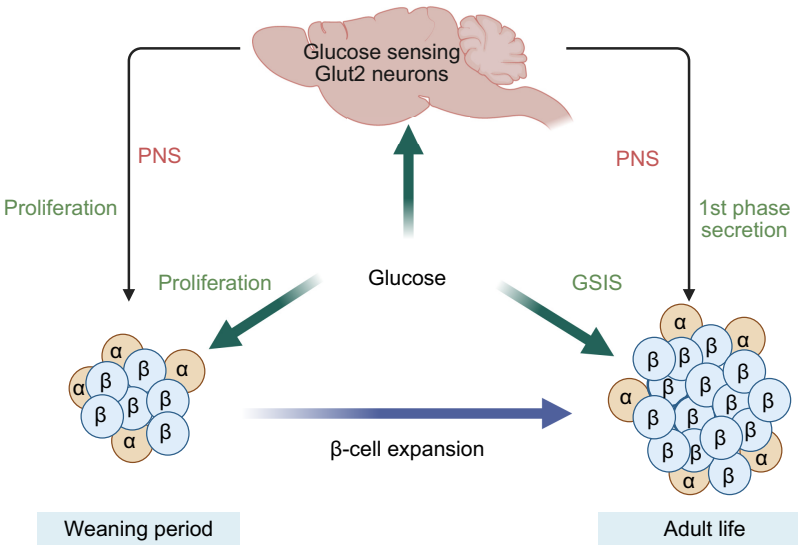


FIGURE 4. Glucose controls β-cell proliferation and mass through Glut2-dependent glucose sensing neurons that control vagal activity. In mice lacking Glut2 expression in neurons, the vagal nerve is no longer activated by glucose, and this leads to reduced β-cell proliferation during the weaning period and lower β-cell mass in adult mice. In adult mice, this is also associated with the absence of first-phase insulin secretion. These combined defects lead to the progressive development of glucose intolerance, even though the direct effects of glucose on β-cell proliferation and insulin secretion are present. Thus, glucose-dependent vagal activity is required for the long-term preservation of glucose homeostasis. GSIS, glucose-stimulated insulin secretion; PNS, parasympathetic nervous system. Figure modified from Ref. 108, with permission from *Trends in Endocrinology & Metabolism*, and generated with BioRender.com, with permission.

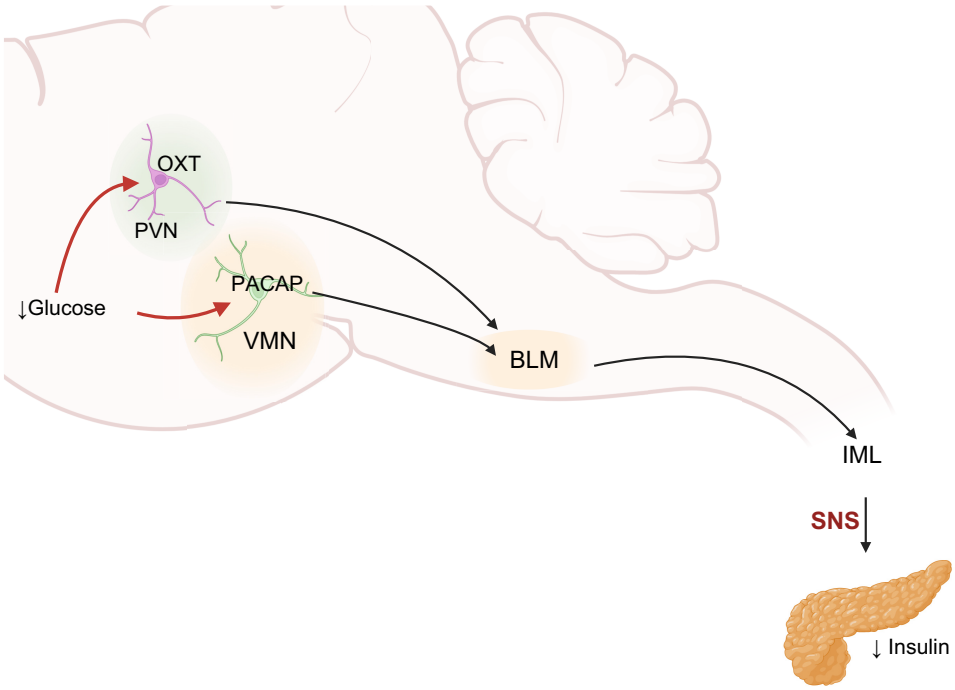


FIGURE 5. Hypoglycemia activates the sympathetic nerve to suppress insulin secretion as part of a global counterregulatory response that restores normoglycemia. Oxytocin (OXT) neurons of the paraventricular nucleus (PVN) are in multisynaptic contact with pancreatic β-cells through the sympathetic nerve. Their activation by hypoglycemia suppresses insulin secretion. Activation of glucose-inhibited (GI) pituitary adenylate cyclase-activating polypeptide (PACAP) neurons of the ventromedial hypothalamus (VMN) also suppresses insulin secretion. BLM, basolateral medulla; IML, intermediolateral column; SNS, sympathetic nervous system.

5. NEURONAL CIRCUITS CONTROLLING GLUCAGON SECRETION

5.1. The Dorsal Vagal Complex

The immediate response to a fall in blood glucose concentrations below the euglycemic level is the secretion of glucagon triggered by vagal nerve activation, and at deeper hypoglycemic levels the sympathetic nervous system also becomes activated (115). The dorsal vagal complex, which comprises the vagal neurons of the DMNX, the AP, and the NTS (**FIGURE 2**), is an important site of hypoglycemia sensing that triggers counterregulation and feeding (116, 117). In the NTS, neurons are directly sensitive to small variations in circulating glucose concentrations (47, 57). For instance, neurons of the NTS that express the glucose transporter Glut2 are activated by hypoglycemia in a concentration- and AMPK-dependent manner when glucose decreases below 5 mM. These neurons send projections to the DMNX, and their optogenetic activation increases vagal nerve firing, leading to increased glucagon secretion. This NTS-DMNX-α-cell circuit links central hypoglycemia sensing to the secretion of glucagon to restore euglycemia (118) (**FIGURE 6**).

