Insulin Action on GABA Neurons Is a Critical Regulator of Energy Balance But Not Fertility in Mice

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Insulin signaling in the brain plays an important role in the central regulation of energy homeostasis and fertility, such that mice exhibiting brain-specific deletion of insulin receptors (InsRs) display a diet-sensitive obesogenic phenotype and hypothalamic hypogonadism. However, the specific neurons mediating insulin’s central effects on fertility remain largely unidentified. The neurotransmitters γ-aminobutyric acid (GABA) and glutamate are important modulators of fertility and energy homeostasis and are widely distributed in the hypothalamus. We therefore investigated whether insulin signaling via GABAergic or glutamatergic neurons plays an important role in the metabolic regulation of fertility. We used the Cre-loxP system to generate mice with a selective inactivation of the Insr gene from GABAergic (Vgat$^{-}$) or glutamatergic (Vglut2$^{-}$) cells by crossing Insr-flox mice with Vgat-Cre or Vglut2-Cre mice, respectively. Multiple reproductive and metabolic parameters were then compared between male and female Insr-flox/Vgat-Cre$^{-}$ (Vgat-IRKO), Insr-flox/Vglut2-Cre$^{-}$ (VglutIRKO), and Insr-flox/Creat-negative control (CON) mice. Female VgatIRKO mice exhibited a significant increase in adult body weight, abdominal fat mass, and fasting plasma insulin and leptin concentrations, but normal fasting glucose concentration and glucose tolerance compared with CON mice. Surprisingly, VgatIRKO and VglutIRKO mice exhibited normal reproductive maturation and function compared with CONs. No differences in the age of puberty onset, estrous cyclicity, or fertility were observed between VgatIRKO, VglutIRKO, and CON mice. However, male VgatIRKO mice exhibited significantly augmented LH concentration and a trend toward reduced seminal vesicle weight compared with CON mice, which may be indicative of primary hypogonadism. Our results therefore demonstrate that insulin signaling via GABAergic and glutamatergic cells is not required for fertility in mice, but show that GABAergic neurons encompass circuitry through which insulin acts to modulate energy homeostasis. (Endocrinology 155: 4368–4379, 2014)

Mammalian reproduction is energetically costly, and sufficient energy availability is thus required for the initiation of puberty and maintenance of reproductive capacity (1, 2). Accordingly, several centrally acting metabolic messengers involved in communicating peripheral energy status to the neuroendocrine reproductive axis have been identified (3). Importantly, the pancreatic peptide insulin is considered a critical central regulator of energy balance and fertility, such that in its absence, metabolic and reproductive impairments arise. For example, mice exhibiting brain-specific (Nestin-Cre-mediated) insulin receptor (InsR) deletion (NIRKO mice) exhibit metabolic dysfunction and subfertility (4). However, the specific neuronal populations involved in critically mediating insulin’s central effects on metabolic and reproductive function remain largely unidentified. Some of the neuronal candidates thought to facilitate insulin’s central actions have already been directly examined, yet knockout (KO) studies have failed to fully recapitulate the metabolic and/or subfertile phenotype observed in NIRKO mice (4–6). Interestingly, although the targeted disruption of both insulin and leptin signaling in pro-opiomelanocortin neurons

Abbreviations: ARC, arcuate nucleus; AUC, area under the curve; BW, body weight; CI, confidence interval; CON, control; CV, coefficient of variance; GABA, γ-aminobutyric acid; GTT, glucose tolerance test; HOMA, homeostatic model assessment; HOMA-%B; HOMA of β-cell function; HOMA-IR, HOMA of insulin resistance; InsR, insulin receptor; KO, knockout; LepR, leptin receptor; MBH, medial basal hypothalamus; pAKT, phospho-AKT; POA, preoptic area; PVN, paraventricular nucleus; qPCR, quantitative RT-PCR; TBS, Tris-buffered saline; TH, tyrosine hydroxylase; VTA, ventral tegmental area; WT, wild-type.
rons compromises metabolic and reproductive function, disruption of insulin signaling alone has no effects (6). Insulin signaling via GnRH neurons is likewise not critically involved in regulating reproductive function, because GnRH-specific InsR-KO mice undergo normal reproductive maturation and are fully fertile (7). Finally, insulin signaling via kisspeptin neurons, which are the most potent known stimulators of GnRH release, does not affect adult fertility and has very little (8) or no (9) effect on the timing of puberty onset. The selective disruption of insulin signaling from specific hypothalamic peptide-expressing neuronal populations has not led to major metabolic (5, 6) or reproductive (7, 8) phenotypes, likely due to both the complexity and redundancy of the insulin-sensitive pathways regulating energy balance and reproductive activity.

