Brain Insulin Action Regulates Hypothalamic Glucose Sensing and the Counterregulatory Response to Hypoglycemia

Running title: Brain Insulin Action Regulates Glucose Sensing

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Objective: An impaired ability to sense and appropriately respond to insulin-induced hypoglycemia is a common and serious complication faced by insulin-treated diabetic patients. This study tests the hypothesis that insulin acts directly in the brain to regulate critical glucose sensing neurons in the hypothalamus to mediate the counterregulatory response to hypoglycemia.

Research Design and Methods: To delineate insulin actions in the brain, neuron-specific insulin receptor knockout (NIRKO) mice and littermate controls were subjected to graded hypoglycemic (100, 70, 50, and 30 mg/dL) hyperinsulinemic (20 mU.kg\(^{-1}\).min\(^{-1}\)) clamps and non-hypoglycemic stressors (e.g., restraint, heat). Subsequently, counterregulatory responses, hypothalamic neuronal activation (with transcriptional marker c-fos), and regional brain glucose uptake (via \(^{14}\)C-2deoxyglucose autoradiography) were measured. Additionally, electrophysiological activity of individual glucose-inhibited (GI) neurons and hypothalamic glucosensing protein expression (GLUTs, glucokinase) were measured.

Results: NIRKO mice revealed a glycemia-dependent impairment in the sympathoadrenal response to hypoglycemia and demonstrated markedly reduced (70%) hypothalamic c-fos activation in response to hypoglycemia, but not other stressors. GI neurons in the ventromedial hypothalamus of NIRKO mice displayed significantly blunted glucose responsiveness (membrane potential and input resistance responses were blunted 66% and 80%, respectively). Further, hypothalamic expression of the insulin-responsive glucose transporter 4, but not glucokinase, was reduced by 30% in NIRKO mice while regional brain glucose uptake remained unaltered.

Conclusions: Chronically, insulin acts in the brain to regulate the counterregulatory response to hypoglycemia by directly altering glucose sensing in hypothalamic neurons and shifting the glycemic levels necessary to elicit a normal sympathoadrenal response.

Intensive insulin therapy markedly increases the risk of severe hypoglycemia in people with type 1 (1) and type 2 (2) diabetes. Thus, hypoglycemia is the rate-limiting step for tight glycemic management in diabetic patients. In response to hypoglycemia, glucose sensors in the central and peripheral nervous system coordinate efferent autonomic responses resulting in the release of key counterregulatory hormones—glucagon, norepinephrine, epinephrine, and cortisol. This coordinated response stimulates hepatic glucose output and restricts glucose utilization in order to increase blood sugar levels. Patients with diabetes often have an impaired ability to sense and respond to hypoglycemia (3-5) because several components of the counterregulatory response have been shown to be either absent (i.e. fall in insulin, rise in glucagon) or markedly blunted (i.e. the sympathoadrenal response) (6;7).

While hypoglycemia is caused by absolute or relative insulin excess, the role of insulin in regulating the counterregulatory response is unclear. Studies have demonstrated that
increased insulin levels may augment (8-11), diminish (12), or not change (13-16) the sympathoadrenal response to hypoglycemia. Given recent evidence indicating that insulin acts in the brain (17), some studies have investigated whether insulin’s putative actions in regulating the counterregulatory response might be mediated via actions in the central nervous system. Again, conflicting reports suggest that insulin may act centrally to enhance (18-20), reduce (21) or not alter (22) the sympathoadrenal response to hypoglycemia. If insulin acts in the brain, its likely site of action is glucose sensing neurons located in the ventromedial hypothalamus (VMH) (21;23-26). These glucose sensing neurons share metabolic similarities to other well-characterized glucose sensing cells (i.e. pancreatic β-cells), especially with regards to glucose transport and metabolism (27-29). Based on the expression of insulin receptors in the majority of glucosensing neurons in the VMH (30), it is postulated that brain insulin action may mediate its effects on central glucose sensing by regulating expression of glucose transporters (GLUTs) and/or glucokinase (GK).

In this study, the neuronal specific insulin-receptor knockout (NIRKO) mouse model, which chronically lacks central nervous system (CNS) insulin signaling (17;31), was used to investigate the role and mechanism by which brain insulin action regulates central glucose sensing and the counterregulatory response to hypoglycemia.

