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Hypothalamic Glut4 neurons and nutrient sensing

Altered Central Nutrient Sensing in Male Mice Lacking Insulin Receptors in Glut4-expressing Neurons

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Insulin signaling in the central nervous system influences satiety, counterregulation, and peripheral insulin sensitivity. Neurons expressing the Glut4 glucose transporter influence peripheral insulin sensitivity. Here, we analyzed the effects of insulin receptor (IR) signaling in hypothalamic Glut4 neurons on glucose sensing as well as leptin and amino acid signaling. By measuring electrophysiological responses to low glucose conditions, we found that the majority of Glut4 neurons in the ventromedial hypothalamus (VMH) were glucose excitatory neurons. GIRKO mice with a combined ablation of IR in Glut4-expressing tissues showed increased counterregulatory response to either 2-deoxyglucose-induced neuroglycopenia or systemic insulin-induced hypoglycemia. The latter response was recapitulated in mice with decreased VMH IR expression, suggesting that the effects on the counterregulatory response are likely mediated through the deletion of IRs on Glut4 neurons in the VMH. Using immunohistochemistry in fluorescently labelled hypothalamic Glut4 neurons, we show that IR signaling promotes hypothalamic cellular signaling responses to the rise of insulin, leptin, and amino acids associated with feeding. We conclude that hypothalamic Glut4 neurons modulate the glucagon counterregulatory response, and that IR signaling in Glut4 neurons is required to integrate hormonal and nutritional cues for the regulation of glucose metabolism.

INTRODUCTION

The re-emerging notion that insulin, acting through the central nervous system (CNS), controls peripheral insulin sensitivity raises the possibility of developing CNS-based approaches to treat insulin resistance, obesity, and type 2 diabetes. IR signaling in the CNS also has roles in the regulation of food intake [1], the counterregulatory response to hypoglycemia [2], gonadotropin release [3], peripheral glucose metabolism [4], leptin sensitivity [5], and neuronal plasticity [6]. We previously identified a subset of neurons characterized by Glut4 glucose transporter expression

that are important for systemic insulin sensitivity [7]. Indeed, mice with a collective ablation of IR in Glut4-expressing tissues (GIRKO), develop highly penetrant diabetes that recapitulates cardinal features of the human disease [7].

In this study, we used GIRKO mice to study the role of CNS IR signaling in the response to glucose deprivation. Hypoglycemia is commonly observed in association with intensive insulin therapy [8,9]. Restoration of normoglycemia following acute hypoglycemia requires effective sensing of low glucose levels and rapid activation of the counterregulatory hormone responses, particularly glucagon and epinephrine [2], in addition to other key metabolic hormones, such as leptin [10]. Moreover, recurrent hypoglycemia can decrease the sympathoadrenal response, resulting in greater risk of severe hypoglycemia and hypoglycemia unawareness [8]. Thus, understanding CNS-based mechanisms that initiate counterregulatory responses can help reduce or prevent hypoglycemia.

It has been shown that neurons in the rodent mediobasal hypothalamus, which consists of the ventromedial (VMN) and arcuate nuclei (ARC), are important for glucose sensing, and that IR signaling modulates the counterregulatory response to neuroglycopenia [11,12]. Nonetheless, the specific neuronal sub-types involved in this response is unclear [13]. We previously showed that Glut4 neurons represent a distinct neuronal population that has a heterogeneous composition of neuronal markers and neurotransmitters [14]. Upon toxin-mediated ablation of these neurons in the hypothalamus, mice develop anorexia and reduced insulin secretion that results in impaired glucose tolerance with fasting hyperglycemia [15]. These data are consistent with the notion that hypothalamic Glut4 neurons are important mediators of insulin action. In this study, we investigated whether insulin receptor signaling on hypothalamic Glut4 neurons participate in the regulation of the counterregulatory response to hypoglycemia, using a combination of electrophysiology, hypoglycemic clamps, targeted neuroglycopenia and analysis of biochemical signaling pathways.

Material and Methods

Mice

Gt(Rosa)26Sor$^{tm9(CAG\text{-}tdTomato)Hze}$, *Gt(Rosa)26Sor*$^{tm3(CAG\text{-}EYFP)Hze}$, ROSA26-EGFP, and wild type B6 mice were obtained from The Jackson Laboratories. Glut4 neurons were fluorescently labelled by breeding *Rosa* reporter mice with *Glut4-Cre* mice. GIRKO mice were generated as previously described [7]. C57BL6 mice for the VMH insulin receptor knockdown studies were procured from Charles River. The procedures were approved by Indiana University, University of Utah, and Columbia University Animal Care and Utilization Committees.

Evaluating the glucose responsiveness of hypothalamic Glut4-Expressing Neurons

To determine whether hypothalamic Glut4-expressing neurons respond to fluctuating glucose levels, we conducted electrophysiological analyses on coronal brain slices (300 um) taken through the hypothalamus of three male *Glut4-EYFP* mice (8-10 weeks old). Briefly, the mice were sacrificed by decapitation, the brains were rapidly removed and slices were cut through the hypothalamus using a Leica VT1200 vibratome in an ice-cold, oxygenated (95% O_2, 5%CO_2) solution (in mM: 10 NaCl, 25 $NaHCO_3$, 2.5 KCl, 10 D-glucose, 1.25 NaH_2PO_4, 195 sucrose, 2 NaN_3, 7 $MgCl_2$, 1 $CaCl_2$, pH 7.3). Three-four slices were prepared from each mouse. Prior to electrical recordings, the brain slices were maintained at room temperature for at least 1 h in oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) (in mM: 120 NaCl, 5 KCl, 26 $NaHCO_3$, 1.2 NaH2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 5 D-glucose, pH 7.4). Mannitol was used to adjust the osmolality of the low glucose aCSF (0.1 mM or no glucose). Whole-cell patch

recordings were performed using borosilicate glass electrodes with resistance between 4 and 6M backfilled with an intracellular solution (in mM: 128 K-gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 2 $MgCl_2$, 3 ATP and 0.1 GTP, pH 7.3). Glut4 neurons in the VMN or POMC neurons in the ARC were identified by fluorescence and patch clamped under IR-DIC optics. Recordings were acquired with an Axopatch 700B amplifier, digitized and analyzed with pClamp10 software (Molecular Devices).