The purpose of this study was therefore to take a more widespread approach and determine whether targeted deletion of InsR signaling from either γ-aminobutyric acid (GABA)- or glutamate-expressing neurons results in disrupted regulation of energy balance and/or reproductive function. GABA- and glutamate-expressing neurons are widely distributed throughout the hypothalamus and are both involved in the regulation of GnRH neuronal activity (10–12) and energy homeostasis (13, 14). Furthermore, electrophysiological evidence indicates fasting affects GABAergic drive to GnRH neurons, and leptin treatment restores this drive in fasted mice (15), suggesting GABA neurons mediate some of leptin’s effects on the neuroendocrine reproductive axis. In further support of this, GABA-specific leptin receptor (LepR) KO mice were recently shown to exhibit profound obesity, delayed puberty onset, and subfertility (16). In contrast, glutamate-specific LepR-KO mice exhibited a more mild body weight (BW) phenotype and were fully fertile (16), highlighting that leptin’s central effects on the neuroendocrine reproductive axis are critically mediated via GABAergic neurons, and not glutamatergic neurons.

Therefore, performing a comparable study to determine whether insulin signals predominantly via GABAergic and/or glutamatergic neurons would help narrow the search for the critical insulin-responsive neuronal populations involved in mediating insulin’s effects on fertility and energy homeostasis. To this end, the Cre-loxP system was used to generate mice exhibiting Insr deletion from either GABAergic or glutamatergic cells. The 2 Cre-expressing mouse lines used to specifically target Insr deletion from GABA and glutamate cells were 1) a vesicular GABA transporter (Vgat)-Cre mouse, which identifies almost all GABAergic neurons (17), and 2) a vesicular glutamate transporter-2 (Vglut2)-Cre mouse (18), which identifies glutamate neurons in the hypothalamus, thalamus, midbrain, and hindbrain (19). Multiple reproductive and metabolic parameters were then assessed in the resultant GABA-specific and glutamate-specific Insr-KO mice to characterize the functional role of insulin signaling via GABAergic and glutamatergic neurons, respectively.

Materials and Methods

Animals
Male and female C57BL/6J wild-type (WT) and gene-targeted mice were obtained from the University of Otago animal breeding facility. Rodents were group-housed and maintained under conditions of controlled lighting (lights on from 6:00 AM to 6:00 PM) and temperature (22°C ± 1°C) and had free access to standard rodent chow and water. The University of Otago Animal Ethics Committee approved all animal protocols.