**RESEARCH DESIGN AND METHODS**

**Animals.** Mice homozygous for the floxed insulin receptor allele (IR<sup>lox-lox</sup>) were bred with transgenic mice that express Cre recombinase cDNA from the rat nestin promoter to generate (IR<sup>lox-lox</sup>:nestin-Cre<sup>+/−</sup>) nervous system specific insulin receptor knockout (NIRKO) mice (17). Genotypes were determined by PCR of tail DNA. Unless otherwise indicated, 2-4 month old NIRKO (IR<sup>lox-lox</sup>:nestin-Cre<sup>+/−</sup>) and littermate control (Control, IR<sup>lox-lox</sup>:nestin-Cre<sup>−/−</sup>) mice were used for these experiments. All mice were housed on a 12-hour light/dark cycle and fed a standard rodent chow (Mouse Diet 9F, PMI Nutrition International, St. Louis, MO) ad libitum. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Animal Studies Committee of Washington University.

**Hypoglycemic-hyperinsulinemic glucose clamps.** Mice anesthetized with ketamine/xylazine (87 and 13.4 mg/kg IP) were implanted with catheters (MRE 025, Braintree Scientific Inc., Braintree, MA) into both the right internal jugular and the left carotid or femoral artery. After a 5-7 day recovery period, hyperinsulinemic (20mU.kg<sup>−1</sup>.min<sup>−1</sup>) hypoglycemic clamps were performed in 5-hour fasted, awake, unrestrained, NIRKO and control mice (n=6-9 per group). To create various degrees of hypoglycemic stress, arterial blood glucose was measured at ten-minute intervals and the rate of intravenous 50% dextrose infusion was carefully adjusted to create equivalent levels of mild (70mg/dL), moderate (50mg/dL), and severe hypoglycemia (30mg/dL) as well as a euglycemic (110mg/dL) control. HPLC-purified [3]<sup>−</sup>H-glucose tracer (NEN Life Science Products Inc., Boston, MA) was infused (10μCi bolus and 0.1μCi/min) for the assessment of hepatic glucose production (32). Three blood samples for hepatic glucose production determination were taken during the basal period and again during the last half hour of the clamp. An additional blood sample was obtained for hormonal measurements during the basal period and at the end of the clamp.
**Brain Glucose Uptake.** Briefly, awake, unrestrained, cannulated NIRKO and littermate control mice (n=6-9 per group) underwent a 2-hour hyperinsulinemic (40mU/kg/min) hypoglycemic (30mg/dL) clamp protocol. At 45 minutes prior to the end of the clamp, a 5μCi bolus of 14C 2-deoxyglucose was rapidly infused intravenously and ten-timed arterial blood samples were collected for analysis of arterial plasma glucose and 14C levels. Isotope concentrations in regions of interest were measured from 20μm thick coronal serial sections after exposure to autoradiograph film via optical densitometry. Regional glucose uptake was calculated according to Sokoloff’s equation utilizing rat rate constants, as mice rate constants have not been established (33).

**Hypoglycemia-induced c-fos expression.** Awake, 5-hour fasted NIRKO (n=4) and littermate controls (n=6) were given a single intraperitoneal (i.p.) injection of high dose insulin (3.0-3.5U/kg) to achieve a consistent and stable hypoglycemic insult (~30 mg/dL) for 2 hours. Euglycemic controls (~110mg/dL, n=4 per group) were given an i.p. injection of saline. After a 2 hour duration of hypoglycemia (or euglycemia), each cryoprotected brain was analyzed for c-fos expression.

**Electrophysiological studies.** Male 14-28 day old NIRKO and littermate control mice were anesthetized and transcardially perfused with ice-cold oxygenated perfusion. Sections (350μm) through the hypothalamus were made on a vibratome (Vibroslice; Camden Instruments). The brain slices were maintained at 34°C in oxygenated high-Mg2+ low-Ca2+ artificial cerebrospinal fluid for 30 minutes and then transferred to normal oxygenated ACSF for the remainder of the day. Viable neurons were visualized and studied under infrared differential-interference contrast microscopy using a Leica DMLS microscope equipped with a 40× long working-distance water-immersion objective. Current-clamp recordings (standard whole-cell recording configuration) from neurons in the VMH were made using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) as previously described (34;35). During recording, brain slices were perfused at 10mL/min with normal oxygenated ACSF. Input resistance was calculated from the change in membrane potential in response to small 500-msec hyperpolarizing pulses (~10 to ~20pA) given every 3 seconds. The membrane potential response was measured only after the membrane response to altered extracellular glucose had stabilized, and this value was compared with controls that were measured immediately before changing extracellular glucose. Individual glucose inhibited (GI) neurons were identified as those neurons that increased their action potential frequency, membrane potential, and input resistance with decreases in extracellular glucose from 2.5 to 0.1mM.

**Restraint Stress.** Awake, 5-hour fasted, control and NIRKO mice (n=6 per group) were placed in a mouse restrainer (Braintree Scientific, Braintree, MA) for 45 minutes to induce restraint stress. Cardiovascular parameters were obtained during the basal period and during the last 20 minutes of restraint stress using a tail-cuff system (Kent Scientific Corporation, Torrington, CT). Blood samples were taken by previously implanted arterial cannula at the beginning and end of the restraint period to measure plasma epinephrine levels.