5.2. Hypothalamic Mechanisms

5.2.1. The VMN.

The VMN is an important nucleus involved in the control of glucose homeostasis (119, 120), which contains functionally well-characterized GE, GI, and glucose-nonresponding neurons (121–123). The role of the VMN neurons in physiological regulation has now been tested with genetic approaches. For instance, Sf1-Cre mice have been used to allow Cre-dependent expression of light-activated or light-inhibited ion channels. Activation of Sf1 neurons by laser light stimulates glucagon secretion and increases glycemia, whereas their silencing markedly delays the glycemic recovery from insulin-induced hypoglycemia (124). Similarly, expression of a magnetically controlled ion channel in glucokinase neurons of the VMN, which also represent a large fraction of the neurons in this nucleus, allows their activation by an external magnetic field (125). This results in a robust increase in blood glucose levels, associated with higher plasma glucagon and reduced insulin secretion. Although these studies provide clear evidence for the role of the VMN neurons in stimulating glucagon secretion, they do not distinguish between the role of GE and GI neurons. A more recent study, however, showed that estrogen receptor-expressing (Esr^+) neurons of the ventrolateral part of the VMN are either GE or GI. The authors showed that the Esr^+ GI neurons require the presence of the chloride channel anoctamine 4 (Ano4) for their activation by hypoglycemia (78). These GI neurons project to the medioposterior part of the ARH, and their stimulation increases glycemia. Interestingly, the Esr^+ GE neurons, whose activation by high glucose depends on the presence of the K_{ATP} channel, project to the dorsal raphe nucleus, and their optogenetic inhibition increases glycemia (78). Thus, hypoglycemia triggers glucagon secretion by a dual action on activating GI neurons and inhibiting GE neurons. However, inactivation of the *Ano4* gene in the VMN,

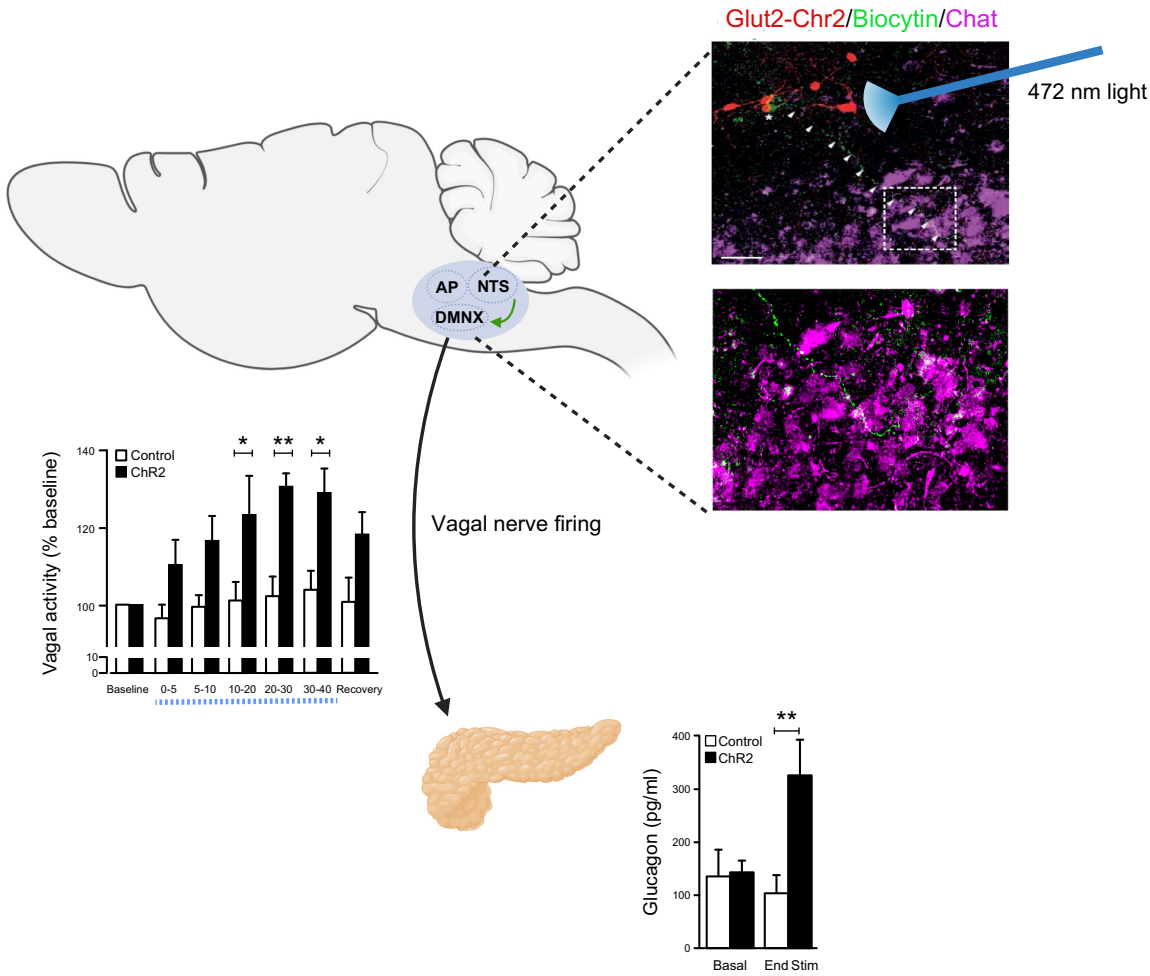


FIGURE 6. Glut2 neurons of the nucleus tractus solitarii (NTS) are connected to dorsal motor nucleus of the vagus (DMNX) neurons to control glucagon secretion through the vagal nerve. Glut2- and channelrhodopsin2 (ChR2)-expressing neurons of the NTS send projections (labeled with biocytin, green) to the DMNX neurons identified by choline acetyltransferase (ChAT) labeling (violet). When stimulated by light, the NTS Glut2 neurons of mice that express ChR2, but not those of control mice, activate vagal nerve firing and glucagon secretion. AP, area postrema. Figure modified from Ref. 118, with permission from *Cell Metabolism*, and generated with BioRender.com, with permission. Significant difference: $*P < 0.05$; $**P < 0.01$.

which suppresses GI neuron activity, is sufficient to reduce glycemia and cause defective glucagon secretion in response to 2-deoxy-D-glucose (2DG)-induced neuroglucopenia or to insulin-induced hypoglycemia (80).

An interesting question is whether the glucose sensing properties of VMN GI neurons are required for the physiological response to hypoglycemia. These VMN neurons receive information from afferent GI neurons located at various anatomical sites, such as the hepatoportal vein area, the NTS, or the parabrachial nucleus (PBN). In the PBN, CCK-expressing neurons are activated by hypoglycemia and project to VMN Sf1 neurons, which then control glucagon secretion through a synaptic relay in the bed nucleus of the stria terminalis (BNST) (124, 126, 127). In this PBN-VMN-BNST circuit (**FIGURE 7**), VMN neurons are required to transmit the information about hypoglycemia. Indeed, genetic inactivation of the vesicular glutamate transporter vGLUT2 in Sf1 neurons prevents synaptic communication, and mice carrying this mutation display defective glucagon response to hypoglycemia, indicating that these neurons form an essential link in this circuit (128). However, suppression of hypoglycemia sensing by these neurons by inactivation of the α1- and α2-subunits of AMPK does not impair hypoglycemia-induced glucagon secretion (82). Thus, the hypoglycemia sensing property of these neurons appears to be dispensable for the physiological response to hypoglycemia; it may nevertheless be recruited in the case of dysfunction of the peripheral glucose sensors or in response to fast development of hypoglycemia (see sect. 6), which would lead to development of central hypoglycemia.

