Functional identification of a neurocircuit regulating blood glucose

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Edited by Gerald I. Shulman, Howard Hughes Medical Institute/Yale University, New Haven, CT, and approved February 16, 2016 (received for review October 28, 2015)

Previous studies implicate the hypothalamic ventromedial nucleus (VMN) in glycemic control. Here, we report that selective inhibition of the subset of VMN neurons that express the transcription factor steroidogenic-factor 1 (VMNSF1) neurons blocks recovery from insulin-induced hypoglycemia whereas, conversely, activation of VMNSF1 neurons causes diabetes-range hypoglycemia. Moreover, this hyperglycemic response is reproduced by selective activation of VMNSF1 fibers projecting to the anterior bed nucleus of the stria terminalis (aBNST), but not to other brain areas innervated by VMNSF1 neurons. We also report that neurons in the lateral parabrachial nucleus (LPBN), a brain area that is also implicated in the response to hypoglycemia, make synaptic connections with the specific subset of glucoregulatory VMNSF1 neurons that project to the aBNST. These results collectively establish a physiological role in glucose homeostasis for VMNSF1 neurons and suggest that these neurons are part of an ascending glucoregulatory LPBN→VMNSF1→aBNST neurocircuit.

Becausethe brain relies exclusively on glucose as a fuel source, brain function is rapidly compromised when circulating glucose levels drop below the normal range. Consequently, hypoglycemia elicits a robust, integrated, and redundant set of counterregulatory responses (CRRs) that ensure the rapid and efficient recovery of plasma glucose concentrations into the normal range (1). Components of the CRR include increased secretion of the hormones glucagon, epinephrine, and glucocorticoids, inhibition of glucose-induced insulin secretion, increased sympathetic nervous system (SNS) outflow to the liver, and increased food intake (1–3). Owing to this redundancy, recovery from hypoglycemia is difficult to block in normal humans and animal models, even when adrenal or glucagon responses are prevented. Only when multiple responses are blocked is the ability to recover from hypoglycemia significantly compromised (4). This arrangement is perhaps unsurprising, given the threat to survival posed by hypoglycemia.

Although glucose sensing can occur at peripheral (e.g., neurons innervating the hepatic portal vein) as well as central sites (3, 5), the brain is the organ responsible both for transducing this information into effective glucose counterregulation and for terminating this response once euglycemia is restored. Of the many brain areas that have been investigated, the hypothalamic ventromedial nucleus (VMN) has emerged as potentially being both necessary and sufficient to elicit this powerful response. This assertion is based on evidence that, whereas electrical stimulation of the VMN activates the CRR and thereby raises circulating glucose levels (6), glucose infusion directly into the VMN can suppress the CRR during hypoglycemia (7) and thereby impair recovery of normal blood glucose levels (8). Moreover, two recent papers identified a circuit comprised of neurons in the lateral parabrachial nucleus (LPBN) that project to the VMN, activation of which seems to be required for effective glucose counterregulation (9, 10). The relevant VMN neurons seem to be glutamatergic because genetic disruption of glutamatergic signaling within a specific subset of VMN neurons [known as steroidogenic factor-1 (SF1) neurons] also attenuates recovery from insulin-induced hypoglycemia (11).

Together, these observations support a model in which, during hypoglycemia, inputs from the LPBN and other glucose-responsive neurocircuits converge upon and activate VMN neurons, and in which this activation is required for effective glucose counterregulation. Whether these VMN neurons or any other specific neuronal subset(s) are truly necessary or sufficient for this important adaptive response has yet to be established, however. Recent technological advances, including the use of optogenetics to selectively activate or inactivate neurons in a regionally and temporally specific manner, now enable such questions to be addressed (12).

A relevant parallel can be drawn to knowledge recently gained regarding control of feeding behavior by neurons that express Agouti-related peptide (Agrp), found in the adjacent hypothalamic arcuate nucleus. Since their discovery more than a decade ago (13, 14), innumerable papers were published implicating these neurons as key drivers of fasting-induced hyperphagia (15, 16), but whether they are in and of themselves necessary or sufficient for normal food intake was unknown until recently. The past few years have shown (among other things) that selective activation of Agrp neurons is sufficient to potently drive intake whereas inhibition of these neurons blunts the effect of fasting to stimulate feeding (17, 18). These findings offer direct, compelling support for the hypothesis that Agrp neurons are primary drivers of need-based feeding. Whether activation of VMNSF1 neurons is both necessary and sufficient to explain the powerful adaptive responses elicited by hypoglycemia is a question of similar importance. Given

 significance

Hypoglycemia is an important and frequently encountered complication of diabetes treatment. Here, we identify a subset of neurons located in the ventromedial hypothalamic nucleus, activation of which is both necessary and sufficient to mediate adaptive counterregulatory responses to hypoglycemia that return low blood glucose levels into the normal range. These neurons receive ascending input from neurons in the lateral parabrachial nucleus and in turn control blood glucose levels via projections to the anterior bed nucleus of the stria terminals. Together, this work identifies a previously unrecognized functional neurocircuit involved in glycemic control.


This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1521160113/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1521160113
the many sites that participate in glucose sensing (5, 19, 20) and how difficult it is to prevent recovery from hypoglycemia (owing to redundancy inherent in the CRR), it seems improbable that the entire response should hinge on activation of a select population of neurons in a relatively small brain area. To test this hypothesis, we first determined whether optogenetic inactivation of VMNSF1 neurons during insulin-induced hypoglycemia blocks increased glucagon and corticosterone secretion, inhibition of glucose-induced insulin secretion, and recovery of normal blood glucose levels. We also asked whether, conversely, optogenetic activation of VMNSF1 neurons elicits hyperglycemia by activating the CRR in otherwise normal mice. Last and perhaps most importantly, we sought to identify projections of VMNSF1 neurons to downstream brain areas involved in glucose counterregulation and to determine whether these VMNSF1 neuronal subsets make synaptic contacts with the previously identified upstream neurons located in the LPBN. Our data collectively indicate that (i) VMNSF1 neurons are key components of a circuit that extends from the LPBN to the VMN and then to the anterior bed nucleus of the stria terminalis (aBNST) (LPBN→VMN→aBNST), (ii) VMNSF1 neurons function as a primary motor output driving the CRR, and (iii) activation of VMNSF1 neurons is required for effective recovery from insulin-induced hypoglycemia.