**Heat Stress.** Awake control and NIRKO mice (n=6 per group) were exposed to an ambient temperature of 42°C for 90 minutes to induce heat stress. Blood samples were taken at the end of the heat stress period to measure catecholamines. Subsequently, cryoprotected
brains were analyzed for heat stress induced c-fos immunostaining.

**Western Blots.** The medial basal hypothalamus, defined anatomically as posterior to the optic chiasm, anterior to the mammillary body, inferior to the thalamus, and ±1 mm lateral to the midline was dissected and frozen for analysis. Homogenized hypothalamic protein extracts (20 μg for GLUT1 and GLUT3; 100 μg for GLUT4 and glucokinase) were fractionated by electrophoresis on a 10% Bis-Tris Criterion XT (Biorad, Hercules, CA) gel and subjected to transfer. The following primary antibodies were used: GLUT1 (1:5000, Chemicon), GLUT3 (1:1000, Chemicon), GLUT4 (1:1000, kindly supplied by Dr. M. Mueckler), Glucokinase (1:1000, Calbiochem). The blots were developed using a horseradish peroxidase-conjugated secondary antibody (1:8000). Primary antibody binding was detected by enhanced chemiluminescence ECL reagents (Perkin Elmer, Wellesley, MA) on ISO-MAX films and quantified by ImageQuant software analysis (Amersham Pharmacia, Piscataway, NJ). An antibody against β-actin (1:2000, Sigma, St. Louis, MO) served as a loading control.

**Immunohistochemistry.** Cryoprotected brains were processed for DAB immunohistochemistry or immunofluorescence. Briefly, free-floating hypothalamic sections (20-30 μm) throughout the VMH/ARC were taken from 1.46 to 1.82 mm caudal to bregma, blocked, and incubated overnight at 4°C in primary antibodies. The following antibody dilutions were used: GLUT4 (1:1000), c-fos (1:2000, Ab-5, Calbiochem). For immunofluorescence, goat anti-rabbit Texas Red (1:200, Molecular Probes) was used as the secondary antibody. Subsequently, the sections were mounted on slides using Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). For DAB immunohistochemistry, immunoreactivity was performed with biotinylated goat anti-rabbit immunoglobulin G (1:200) using the Elite ABC kit (Vector Laboratories). As a negative control, alternative sections were incubated without primary antibodies. Regions of interest were identified using anatomical landmarks (36) and positively stained cells were counted by a blinded investigator. Four to six brain sections per mouse were quantified for statistical purposes.

**RT-PCR.** Sections (400 μm) were taken from brain sections 1.46 to 1.86 mm caudal to bregma. 0.5 mm bilateral punch biopsies from the VMH and ARC (0.75 mm from the piriform cortex) were collected from NIRKO mice and littermate controls (n=7-8 per group). The mRNA extracted with Trizol (Invitrogen Corporation, Carlsbad, CA) was subject to quantitative two-step RT-PCR reaction performed in triplicate in a fluorescent temperature cycler (GeneAmp 7700 Sequence Detector, Applied Biosystems) with glucokinase primers (glucokinase probe 5’-/56-FAM/ACC GCC AAT GTG AGG TCG GCA /3BHQ_1/-3’; glucokinase reverse 5’- AGC CGG TGC CCA CAA TC- 3’; and glucokinase forward 5’- CCA CAA TGA TCT CCT CTT GCT ACT ATG A-3’). The results were quantified after normalizing to ribosomal RNA L32mRNA.

**Plasma Assays.** Blood glucose was measured by a glucometer (Becton, Dickinson and Company, Franklin Lakes, NJ) while plasma glucose was assayed by the glucose oxidase method and a spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Radioimmunoassays were performed for glucagon (LINCO Research, Inc., St. Charles, MO) and corticosterone (ICN Biomedicals, Inc., Costa Mesa, CA). Insulin was assayed by ELISA (Chrysal Chem. Inc., Downers
Grove IL). Plasma epinephrine and norepinephrine were measured with a single isotope derivative (radioenzymatic) method (37).

**Statistics.** All values are presented as the mean ± standard error of the mean (SEM). Statistical significance was set at p<0.05, as determined by Student’s t test.

**RESULTS**

**Brain Insulin Action is Necessary for Full Sympathoadrenal Response to Hypoglycemia.**

To characterize the counterregulatory response to hypoglycemia, a series of hyperinsulinemic glucose clamps were performed. Blood glucose was clamped at 110, 70, 50 and 30mg/dL in control and NIRKO mice to induce various degrees of hypoglycemia (mild=70mg/dL, moderate=50mg/dL, and severe hypoglycemia=30mg/dL) or no hypoglycemia (euglycemic clamp=110mg/dL) (Figure 1). In response to insulin infusion, plasma insulin levels were similarly elevated in NIRKO and control mice (Figure 2 A). Severe hypoglycemia (30mg/dL) resulted in a 6-fold increase in glucagon levels and ~60% increase in corticosterone levels, but these increases were similar in both groups (Figure 2 B-C).