6. HEPATOPORTAL GLUCOSE SENSORS AND AUTONOMIC CONTROL OF PANCREATIC ISLETS

The hepatoportal vein area is richly innervated by nutrient-sensitive vagal and spinal afferents (**FIGURE 8**) that

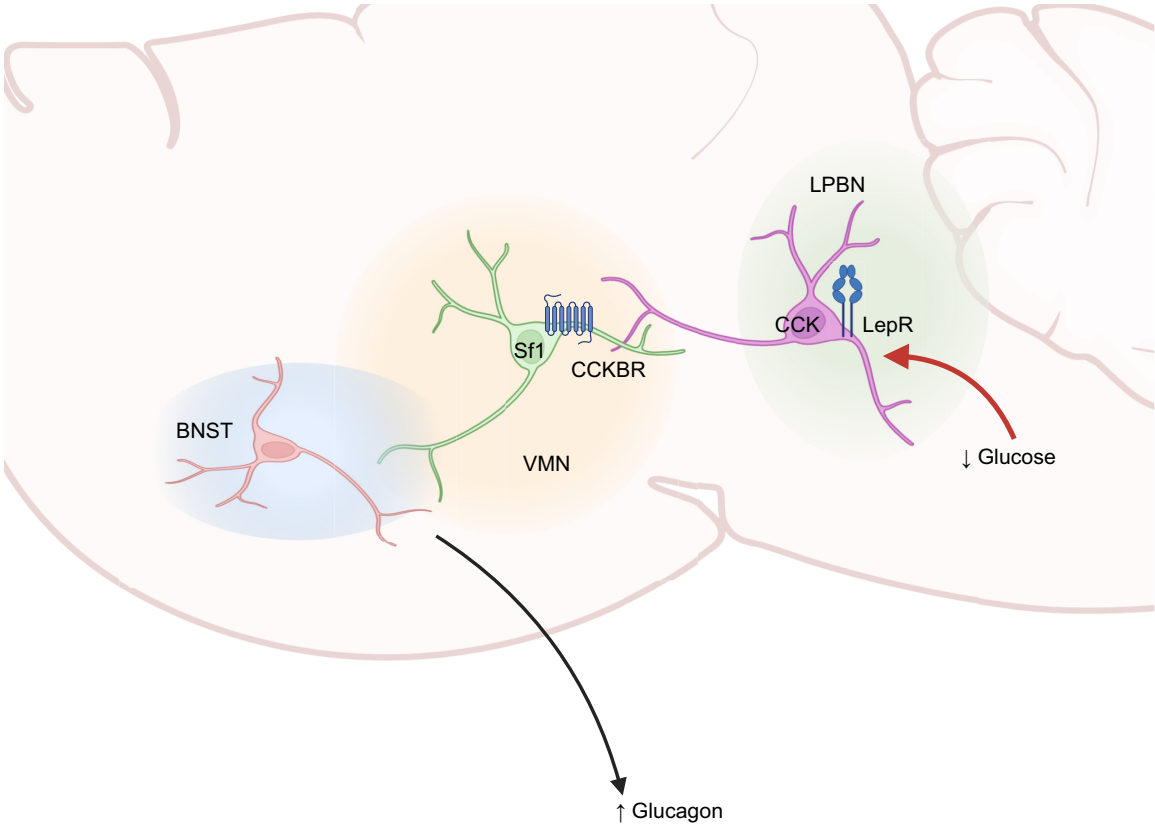


FIGURE 7. A lateral parabrachial nucleus (LPBN)-ventromedial hypothalamus (VMN)-bed nucleus of the stria terminalis (BNST) circuit that controls hypoglycemia-induced glucagon secretion. CCK-expressing neurons of the PBN are activated by hypoglycemia, an activation countered by leptin action. These neurons project to Sf1 neurons of the VMN that express the CCKB receptor (CCKBR). When activated, these VMN neurons trigger glucagon secretion through a relay in the BNST. Whether the BNST activates the sympathetic nervous system (SNS) or parasympathetic nervous system (PNS) to control glucagon secretion is not established. LepR, leptin receptor. Figure generated with BioRender.com, with permission.

monitor the local concentrations not only of glucose but also of amino acids and lipids. These nutrient sensing afferents have profound effects on feeding behavior and glucose homeostasis, in part by modulating autonomic nervous activity (129–134).

Vagal afferents have their cell bodies mainly in the left nodose ganglion and project to the DMNX, the NTS, and the AP; the projections to the DMNX can activate a vago-vagal reflex to rapidly control pancreatic islet hormone secretion (132). The spinal sensory fibers of the hepatoportal vein area originate from dorsal root ganglion bipolar neurons, which are connected to second-order neurons present in the spinal cord; these project to the DVC and to other central regions (129); the projections to the DMNX indicate that spino-vagal connections can control islet hormone secretion (135).

Vagal sensory fibers are responsive to glucose injections in the portal vein (136). When glucose is infused in the portal vein at a relatively high rate, corresponding to endogenous glucose production, it triggers glucose uptake in muscle, heart, and brown fat by an insulin-independent mechanism (137–139). Both pharmacological and genetic studies showed that glucose sensing by this system depends on the expression of Glut2 and of the Glp-1R and can be inhibited by somatostatin (140, 141) (**FIGURE 8**), thereby sharing similarities with glucose sensing by pancreatic β-cells. Notably, this sensing system also controls the first phase of insulin secretion induced by an intraperitoneal glucose injection. The necessity for Glut2 expression was demonstrated by studies revealing suppressed first-phase insulin secretion in mice lacking Glut2 in their nervous system (107). Activation of a first phase of insulin secretion can also be induced by portal infusion of GLP-1, a response triggered by a hepatopancreatic vago-vagal reflex (142–144).

Slow-onset hypoglycemia (reaching ~2.5 mM over 75 min) activates spinal afferents and induces expression of c-Fos (a marker of neuron activation) in the AP, the NTS, and the DMNX (145) and triggers the counterregulatory secretion of epinephrine, norepinephrine, and glucagon (146, 147) (**FIGURE 8**). This response is prevented by celiac-superior mesenteric ganglionectomy but not by vagotomy or cooling-induced inhibition of vagal nerve firing (146, 148). Notably, if hypoglycemia develops faster (reaching 2.5 mM within 20 min), a normal CRR develops