Results
VMNSF1 Neurons Are Required for Intact Responses to Insulin-Induced Hypoglycemia. Because of the threat to survival posed by inadequate glucose delivery to the brain, the CRR to hypoglycemia is mediated by a highly integrated, redundant, and powerful combination of behavioral, autonomic, and neuroendocrine responses that are conserved across mammalian species. To investigate the physiological role played by VMNSF1 neurons in this response, we sought to determine whether activation of these neurons is required for the ability to recover from hypoglycemia. To this end, we expressed a light-activated inhibitory channel selectively in VMNSF1 neurons. This objective was achieved through bilateral stereotaxic microinjection of an adeno-associated viral construct (AAV) into the VMN of SF1-Cre-positive (SF1-Cre+) mice (in which Cre recombinase is expressed under the control of the VMN-specific SF1 promoter) (21). The AAV expresses a modified channelrhodopsin chloride-conducting anion channel fused with the fluorescent reporter EYFP (AAVDJ8-DIO-SwiChRCA-EYFP, referred to hereafter as “SwiChRCA virus”) and is “Cre-inducible,” meaning that viral expression is restricted to cells that also express Cre recombinase. Through an optic fiber implanted immediately dorsal to each VMN injection site (Fig. 1A–C), delivery of light (“Laser On”) causes hyperpolarization of VMNSF1 neurons, but not other neurons in the VMN or surrounding brain areas (Fig. 1C).

We report that, whereas photoinhibition of VMNSF1 neurons had no effect on fasting blood glucose levels in the basal state relative to the “Laser-Off” condition (Fig. 1G), the ability of mice to recover from insulin-induced hypoglycemia was severely impaired by light-induced silencing of VMNSF1 neurons (Fig. 1D). Thus, activation of a discrete and uniquely identified subpopulation of VMN neurons is required to effectively recover from hypoglycemia. By comparison, VMNSF1 neuron activation is not required for maintenance of normal blood glucose levels in the basal state (e.g., in the absence of a threat to brain glucose delivery).

The striking inability of mice to respond to insulin-induced hypoglycemia when VMNSF1 neurons are silenced implies that their activation is required for secretion of counterregulatory hormones in this setting. Consistent with this hypothesis, we found, in a separate experiment, that the increase of circulating glucagon and corticosterone levels that normally occurs during hypoglycemia was blocked when VMNSF1 neurons were silenced (Fig. 1E and F). These data demonstrate that activation of VMNSF1 neurons is indispensable for an intact CRR to hypoglycemia, as previously hypothesized (11), and thus constitute direct evidence of a physiological role for these neurons in glucose homeostasis.

Photoactivation of VMNSF1 Neurons Induces Hyperglycemia. Based on our finding that activation of VMNSF1 neurons is required for an intact response to hypoglycemia, we hypothesized that activation of these neurons in otherwise normal mice will trigger the CRR and thereby cause blood glucose levels to increase. To test this
hypothesis, we expressed a light-activated excitatory channel selectively in VMN SF1 neurons through unilateral stereotaxic microinjection of a Cre-dependent, AAV-expressing channelrhodopsin-EYFP virus (AAV5-DIO-ChR2-EYFP) into the VMN of SF1-Cre+ mice, followed by implantation of an optic fiber above the injection site (Fig. 2A and B). As expected, EYFP fluorescence was restricted to the VMN of SF1-Cre+ mice, and, 1 h after photostimulation of these neurons, a robust induction of c-Fos (a marker of neuronal activation) was detected in the VMN and surrounding hypothalamic areas (Fig. 2C). As predicted, photostimulation of VMN SF1 neurons raised blood glucose levels rapidly and markedly, with glycemia returning to baseline values within 1 h after cessation of photostimulation (Fig. 2D). Because this effect was not observed in Cre-negative littermate controls (VMNControl) that underwent the same viral microinjection and light exposure procedure (Fig. 2E), we conclude that the observed hyperglycemic response was a specific consequence of VMN SF1 neuron activation. To further characterize the effect of VMN SF1 neuronal activation on glucose homeostasis, we performed an intraperitoneal (i.p.) glucose tolerance test (IPGTT) in both the presence and absence of unilateral photostimulation of VMN SF1 neurons. Glucose tolerance was markedly impaired by VMN SF1 neuron activation (Fig. 2F) (GluAUC; Laser Off, 21,560 ± 904 vs. Laser On, 39,659 ± 4,253; *P < 0.05), further establishing its powerful diabetogenic effect. To determine whether increased hepatic gluconeogenesis contributes to this impairment of glucose tolerance, we performed a pyruvate tolerance test (PTT) in a separate cohort of SF1-Cre+ mice in both the presence and absence of VMN SF1 neuron photostimulation. Our finding that the rise of blood glucose levels in response to a pyruvate challenge was markedly increased during activation of VMN SF1 neurons (Fig. 2G) (GluAUC; Laser Off, 13,723 ± 897 vs. Laser On, 20,855 ± 903; *P < 0.05) implicates increased hepatic gluconeogenesis in the hyperglycemia and glucose intolerance observed in this setting.