Evaluating the counterregulatory response to neuroglycopenia

Stereotaxic injections were performed as described previously [16]. Briefly, 3-4 months old wild type (N=6) and GIRKO (N=6) mice were anesthetized and a unilateral microinjection guide cannula was stereotaxically implanted into the lateral ventricle (-0.3mm anterior and 1mm lateral to bregma and 2.2mm below the skull surface). The mice were then allowed 1 week for recovery. We verified correct positioning of the cannula by injecting methylene blue (1%) after the animals were euthanized. We induced neuroglycopenia by acutely injecting the non-metabolizable glucose analog, 2-deoxyglucose (2-DG; 1mg/mouse) intracerebroventricularly (*icv*) into the lateral ventricle. In the first experiment, we monitored blood glucose from a tail nick over the course of 5 hours following injection of 2-DG. In the second experiment, we collected blood samples to measure plasma glucagon levels 30 minutes after *icv* 2-DG injection.

Evaluating the counterregulatory hormone response to systemic insulin-induced hypoglycemia in GIRKO mice

Having demonstrated that hypothalamic Glut4 neurons are in fact glucose responsive, we proceeded to establish whether loss of insulin signaling in Glut4 tissues altered the hormone response to hypoglycemia. Approximately 4-5 days prior to the clamp procedure, wild type (N=6) and GIRKO (N=6) mice were anesthetized with isoflurane and a single catheter (MRE-25) was implanted into the jugular vein and tunneled subcutaneously as described previously [17]. Four to five days later, hypoglycemic clamp studies were performed as described previously [11]. Briefly, overnight fasted mice were connected to infusion pumps and allowed to recover for a period of ~2hrs prior to obtaining baseline blood samples through a tail nick. Subsequently, a bolus-constant insulin (20mU/kg/min) and variable 20% dextrose solution were infused intravenously to lower and maintain plasma glucose levels at ~50 mg/dl for 90 minutes. Blood samples were collected at 30 min intervals throughout the hypoglycemic clamp portion of the study for measurement of plasma glucagon and catecholamine responses, and at 90 min for plasma insulin levels. Erythrocytes from a donor mouse were washed, suspended in heparinized-saline and infused over the course of the study to prevent volume depletion and anemia. At the end of the study, the animals were euthanized with an overdose of sodium pentobarbital.

Identifying whether loss of insulin receptors in the VMH recapitulates the counterregulatory defects observed in GIRKO mice

To better establish whether loss of insulin receptors in the VMH contributed to the amplified glucagon response observed in GIRKO mice, we locally knocked down insulin receptor expression in the VMH using an adeno associated viral vector that expresses an insulin receptor shRNA and enhanced GFP reporter under the control of a U6 promoter (Vector Biolabs, cat# shAAV-262287). This viral vector was shown to reduce insulin receptor expression in the VMH by ~45% (Supplemental Figure 2) [18]. 0.5ul of the IR shRNA AAV was bilaterally microinjected into the VMH (-1.46mm AP, ±0.4mm ML and -5.2mm DV at an angle of 0°) of C57BL6 mice (N=6) under isoflurane anesthesia. An AAV expressing a non-specific scrambled RNA sequence was used as a negative control (N=5). Three weeks after viral inoculation, a vascular catheter

was implanted into the jugular vein were implanted. The animals were then recovered for 4-5 days before undergoing a hypoglycemic clamp as described above.

Hormone analyses

We used ELISA for insulin measurements (Millipore), and colorimetric assays for metabolite measurements [16]. Plasma insulin and glucagon in blood samples collected during the clamp procedures were measured by radioimmunoassay (Millipore) [19] or ELISA (Mercodia, Winston Salem, NC). Catecholamines were measured by ELISA (Eagle Biosciences).

Evaluating whether the hypothalamic Glut4 neurons response to insulin, leptin and amino acids signaling during feeding is affected in GIRKO mice.

To determine whether loss of insulin receptor in hypothalamic Glut4-expressing neurons alters their ability to respond to insulin, leptin and amino acids, we used immunohistochemistry to quantify the activated downstream signaling targets, i.e. phospho-Akt (pAkt), phosphor-Stat3 (pStat3), and phosphor-S6 (pS6), respectively, in the arcuate nucleus. Both wild type and GIRKO mice were fasted and refed before they were sacrificed. Their brains were harvested and immunohistochemically processed as previously described [16]. We quantified the fluorescent signal intensity of pAkt/pStat3/pS6 and GFP from individual cells in the arcuate nucleus. Image J was used to quantify the fluorescence intensity and mark individual neurons. Glut4 neurons were identified by the green fluorescence, where the GFP signal intensity was used to identify $Glut4^+$ neurons versus non-Glut4 neurons. Neurons were considered $Glut4^+$ if the GFP integrated density (IntDen) exceeded 0.300 (arbitrary units, AU), while $Glut4^-$ neurons had a GFP IntDen less than or equal to 0.300. Phosphorylated Akt, Stat3, and S6 were used to gauge the activity of insulin, leptin, and amino acid signaling pathways, respectively. Plotting the results in groups of $Glut4^+$ neuron versus non-Glut4 neurons from WT and GIRKO mice allowed us to quantify the signaling activity in individual cells, which serves as an unbiased way to assay hormone and nutrient sensitivity in $Glut4^+$ versus non-Glut4 neurons. The antibodies used were procured from Cell Signaling Technology and ThermoFisher Scientific and include phosphor-Akt (Ser473) (D9E) XP Rabbit mAb (#4060) (RRID: AB_2315049) [20], phosphor-S6 ribosomal protein (Ser235/236) (D57.2.2E) XP Rabbit mAb (#4858) (RRID: AB_916156) [21], phosphor-Stat3 (Tyr705) (D3A7) XP Rabbit mAb (#9145) (RRID: AB_2491009) [22], Alexa Fluor 555 goat anti-rabbit IgG secondary antibody (#A27039) (RRID: AB_2536100) [23]. **Food intake.** We measured food intake and feeding response as previously described [24,25].

Statistical analyses

We analyzed data with Student's t–test or one–way or two-way ANOVA using GraphPad Prism software. We used the customary threshold of $p < 0.05$ to declare statistical significance.