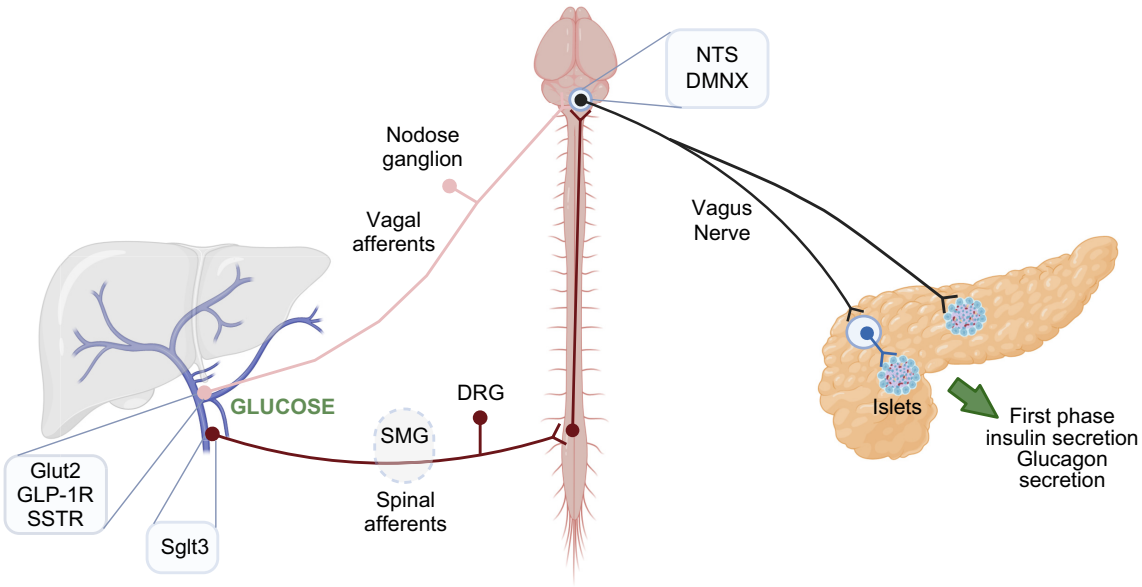


FIGURE 8. The hepatoportal glucose sensors and islet hormone secretion. The hepatoportal vein area contains glucose sensors that are linked to vagal and spinal afferents. Vagal afferent activity is controlled by sensors that depend on the expression of Glut2 and the glucagon-like protein-1 (GLP-1) receptor (GLP-1R) and are inhibited by somatostatin. The signal transmitted to the dorsal motor nucleus of the vagus (DMNX) can induce a vago-vagal reflex that is responsible for glucose and GLP-1 induction of first-phase insulin secretion. Activation of these sensors also increases insulin-independent glucose uptake by muscle, heart, and brown adipose tissue (see text). Spinal afferents are activated by slow-developing hypoglycemia. The signal generated is transmitted to the DMNX and to hypothalamic sites. The connection to the DMNX can induce a spino-vagal reflex to stimulate glucagon secretion. DRG, dorsal root ganglia; NTS, nucleus tractus solitarii; SMG, superior mesenteric ganglia; SSTR, somatostatin receptor. Figure generated with BioRender.com, with permission.

even upon ganglionectomy (149, 150). This may be explained by the recruitment of central glucose sensors, possibly the GI neurons of the VMN that are part of a neuronal circuit that controls glucagon secretion, as discussed above.

The mechanism of hypoglycemia sensing that activates spinal afferent is not established. It may depend on Sglt3 (151), an isoform of the Na^+-glucose symporter family that does not transport glucose but induces sodium currents upon glucose binding (152). Genetic evidence for the role of this symporter in glucose sensing is, however, still to be obtained.

Collectively, the above information indicates that the hepatoportal vein area contains multiple glucose sensing mechanisms that activate vagal and spinal afferents (**FIGURE 8**). These are connected to the dorsal vagal complex, which can transfer information to hypothalamic nuclei or other central areas. Importantly, vagal and spinal afferents projecting to the DMNX can initiate vagal reflexes to rapidly control the secretion of pancreatic islet hormones.

Through stimulating first-phase insulin secretion and enhancing peripheral tissue glucose uptake, vagal afferents play a critical role in the anticipatory adjustment of the body to incoming glucose loads. Conversely, during slow development of hypoglycemia spinal afferents are essential to trigger the secretion of counterregulatory hormones.

7. GENETIC IDENTIFICATION OF HYPOTHALAMIC MECHANISMS CONTROLLING THE CRR

As described above, the DMNX neurons receive input from second-order neurons located not only in the NTS but also in several hypothalamic nuclei where glucose sensing neurons are also located (15, 45). Thus, activation of DMNX vagal neurons may also be controlled by hypoglycemia sensing by these hypothalamic neurons. To identify, in an unbiased manner, novel hypothalamic regulators of glucagon secretion, Picard et al. (153, 154) performed genetic screens. They used a panel of recombinant inbred BXD mice, derived from the cross of C57BL/6 and DBA/2 mice (155), and induced glucagon secretion by intraperitoneal injection of 2DG or of insulin (insulin-induced hypoglycemia, IIH) (**FIGURE 9**). Interestingly, the two screens revealed different quantitative trait loci (QTLs) for the control of glucagon secretion, indicating that the response to 2DG-induced neuroglucopenia and IIH depends on different molecular mechanisms (**FIGURE 9**). The screen for 2DG-induced glucagon secretion led to the identification of a single QTL on the distal part of chromosome 7. Combining this information with RNA sequencing data from the hypothalami of the BXD mice identified *Fgf15* as the mRNA whose hypothalamic level of expression correlated most, and negatively, with the glucagon trait (153). In the IIH screen, two QTLs were identified, one on

chromosome 8 and one on chromosome 15. On chromosome 8 *Agpat5* and on chromosome 15 *Irak4* and *Tmem117* were the best candidates (154). Analysis of these genes yielded new information about the complexity of the neuronal mechanisms and circuits that regulate glucagon secretion. These genes are described below (and see **FIGURE 9**).

7.1. *Fgf15 in Neurons of the DMH*

Fgf15 mRNA was found to be expressed in the perifornical area (PeF) and by glutamatergic neurons of the dorsomedial hypothalamus (DMH). Intracerebroventricular injection of FGF19, the human ortholog of Fgf15, decreases hypoglycemia-induced activation of NTS and DMNX neurons as measured by c-Fos immunostaining and reduces glucagon secretion (153), in line with the negative correlation between *Fgf15* expression and plasma glucagon levels. *Fgf15-Cre* mice were then generated to allow for virus-directed expression of hM3Dq receptors, which can be activated by intraperitoneal clozapine or clozapine-*N*-oxide, specifically in Fgf15 neurons of the DMH (156, 157). Chemogenetic activation of the Fgf15 neurons reduces hypoglycemia-activated vagal activity and glucagon secretion, confirming the negative role of these neurons in vagal nerve activation. However, activation of the Fgf15 neurons induces intraperitoneal glucose intolerance. This effect is not caused by reduced insulin secretion or by increased insulin resistance. Instead, it is caused by a strong activation of the parasympathetic nerve leading to phosphorylation of the transcription factor CREBP in the liver, increased expression of Pepck, a rate-limiting enzyme in gluconeogenesis, and increased hepatic glucose production (**FIGURE 10**). In basal conditions activation of Fgf15 neurons also led to an ~1-mM increase in glycemia (156). Thus, Fgf15 neurons of the DMH suppress hypoglycemia-induced vagal nerve activity and glucagon secretion, but, at the same time, they activate hepatic glucose production through a direct sympathetic pathway. Only a small fraction of Fgf15 neurons of the DMH are GI, a higher proportion are GE, and the majority are glucose nonresponders, and IIH does not induce c-Fos expression in these neurons. Thus, these Fgf15 neurons may not directly respond to hypoglycemia. However, viral tracing