The VMN comprises a heterogeneous population of neurons, many of which express SF1. A large subset of VMN neurons also express the long form of the leptin receptor (LepRb), and a majority of these neurons also express SF1 (21, 22). To determine whether activation of LepRb-expressing neurons in the VMN (VMNLepRb) also causes hyperglycemia, we microinjected the Cre-dependent channelrhodopsin virus into the VMN of leptin receptor-IRESCre (LeprCre-+) mice (Fig. 3A–C) so as to enable photoactivation of VMNLepRb neurons. Successful transduction of these neurons was confirmed by observing EYFP fluorescence in the VMN, with EYFP fluorescence also detected in a few cells in adjacent areas, including the dorsomedial hypothalamus (DMH) and arcuate nucleus (ARC) (Fig. 3C). However, little or no c-Fos was colocalized with transduced LepRb neurons outside the VMN after photostimulation (Fig. 3C). Thus, our photostimulation protocol seems to have effectively targeted only VMNLepRb neurons and not adjacent hypothalamic LepRb subsets.

In contrast to the potent diabetogenic effect of VMN SF1 neuron activation, photostimulation of VMNLepRb neurons did not affect blood glucose levels (Fig. 3D), despite the fact that VMNLepRb neurons were clearly activated by the photostimulation protocol and that many VMNLepRb neurons coexpress SF1 (21, 22). These observations suggest that a subset of VMN SF1 neurons that do not express LepRb are responsible for the hyperglycemic effect observed during VMN stimulation. Alternatively, it is possible that VMN, ventromedial hypothalamic nucleus. Blood glucose levels before (Pre), during (Stim) and 1 h after (Post) unilateral stimulation of VMN SF1 neurons in (D) SF1-Cre+ mice and (E) VMNControl animals (Cre-negative) (n = 5–8 per group). *P < 0.05 vs. Laser Off. All data are expressed as mean ± SEM.
activation of a subset of VMN<sub>lepRb</sub> neurons that do not express SF1 exerts an inhibitory effect on those neurons that do, and thereby blocks the activation of responses underlying hyperglycemia.

Neuroendocrine Effects of VMNSF1 Photostimulation. To explain how VMNSF1 neuron photostimulation elicits its robust diabetogenic effect, we hypothesized a role for both an autonomic mechanism, whereby glucose-induced pancreatic insulin secretion is blocked, and a neuroendocrine component involving increased secretion of counterregulatory hormones. To test the former hypothesis, we measured plasma insulin levels before and during photostimulation of VMNSF1 neurons. Despite the marked hyperglycemia elicited by activation of VMNSF1 neurons (Fig. 4E and F), plasma insulin levels remained at their normoglycemic baseline (Laser Off) (Fig. 4B). As expected, neither blood glucose nor plasma insulin levels were affected by light stimulation of the VMN of either VMN<sup>lepRb</sup> or VMN<sup>control</sup> mice (Fig. 4A). Neuropeptide expression and response to photostimulation of VMNSF1 neurons were also detected in both the CeA and PVN; in the latter, innervation was particularly robust in medial regions along the border of the third ventricle (Fig. 5E–F). Because these findings are consistent with previous work using standard tract-tracing methods to detect projections from unspecified VMN neurons (27), the distribution of projections from the subset expressing SF1 does not seem to differ significantly from the population of VMN neurons overall.

To investigate whether functional connectivity exists between cell bodies of SF1 neurons in the VMN and any of these four downstream innervation sites, we used an optogenetics approach in which a ChR2-expressing virus was unilaterally microinjected into the VMN of SF1<sup>Cre</sup> mice, followed by histological imaging to detect the EYFP reporter in axonal projections (Fig. 5A–E). Labeled projections of VMNSF1 neurons were detected in both ipsilateral and contralateral PAG, particularly in the dorsal region superior to the cerebral aqueduct. In the BNST, fibers arising from VMNSF1 neurons were detected in the dorso-medial region, along with a dense plexus in the anterior BNST and the anterior-medial subdivision (aBNST). Projections of VMNSF1 neurons were also detected in both the CeA and PVN; in the latter, innervation was particularly robust in medial regions along the border of the third ventricle (Fig. 5B–E). Because these findings are consistent with previous work using standard tract-tracing methods to detect projections from unspecified VMN neurons (27), the distribution of projections from the subset expressing SF1 does not seem to differ significantly from the population of VMN neurons overall.
Collectively, these findings suggest that the glucoregulatory subset of SF1 neurons project specifically to the aBNST.

Consistent with the interpretation that VMNSF1→aBNST stimulation recapitulates the glycemic response to VMNSF1 neuronal activation, this effect was accompanied by elevated plasma levels of both corticosterone and glucagon (Fig. 5 N and R) and with inhibition of glucose-induced insulin secretion, such that plasma insulin levels did not increase despite the rise of blood glucose concentrations (Fig. 5 F and J). By comparison, no effect on levels of blood glucose, plasma insulin, or plasma glucagon was detected during stimulation of the VMNSF1 neuron fibers supplying the PVN although a nonsignificant increase of plasma corticosterone levels was observed (Fig. 5 F–R). Collectively, these observations implicate the subset of VMNSF1 neurons that project to the aBNST, but not to the PAG, CeA, PVN, or mBNST, as components of a functional glucoregulatory circuit.

To localize the cell bodies of VMNSF1 neurons that project to the aBNST, we used a two-pronged strategy. First, we injected cholera toxin subunit B (CTB), a fluorescently labeled retrograde neuronal tracer, into the aBNST and examined fluorescence within the VMN (Fig. 6 A and B). As expected, we found dense CTB expression throughout the VMN, supporting previous evidence that axonal projections from neuronal cell bodies of aBNST-projecting VMNSF1 neurons. CTB was also detected during stimulation of the VMNSF1 neuron fibers supplying the PVN although a nonsignificant increase of plasma corticosterone levels was observed (Fig. 5 F–R). Collectively, these observations implicate the subset of VMNSF1 neurons that project to the aBNST, but not to the PAG, CeA, PVN, or mBNST, as components of a functional glucoregulatory circuit.