RESULTS

Glut4 neurons in the hypothalamus are glucose responsive

We characterized the glucose responsive nature of the Glut4 neurons using electrophysiology. We first validated the whole-cell patch-clamp technique using hypothalamic proopiomelanocortin (POMC) neurons (Supplemental Figure 1) [18], an established type of glucose excited neurons[26-28]. GFP labelled POMC neurons respond to glucopenia with hyperpolarization and reduced spontaneous firing frequency (Supplemental Figure 1A-D) [18]. This is consistent with previous reports that identified POMC neurons as glucose-excitatory neurons [26-28], thus validating our experimental system. Next, we measured the electrophysiological response of Glut4 neurons to changing glucose concentrations by patch-clamping EYFP-labeled Glut4

neurons (with intact IR signaling) in the ventromedial nucleus of the hypothalamus (VMN) (Figure 1). When baseline glucose (5mM) was lowered to 0.1mM, Glut4 neurons responded with hyperpolarization and reduced spontaneous firing frequency (Figure 1A-B), which was restored to baseline measurement during the washout phase (Figure 1C-D). More measurements revealed that hypothalamic Glut4 neurons are a mixed population, the majority of these neurons are glucose-excited neurons (61.5%), while a smaller fraction of these Glut4 neurons are either glucose-inhibited (15.4%) or non-responsive (23.1%).

Augmented hyperglycemic response to neuroglycopenia in GIRKO mice

We also assessed the glucagon response to neuroglycopenia in GIRKO mice following an intracerebroventricularly (*icv*) injection of 2-deoxyglucose (2-DG). After *icv* injection of 2-DG, WT mice developed hyperglycemia, a response that was greatly amplified in GIRKO mice throughout the 5-hr observation period (Figure 2A). The rise in glucose was accompanied by a nearly 50% increase in glucagon levels in the GIRKO mice, compared to WT mice (Figure 2B).

To examine the signaling pathways mediating the amplified counterregulatory response seen in GIRKO mice, we measured levels of phospho-(p)Akt by western blotting of hypothalamic extracts dissected following *icv* 2-DG injection. Unexpectedly, overall pAkt was markedly increased in GIRKO hypothalamic samples (Figure 2C). In contrast, hippocampi collected from the GIRKO and control mice showed comparable amount of pAkt (Figure 2C). This supports the notion that hypothalamus is a critical site for sensing glucose. Next, we examined pAkt by immunohistochemistry, to localize the activation to a specific hypothalamic cell type. In these experiments, we used GIRKO mice in which Glut4 neurons had been labelled with *Rosa26-tdTomato* [14]. Using double staining with pAkt and Tomato (to label Glut4 neurons), we found that the increase in pAkt occurred largely, if not exclusively, in non-Glut4 neurons of GIRKO mice. Quantitative analysis of the results showed the percentage of pAkt-positive Glut4 neurons were ~one-third lower in the arcuate nucleus (ARC) of GIRKO mice, whereas the percentage of pAkt-positive *non*-Glut4 neurons was ~6-fold greater in GIRKO mice (Figure 2D). The aberrant Akt signaling in the non-Glut4 neurons may explain the exaggerated counterregulatory response in GIRKO mice experiencing neuroglycopenia.

GIRKO mice have an enhanced counterregulatory response to systemic insulin-induced hypoglycemia

Glut4 is expressed in glucose-sensing neurons [29], but it's not known whether IR signaling in Glut4 neurons regulates glucose sensing. To address this question, we investigated the hormonal response to systemic insulin-induced hypoglycemia in GIRKO mice. Our previous work showed that GIRKO mice develop diabetes with age [7]. To avoid the potential confounders of hyperglycemia and beta cell failure, we selected chow-fed euglycemic GIRKO and littermate controls for these studies.

Despite matched plasma glucose and insulin concentrations during the hypoglycemia clamp (Figure 3A-B), the glucose infusion rate (GIR) needed to maintain plasma glucose levels at ~ 50 mg/dl in the GIRKO mice was nearly 75% lower compared to the WT mice (Figure 3C). The decrease in the GIR corresponded to an increase in glucagon secretion (Figure 3D) during the hypoglycemic clamp, but the epinephrine and norepinephrine responses were not affected.

Improvements in the glucagon response to hypoglycemia stem from a reduction in VMH insulin receptor expression

Our results show that GIRKO mice responded to insulin-induced hypoglycemia with an increased counterregulatory response. However, GIRKO mice have global IR deficiency and the defect is not confined to the brain. In order to rule out the possibility that systemic insulin

resistance led to improvements in glucagon secretion during hypoglycemia in GIRKO mice, we performed hypoglycemic clamp studies on mice with VMH-specific IR knockdown. This nucleus was targeted because our previous work showed that, within the hypothalamus, Cre activity of the *Glut4-Cre* transgene was concentrated in the VMH [15]. Therefore, we selectively knocked down IR in the VMH (IR-KD). Despite similar plasma glucose levels (Figure 4A), VMH IR-KD mice required significantly less exogenous glucose during the clamp (Figure 4B). The reduction in GIR observed in the VMH IR-KD mice corresponded to improvements in the glucagon response (Figure 4C). Plasma epinephrine responses were not significantly different during the clamp (Figure 4D). These results were consistent with those from the clamp studies in GIRKO mice (Figure 3). Therefore, we conclude that the exaggerated glucagon response to hypoglycemia observed in GIRKO mice is most likely due to a loss of insulin signaling in the VMH rather than increased peripheral insulin resistance.

Regulation of feeding associated hormonal and nutrient signaling by IR in Glut4 neurons

Thus far, we established that Glut4 neurons play an important role in regulating the glucagon response to hypoglycemia. We then asked whether Glut4 neurons respond to other hormonal and nutrient-related signals associated with feeding, such as leptin and amino acids. We took advantage of the ability to label Glut4 neurons in WT and GIRKO mice using a *Rosa-GFP* reporter allele to investigate these questions. During fasting, leptin and amino acid levels are low, resulting in low basal signaling activity. After refeeding, the increased availability of nutrients triggers hormone release, therefore how the animals respond to the surge reflects the nutritional and hormonal sensitivity. We decided to use the fast-refeeding paradigm as opposed to injecting hormones directly because this is a more physiologically relevant setting to capture the *in vivo* response. We subjected mice to fasting and re-feeding, then performed immunohistochemistry on hypothalamic sections using readouts of insulin (pAkt), leptin (pStat3), or amino acid signaling (pS6) (red), as well as GFP to identify Glut4-expressing neurons (green). After re-feeding, we detected pAkt (red) in Glut4 neurons (green) of WT mice (Figure 5A, yellow arrowhead in the inset), whereas in GIRKO mice the intensity and co-localization of pAkt in Glut4 neurons was markedly diminished (Figure 5A lower panel, separate green and red arrowheads, inset). These data indicate that most of the pAkt detected following re-feeding localizes to IR-containing Glut4 neurons in the arcuate nucleus (ARC) of WT mice, and that ablation of IR markedly blunts this response, thereby helping to validate the model's utility to investigate the effects of IR signaling on other signaling pathways.