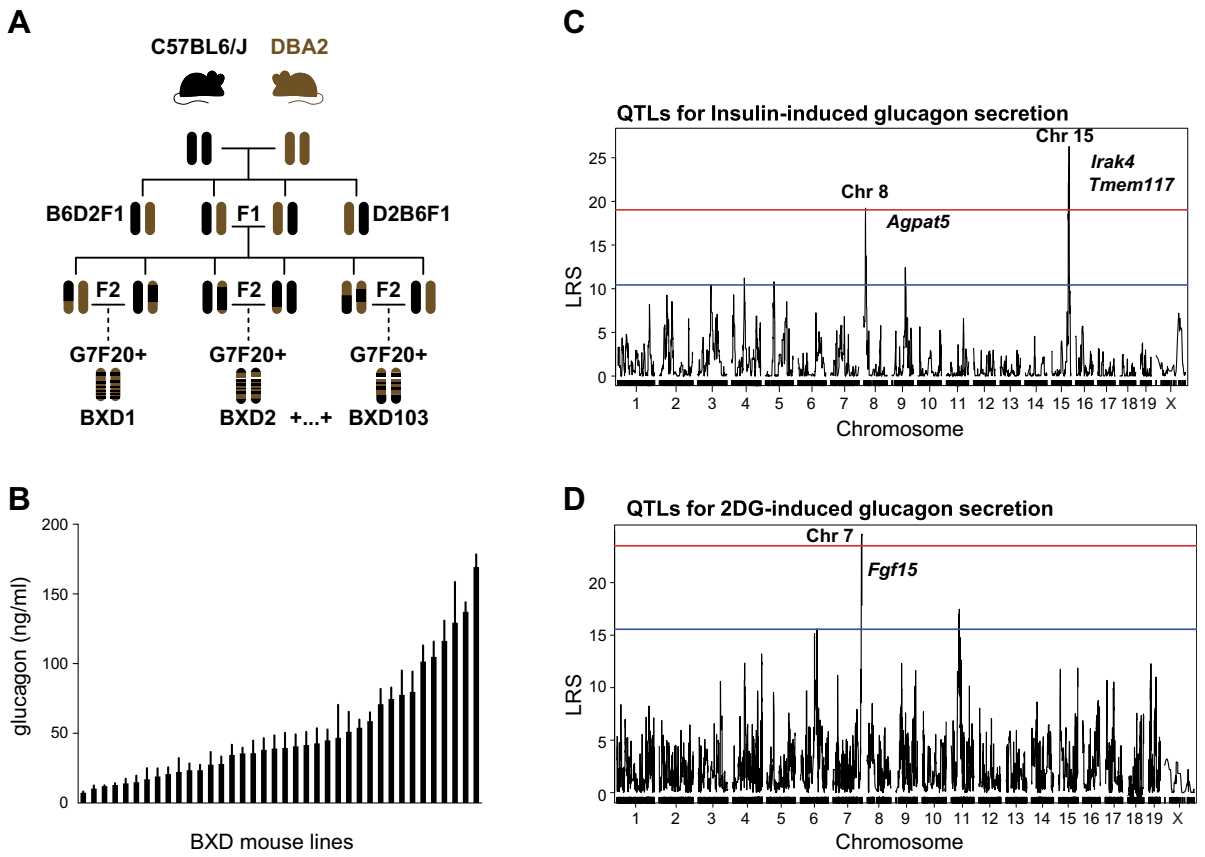


FIGURE 9. Recombinant inbred mice for the identification of gene loci controlling glucagon secretion. *A*: recombinant inbred BXD mice were generated from the breeding of C57BL/6 and DBA/2 mice. Mice with unique combinations of the parental chromosomes are obtained after several backcrossings; their genotype has been established, allowing identification of quantitative trait loci (QTLs) for 2-deoxy-D-glucose (2DG)- or insulin-induced glucagon secretion. *B*: illustration of the variability of the glucagon response to insulin-induced hypoglycemia across all tested BXD lines. *C*: identification of QTLs for insulin-induced glucagon secretion. The candidate genes are indicated. *D*: identification of a QTL for 2DG-induced glucagon secretion and of Fgf15 as the candidate gene. LRS, likelihood ratio statistic.

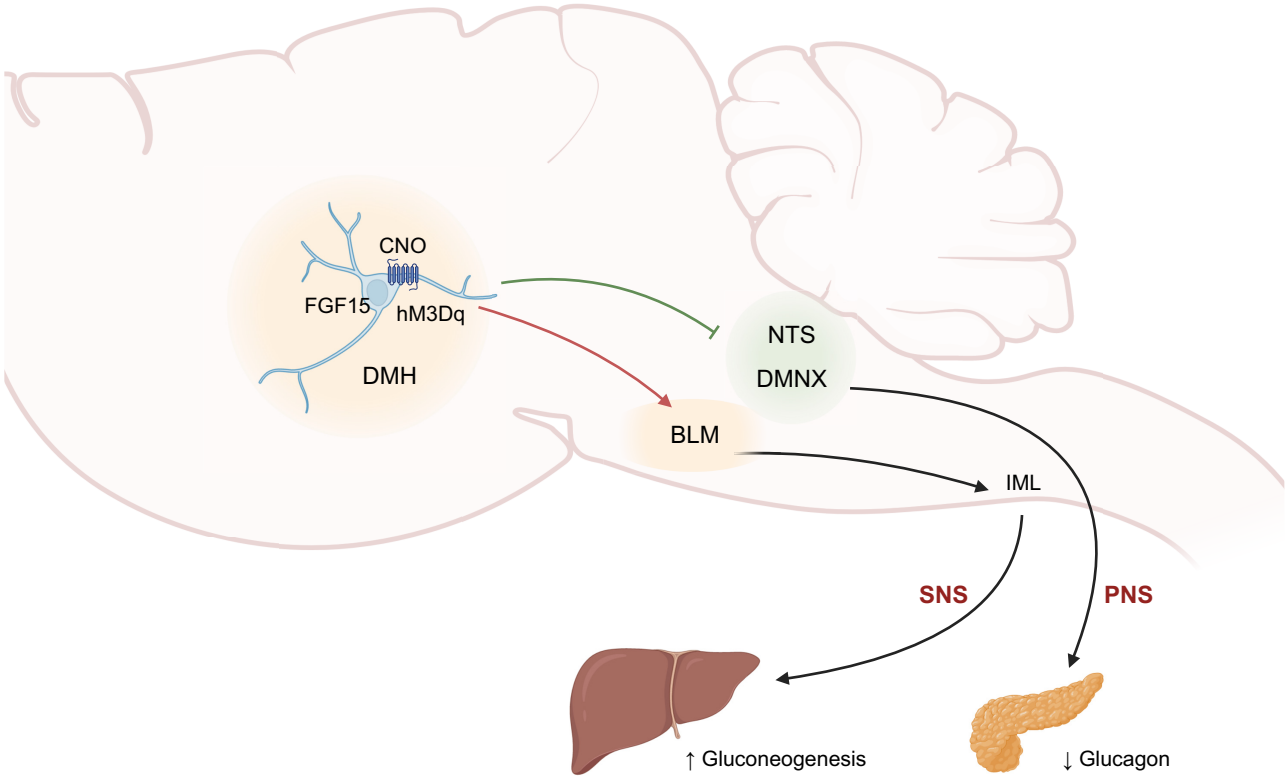


FIGURE 10. Fgf15 neurons of the dorsomedial hypothalamus (DMH) that express the hM3Dq receptor can be activated by intraperitoneal injection of clozapine-*N*-oxide (CNO). This suppresses hypoglycemia-induced dorsal motor nucleus of the vagus (DMNX) neuron activation and vagal nerve firing but increases sympathetic nerve activity, and that stimulates hepatic glucose output. BLM, basolateral medulla; IML, intermediolateral column; NTS, nucleus tractus solitarii; PNS, parasympathetic nervous system; SNS, sympathetic nervous system. Figure generated with BioRender.com, with permission.

experiments showed that they receive inputs from neurons in the ARH, the VMN, the PVN, and the LH and may thus be activated indirectly by hypoglycemia. In addition, viral tracing showed that these neurons send projections to the medial preoptic nucleus (MPO), the ARH, and the LC. The latter is a site that controls activation of the sympathetic nerve and inhibition of the parasympathetic nerve (158); it may thus form an important relay for the glucoregulatory effect of the Fgf15 neurons. The dual effect on both branches of the ANS may be required when a rapid hepatic glucose release is required without triggering other glucagon responses, for instance, a reduction in food intake (159).