We next identified cell bodies of neurons in the VMN that were activated (as assessed by expression of c-Fos) after photostimulation of axonal projections of VMNSF1 neurons supplying the aBNST (Fig. 6 E–H). The c-Fos was somewhat widespread after activation of the aBNST projections, mimicking channelrhodopsin expression, with little c-Fos staining observed in the absence of light treatment (Fig. 6I). The resultant c-Fos (+) neurons were concentrated in the central (c) and dorsomedial (dm) regions of the VMN (Fig. 6 J–N), areas previously implicated in metabolic regulation (30, 31). By comparison, few activated neurons were present in the ventrolateral (vl) portion of the VMN, an area associated with reproduction and aggressive behavior (32–35). Together, these findings implicate subsets of SF1 neurons located in central and dorsomedial regions of the VMN in glycemic control (10).

The LPBN Sends Afferents to aBNST-Projecting VMNSF1 Neurons. In addition to the VMN, recent evidence suggests that the CRR to hypoglycemia involves a subset of neurons in the LPBN marked by coexpression of leptin receptor and CCK (9, 10). Because these LPBN LepRbCCK neurons also project to the VMN, we sought to determine whether they are anatomically coupled to VMNSF1 neurons identified herein as having a physiological role in glucose counterregulation. To first verify that neurons in the LPBN project to VMN, we injected CTB to the VMN and, 4 d later, examined the brain for alexa-488 fluorescence label. Included among several sites upstream of the VMN identified by this approach were the medial preoptic area, amygdala, and BNST (Fig. S3), as well as the LPBN (Fig. 7 A–C).

We next sought to determine whether the subset of VMNSF1 neurons that project to the aBNST are synaptically contacted by LPBN neurons. Accomplishing this goal was made possible through the use of a two-virus strategy. The first virus is a modified rabies viral construct, SADΔG-EGFP, engineered to lack the gene encoding rabies glycoprotein G that is required for transsynaptic spread (36), and packaged in a single envelope glycoprotein (EnvA). The second virus is a Cre-dependent AAV-expressing TVA construct (that expresses the receptor for the avian sarcoma leucosis virus glycoprotein, EnvA) linked with RG (rabies envelope glycoprotein) using a 2A-cleavage peptide, AAV8-DIO-TVA-RG (TVA-RG). With this strategy, microinjection of TVA-RG into the VMN of SF1-Cre+ mice selectively renders VMNSF1 neurons (i) uniquely susceptible to infection by EnvA and (ii) capable of retrograde monosynaptic spread due to the presence of RG (Fig. 7D). By subsequently injecting the SADΔG-EGFP virus (EnvA) into the aBNST of the same mice, infection by the latter virus is restricted initially to VMNSF1 terminals projecting to this region previously transsected by the TVA-RC virus. Subsequently, the SADΔG-EGFP virus is able to cross a single synapse and thereby infect neurons upstream of aBNST-projecting VMNSF1 neurons.
Fig. 5. Photoactivation of VMN^SF1^→aBNST projections selectively promote hyperglycemia in nondiabetic mice. (A) Schematic showing unilateral stereotaxic micro-injection of the Cre-dependent light-activated channelrhodopsin (ChR2-EYFP) virus into the VMN of SF1-Cre^+^ mice (i.e., VMN^SF1^ neurons) and implantation of the optic fiber above each of four projection sites in separate cohorts of animals. Image adapted from connectivity.brain-map.org; experiment ID 182337561. aBNST, anterior bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; PAG, periaqueductal gray; PVN, paraventricular nucleus; VMN, ventromedial nucleus. Fluorescently labeled projections of channelrhodopsin in VMN^SF1^ neurons detected in terminal targets including the (B) aBNST, (C) PVN, (D) CeA, and (E) PAG. (Magnification: 4×) 3V, third cerebral ventricle; CA, cerebral aqueduct. (F–I) Blood glucose, (J–M) plasma insulin, and (N–Q) plasma corticosterone (CORT) levels before (Pre), during (Stim), and 1 h after (Post) photoactivation of VMN^SF1^ neuron axon projection fields. (aBNST, n = 7; PVN, n = 6; CeA, n = 5; and PAG, n = 6 per group). ^*^P < 0.05 vs. Pre. (R) Plasma glucagon 1 h after photoactivation of each site. ^*^P < 0.05 vs. CeA and PAG; no significant difference from PVN. All data are expressed as mean ± SEM.
As expected, analysis of the brains of these mice revealed large numbers of EGFP (+) cells in the VMN, potentially representing not only those VMNSF1 neurons initially infected with TVA-RG but also local VMN cells that synapse onto these neurons (Fig. 7E). We also detected EGFP (+) neurons in the LPBN as well as several other brain regions (Fig. S4). Specifically, labeled neurons within the PBN were limited to the central, external, and ventral aspects of the LPBN, with none detected in either the superior LPBN or the mediobasal PBN (Fig. 7F). These observations demonstrate that a subset of LPBN neurons make synaptic connections with those VMNSF1 neurons that project to the aBNST.

**Discussion**

To cope with the brain’s exclusive reliance on glucose as a fuel, a sophisticated system has evolved to detect and respond to the threat posed by reduced glucose availability. The brain clearly plays a central role in this regulatory system, and, whereas previous studies have implicated a role for the VMN, the underlying neurocircuitry remains largely unidentified. Here, we report a crucial role for VMNSF1 neurons in the physiological response to hypoglycemia and identify them as part of an ascending glucoregulatory network (Fig. S5). These observations offer direct evidence of a previously unrecognized neurocircuit involved in glycemic control.