Generation of Vgat- and Vglut2-specific Insr-KO mice

To generate Vgat-specific Insr-KO (VgatIRKO) and Vglut2-specific Insr-KO (VglutIRKO) mice, homozygous Insr-flox mice of C57BL/6N genetic background (4) were bred to Vgat-Cre or Vglut2-Cre mice of mixed FVB and C57BL/6J background (18) and then backcrossed to homozygous Insr-flox mice to produce homozygous Vgat-Cre/Insr-flox and Vglut2-Cre/Insr-flox mice. Littermates lacking Cre expression from both of these breeding programs were used as the respective controls (CONs). Transgenic mice were identified using PCR analysis of genomic DNA isolated from tail tips using the following primer sets and annealing temperatures: Cre primers, 5’-GCCGGTCTGGACGTTAAAAACTATC-3’, 5’-GTGAAACAGCATGTGCATTGCTGT-CACCT-3’, 5’-CTAGGCCACAGGATTTGAGATCT-3’, and 5’-GTAGGTGAAATTCTGACATCATC-3’ (52°C); and Insr-flox primers, 5’-GATGTGCACCCCATGTGTCTG-3’ and 5’-CTGAATAGCTGAGCACAC-3’ (56°C). PCR was also performed on DNA from the hypothalamus, cortex, pancreas, adipose tissue, and gonads of VgatIRKO, VglutIRKO, and CON male mice using the following primer sets and annealing temperature: 5’-ACCGTGCTTAGAGACTCCAA-3’ and 5’-CTGAATAGCTGAGCACAC-3’ (56°C). As seen in Figure 1, a 220-bp product indicating recombination and gene deletion was produced from the hypothalamus and cortex of VgatIRKO and VglutIRKO, and also to a small degree from the gonads of male VgatIRKO mice. A 300-bp product indicating the presence of the nonrecombined floxed Insr gene was produced in all tissues from both CON and KO mice (Figure 1).

Reproductive assessment

For assessment of reproductive function in males and females, VgatIRKO (females, n = 8; males, n = 11), VglutIRKO (females, n = 5; males, n = 4–8) and CON (females, n = 10–12 per group; males, n = 7–8 per group) mice were first monitored for puberty onset and estrous cyclicity. To determine puberty onset in females, mice were examined for vaginal opening from 25 days of age and vaginal smears were collected from the day of vaginal opening for determination of first estrus occurrence by cytological examination. Beginning at least 7 days after first estrus was observed, vaginal cytology was used to monitor estrous cyclicity. Once regular estrous cyclicity was established, daily cycle stages...
were monitored for 14 days, which included at least 2 full cycles per animal (aged 7–9 weeks). Mean cycle length was considered the average estrus-to-estrus interval. The experimental male mice were paired with mature WT females from 35 days of age and the age of the first fertile mating was determined by back-dating 20 days (gestation length) from the date of birth of the first litter. To assess adult fertility, VgatIRKO (females, n = 8; males, n = 11), VglutIRKO (females, n = 5; males, n = 8) and CON (females, n = 10 per group; males, n = 7–8 per group) mice were individually paired with fertile WT mates and mean interlitter intervals and size of litters produced were recorded over at least 100 days.

Metabolic assessment

The same cohort of mice used for fertility assessment was used for assessment of metabolic phenotype. Postweaning BWs were recorded every 2 weeks, except female mice were not weighed during the period of fertility assessment (age 10–26 weeks). Final adult BWs and glucose tolerance tests (GTTs) were performed on male (aged 23 weeks) and female (aged 30 weeks) mice after an 18-hour overnight fast (4:00 PM to 10:00 AM). For the GTT, fasted blood glucose concentrations were measured, and animals were then injected sc with a bolus of 1 g/kg glucose in 100 μL physiological saline. Blood glucose concentrations were measured at 15, 30, 45, 60, 90, 120, and 180 minutes thereafter. Blood glucose values were determined from tail vein blood using an automatic glucose monitor (Roche; Accu-Chek Performa). A modified trapezoidal integrated area under the curve (AUC) technique was used to compare differences in glucose tolerance between groups. Homeostatic model assessment (HOMA) scores were calculated according to Matthews et al (20) to further investigate insulin resistance (HOMA-IR) and pancreatic β-cell function (HOMA-%β).

Tissue collection

Female mice

At the end of the study, female mice were fasted overnight, anesthetized with sodium pentobarbital (240 mg/kg ip), and transcardially perfused with 4% paraformaldehyde. To allow for insulin-induced phospho-AKT (pAKT) immunohistochemistry, VgatIRKO and CON mice were given 200 mU porcine insulin and 90 mg glucose sc (n = 4–5 per group) or 200 μL saline vehicle alone (n = 3–5 per group) 30 minutes before perfusion, as described previously (9). Due to the small group size (n = 5), VglutIRKO females did not receive the insulin challenge. Brains were postfixed for at least 1 hour in 4% paraformaldehyde and then transferred to 30% sucrose solution and stored at 4°C until the brains sank. Coronal (30 μm thick) sections throughout the hypothalamus were then cut on a sliding microscope to provide 3 sets of consecutive sections (90 μm between sections within each set). Atrial blood samples were collected just before perfusion with a heparinized syringe and the plasma harvested and stored at −20°C for later hormone immunooassay. Abdominal fat and uteri from animals in metestrus or diestrus were dissected and the wet weight recorded.