7.2. Agpat5 in AgRP Neurons

Agpat5 encodes acylglycerolphosphate-acyltransferase 5, an enzyme associated with the outer mitochondrial membrane, which catalyzes the formation of phosphatidic acid from lysophosphatidic acid and fatty acyl-CoA. It is ubiquitously expressed, including in the ARH and PVN, two nuclei that show intense c-Fos labeling upon insulin-induced hypoglycemia (154). Knockout of *Agpat5* in PVN neurons had no effect on any measured glucose homeostasis parameters. Its inactivation in AgRP neurons of the ARH, in contrast, reduced the number of AgRP GI neurons, decreased hypoglycemia-induced vagal nerve activity, and blunted glucagon secretion (160) (**FIGURE 11**). Why is this lipid-modifying enzyme required to preserve hypoglycemia sensing? Activation of AgRP GI neurons by hypoglycemia is triggered by a fall in intracellular ATP concentration, which reduces the activity of the Na^+-K^+-ATPase, leading to membrane depolarization and neuron firing (161). Thus, for the intracellular ATP levels to reflect a fall in extracellular glucose concentrations, ATP should not be produced from other substrates, in particular from free fatty acids, whose circulating concentrations augment during fasting, i.e., when glucagon secretion is needed. In this context, Agpat5, whose expression level increases in AgRP neurons during fasting (162), converts fatty acyl-CoAs into phosphatidic acid and thus diverts them away from

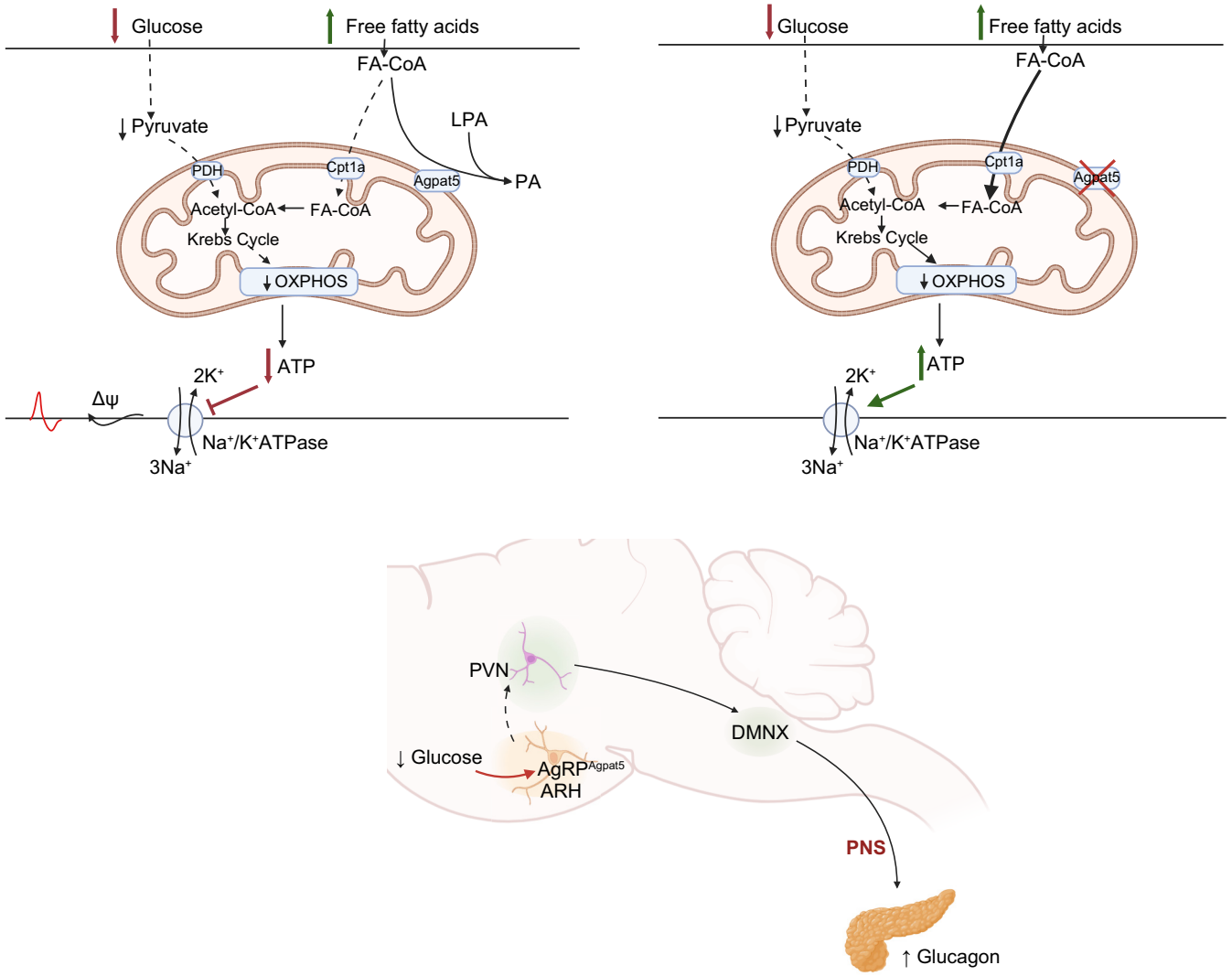


FIGURE 11. Agpat5 is required for the response to hypoglycemia in a subset of AgRP neurons. *Top*: the schemes illustrate the role of Agpat5 in preventing fatty acyl-CoAs (FA-CoA) from entering the mitochondria to generate β-oxidation-derived ATP. In the absence of Agpat5, FA-CoAs can enter the mitochondria through Cpt1a, leading to increased ATP levels. This prevents ATP levels from decreasing in proportion to the developing hypoglycemia and activating AgRP neuron firing. *Bottom*: when activated by hypoglycemia, Agpat5-expressing AgRP neurons trigger dorsal motor nucleus of the vagus (DMNX) neuron activity, increase vagal nerve firing, and increase glucagon secretion. ARH, arcuate nucleus; LPA, lysophosphatidic acid; OXPHOS, oxidative phosphorylation; PA, phosphatidic acid; PDH, pyruvate dehydrogenase; PNS, parasympathetic nervous system; PVN, paraventricular nucleus. Figure generated with BioRender.com, with permission.

Cpt1a-dependent entry into the mitochondria and β-oxidation-directed ATP production (see **FIGURE 3*B***). This role was proven in the GT1-7 cell line by showing that ATP production is increased by silencing Agpat5 expression and that simultaneous silencing of Cpt1a returned ATP to basal levels. Similarly, in AgRP^{Agpat5KO} mice the loss of GI neurons is fully reversed by additional inactivation of the *Cpt1a* gene. Thus, Agpat5, in a subset of GI AgRP neurons, is required for proper hypoglycemia sensing, induction of vagal activity, and full glucagon response.