Amino acid sensing in the CNS occurs primarily through the mTOR-S6K-S6 pathway [30] and thus we assessed pS6 immunohistochemistry in WT and GIRKO samples. Similar to the pStat3 results, we detected a strong correlation between pS6 and Glut4 neurons in re-fed WT mice (Figure 5B, yellow arrowhead in the inset), whereas in GIRKO mice samples, pS6 immunoreactivity was markedly attenuated, with a trend of increase in a subset of non-Glut4 neurons (Figure 5B, separate green and red arrowheads, inset).

Next, we analyzed pStat3 as a means to gauge leptin sensitivity. While pStat3 and Glut4 (GFP) immunoreactivity showed substantial overlap (Figure 5C, yellow arrowhead in the inset), we observed a bimodal pStat3 pattern in re-fed GIRKO mice. pStat3 immunoreactivity was still detected in Glut4 neurons, whereas there was a marked activation in a subset of non-Glut4 neurons (Figure 5C, separate green and red arrowheads, inset). The increased leptin sensitivity in non-Glut4 neurons of GIRKO mice might reflect: (*i*) a compensatory increase, or (*ii*) removal of an IR-dependent inhibitory signal arising from Glut4 neurons.

Given the bimodal response of pStat3 to leptin (Figure 5D) in GIRKO mice, we assessed leptin responsiveness *in vivo* by measuring food intake after leptin injection in euglycemic WT and GIRKO mice. The GIRKO mice exhibited a blunted response to leptin (Figure 5D), suggesting that ablation of IR signaling in Glut4 neurons impairs leptin sensitivity despite the changes in non-Glut4 neurons.

Taken together, GIRKO mice appear to have an abnormal response to nutrient availability, which may lead to aberrant feeding behavior. For instance, while neuroglycopenia increased food consumption in WT mice, the food consumption of GIRKO mice in response to both central saline and 2DG administration were similar (Figure 5E).

Conclusion

The findings of the present work contribute to our understanding of the contribution of insulin and Glut4 towards the counterregulatory response to hypoglycemia and nutrient sensing. We conclude that (*i*) Glut4 neurons are glucosensing neurons in the CNS; (*ii*) a decrease in IR signaling in Glut4 neurons blunts the glucagon response to hypoglycemia; and (*iii*) IR signaling in Glut4 neurons has unexpected effects on leptin and amino acid signaling in hypothalamic Glut4 as well as non-Glut4 neurons.

DISCUSSION

In previous studies, we showed that Glut4 neurons are indeed insulin-sensitive neurons and that they may play a role in integrating olfacto-sensory responses with metabolic cues [14]. Moreover, in cell ablation experiments, we showed that hypothalamic Glut4 neurons regulate satiety, glucose homeostasis, and CNS sensing of hormones and nutrients [15]. The present findings extend our previous work showing that hypothalamic Glut4 neurons are mostly glucose- excited neurons and that inhibition of IR function in the VMN of rodents increases the glucagon response to hypoglycemia [11].

To better understand the characteristic of hypothalamic Glut4 expressing neurons, we performed electrophysiological studies to determine whether they were glucose responsive. The majority of these Glut4 neurons in the VMN decreased their activity in response to a reduction in glucose in the medium, suggesting they are glucose-excited neurons. A previous study has shown that insulin stimulates glucose uptake and promotes translocation of Glut4 to the neuron surface[31]. This suggests that during the early physiological response to hypoglycemia, when insulin secretion declines, a reduction in insulin action can decrease the translocation of GLUT4 glucose transporters to the membrane in hypothalamic Glut4 neurons, thereby reducing glucose uptake and silencing neuronal activity. If these hypothalamic glucose excited neurons are indeed inhibitory GABAergic neurons, as has been shown previously [32], then a decrease in inhibitory output can enhance the counterregulatory hormone response. In the case of the GIRKO mice, a chronic reduction in IRs may decrease the activity of these glucose excited GABAergic neurons and ultimately, augment the counterregulatory response during hypoglycemia.

The current data provides evidence that insulin may influence the activity of glucose responsive Glut4 neurons in the hypothalamus to modulate the counterregulatory hormone response to hypoglycemia. Specifically, loss of insulin receptors and signaling on Glut4 neurons in GIRKO mice results in an exaggerated glucagon response to insulin-induced hypoglycemia. As IRs are also knocked out in peripheral tissues of GIRKO mice, peripheral insulin resistance becomes a confounding factor. For this reason, we knocked down IR expression in the VMH of C57BL6 mice and found that this manipulation recapitulated the enhanced glucagon response

observed in the GIRKO mice. It is important to note that the chronic loss of IRs in the VMH can lead to impaired glucose tolerance after twelve weeks of knockdown, but not after 6 weeks of knockdown [33]. In our VMH IR-KD mice, IR knockdown was only maintained for a period of 4 weeks and therefore, the development of peripheral insulin resistance and impaired glucose tolerance was likely not a factor that augmented the glucagon response. Moreover, when we centrally administered the glucoprivic agent, 2-DG, into the brains of GIRKO mice, we observed an exaggerated hyperglycemic response that was coupled with an augmented glucagon response. These observations are consistent with the idea that the loss of insulin receptors in the brain and more specifically, in hypothalamic Glut4 neurons, leads to a decrease in inhibitory output which enhances glucagon secretion.

In addition, these studies also show that in response to neuroglycopenia, there was greater phosphorylation of Akt in the hypothalamus of GIRKO mice compared to the hippocampus. Interestingly, while the cell-autonomous decrease of pAkt in Glut4 neurons of GIRKO mice was expected in this scenario, the marked increase of pAkt in non-Glut4 neurons has not been described. This change in pAkt could be engaged independently of the IR and may be due to many factors. For instance, this may be a result of the hormonal/metabolites feedback inputs to these non-Glut4 neurons. Nevertheless, further studies will be required to ascertain whether this ectopic Akt activation in non-Glut4 neurons is either necessary or sufficient to initiate the counterregulatory response.

An unexpected finding of this work is the paradoxical activation of Stat3 and S6 signaling in non-Glut4 neurons following IR ablation in Glut4 neurons, suggesting that insulin signaling in Glut4 neuron dampens leptin and amino acid signaling in a cell-autonomous fashion. The finding emphasizes the integrative role of Glut4 neurons in energy homeostasis [34], while raising the possibility that the identification of the additional feedback cues emanating from these neurons will provide actionable points to increase leptin or nutrient sensitivity in the treatment of metabolic disorders [35].