A key finding is that, whereas inhibition of VMNSF1 neurons has no glucose-lowering effect under basal conditions, it severely impairs recovery from hypoglycemia because it blocks the powerful CRRs that normally restore low blood glucose levels into the normal range. Conversely, activation of VMNSF1 neurons in otherwise normal mice causes marked hyperglycemia, it makes physiological sense for these neurons to remain inactive unless blood glucose levels are threatened. In the latter setting, however, inhibition of VMNSF1 neurons has devastating consequences because it blocks the powerful CRRs that normally restore blood glucose levels into the normal range (11, 30), as expected for neurons that function as a primary motor output for raising blood glucose levels when glucose availability is threatened.

Details regarding the cellular phenotype of VMNSF1 neurons involved in this response await further analysis. Recent work has focused on the role of intracellular glucose sensors, neurotransmitters such as gamma-aminobutyric acid (GABA), glutamate, serotonin, and neuropeptides [corticotrophin-releasing hormone (CRH) and urocortins] (1, 30). Most VMNSF1 neurons are glutamatergic (39, 40), and previous evidence points to a role for glutamate release from these neurons in the CRR response (11). However, our data suggest that not all VMNSF1 neurons participate in glucose counterregulation because photoactivation of VMNSFLepRd5 neurons, many of which express S1 and are also glutamatergic (21, 22), was without effect on either glycemia or secretion of counterregulatory hormones. To shed additional light on the subset of VMNSF1 neurons involved in glucose homeostasis, we used fluorescently tagged channelrhodopsin to characterize the circuit architecture. Consistent with previous studies (27), we found that the heaviest projections of VMNSF1 neurons terminate in the PAG, CeA, PVN, and aBNST. Our finding that photocistimulation of VMNSF1 neurons that project to the aBNST, but not to the PAG, PVN, CeA, or mBNST, mimics the glycemic response elicited by VMNSF1 neuronal stimulation offers clear evidence that projections of VMNSF1 neurons to the aBNST contribute to observed effects on glucose homeostasis. Moreover, the glucose-raising effect of VMNSF1→aBNST fiber activation was accompanied by hormonal responses similar to those induced by stimulation of VMNSF1 cell bodies, including increases of plasma glucagon and corticosterone levels and inhibition of glucose-stimulated insulin secretion. Collectively, these observations implicate axonal projections to the aBNST in the glycemic response observed after photocistimulation of VMNSF1 cell bodies.

Our finding that photocistimulation of VMNSF1→aBNST axons induces activation of VMNSF1 neuron cell bodies predominantly in the dmVMN and cVMN, and not the vlVMN, is consistent with established evidence implicating the dmVMN and cVMN in autonomic control of metabolism whereas neurons in the vlVMN participate in the control of aggression and reproduction.
These findings collectively support the hypothesis that VMNSF1 neurons involved in glucose homeostasis (i) are located in dmVMN and cVMN, (ii) project to the aBNST, and (iii) are distinct from those neurons expressing leptin receptors. In addition to the VMN, recent work implicates a subset of neurons situated in the LPBN in the CRR to hypoglycemia. These LPBN neurons express both leptin receptor and CCK, and, like photoactivation of VMNSF1 neurons, pharmacogenetic activation of LPBN LepRbCCK neurons raises blood glucose levels in association with increased secretion of glucagon and corticosterone. Conversely, inhibition of LPBN LepRbCCK neurons blunts the glycemic response to glucoprivation (9, 10), an effect resembling the consequences of optogenetic silencing of VMNSF1 neurons during insulin-induced hypoglycemia in the current studies. Because these LPBN LepRbCCK neurons project to the dmVMN and cVMN and because the induction of CRRs after activation of PBN LepRbCCK neurons is attenuated by pharmacogenetic inhibition of VMNSF1 neurons (10), we hypothesized that the former neurons provide ascending, stimulatory input to the subset of VMNSF1 neurons that project to the aBNST.

In support of this hypothesis, we found that aBNST-projecting VMNSF1 neurons are synaptically connected to neurons in the LPBN, among other regions. These data offer direct evidence in support of an LPBN→VMNSF1→aBNST neurocircuit involved in glycemic regulation, and we anticipate that future studies will demonstrate that LepRbCCK neurons are among those LPBN neurons involved in this circuit. Interestingly, several areas additional to the LPBN send afferents to the subset of VMNSF1 neurons that project to the aBNST. How this information is processed and the extent to which these projections contribute to glucose homeostasis are questions that await additional study.

The liver plays a key role as the primary source of circulating glucose mobilized by CRRs to hypoglycemia. Activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased glucagon secretion (41) each raise blood glucose levels by increasing HGP via increases of both glycogenolysis and gluconeogenesis. The latter can be assessed indirectly by the expression of the hepatic gluconeogenic genes G6Pase and Pepck, and we found that hyperglycemia induced by photoactivation of VMNSF1 neurons is associated with increased hepatic expression of both genes, suggesting that increased hepatic gluconeogenesis may have contributed to hyperglycemia elicited by photoactivation of VMNSF1 neurons. Consistent with this hypothesis, we found that the glycemic excursion in response to a pyruvate challenge was also markedly increased by photoactivation of VMNSF1 neurons.