Male mice

Male mice were fasted overnight and killed by decapitation. Trunk blood samples were collected and the serum harvested and stored at −20°C for later hormone immunooassay. Fresh brains and pancreata were dissected out and immediately put on dry ice before being transferred to −80°C for storage. Paired testes, seminal vesicles, and all abdominal fat was dissected out and the wet weight recorded.

Hormone measurements

All hormones were measured in female plasma samples and male serum samples collected from saline-treated or untreated animals after an overnight fast. Insulin and leptin concentrations were measured using commercially available ultrasensitive mouse insulin and mouse leptin ELISA kits (Crystal Chem, Inc). The sensitivities of the assays were 0.16 ng/mL insulin and 0.2 ng/mL leptin, respectively. The mean intra-assay coefficients of variance (CV) were 3.8% and 2.9%, respectively.

A sandwich ELISA, adapted from Steyn et al (21), was used to measure LH concentration. Monoclonal bovine LHβ 518 B7 (1:1000 dilution) was used as the capture antibody (obtained from Lillian Sibley at University of California Davis), and the standard was NIDDK mouse LH reference preparation from A. F. Parlow (AFP5306A mouse RIA kit). Bound standards and samples were incubated with the detection antibody (rabbit polyclonal LH antibody AFP240580Rb; final dilution 1:1000) followed by horseradish peroxidase-conjugated secondary antibody (DAKO Cytomation polyclonal goat antirabbit IgG/ horseradish peroxidase; final dilution 1:1000). Addition of o-phenylenediamine (Life Technologies) substrate resulted in the colorimetric reaction, and the absorbance was read at 490 nm. The sensitivity was 0.054 ng/mL and the mean intra-assay CV was 2.9%.

Total testosterone was measured in 50-μL aliquots extracted in diethyl ether, using a commercially available enzyme immunooassay (Cayman Chemical Company) according to the manufacturer’s instructions. The sensitivity was 3.9 pg/mL, and the mean intra-assay CV was 1.3%.

Characterization of VgatIRKO and VglutIRKO mice

Insr mRNA analysis by quantitative RT-PCR

To compare relative levels of Insr expression in discrete brain regions between KO and CON mice, quantitative RT-PCR (RT-
qPCR) was performed. Briefly, 300-μm coronal brain sections were cut from VgatIRKO (n = 10), VglutIRKO (n = 8), and CON (n = 4–6 per group) male mouse brains using a cryostat, and tissues were micropunched from the preoptic area (POA) (2 consecutive sections between +0.2 and +0.8 mm relative to bregma), paraventricular nucleus (PVN) (1 section between −1.4 and −2.0 mm relative to bregma) with blunted stainless-steel 21-gauge needles with a 500-μm diameter. Tissues were placed in 350 μL RLT buffer (from RNeasy Plus Micro spin columns kit; QIAGEN) containing 3.5 μL β-mercaptoethanol. Total RNA was extracted using the RNeasy Plus Micro spin columns kit according to the manufacturer’s instructions. Extracted RNA (70 ng per sample) was subjected to RQ1 (RNA-Qualified) Promega deoxyribonuclease treatment (Promega Corporation). Total RNA was then reverse transcribed with SuperScript III reverse transcriptase (Invitrogen) using random hexamers as primers, as described previously (22).