Future work is warranted to investigate the circuitry of Glut4 neurons mediating the response to hypoglycemia, as Glut2 neurons of the nucleus tractus solitarius (NTS) are reported to link hypoglycemia detection to counterregulatory response [36]. The finding that hyperglycemic response to 2DG is not concordant with blunted feeding response in the GIRKO is surprising. Similar findings were reported in the hindbrain catecholamine neurons [37]. Therefore, whether the hindbrain is involved in the circuitry of Glut4 neurons mediating the hypoglycemia response will also be investigated. In conclusion, the findings of the present work contribute to mapping neural entities contributing to the regulation of energy homeostasis, as well as new insight into the pathophysiology of the counterregulatory response.

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AUTHORS' CONTRIBUTIONS

D.A., H.R. and O.C. designed and conducted experiments, analyzed data, and wrote the article; S.Y., A.V., and A.M.R. conducted experiments and analyzed data. A.M.R., H.R. bred the mice, performed the GIRKO neuroglycopenic experiments, and analyzed the hypothalamic signaling pathways. S.Y. conducted the electrophysiology experiments. A.V. and O.C. validated the brain specific IR knockdown using viral approach, performed the hypoglycemic clamp studies and measured the counterregulatory hormones during the clamp.

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Declarations of no interest.

Data Availability

All data generated or analyzed during this study are included in this published article or in the data repositories listed in References.

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Figure 1. Glut4 neurons are glucose responsive neurons. (A) Glut4 neurons were identified using a fluorescent microscope. (B) Spontaneous action potentials (sAPs) were recorded in the whole-cell current-clamp mode in Glut4 neurons perfused sequentially with aCSF containing 5 mM glucose (baseline), 0.1 mM glucose and 5 mM glucose (washout). Representative 30 s sections of a current-clamp recording from the same neuron in aCSF containing different extracellular glucose concentration were shown below. (C) Mean changes in membrane potential with changes in extracellular glucose concentration. (D) Mean fold changes in sAP frequency with changes in extracellular glucose concentration. Data show means ± SEM (n=8) (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, paired t-test).

Figure 2. pAkt signaling after neuroglycopenia challenge. (A) Time course study of the hyperglycemic response after 2-DG *icv* injection in WT and GIRKO mice (n=7-10 per group). (B) Serum glucagon measurement after saline or 2-DG *icv* injection. Data show means ± SEM (n=9). (C) Western blot showing increased pAkt in GIRKO hypothalamus. (D) Quantification of the distribution of pAkt+ cells and Glut4 neurons in WT and GIRKO mice after neuroglycopenia. Data show means ± SEM (n=5) (*= $p<0.05$, **= $p<0.01$, ****= $p<0.0001$, 2way ANOVA Sidak's multiple comparison test).

Figure 3. Increased counterregulatory response in GIRKO mice in hypoglycemic clamp studies. Plasma glucose (A) and insulin (B) concentration during the hypoglycemic clamp procedure were well matched between WT and GIRKO mice. Despite this, glucose infusion rates (C) were significantly lower in the GIRKO mice compared to WT. The glucagon response (D) was significantly increased in the GIRKO mice, but plasma epinephrine (E) and norepinephrine (F) responses were not significantly different. Data show means ± SEM (n=6) (*= $p<0.05$, ***= $p<0.001$, ****=P<0.0001, 2way ANOVA Sidak's multiple comparison test).

Figure 4. Increased counterregulatory response in IR-KD mice in hypoglycemic clamp studies. Plasma glucose (A) concentrations were matched between Control and VMH IR knockdown (IR-KD) mice during the hypoglycemic clamp. Glucose infusion rates (B) were significantly lower in IR-KD mice. Plasma glucagon (C) and epinephrine (D) were measured during the clamp. Data show means ± SEM (n=5-6 per group) (*= $p<0.05$, 2way ANOVA Sidak's multiple comparison test).

Figure 5. Hormone and nutrient signaling in Glut4 neurons from GIRKO mice. (A-C) Immunohistochemistry with pAkt (A), pS6 (B), and pStat3 (C) and GFP in the ARC of WT and GIRKO mice. Following refeeding, integrated density of the fluorescent signal in Glut4- (black bars) and Glut+ (green bars) neurons was measured and plotted on the right. Data show means ± SEM (n=21-138 cells measured per group) (*= $p<0.05$, **= $p<0.01$, ****= $p<0.0001$, one-way ANOVA Tukey's multiple comparisons test). (D) 24-hr food intake following leptin injection. Data show means ± SEM (n=7) (*= $p<0.05$, 2way ANOVA Bonferroni multiple comparisons). (E) Food intake measurement after saline or 2-DG ip injection. Data show means ± SEM (n=6) (***= $p<0.001$, 2way ANOVA Bonferroni multiple comparisons).