The epinephrine response was significantly impaired in NIRKO mice during moderate (50mg/dL) and severe (30mg/dL) hypoglycemia (Figure 3 A). The epinephrine response was highly correlated to glycemia levels in both control (R²=0.76) and NIRKO (R²=0.75) mice and indicated as a shift in the hypoglycemia-epinephrine response curve (Figure 3 A-inset). Norepinephrine levels trended lower in NIRKO mice during moderate (50mg/dL) and severe (30mg/dL) hypoglycemia, but the difference did not reach significance (Figure 3 B). In response to the high dose of insulin (20mU.kg⁻¹.min⁻¹), hepatic glucose production was completely inhibited during the hyperinsulinemic clamp at glycemic levels of 100, 70, and 50mg/dL.

During severe hypoglycemia (30mg/dL), hepatic glucose production rose significantly; however, the rise in hepatic glucose production was significantly blunted in NIRKO mice (Figure 3 C).

**Absent CNS Insulin Action Impairs Hypothalamic Neuronal Activation to Hypoglycemia.** In order to assess the brain’s response to hypoglycemia, c-fos-based functional mapping was used to demonstrate activated neurons and functional circuits that respond to hypoglycemic stress (38). Euglycemic (~110mg/dL) controls displayed low c-fos expression in the hypothalamus (Figure 4 B). In response to insulin-induced hypoglycemia (31.5±3.1mg/dL), both NIRKO and control mice markedly increased c-fos expression within the paraventricular nucleus (PVN) of the hypothalamus (Figure 4 A). However, NIRKO animals showed a 3-fold impairment in c-fos activation as compared to controls (Control: 99±16 vs. NIRKO: 31±5, p<0.01) (Figure 4 B).

**Impaired Glucose Sensing in Individual Glucose-Inhibited Neurons.** Whole-cell current clamp recordings were performed to evaluate the glucose sensitivity of individual glucose-inhibited (GI) neurons in the ventromedial hypothalamus (VMH). As expected for VMH GI neurons bathed in sufficient 2.5mM glucose, action potentials in this basal state were absent in recordings from both control and NIRKO mice. There were also no group differences in membrane potential (MP) or input resistance (IR) in 2.5mM glucose (Control: MP -57±4 mV, IR 1209±272 MΩ; NIRKO: MP -59±3 mV, IR 1016±162 MΩ). Further, no group differences were observed in GI neurons in response to a maximal glucose decrease from 2.5 to 0.1mM (not shown). In contrast, GI neurons in NIRKO mice had a significantly impaired change in membrane potential and input resistance (66% and 80% impairment,
respectively) in response to a glucose decrease from 2.5 to 0.5mM (Figure 4 C-D).

**Absent CNS Insulin Signaling does not Influence Response to Restraint or Heat Stress.** NIRKO and control mice were subjected to a mild stressor (restraint stress) and a more profound stressor (heat stress) to evaluate sympathoadrenal activation in response to glycemia-independent stress. In response to milder restraint stress, plasma epinephrine levels rose similarly 2-fold in both littermate controls and NIRKO mice (Figure 5 A). The physiological increased heart rate to restraint stress was also similar in control and NIRKO mice (Figure 5 B). Heat stress induced a more pronounced catecholamine elevation than restraint stress (to levels observed with hypoglycemia), but the rise in both epinephrine and norepinephrine in response to heat stress was again not significantly different between groups (Figure 6 C-D). To determine whether this defect in neuronal activation was unique to hypoglycemia, *c-fos* expression was also assessed in response to heat stress. Increased *c-fos* expression was again noted in the PVN in response to heat stress (Figure 6 A), to levels observed with hypoglycemia; however, in response to heat stress, there was no difference in *c-fos* expression between groups (Control: 123±4 vs. NIRKO: 129±10, p=NS) (Figure 6 B).