Triplicate uniplex reactions for measurement of Insr levels were carried out on cDNA samples using the following primers: Insr forward, 5′-TATGCTAGTTCCCGTATCC-3′ and reverse 5′-ACAGACAGTCCCCCATCTCCA-3′ (NCBI reference sequence NM_010568). The reference gene Actb was chosen in this experiment due to its minimal variation across experimental groups: forward 5′-AGGCCAACGTTGAAGATG-3′ and reverse 5′-GCCTTGATGGCTACGTACATG-3′ (NM_007393). Reactions (20 μL) were prepared using SYBR Green PCR Master Mix (Applied Biosystems). Using a LightCycler 480 qPCR thermo-cycler (Roche), samples were heated to 50°C for 2 minutes and then 95°C for 10 minutes before 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Quantitative PCR data were analyzed using the comparative Cq method in which Cq is the quantification cycle number at which the fluorescence reading is first recorded above background levels. Subtracting the average Cq value for the gene of interest from the average Cq value for the reference gene gave the ΔCq. Subtracting the mean ΔCq value from the ΔCq for each sample gave the relative change (ΔΔCq). Finally, the arithmetic formula \[2^{-\Delta\Delta Cq}\] was used to achieve relative quantitation. These \(2^{-\Delta\Delta Cq}\) values were used for statistical analysis and graphical presentation.

**Chromogen immunohistochemistry for Cre recombinase**

All immunohistochemistry steps were separated by 3 washes in 0.05M Tris-buffered saline (TBS). Free-floating sections were incubated for 30 minutes in 1% hydrogen peroxide and then blocked for at least 30 minutes in blocking solution (2% normal goat serum in TBS with 0.5% Triton X-100 and 1% BSA). Sections were then incubated overnight at 4°C in rabbit anti-Cre (1:10 000; a kind gift from G. Schütz, Heidelberg, Germany, and A. Herbsion, Dunedin, New Zealand), followed by a 90-minute incubation with secondary antibody (biotinylated goat anti-rabbit IgG, dilution 1:1000; Vector Laboratories) in TBS at room temperature. Sections were then incubated in Vectastain Elite ABC reagent (Vector Laboratories) in TBS for 60 minutes at room temperature. Finally, sections were developed by incubating in a nickel-enhanced diaminobenzidine solution, giving a dark purple Cre label. Chromogen sections were mounted on gelatin-coated glass slides and coverslipped with DPX mounting media.

**Fluorescent immunohistochemistry for InsR-β and pAKT**

To investigate whether the metabolic phenotype observed in the VgatIRKO female mice was associated with altered insulin signaling in the ARC, immunohistochemistry was performed on free-floating tissue sections from the arcuate nucleus (ARC) of VgatIRKO and CON mice (n = 3 per group) to label both InsR-β and pAKT. Sections underwent an antigen retrieval step (15 minutes; 1mM EDTA [pH 8.0] at 90°C) and were then incubated in AffiniPure Fab fragment goat antimouse IgG (1:100; Jackson ImmunoResearch Laboratories) overnight at 4°C to prevent nonspecific mouse-on-mouse binding. Sections were then blocked for at least 30 minutes in TBS/Triton X-100/BSA blocking solution before incubation in monoclonal mouse anti–InsR-β (1:1000; Thermo Scientific) and monoclonal rabbit anti-pAKT (1:2000; Cell Signaling catalog item 4060) in blocking solution for 72 hours at 4°C. This was followed by a 90-minute incubation in biotinylated goat antimouse IgG (1:500; Jackson ImmunoResearch Laboratories) in TBS and then a final 90-minute incubation with streptavidin 568 (1:500; Molecular Probes, Life Technologies) and Alexa Fluor 488 goat antirabbit IgG (1:500; Molecular Probes, Life Technologies) in TBS.

Immunofluorescent sections were mounted on glass slides and coverslipped with Vectashield (Vector Laboratories). Staining was examined on a Zeiss LSM 710 upright confocal laser scanning microscope. The fluorochromes were detected with 488-nm (pAKT) and 543-nm (InsR-β) laser excitation lines and filters. Omission of primary antibodies resulted in a complete absence of staining. Inclusion of brain sections from a forebrain neuron-specific InsR-KO (Cam-kinase IIα-Cre, Insr-flox) mouse as an additional negative CON confirmed the specificity of the immunoreactivity (Supplemental Figure 1). All images were examined using ImageJ software (National Institutes of Health). A cell was considered positive for InsR-β or pAKT only if the cell shape could be identified based solely on InsR-β or pAKT immunoreactivity, respectively. For assessment of InsR-β and pAKT immunohistochemistry, the separately recorded green and red images were merged for analysis of colocalization (yellow pixels). The number of dual-labeled soma were counted from the ARC of at least 3 tissue sections per animal. Mean percentages of dual-labeled soma were quantified for each animal, and data were grouped to provide mean ± SEM values.