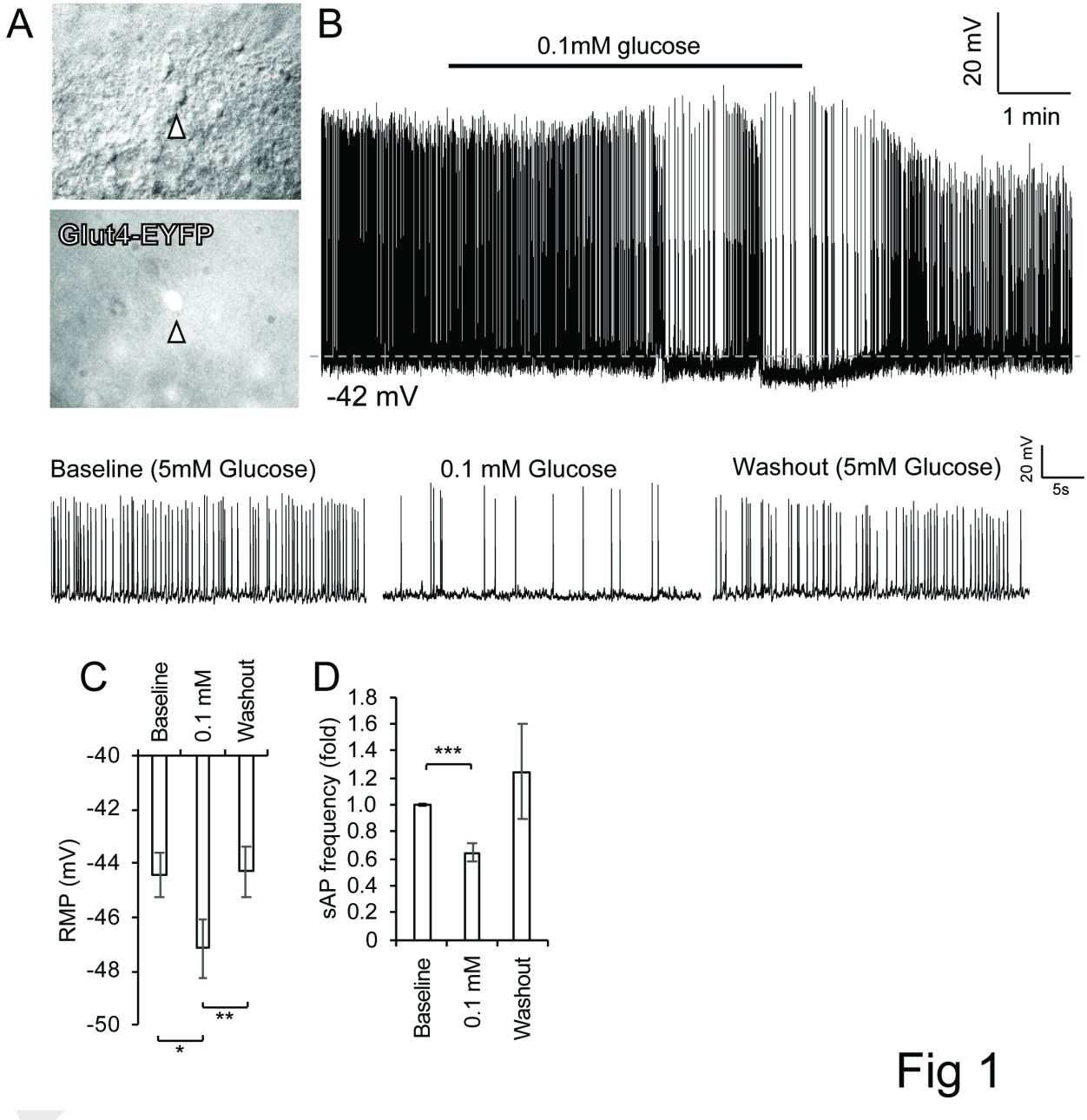
A
Glut4-EYFP
B
0.1mM glucose
20 mV
1 min
-42 mV
Baseline (5mM Glucose)
0.1 mM Glucose
Washout (5mM Glucose)
20 mV
5s
C
Baseline
0.1 mM
Washout
RMP (mV)
-40
-42
-44
-46
-48
-50
*
**
D
sAP frequency (fold)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
***
Baseline
0.1 mM
Washout

Fig 1

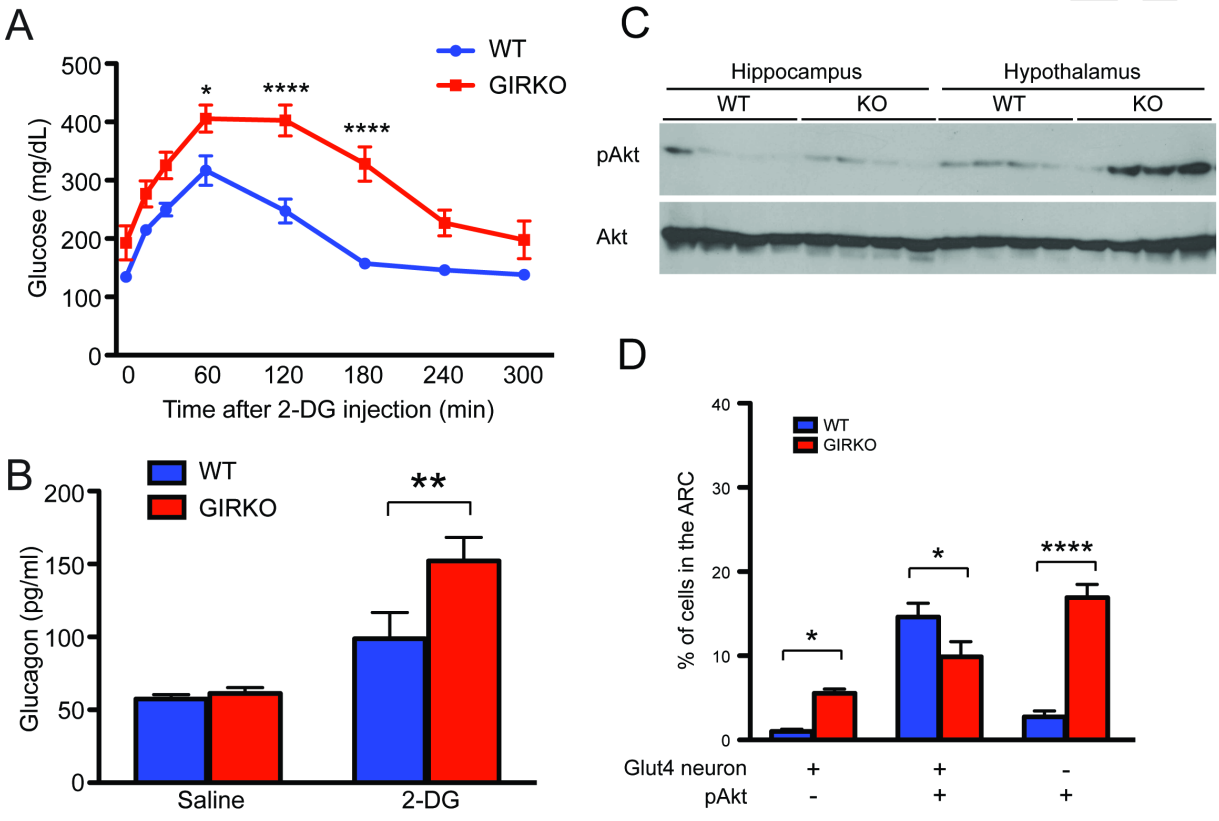
A
WT
GIRKO
Glucose (mg/dL)
500
400
300
200
100
0
*
****
****
0
60
120
180
240
300
Time after 2-DG injection (min)
B
WT
GIRKO
**
Glucagon (pg/ml)
200
150
100
50
0
Saline
2-DG
C
Hippocampus
Hypothalamus
WT
KO
WT
KO
pAkt
Akt
D
WT
GIRKO
% of cells in the ARC
40
30
20
10
0
*
*
****
Glut4 neuron
pAkt
+
-
+
+
-
+