**Abrogated Brain Insulin Action and Expression of Hypothalamic Glucose Sensors.** To assess whether CNS insulin action regulates glucose transporters (GLUTs) and glucokinase (GK) in the brain, hypothalamic protein and mRNA expression was assessed. GLUT1 and GLUT3 hypothalamic protein expression were 3-fold higher than either GLUT4 or glucokinase. GLUT1 protein levels in the hypothalamus were similar in control and NIRKO mice (Figure 7 A-B). Hypothalamic GLUT3 protein levels in NIRKO mice were slightly (80.5±9.8% of control), but not significantly (p=0.08) reduced (Figure 7 A-B). GK protein levels were also similar in control and NIRKO mice (Figure 7 A-B). GK mRNA expression was preferentially expressed in the VMH and arcuate nucleus, but there was no difference in expression levels between experimental groups (Figure 7 C), consistent with the glucokinase protein expression findings. Interestingly, insulin-regulated glucose transporter, GLUT4, protein levels were significantly reduced (68.5±5.5% of control, p<0.05) in the hypothalamus of NIRKO mice (Figure 7 A-B). To assess regional localization, immunohistochemistry results demonstrated that GLUT4 protein was highly enriched in the ventromedial hypothalamus (VMH) and the arcuate nucleus (ARC) of control mice. In NIRKO mice, GLUT4 protein expression was markedly reduced in these regions (Figure 7 D). In spite of reductions in glucose transporter expression in NIRKO mice, regional brain glucose uptake, as assessed during hyperinsulinemic-hypoglycemic clamps, was not different between experimental groups (Figure 7 E).

**DISCUSSION**

Insulin’s role in regulating the counterregulatory response to hypoglycemia is an area of active investigation. Insulin has been shown to increase (8-11;18-20), diminish (12;21), and not alter (13-16;22) the sympathoadrenal response to hypoglycemia. In this study, using a model of chronic brain insulin receptor deficiency, it was demonstrated that insulin action in the brain, 1) regulates the glucose sensitivity of glucose sensing neurons in the VMH, 2) regulates hypothalamic neuronal activation uniquely due to hypoglycemic stress, and 3) modulates the sympathoadrenal response to hypoglycemia by altering the glycemic level required to elicit appropriate sympathoadrenal responses.

In these studies, a ~60% rise in corticosterone was observed in response to severe hypoglycemia in NIRKO and control mice.
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(Figure 2 B). Although not well characterized in mice, this degree of hypothalamic-pituitary-adrenal (HPA) induced increment in corticosterone is consistent with other groups (39;40). Contrary to the stimulatory effect of insulin on the cortisol response to hypoglycemia observed in canine models (18;19), these studies in mice demonstrate that the absence of brain insulin action does not impair the HPA axis response to hypoglycemia.

Reports of insulin action’s in the CNS in modulating the glucagon response to hypoglycemia are variable, with studies demonstrating insulin to increase (18;19), decrease (21;41), or not effect (20) the glucagon response to hypoglycemia. In the current studies, the pancreatic α-cell response to severe hypoglycemia showed a 6-fold increase in plasma glucagon levels that was not altered by the absence of CNS insulin receptors in NIRKO mice. Interestingly, although catecholamines stimulate the α-cell, the impaired catecholamine response to hypoglycemia did not diminish the full glucagon response in NIRKO mice (Figure 2 C). These results indicate that factors other than central insulin action and systemic catecholamine responses (perhaps, local glycemia, intra-islet insulin/zinc, direct innervations, etc.) are more important mediators of the glucagon response to hypoglycemia.

The absence of brain insulin receptors resulted in a significantly impaired epinephrine response in NIRKO mice during moderate (50mg/dL) and severe (30mg/dL) hypoglycemia. However, the absence of brain insulin signaling did not result in total deficiency of hypoglycemic counterregulation. An epinephrine response of ~1500 pg/mL, which was achieved during moderate hypoglycemia (50mg/dL) in controls, was also elicited at a lower blood glucose level (30mg/dL) in NIRKO mice (Figure 3 A). Consistent with this finding, the shift in the hypoglycemia-epinephrine response curve (Figure 3 A-inset) indicates that in the absence of insulin signaling, NIRKO mice needed to reach lower glycemic levels to appropriately activate their adrenomedullary response. While insulin infusion suppressed hepatic glucose production during the clamps, only during severe hypoglycemia (30mg/dL) was the counterregulatory response of a sufficient magnitude to overcome the suppressive effects of insulin and significantly increase hepatic glucose production. In NIRKO mice, however, the counterregulatory-induced stimulation of hepatic glucose production was significantly blunted during severe hypoglycemia (Figure 3 C), consistent with an impaired sympathoadrenal response. These findings indicate that chronic lack of CNS insulin action alters glucose sensing and/or responsiveness, leading to an impaired sympathoadrenal response and an impaired ability to defend against iatrogenic hypoglycemia.