**Statistical analysis**

Repeated-measures two-way ANOVA was used to compare changes over time between 2 groups, and if a significant main effect or interaction was found, Sidak’s multiple comparisons post hoc testing was used to identify the time points at which significant differences occurred. Single time-point comparisons were analyzed using unpaired Student’s t tests unless the data were not equally distributed or the sample size was fewer than 6, in which case nonparametric Mann-Whitney U tests were used instead. Additionally, 95% confidence intervals (CI) were calculated for nonsignificant hormone measurements when the sample size was fewer than 6 per group. Results are presented as mean ± SEM, and a value of P < .05 was considered to be significant.
Results

Characterization of VgatIRKO and VglutIRKO mice

To characterize differences in \textit{Insr} mRNA expression levels of VgatIRKO, VglutIRKO, and CON male mice in different hypothalamic nuclei, real-time qPCR was performed on the following micropunched hypothalamic regions: the POA (including the rostral POA and anteroventral periventricular nucleus), PVN, and MBH (including the ARC and ventromedial hypothalamic nuclei). Representative photomicrographs of the Cre expression in the VgatIRKO and VglutIRKO mice in these sampled regions are shown in Figure 2, A and B. As seen in Figure 2, C and D, there was a significant decrease in \textit{Insr} mRNA expression in the POA of both VgatIRKO (\(P = .015\)) and VglutIRKO (\(P = .017\)) mice and a nonsignificant decrease in \textit{Insr} mRNA expression in the MBH of VgatIRKO mice (\(P = .08\)) vs CON mice. To further examine the nonsignificant trend toward reduced \textit{Insr} mRNA in the MBH of VgatIRKO mice, dual-label immunohistochemistry for insulin-induced pAKT and InsR-\(\beta\) was performed. As seen in Figure 3, A and B, VgatIRKO mice exhibited significantly reduced InsR-\(\beta\) expression and insulin signaling in the ARC in comparison with CON females, as evidenced by the significant decrease in insulin-induced pAKT and InsR-\(\beta\) immunolabeling (7.6 \(\pm\) 1.0 vs 30.0 \(\pm\) 2.5, \(P = .0002\)). No pAKT-immunolabeled soma were present in the ARC of vehicle-treated animals (data not shown), and no InsR-\(\beta\) staining was observed in the ARC of a forebrain neuron-specific InsR-KO mouse (Supplemental Figure 1).

VgatIRKO mice

Reproductive phenotype

Surprisingly, given the widespread nature of GABA expression in the hypothalamus, VgatIRKO mice (11 males, 8 females) exhibited normal reproductive maturation and fertility compared with CON mice (7 males, 12 females). No differences in the age of male reproductive competency (Figure 4A) or female puberty onset (Figure 4B) were observed, and during a 115-day period of fertility assessment, VgatIRKO males and females exhibited normal interlitter intervals and litter sizes in comparison with CON mice (Figure 4, C and D). Furthermore, no differences were observed in the percent time spent in each estrous cycle phase (Figure 4E) or in mean estrous cycle length (5.3 \(\pm\) 1.1 vs 5.4 \(\pm\) 1.4 days, \(P = .818\)) between VgatIRKO and CON females (aged 7–9 weeks).