7.3. Irak4 and Il-1β Signaling

Irak4 is a kinase that acts downstream of the Il-1βR or the Toll-like receptors (TLRs) and upstream of the transcription factor NF-κB (163). Its expression in the hypothalamus is negatively correlated with the glucagon response, and studies of C57BL/6 and DBA/2 mice showed that *Irak4* is much more expressed in the hypothalamus of DBA/2 mice than in that of C57BL/6 mice and that insulin, despite inducing the same hypoglycemia in both strains, triggers a lower glucagon response in DBA/2 mice (154). The relative unresponsiveness of DBA/2 mice is also associated with higher hypothalamic Il-1β expression. Intracerebroventricular injection of an Il-1R antagonist (anakinra) increases hypoglycemia-induced c-Fos expression in the ARH but not in the PVN, vagal activity, and glucagon secretion. These effects are seen in DBA/2 mice but not in C57BL/6 mice. Thus, *Irak4* expression level is genetically determined and

controls Il-1β production and Il-1β signaling (164), which controls the response to hypoglycemia and glucagon secretion. How does Il-1β decrease hypoglycemia sensing and the CRR? Il-1β can be produced by neurons, astrocytes, or microglial cells, and microglia-produced Il-1β has been reported to decrease AgRP neuron activation and glucagon secretion (165). Il-1β increases glucose uptake and glycolysis in neurons and astrocytes and thus shifts the relationship between the fall in extracellular glucose concentration and the decreased production of ATP, thereby dampening the normal induction of GI neuron firing (166).

7.4. *Tmem117, AVP Secretion, and Glucagon Response*

Tmem117 is an eight transmembrane domain-containing protein (167), which regulates ROS production and endoplasmic reticulum (ER) stress (168) and whose expression is inversely correlated with IIH-induced glucagon secretion (154). In the hypothalamus, Tmem117 is expressed in AVP magnocellular neurons of the PVN and supraoptic nucleus (SON) (169). Its knockout in AVP neurons increases ROS production and AVP mRNA expression, in agreement with studies showing transcriptional upregulation of this gene by ROS (170, 171). In addition, knockout of *Tmem117* in AVP neurons increases their intracellular Ca^{2+} response to insulin-induced hypoglycemia as measured by fiber photometry in living mice. Noteworthy, in female mice the effect of *Tmem117* inactivation on glucagon secretion was only observed during the proestrus phase, indicating a strong sex-dependent role of this protein. Because *Tmem117* was identified in an unbiased genetic screen for hypothalamic regulator of glucagon secretion, this highlights the role of the AVP neurons in the physiological response to hypoglycemia. This is in line with previous studies showing that hypoglycemia triggers AVP secretion and that AVP can stimulate glucagon secretion by binding to the pancreatic α-cell AVP1b receptor (172–174). A more recent study further showed that AVP is a physiological regulator of glucagon secretion in humans and that part of the defective CRR observed in individuals with type 1 diabetes is linked to a defect in the AVP response to hypoglycemia (175). This study also provided evidence that activation of AVP neurons is, at least in part, secondary to activation of GI neurons of the basolateral medulla (BLM), which send projection to these neurons in the SON, thus forming a BLM-SON-posterior pituitary axis for the controlled release of AVP (**FIGURE 12**). It is also interesting to note that in a mouse model of type 2 diabetes with defective CRR induced by repeated IIH, which displays reduced glucagon secretion, *Avp* is one of the most downregulated mRNAs compared with gene expression in the hypothalamus of control mice (176).

The integration of AVP magnocellular neurons/AVP as a physiological axis in the control of glucagon secretion is of significant importance. It expands the diversity of the central mechanisms that control the CRR to hypoglycemia and more specifically of glucagon. This axis comes in addition to both branches of the autonomic nervous system and of the sympatoadrenal axis that releases epinephrine, which secondarily stimulates glucagon secretion.

8. INTEGRATION OF NEURONAL GLUCOSE SENSING IN THE CONTROL OF INSULIN AND GLUCAGON SECRETION

Glucose sensing cells regulate insulin and glucagon secretion through the modulation of sympathetic and parasympathetic nerve activity and by controlling the secretion of the neurohormone AVP. These sensing cells are situated in the hepatoportal vein area and in several nuclei of the brain stem and hypothalamus. An often-discussed question is whether this glucose sensing system has a hierarchical organization, with one region exerting a dominant role in triggering islet hormone secretion or, alternatively, whether glucose sensing neurons form a distributed system that monitors local glucose concentrations and integrate this information at a preautonomic level to trigger hormone secretion.

In anticipation of food absorption, the sight or smell of food or the initial presence of sugar-containing food in the oral cavity triggers a CPIR, a response that is vagally controlled. Then, appearance of glucose in the portal vein activates sensors that trigger a vagal-dependent first phase of insulin release and an insulin-independent stimulation of glucose uptake by peripheral tissues. These anticipatory, neuronally mediated responses are activated sequentially. Their overall goal is to minimize postabsorptive glucose excursions and ensure normal glucose tolerance.

The neural response to hypoglycemia involves glucose sensing cells in the hepatoportal vein area, the brain stem, and the hypothalamus. Early studies showed that hypoglycemia-induced CRR is suppressed by infusion of glucose in the VMN and, conversely, that administration of 2DG in the VMN of euglycemic rats stimulates glucagon secretion (177, 178). These observations suggested a primordial role of the VMN in CRR. However, other experiments showed that glucose infusion in the portal vein of hypoglycemic rats reduces the CRR and that spinal denervation impairs the CRR induced by hypoglycemia (146, 179), thereby supporting a crucial role of the hepatoportal vein sensors. More recently, these sensors were shown