Additional evidence of a role of the VMN in these responses (42, 43) includes the finding that electrical stimulation of the mediobasal hypothalamus elicits hyperglycemia (6), driven in
part by increased glucagon secretion (44). Similarly, glucagon release is triggered by administration of 2-deoxyglucose (2-DG) locally into the VMN to induce neuroglucopenia in this brain area (45). Conversely, the glucagon response to systemic hypoglycemia is blocked by intra-VMN administration of glucose (7) and in rats with bilateral VMH lesions (46). Combined with our findings that photoinhibition of VMNSTF1 neurons blocks the plasma glucagon and corticosterone response to hypoglycemia, whereas photoactivation of these neurons has the opposite effect, we conclude that a neuronal network involving VMNSTF1 neurons is critically involved in the CRR to hypoglycemia.

In addition to increased counterregulatory hormone secretion, plasma insulin levels were unchanged during photoactivation of VMNSTF1 neurons, despite an associated, dramatic increase of blood glucose levels. Although this failure to raise plasma insulin levels may seem unimpressive, the observed increase of blood glucose constitutes a powerful stimulus to insulin secretion, and increased SNS outflow to the pancreas is among very few mechanisms capable of blocking this response in normal animals. From a teleological perspective, it makes intuitive sense that, when glucose availability is threatened, responses that increase glucose production would be activated (e.g., elevated glucagon and corticosterone levels) in concert with responses that reduce glucose clearance (e.g., inhibition of insulin secretion), and much of the literature indicates that pancreatic beta cells are subject to inhibitory control via the sympathetic nervous system (SNS) (47, 48). For example, bilateral VMN lesioning induces hyperinsulinemia (49, 50) whereas electrical stimulation of the VMN suppresses glucose-induced insulin secretion (51). Further, the VMN is known to regulate autonomic outflow to the pancreas (52, 53), and activation of islet sympathetic nerves during glycopenic stress (54) inhibits insulin secretion via a mechanism involving activation of α2-adrenoceptors on the beta cell (47, 48). However, the neurocircuity that underlies inhibitory control of insulin secretion by the brain remains unknown.

In conclusion, we report that optogenetic silencing of VMNSTF1 neurons profoundly impairs recovery from insulin-induced hypoglycemia by blocking powerful neuroendocrine and autonomic components of the CRR. These findings offer unambiguous evidence that activation of VMNSTF1 neurons is required for effective glucose counterregulation. At the same time, tonic inhibition of these neurons seems to be permissive for maintenance of glucose homeostasis under usual conditions because photoactivation of VMNSTF1 neurons rapidly induces diabetes-range hyperglycemia with impaired glucose tolerance, owing to a combination of increased CRR hormone secretion and inhibition of glucose-induced insulin secretion. A subset of VMNSTF1 neurons that project to the aBNST and are anatomically linked to upstream neurons in the LPBN are implicated in this glucoregulatory neurocircuit. An improved understanding of the functional organization of this neurocircuit may help to identify future strategies for prevention of both insulin-induced hypoglycemia and hypoglycemic unawareness, two of the most common and costly complications of diabetes treatment (55).

**Experimental Procedures**

**Animals.** All procedures were performed in accordance with NIH guidelines for the care and use of animals and were approved by the Animal Care Committee at the University of Washington. All studied animals were individually housed in a temperature-controlled room with a 12:12-h light:dark cycle under specific-pathogen free (SPF) conditions and provided with ad libitum access to water and chow unless otherwise stated (PMI Nutrition). SFlCre mice (approved mouse gene name, Nr5a1) have been generated and described previously (21, 22) and were bred in our colony (56) whereas Lepr-IRESCre mice were purchased from The Jackson Laboratory (stock no. 008320).

**Viral Generation, Injections, and Fiber Placement.** The viral vectors AAV5-EF1α-DIO-HChR2(H134R)-EYFP and AAVJD8-EF1α-DIO-SwiChR3-TS-EYFP-WPRE used in these studies have been described previously (57–59). All viruses were packaged at the Gene Therapy Center at the University of North Carolina, except the SwiChR2 virus, which was packaged into a D28 AAV vector by the University of Washington Diabetes Research Center Viral Vector and Transgenic Mouse Core. The Cre-inducible EF1α-DIO-SwiChR3-TS-EYFP-WPRE construct was kindly provided by Karl Deisseroth (Stanford University, Stanford, CA) (57). Details regarding stereotaxic delivery of viruses to specific brain areas is provided in SI Experimental Procedures.

**Optogenetic in Vivo Photostimulation and Photoinhibition.** Optogenetic studies were supported by the Nutrition Obesity Research Center (NORC) Energy Balance and Glucose Metabolism Core at the University of Washington. For delivery of light pulses with millisecond precision, the output beam from a diode laser (473 nm, DPSS continuous wave laser system; Laserglow) was controlled using an AMPI Master-9 stimulator (Laserglow) through a single fiber port (17, 60). The light was then split using multimode optical fibers with a 200-μm diameter core, N.A. 0.22 (Thor Laboratories), and passed through a fiber optic rotary joint (Thor Laboratories). A terminal fiber attached to the rotary joint was fixed to a 230-μm-bore ceramic ferrule and a mating sleeve that allowed delivery of light to the brain through coupling with a 200-μm ferrule-capped fiber implanted within the mouse brain. Unless otherwise indicated, for all in vivo photostimulation experiments, 5-ms pulses, 40 pulses per s, were used for 1 h, which is slightly higher than the traditional stimulation patterns used with electrical activation of the VMN (6, 61). Photoinhibition experiments used a constant beam of light for 1–2 h as indicated for each experiment. Irradiance at target regions was estimated at 21 W/mm² for photostimulation and 10 W/mm² for photoinhibition experiments based upon the previously described relationship of light scattering in the brain of mammals with light power exiting the fiber tip corresponding to 8 mW and 4 mW for activation and inhibition, respectively (web.stanford.edu/group/diabregi/graphic/chart.php). The nature of the numerical aperture (N.A. 0.22), a measurement dictating the range of angles light is transmitted along, allowed a greater depth of light penetration into tissue while resulting in a narrow illumination cone. Based on these calculations and the placement of the optic fiber (0.4 mm dorsal to the VMN), the intensity of light reaching the lateral ventral portion of the VMN likely approached the minimum threshold to result in regular burst firing (1 W/mm²) (17, 60). However, in regions that receive an asymmetric distribution of VMN terminals along a transverse plane, the stimulation of fibers adjacent rather than directly below the fiber would have been limited.