Since the adrenomedullary response to hypoglycemia was not impaired during mild hypoglycemia in NIRKO mice, it was speculated that mild (restraint) stress, as noted by modest elevations in epinephrine levels (Figure 5 A), might not have been of sufficient magnitude to detect a differential response between control and NIRKO mice. However, by achieving comparable epinephrine levels during severe stress (heat) and severe hypoglycemia (30mg/dL) and finding a normal catecholamine response to heat stress in NIRKO mice, these findings indicate that absent CNS insulin signaling does not impair the normal adrenomedullary response even to severe non-hypoglycemic stress (Figure 3 A, Figure 6 A).
Increased *c-fos* expression in the paraventricular nucleus (PVN) has been used as a marker of transcriptional activity in stress-related neural circuitry (38;42-44). Expression of *c-fos* was therefore measured to determine whether the impaired sympathoadrenal response in the NIRKO mice was related to impaired activation of hypothalamic sensing neurons. During hypoglycemia, increased *c-fos* expression was predominantly observed in the PVN and not seen in the VMH, consistent with other studies (45). Hypoglycemia-induced *c-fos* activation in the PVN may represent direct activation in response to hypoglycemia or indirect activation in response to afferent input from other areas containing glucose sensing neurons. Thus, the impaired *c-fos* activation in the PVN of NIRKO mice in response to hypoglycemia could represent reduced glucose sensing of PVN neurons; or given the abundance of insulin receptors in important VMH glucose sensing neurons, an indirect reduction in afferent inputs from glucose sensing neurons in the VMH. Whether this defect indicates impaired direct or indirect glucose sensing, the reduced *c-fos* activation in NIRKO mice was profound and consistent with other models of impaired glucose sensing and impaired counterregulation (44;46). Further, in response to a non-hypoglycemic stressor, heat stress increased *c-fos* expression to a similar magnitude as observed during severe hypoglycemia (30mg/dL); however, no difference in heat-induced *c-fos* expression was noted between control and NIRKO mice (Figure 4, Figure 6 C-D). These results indicate that NIRKO mice have an intact neuronal circuitry for sensing and responding to non-hypoglycemic stress; therefore, the impaired responses to hypoglycemia in the NIRKO mice appear to be unique to hypoglycemic stress and/or glucose sensing.

Whole cell current clamp recordings of spontaneous electrical activity were made in individual glucose-inhibited (GI) neurons to assess responses of individual glucose sensing neurons in the VMH. While a direct relationship between glucose sensing neurons and sympathoadrenal activation has yet to be definitively established, it is noteworthy that the ability of VMH GI neurons to sense a fall in ambient glucose levels is impaired under several conditions where the sympathoadrenal response to hypoglycemia is also impaired (i.e. rats treated with recurrent hypoglycemia or streptozotocin-induced diabetes) (35;47;48). In NIRKO mice, the observed impaired response of VMH GI neurons to reductions in glucose levels (Figure 4 C-D) is entirely consistent with the impaired neuronal (*c-fos*) activation (Figure 4 A) and the impaired sympathoadrenal activation (Figure 3 A). Further, the electrophysiological findings that NIRKO GI neurons respond normally to maximal glucose deprivation (0.1mM), but show impaired responses at 0.5mM are consistent with a relative, not absolute, impairment in glucose sensing. These results indicate that insulin acts directly in the brain to regulate the glucose sensing ability of hypothalamic GI neurons that are critically important and functionally linked in mediating the sympathoadrenal response to hypoglycemia. Of particular interest is that GI neurons of NIRKO mice have an impaired ability to respond to a fall in glucose even in the absence of insulin administration. Combining these *in vitro* findings to the *in vivo* findings suggest that it may not solely be a failure of insulin to acutely activate its receptor that leads to impaired glucose sensing and altered neuronal responses; but rather, we propose that the chronic lack of insulin signaling in NIRKO mice causes long-term adaptations in gene
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transcription/transduction (i.e., decrease in GLUT4—see Figure 7), leading to impaired glucose sensing. Alternatively, since neuronal nitric oxide production is required for GI neurons to sense decreased glucose (49;50) and insulin enhances nitric oxide production in VMH GI neurons (49), the chronic lack of insulin signaling in NIRKO mice may lead to impaired glucose sensing by impairing nitric oxide production. It is entirely plausible that the chronic actions of insulin may be mechanistically very different from the acute actions of insulin in regulating neuronal glucose sensing and the counterregulatory response to hypoglycemia.