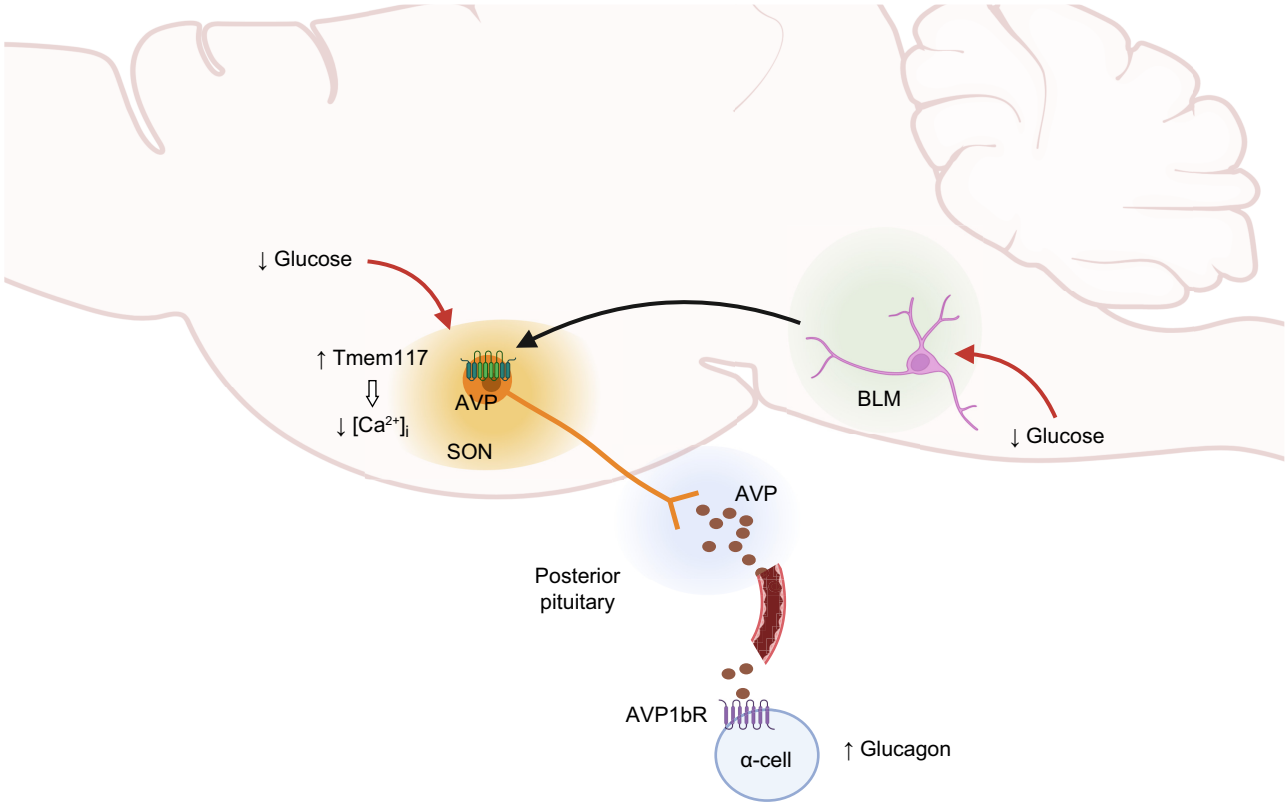


FIGURE 12. Vasopressin (AVP) neurons of the supraoptic nucleus (SON) can be activated by hypoglycemia, probably directly but also indirectly by glucose-inhibited (GI) neurons of the basolateral medulla (BLM). They then secrete AVP in the blood at the level of the posterior pituitary. AVP binding to AVP1b receptors present at the surface of pancreatic α-cells stimulates glucagon secretion. Tmem117, an 8 transmembrane domain-containing protein, negatively controls AVP secretion at least in part by regulating intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Figure generated with BioRender.com, with permission.

to be essential for the CRR induced by slow- but not fast-onset hypoglycemia. Thus, depending on the experimental conditions, a primary role in the CRR can be attributed to central or peripheral sensors.

Additionally, not all central sensing units are equal. For instance, hypoglycemia sensing neurons of the NTS are directly sensitive to small blood glucose variations (47); they may thus participate in the CRR response to slow-onset hypoglycemia. Also, their presence can explain why celiac-superior mesenteric ganglionectomy does not completely suppress the counterregulatory hormone secretion (146). In contrast, VMN GI neurons, which are located within the blood-brain barrier, are activated when parenchymal glucose concentrations decrease below a certain threshold, well under the blood glucose concentrations. Such low central glucose concentrations probably occur rarely, possibly when peripheral sensors dysfunction or, experimentally, during fast-onset hypoglycemia. The central GI neurons located within the blood-brain barrier may thus be a fail-safe system activated only when central hypoglycemia develops.

Additional differences between glucose sensing neurons are their sensitivities to metabolites, immune, and hormonal signals. For instance, the CCK GI neurons of the PBN express the leptin receptor (LepRb) whose activation reduces hypoglycemia-induced CRR (127); the response to hypoglycemia of ARH AgRP neurons is impacted by circulating free fatty acid levels (160); Il-1β decreases the responsiveness of ARH neurons and the activation of the vagal nerve and glucagon secretion (154); and Tmem117, a regulator of ER stress and ROS production, modulates the secretory activity of AVP neurons and the glucagon response in a sex-specific manner (169). Thus, although the CRR is triggered by a fall of glycemia it is also modulated by information about the body energy level and the local or global inflammatory state and is influenced by sex hormones, all acting on different glucose sensing neurons. Another aspect of the CRR is that it not only induces counterregulatory hormone secretion but also suppresses insulin secretion, as controlled by oxytocin neurons of the PVN and PACAP neurons of the VMN.

Collectively, the above information supports a model in which the CRR is under the control of a distributed glucose sensing system. Information collected by portal vein sensing neurons is directed to the DVC and hypothalamic nuclei. From the DMNX a spino-vagal reflex can directly control glucagon secretion, although this

has not yet been formally demonstrated. The information conveyed from portal glucose sensors to hypothalamic nuclei utilizes these central neurons as a relay for the subsequent activation of sympathetic and parasympathetic nerves innervating pancreatic islets. This relay function allows these centrally located glucose sensing neurons to fine-tune the CRR to the metabolic status of the organism. Their glucose sensing properties may, however, only be recruited when local hypoglycemia develops, which must remain a rare event. Additionally, AVP secretion triggered, at least in part, by GI neurons of the basolateral medulla represents a parallel, neurohormonal system to restore normoglycemia.

9. CONCLUSIONS

The existence of a link between the central nervous system and the control of glucose homeostasis was described in the middle of the nineteenth century by Claude Bernard (180). Since then, glucose-responsive neurons have been identified that are excited either by hyperglycemia or by hypoglycemia and that are localized not only centrally but also at peripheral sites such as the hepatoportal vein area. The diversity of glucose sensing mechanisms used by GE and GI neurons is still not fully described. Initial investigations relied mostly on electrophysiological recordings of neurons exposed to different concentrations of glucose in combination with various pharmacological inhibitors. Newer technologies now allow for the genetic identification of specific genes required for GE or GI glucose sensing. Virus-based neuronal circuit mapping, together with chemogenetic and optogenetic techniques, allow precise characterization of the neuronal circuits that functionally link glucose sensing neurons to the control of pancreatic islet cells. With these tools and the use of single-cell genomic and spatial transcriptomic technologies (181), it can be expected that the diversity of the glucose sensing mechanisms can not only be expanded but also be attributed to specific neurons, neuronal circuits, and physiological functions. Another frontier in this field of research will be to understand the molecular basis for defective CRR induced by insulin treatment of patients with diabetes and whether it can be prevented or reversed. Initial studies using single-cell genomics analysis of the hypothalamus have revealed that impaired glucagon response to hypoglycemia is associated with multiple defects in neurons, astrocytes, and oligodendrocytes that explain defective hypoglycemia sensing and indicate global impairment in synaptic transmission with signs of neurodegeneration (176). Further studies along these lines are expected to better define the molecular and cellular origin of defective CRR. This could then lead to the prevention and better management of this dangerous condition. Finally, the progressive loss of glucose-stimulated insulin secretion that is associated with, and characterizes, the development of type 2 diabetes may be caused by dysfunctions of not only pancreatic β-cells but also of the numerous extra-pancreatic glucose-sensing cells discussed here that indirectly control β-cell mass and function. This strongly argues for further extensive characterization of this integrated network of glucose-sensing cells to better understand the pathogenesis of diabetes.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

B.T. drafted manuscript; edited and revised manuscript; and approved final version of manuscript.

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