LH concentration was measured in plasma collected from adult female mice and serum collected from adult male mice after completion of the fertility study, and although no significant differences were found between VgatIRKO and CON female mice (95% CI = 0.00–1.702 vs 0.00–2.658 ng/mL, \(P = .51\)), a significant increase was observed in male VgatIRKO vs CON mice (\(P < .01\)) (Figure 4F). Additionally, a trend toward reduced seminal vesicle weight (\(P = .07\)) was observed in the VgatIRKO male vs CON mice (Figure 4H). However, no differences in paired testes weight (Figure 4H) or testosterone concentration (Figure 4G) were observed between VgatIRKO and CON males. Lastly, no differences in uterine weights (Figure 4H), which were used as proxy indicators of estrogen status (23), or plasma testosterone concentration (95% CI = 5.88–42.85 vs 11.96–
Metabolic phenotype

Postweaning BW gain did not differ between VgatIRKO and CON male and female mice (Figure 5, A and B). However, a trend toward increased BW (P = .08) was observed in VgatIRKO females at 29 weeks of age, and by 30 weeks of age, the VgatIRKO females weighed significantly more (P < .01; Figure 5C) due to increased adiposity (P = .043; Figure 5D). Furthermore, although no significant differences in fasting insulin or leptin concentrations were observed in male VgatIRKO vs CON mice (P = .891 and 0.747, respectively), female VgatIRKO mice exhibited significantly increased fasting insulin (P < .05) and leptin (P < .01) concentrations (Figure 5, G and H). However, both male and female VgatIRKO mice exhibited normal fasting blood glucose concentrations (P = .761 and 0.427, respectively) and glucose AUC during the glucose tolerance assessment period (P = .266 and 0.792, respectively) (Figure 5, E and F). However, although no differences were found in glucose AUC, repeated-measures two-way ANOVA revealed that male VgatIRKO mice, but not females, exhibited impaired glucose tolerance. Although a significant group effect was not found (P = .36), a significant interaction was observed (P < .02), and post hoc testing revealed the VgatIRKO males exhibited significantly elevated blood glucose concentration at the 60-minute time point vs CON males. However, both male and female VgatIRKO mice exhibited normal HOMA-IR scores (P = .799 and 0.986, respectively) and HOMA-%β scores (P = .964 and 0.786, respectively) (Figure 5, I and J).

VglutIRKO mice

Reproductive phenotype

Male VglutIRKO (n = 8) and CON (n = 8) mice reached reproductive competency at the same age (47.4 ± 1.9 vs 44.1 ± 1.9 days, P = .240). Due to a mouse identification number error, puberty onset and estrous cyclicity data for VglutIRKO females could not be statistically compared. Nevertheless, all females (including 5 VglutIRKO and 10 CON mice) exhibited vaginal opening between the ages of 29 and 36 days (32.6 ± 0.4 days), followed by first estrus between the ages of 32 and 40 days (36.7 ± 0.7 days). Furthermore, all females exhibited normal estrous cyclicity (at least 2 estrous cycles). Similar to VgatIRKO mice, VglutIRKO male and female mice exhibited normal fertility compared with CON mice (Figure 6, A and B). No differences between VglutIRKO and CON males or females were observed in interlitter interval (P = .301 and P = .586, respectively) or litter size (P = .351 and P = .563, respectively).

In contrast to the VgatIRKO male mice, VglutIRKO males exhibited normal LH levels vs CON males (95% CI = 0.106–0.407 vs 0.024–0.662 ng/mL, P = .45) (Figure 6C). Female VglutIRKO mice likewise exhibited normal LH levels vs CON females (95% CI = 0.294–1.891 vs 0.132–2.263 ng/mL, P = .87) (Figure 6c). Furthermore, as seen in Figure 6D, no differences in testosterone concentration were observed between VglutIRKO vs CON males (95% CI = 66.6–158.9 vs 15.3–231.6 pg/mL, P = .77) or females (95% CI = 4.82–29.50 vs 15.31–29.35 pg/mL, P = .15). Lastly, no differences in uterine weight (P = .388), paired testes weight (P = .623), or seminal vesicle weight (P = .541) were observed between VglutIRKO and CON mice (Figure 6E).