Fig 2

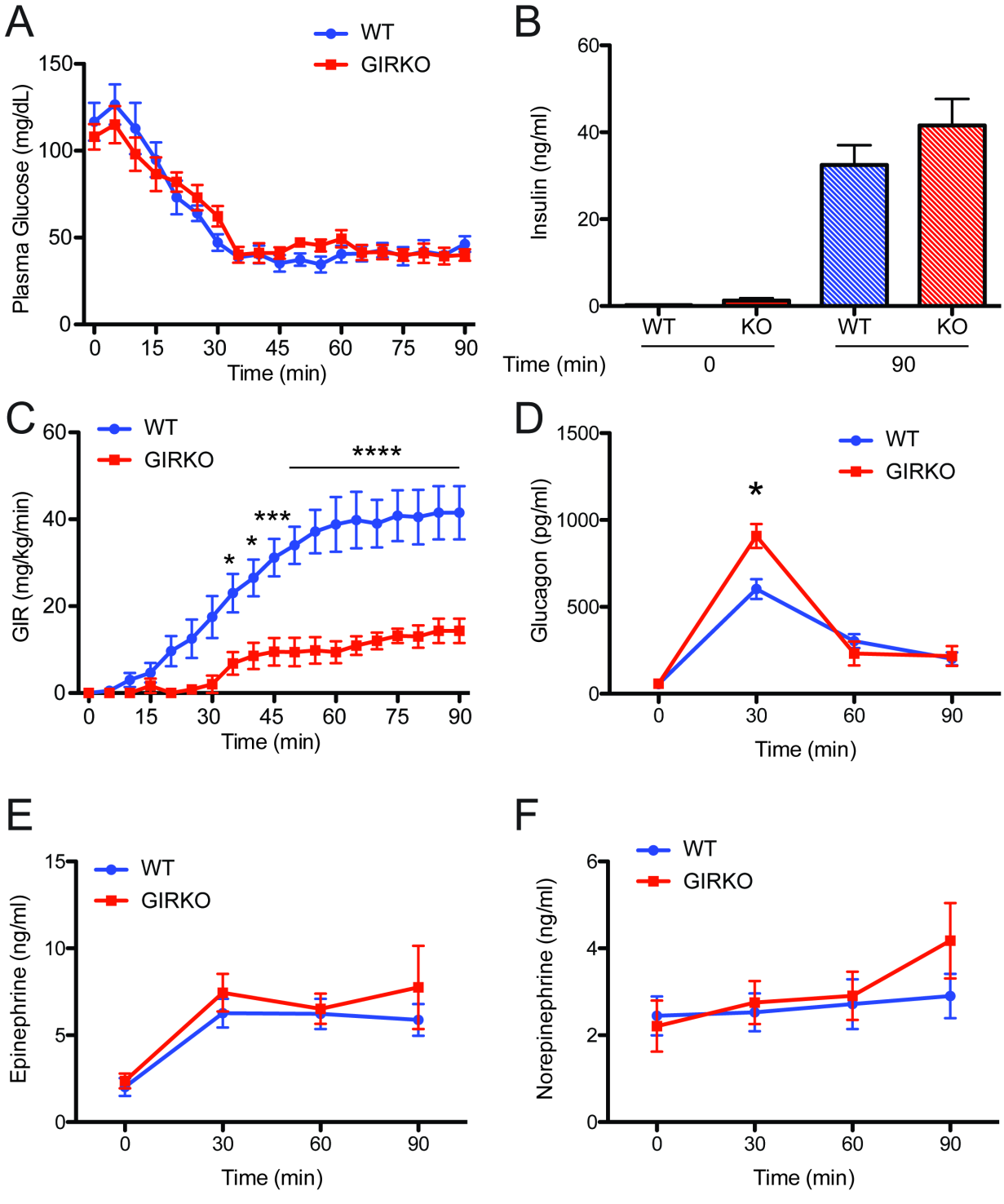
A
WT
GIRKO
Plasma Glucose (mg/dL)
150
100
50
0
0
15
30
45
60
75
90
Time (min)
B
Insulin (ng/ml)
60
40
20
0
WT
KO
WT
KO
Time (min)
0
90
C
WT
GIRKO
GIR (mg/kg/min)
60
40
20
0
****
***
*
*
0
15
30
45
60
75
90
Time (min)
D
WT
GIRKO
Glucagon (pg/ml)
1500
1000
500
0
*
0
30
60
90
Time (min)
E
WT
GIRKO
Epinephrine (ng/ml)
15
10
5
0
0
30
60
90
Time (min)
F
WT
GIRKO
Norepinephrine (ng/ml)
6
4
2
0
0
30
60
90
Time (min)