Similar to its well-characterized actions in muscle and fat, insulin-mediated GLUT4 translocation has been demonstrated in neuronal cell lines (51), hippocampus (52), and hypothalamus (53). GLUT4 mediated glucose sensing has been speculated to be important at low glucose concentrations, where insulin-mediated glucose transport may act to supplement low intracellular glucose levels in hypothalamic glucose sensing neurons (24;26). Indeed, supporting a glucosensing role for insulin receptors and GLUT4 is their coexpression in up to 75% of glucose responsive neurons in the VMH (30). Further, neuronal GLUT4 has recently been shown to be an important mediator of hypoglycemic counterregulation and glucose sensing, as noted in neuronal GLUT4 knockout mice (54). During hypoglycemia, when glucose transport becomes rate-limiting, it was speculated that decreased GLUT4 expression and/or deficient insulin action would result in reduced glucose uptake in critical glucose sensing regions of NIRKO mice. This study, however, noted equal regional brain glucose uptake during the hyperinsulinemic-hypoglycemic clamp (Figure 7 E), indicating that neither deficient insulin signaling nor the reduced GLUT4 levels altered glucose uptake in these brain areas. Since brain GLUT4 expression is much lower than other glucose transporters, it is likely that glucose uptake was primarily regulated by the more abundant GLUT1 and GLUT3, thus masking any subtle effect caused by decreased GLUT4. While regional brain glucose uptake was not altered in NIRKO mice, an effect of insulin signaling and/or GLUT4 availability on mediating glucose uptake in individual glucose sensing neurons cannot be ruled out.

In summary, it is shown that the chronic lack of insulin receptor signaling in the central nervous system, 1) decreases hypothalamic glucose transporter 4 expression, 2) attenuates individual hypothalamic GI neuronal responses to low glucose, 3) impairs hypothalamic neuronal activation in response to hypoglycemia, and 4) reduces the sympathoadrenal response to hypoglycemia by shifting the glycemic level necessary to elicit appropriate sympathoadrenal responses. These defects are specific for glucose sensing, as the lack of CNS insulin signaling does not restrict neuronal activation or the adrenomedullary response to restraint or heat stress.

It is concluded that insulin acts directly in the brain to regulate both glucose sensing in hypothalamic neurons and the counterregulatory response to hypoglycemia. Since insulin-treated diabetic patients have an impaired ability to sense and appropriately respond to insulin-induced hypoglycemia, the mechanisms by which insulin regulates central nervous system glucose sensing need to be actively investigated as research scientists endeavor to supplant insulin-induced hypoglycemia as the rate-limiting factor in the glycemic management of diabetes.
Author Contributions: K.A.D.A. wrote the manuscript, researched data, contributed to the discussion, and reviewed/edited the manuscript. X.Z., Z. S., and D.D.I. researched data. V.H.R. contributed to the discussion and reviewed/edited the manuscript. S.J.F. researched data, contributed to the discussion, and reviewed/edited the manuscript.

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**Figure legends**

**Figure 1.** Hyperinsulinemic, graded hypoglycemic clamps. Blood glucose levels are shown for NIRKO (filled circles) and control (open circles) mice (n=6-8 mice per group). After basal sampling, insulin was infused (20 mU.kg⁻¹.min⁻¹) and blood glucose levels were measured at ten-minute intervals via arterial sampling. By adjusting the rate of intravenous glucose infusion, glucose levels were carefully lowered, then clamped at matched, pre-determined glycemic levels (110, 70, 50, and 30 mg/dL) to create various degrees of hypoglycemic stress (none, mild, moderate, and severe, respectively).

**Figure 2.** Hormone levels during graded hyperinsulinemic glucose clamps. Results are shown for NIRKO (closed bars) and control (open bars) mice (n=6-8 mice per group). A. By experimental design, insulin levels rose markedly, and similarly between groups during the hyperinsulinemic clamps. B&C. Glucagon (B) and corticosterone (C) levels in both treatment groups rose significantly above basal levels (p<0.05) during moderate and severe hypoglycemia but similarly between treatment groups.

**Figure 3.** Catecholamine and hepatic glucose production levels in a series of hyperinsulinemic glucose clamps. Results are shown for NIRKO (closed bars) and control (open bars) mice (n=6-8 mice per group). A. The epinephrine response was significantly impaired in NIRKO mice (p<0.05) during moderate (50 mg/dL) and severe (30 mg/dL) hypoglycemia. The inset picture demonstrates a shift in the hypoglycemia dose-response curve by the solid (controls) versus dashed (NIRKO) line. B. Norepinephrine levels in both treatment groups rose significantly higher from the basal period during moderate and severe hypoglycemia but there was no difference between NIRKO and control responses. C. Hepatic glucose production (HGP), in the basal period prior to insulin infusion, was the same in control and NIRKO mice. During the hyperinsulinemic glucose clamps at mild and moderate hypoglycemia, HGP was suppressed. In spite of the hyperinsulinemia, during severe hypoglycemia (30 mg/dL), HGP rose significantly, but remained lower in NIRKO as compared to control mice. (*p<0.05)