**Viral Track-Tracing Studies.** To investigate the origin of BNST or VMN afferent projections, 150 nL of 1 mg/mL cholera toxin b subunit conjugated with Alexa dye 488 (CTB-488; Life Technologies) in PBS was injected into the region of interest at 50 nL/min. Animals were perfused 4 d after injections to harvest brains for histological analysis.

For retrograde rabies-tracing studies, a modified rabies viral construct, AAV-DIO-TVA-KG (TVA-RG), serotype 8, was injected unilaterally into the VMN (200 nL) of 7- to 8-wk-old mice (titer 1.1 × 10¹⁵ genome copies per milliliter). Twenty-one days later, SADΔEG-EFP (EnvA) rabies was unilaterally injected into the aBNST (100 nL) followed by a 10-min wait. One week after SADΔEG-EFP (EnvA) rabies injection, mice were perfused, and brains were mounted for sectioning as described below.

**Metabolic Studies and Tissue Processing.** Intraperitoneal glucose tolerance tests (GTTs) (2 g/kg body weight) or pyruvate tolerance tests (PTTs) (2 g/kg body weight) were conducted in 6-h fasted animals. Tail vein blood was collected for measurement of blood glucose levels at the times indicated and as described in the SI Experimental Procedures.

**Immunohistochemistry and RT-PCR.** Details for immunostaining and gene expression estimation can be found in SI Experimental Procedures and as previously described (62, 63).

**Statistical Analyses.** All results are presented as means ± SEM. Statistical analyses were performed using PASW Statistics (version 18; IBM SPSS). P values for pairwise comparisons were calculated by two-tailed Student’s t test. Data for comparisons across more than two groups were analyzed using a one-way ANOVA with post hoc comparisons, where appropriate. Time course comparisons between groups were analyzed using a two-way repeated-measures ANOVA with main effects of light (laser on/off) and time (6–8 instances). All post hoc comparisons were determined using Sidak’s correction for multiple comparisons. In all instances, probability values of <0.05 were considered significant.

**ACKNOWLEDGMENTS.** We acknowledge the technical expertise provided by Alexis Cubelo, Annelisse Paige Matsuo, Justin Ngoc Lam, Loan Nguyen, and...
Jonathan D. Fischer (University of Washington). This work was supported by National Institutes of Health Grants F32 DK083042, DK090320, and DK101997 (to M.W.S.), and DK098853 (to M.G.M.), by NIH K01 Career Development Award DK097859 (to T.H.M.), by the Nutrition Obesity Research Center (NORC Grant DK053816), by the Diabetes Research Center (DRC Grant DK70471), and by Diabetes and Metabolism Training Grants F32 DK097859 and T32 DK000724 (University of Washington) and the Michigan Diabetes Research Center (DRC Grant DK20572).


Animals were placed in a stereotaxic frame (Kopf 1900; Cartesian Research Inc.) under isoflurane anesthesia, and the skull was exposed via a small incision. A small hole was drilled for unilateral 400-nL injection volume of AAV5-EF1α-DIO-hChR2(H134R)-EYFP or AAVD8-EF1α-DIO-SwiChR2CA-TS-EYFP-WPRE virus as previously described (62, 64) using a Hamilton syringe (80030) with a 33-gauge needle at a rate of 100 nL/min (Micro4 controller) followed by a 7-min wait before needle withdrawal. All injections were directed toward the VMN of SF1-Cre (VMN$^{SF1}$), Lepr-ires-Cre (VMN$^{Lepr}$), or WT littermate control mice (VMN$^{Control}$) at stereotaxic coordinates from bregma based on coordinates from the Mouse Brain Atlas (65): anterior-posterior (AP), −1.4 mm; lateral, 0.5 mm right side only, dorsal-ventral (DV), 5.3 mm. After viral injections, the fiber-ferrule was then placed above the VMN (AP, −1.4 mm; lateral, 0.5 mm right side only, DV, 4.9 mm). For postoperative care, mice were injected intraperitoneally with buprenorphine hydrochloride (0.05 mg/kg; Reckitt Benckiser). Mice were then allowed 2 wk to recover and acclimated to handling for 1 wk before the start of any in vivo studies. Fiber placement was verified in all animals in which glucose metabolic data were generated, and any data from animals in which the fiber was located outside the targeted area were excluded from the analysis.

**Metabolic Studies.** To investigate the effect of selective activation or inhibition of VMN neurons on glycemic control, we used a 3-h approach with alternating light off/on sequences in SF1-Cre (VMN$^{SF1}$), Lepr-ires-Cre (VMN$^{Lepr}$), and WT littermate control mice (VMN$^{Control}$) mice. After a week of acclimation, food was removed, and mice were attached to a tethering cable for 1 h. Blood glucose was then determined using a hand-held glucometer (AP, −15, 0, 15, 30, 60, 90, and 120 min from the tail vein after insulin injection (64, 66, 67).

**Blood Collection and Tissue Processing.** At study completion, after 1 h of light photostimulation or inhibition, animals were decapitated, trunk blood was collected in appropriately treated tubes, and liver samples were harvested. Blood samples were centrifuged, and the plasma was removed, aliquotted, and stored at −80 °C for subsequent assay whereas liver samples were immediately frozen on dry ice and kept at −80 °C. Plasma insulin (Crystal Chem), corticosterone levels (Alpc0), and glucagon (Mercodia) were determined by ELISA.