Fig 3

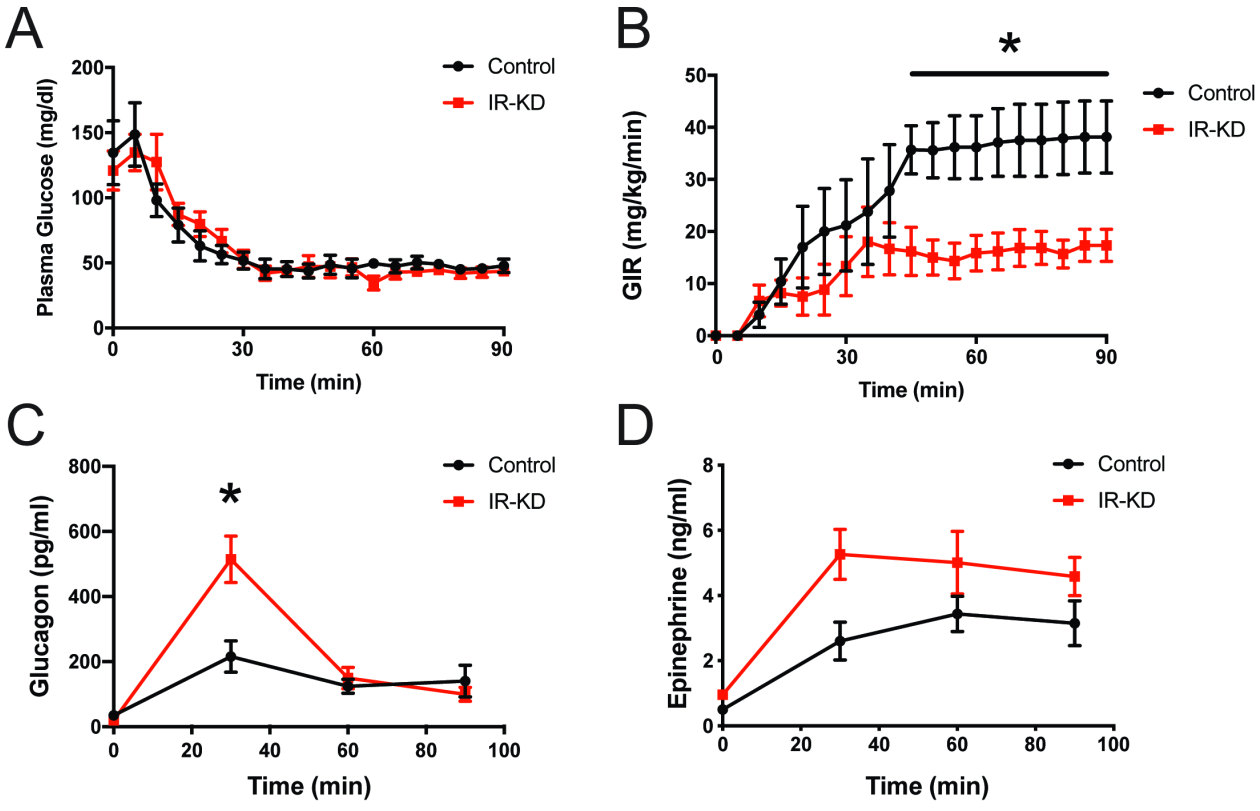
A
Plasma Glucose (mg/dl)
200
150
100
50
0
0
30
60
90
Time (min)
Control
IR-KD
B
*
GIR (mg/kg/min)
50
40
30
20
10
0
0
30
60
90
Time (min)
Control
IR-KD
C
Glucagon (pg/ml)
800
600
400
200
0
*
0
20
40
60
80
100
Time (min)
Control
IR-KD
D
Epinephrine (ng/ml)
8
6
4
2
0
0
20
40
60
80
100
Time (min)
Control
IR-KD

Fig 4

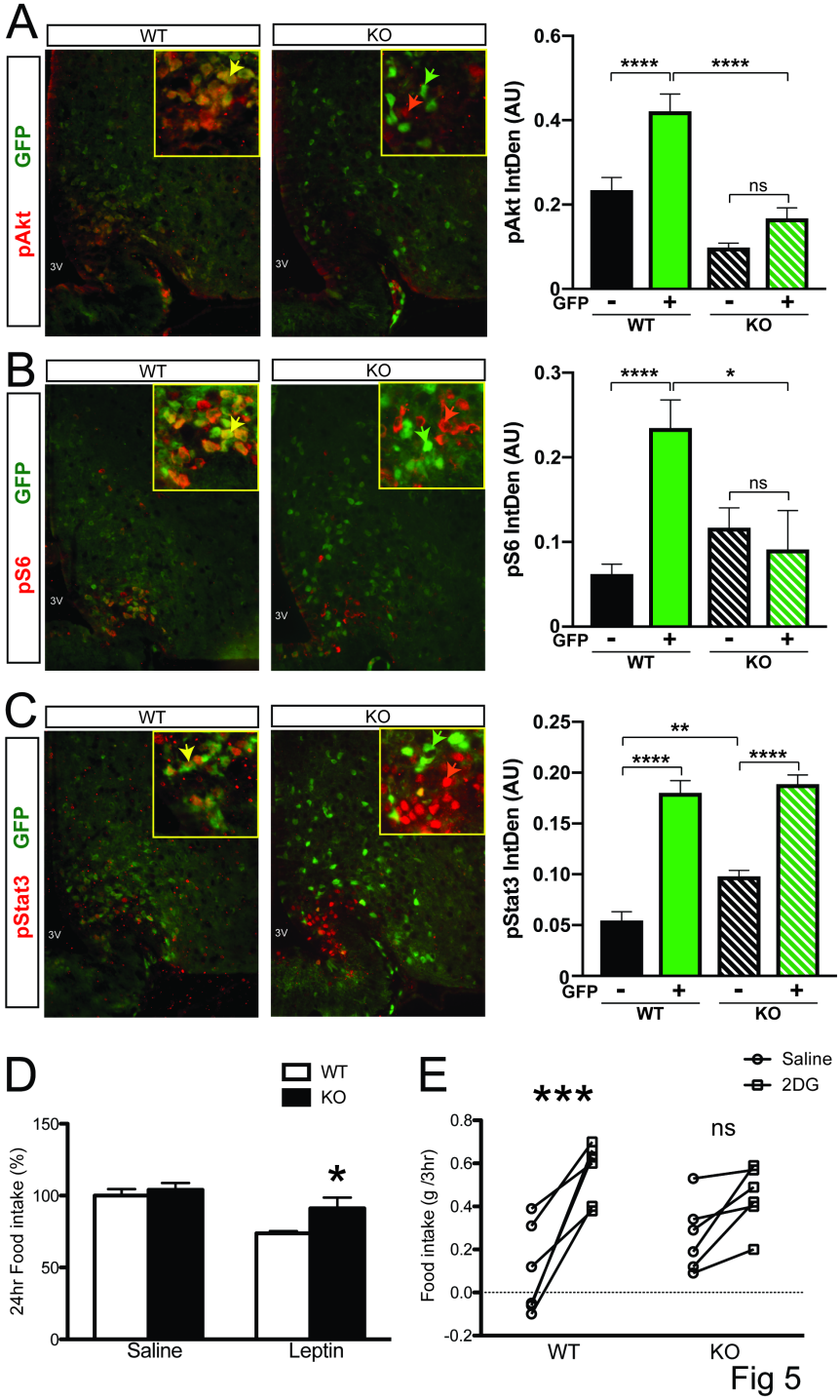
A
WT
KO
pAkt
GFP
3V
pAkt IntDen (AU)
0.6
0.4
0.2
0
****
****
ns
GFP - + - +
WT
KO
B
WT
KO
pS6
GFP
3V
pS6 IntDen (AU)
0.3
0.2
0.1
0
****
*
ns
GFP - + - +
WT
KO
C
WT
KO
pStat3
GFP
3V
pStat3 IntDen (AU)
0.25
0.20
0.15
0.10
0.05
0
**
****
****
GFP - + - +
WT
KO
D
WT
KO
24hr Food intake (%)
150
100
50
0
*
Saline
Leptin
E
Saline
2DG
***
ns
Food intake (g /3hr)
0.8
0.6
0.4
0.2
0.0
-0.2
WT
KO

Fig 5