**Figure 4.** Blunted neuronal activation and glucose responsiveness in NIRKO glucose sensing neurons. A. Following a 2-hour hypoglycemic insult, neuronal activity was assessed using the marker, *c-fos*. Representative images of matched hypothalamic sections highlighting *c-fos* staining in the paraventricular nucleus (PVN) in NIRKO (right image) and littermate controls (left image). B. The quantity of *c-fos* positive cells located within the PVN was minimally induced in both groups during euglycemia (n=4 mice per group). During matched hypoglycemia, the number of *c-fos* positive cells was significantly less in NIRKO (n=4, closed bars) as compared to littermate control (n=6, open bars) mice. C&D. Individual glucose inhibited (GI) neurons were identified as those neurons that increased their action potential frequency, membrane potential, and input resistance with decreases in extracellular glucose from 2.5 to 0.1 mM glucose. In response to lowering extracellular glucose levels from 2.5 to 0.5 mM glucose (G), the percentage change in membrane potential (C) and input resistance (D) of glucose-
inhibited neurons was significantly lower in NIRKO (n=7, closed bars) compared to littermate controls (n=6, open bars). (*p<0.05, **p<0.01)

**Figure 5.** NIRKO mice have a normal physiological response to restraint stress. NIRKO (n=6, closed bars) and littermate control (n=6, open bars) mice were placed into a confining restraint device for 45 minutes. **A&B.** Plasma epinephrine levels (A) and heart rates (B) were elevated in response to restraint stress, but equally in control and NIRKO mice. (*p<0.05 vs. basal)

**Figure 6.** NIRKO mice display normal physiological responses to heat stress. NIRKO (n=6, closed bars) and littermate controls (n=6, open bars) were subjected to heat stress for 90 minutes. **A&B.** Plasma epinephrine (A) and norepinephrine (B) levels were not significantly different between NIRKO and control mice. **C.** Representative images of matched hypothalamic sections highlighting heat stress induced *c-fos* staining in the paraventricular nucleus (PVN). **D.** The quantity of *c-fos* positive cells was similar between NIRKO and controls.

**Figure 7.** Glucose transporter 4, not GLUT1, GLUT3, or glucokinase expression, is reduced in NIRKO brains. **A&B.** Western blots of whole hypothalamic extracts from NIRKO and littermate controls (n=5-6 mice per group) were performed. Representative images (A) and graph (B) quantifying protein expression of glucose transporters (GLUT1, GLUT3, and GLUT4) and glucokinase are shown. **C.** Although glucokinase mRNA was highly expressed in the ventromedial hypothalamus (VMH) and arcuate nucleus (ARC) as compared to the cortex, there was no difference between NIRKO (n=7, closed bars) and controls (n=8, open bars). **D.** Regional localization of GLUT4 protein content, as determined by hypothalamic DAB staining (left) and immunofluorescence (right) of control (above) and NIRKO (below) mice, show enriched GLUT4 protein content in the ventromedial hypothalamus (VMH, circled) and arcuate nucleus (triangle) of control mice. GLUT4 protein content was 62±6% lower in NIRKO mice. **E.** Regional brain glucose uptake was quantified using $^{14}$C 2-deoxyglucose during a hyperinsulinemic hypoglycemic (~30mg/dL) clamp. Results show that regional brain glucose uptake in all regions measured [hippocampus (CA1, CA3, DG), hypothalamus (VMH, ARC, PVN), and hindbrain (NTS)] are similar among NIRKO (n=6, closed bars) and controls (n=9, open bars).

**Figure 1.**
Figure 2.

A

Insulin (ng/mL)

B

Glucagon (pg/mL)

C

Corticosterone (ng/mL)

Basal 110 70 50 30

Clamp Glycemia Achieved (mg/dL)

0 100 200 300 400 500

0 100 200 300 400

0 100 200 300 400 500

0 100 200 300 400 500
Figure 3.

A

Epinephrine (pg/mL)

B

Norepinephrine (pg/mL)

C

Glucose Production (mg.kg⁻¹.min⁻¹)

0 5 10 15 20 25

0 200 400 600 800 1000 1200

0 1000 2000 3000

Basal 110 70 50 30

Clamp Glycemia Achieved (mg/dL)

*
Figure 4.

A

![Brain images showing control and NIRKO conditions.](image)

B

![Bar graph showing the number of c-fos positive cells (per PVN hemisphere) under euglycemia and hypoglycemia conditions.](image)

C

![Bar graph showing membrane potential (percent change) for 2.5 to 0.5 mM glucose.](image)

D

![Bar graph showing input resistance (percent change) for 2.5 to 0.5 mM glucose.](image)
Figure 5.

A

![Bar graph showing Epinephrine levels in Basal and Restraint conditions.](image)

B

![Bar graph showing Heart Rate in Basal and Restraint conditions.](image)
Figure 6.

A

B

C

D

Control

NIRKO

Control

NIRKO

Brain Insulin Action Regulates Glucose Sensing
Figure 7.

A) Glucokinase mRNA (RT-PCR)

B) Band intensity (% of control)

C) Glucokinase mRNA (RT-PCR)

D) Control and NIRKO images

E) Brain glucose uptake (μmol/100g/min)