**Immunohistochemistry.** For brain immunohistochemical (IHC) studies, anesthetized animals were perfused with PBS followed by 4% (vol/vol) paraformaldehyde (PFA) in 0.1 M PBS. Brains were removed, postfixed in 4% PFA, sucrose (25%)-embedded, and snap-frozen in isopentane cooled with dry ice. Brains were sectioned at 25 μM in the coronal plane throughout the region of interest, slide-mounted, and stored at −80 °C for IHC staining.

c-Fos immunostaining was carried out on perfused-fixed sections as previously published (62, 63). Briefly, slides were washed at room temperature with Tris PBS (TBS) followed by a blocking buffer (5% normal goat serum in 10 mM PBS) for 90 min, and by additional buffer washes. The primary antibody was rabbit polyclonal anti-c-Fos (Calbiochem) diluted 1:5,000 in 0.1% BSA in 10 mM PBS, and the secondary antibody was donkey anti-rabbit Alexa 594 (Jackson ImmunoResearch Laboratories) diluted 1:200 in 0.1% BSA in 10 mM PBS. cFos was enhanced with a goat polyclonal antibody (Fitzgerald) diluted 1:1000 in 0.1% BSA in 10 mM PBS and the secondary antibody was donkey anti-goat Alexa 488 diluted 1:500. Histochemical images were then captured using a Nikon Eclipse E600 upright microscope (Nikon) equipped with a Diagnostic Instruments Spot RT Color digital camera.

**RT-PCR.** Total RNA was extracted from liver using TRIzol B according to the manufacturer’s instructions (MRC). RNA was quantitated by spectrophotometry at 260 nm (Nanodrop 1000) and reverse-transcribed with AMV reverse transcriptase (Promega), and real-time PCR was performed on an ABI Prism 7900 HT (Applied Biosystems) as described previously (66). Expression levels of each gene were normalized to a housekeeping gene (18S RNA) and expressed as arbitrary units (A.U.) relative to vehicle controls.
Fig. S1. Activation of VMN projections selectively to the aBNST induces hyperglycemia. (A) Diagram showing a coronal section of the mouse brain at the level of the aBNST. (B) Representative image (magnified 4×) depicting scar tissue after an intraparenchymal injection at the coordinates used for aBNST fiber placement. (C, Left) Expression of fluorescently labeled neuronal tracer cholera toxin subunit B (CTB) in green at the injection site and along the needle track (indicated by white arrows). CTB infects all neurons (not cre-dependent) at the site of injection and is transported in the retrograde direction. (Middle) Red autofluorescence in the same tissue section, confirming the tissue damage seen under light microscopy. (Right) A merge of green and red filters. AC, anterior commissure; LV, lateral cerebral ventricle. Blood glucose before (Pre), during (Stim), and 1 h after (Post) unilateral (D) anterior BNST and (E) medial BNST stimulation of ChR2-EYFP 6 wk after viral injection into the VMN of SF1-Cre+ mice (n = 4 per group). *P < 0.05 vs. Pre. All data are expressed as mean ± SEM.
Fig. S2. Distribution of cholera toxin B throughout the brain after aBNST injection. (A) Schematic of cholera toxin B (CTB) injection into the aBNST of WT mice and tracing of the Alexa-488 fluorophore throughout the brain. See corresponding Fig. 6 for images of mediobasal hypothalamic areas. CTB within (B) the anterior olfactory nucleus (OFN), (C) the lateral septal nucleus (LSN), and (D) the medial preoptic nucleus (MPO) (at both 4x and 10x magnification). (E) CTB at the injection site within the BNST (4x magnification), (F) the medial amygdala (mAMG), (G) the paraventricular thalamic nucleus (PVT), and (H) the central gray (CG) (at both 4x and 10x magnification).
Fig. S3. Distribution of cholera toxin B in upstream brain regions after a VMN injection. (A) Schematic of cholera toxin B (CTB) injection into the VMN of WT mice and tracing of the Alexa-488 fluorophore throughout the brain. See corresponding Fig. 7 for images for lateral PBN. CTB within (B) the lateral septal nucleus (LSN), (C) the medial preoptic nucleus (MPO), (D) the BNST, (E) the medial amygdala (mAMG), and (F) the peripeduncular nucleus (PP) (at both 4x and 10x magnification).
Fig. S4. Distribution of rabies virus throughout the brain after infection of aBNST-VMN$^{SF1}$ efferent neurons. (A) Schematic of rabies virus procedure and outcome. See corresponding Fig. 7 for images of VMN$^{SF1}$ efferent neurons located within the LPBN. Rabies virus within (B) the lateral septal nucleus (LSN) (4x and 10x), (C) the pre-mammillary nucleus (PMN) (4x and 10x), (D) the peripeduncular nucleus (PP), and subiculum (S) (4x and 10x), and (E) the PVN, anterior hypothalamic area (AHA), and the medial amygdala (mAMG) (4x shows all three regions simultaneously, and 10x of each individually).
Fig. 55. Model for a physiological role for VMN<sup>SF1</sup> neurons in glucose homeostasis. Our data suggest that activation of VMN<sup>SF1</sup> neurons is necessary for counterregulatory responses to insulin-induced hypoglycemia, including increases in corticosterone and glucagon secretion and inhibition of glucose-induced insulin secretion. In addition, activation of VMN<sup>SF1</sup> neurons is sufficient to elicit hyperglycemia in otherwise normal mice. Moreover, these neurons receive ascending input from neurons in the lateral parabrachial nucleus (LPBN) and in turn control blood glucose levels via projections selectively to the anterior bed nucleus of the stria terminalis (aBNST).