Metabolic phenotype

In contrast to the VgatIRKO mice, no differences in any metabolic parameters assessed were observed between VglutIRKO and CON males and females (Figure 7). Postweaning BW gain was not different in male VglutIRKO vs CON mice, and as seen in Figure 7, A and B, no differences in adult BW were found in either male (aged 23 weeks) or female (aged 30 weeks) VglutIRKO vs CON mice (P = .687 and P = .269, respectively). Glucose tolerance, assessed by GTT, was likewise not different between VglutIRKO and CON males and females (P = .631 and P = .816, respectively) (Figure 7, C and D). Furthermore, no differences in fasting insulin levels were observed between VglutIRKO and CON mice (males, 95% CI...
0.147–0.881 vs 0.00–2.338 ng/mL, \( P = .704 \); and females, 95% CI 0.199–0.691 vs 0.071–0.434 ng/mL, \( P = .166 \), or in fasting leptin concentrations (males, 95% CI 2.30–7.56 vs 2.36–8.62 ng/mL, \( P = .722 \); and females, 95% CI 0.94–7.46 vs 0.00–7.02 ng/mL, \( P = .594 \)). Lastly, HOMA scores between VglutIRKO and CON males and females were likewise not different (Figure 7, G and H).

**Discussion**

Nutritional status has been recognized as a key modulator of pubertal timing (24) and fertility (25) since the 1970s, yet the specific mechanisms mediating the integrated control of nutritional status and reproductive function are only recently emerging. In 2000, Brüning et al (4) showed that mice with InsR deletion from all neural progenitor cells (using Nestin-Cre mice) exhibited hypothalamic hypogonadism and subfertility. However, the specific neuronal target cells mediating insulin’s central effects on fertility remain largely unknown. It has been shown that GABAergic and glutamatergic afferents compose a major group of synaptic inputs to GnRH neurons (10, 11), and these afferents themselves are modulated by metabolic cues (15, 26). It was therefore hypothesized that insulin signaling via GABAergic and/or glutamatergic neurons plays an important role in the central regulation of GnRH neuronal activity. To test this hypothesis, the metabolic and reproductive phenotypes of GABA-specific (Vgat\(^+\)) and glutamate-specific (Vglut2\(^+\)) InsR-KO mice were characterized. Surprisingly, the male or female VgatIRKO and VglutIRKO mice were fully fertile in comparison with CON mice. However, the VgatIRKO females exhibited significantly increased BW, ad-
iposity, and fasting insulin and leptin concentrations, suggesting insulin signaling in GABAergic neurons does play an important role in modulating energy homeostasis.

Confirming the efficacy of the Cre recombinase-mediated Insr excision, significant reductions in Insr mRNA expression levels in the POA of VgatIRKO and VglutIRKO mice were observed. This indicates that both GABAergic and glutamatergic neurons near the GnRH neurons express InsR. However, even though GABA and glutamate have been identified as key players in the mechanism whereby the GnRH neuronal network becomes activated in pubertal animals (11, 27), no differences in the age of reproductive maturation in either VgatIRKO or VglutIRKO mice were observed. This suggests insulin modulation of GABAergic and glutamatergic neurons is not critically involved in the mechanism whereby nutritional status is conferred to the GnRH neurons to regulate pubertal timing. Furthermore, all females exhibited normal estrous cyclicity over a 14-day monitoring period, and both male and female VgatIRKO and VglutIRKO mice exhibited normal adulthood reproductive competency, despite the fact GABA and glutamate are important modulators of GnRH neuronal activity in adulthood (10, 12).

Interestingly, although male VgatIRKO mice did not exhibit functional reproductive impairments, a possible mild primary hypogonadic phenotype (i.e., a trend toward reduced seminal vesicle weight as well as significantly elevated LH levels) was observed, which could possibly be due to disrupted InsR signaling in the gonads. Although this remains speculative, an excision band was observed in DNA extracted from VgatIRKO testes, and insulin signaling in the gonads has been implicated in the regulation of gonadal steroid production (28, 29).

**Figure 5.** GABA-specific Insr-KO (VgatIRKO) mice exhibited a significant metabolic phenotype compared with CONs. A–D, No differences in postweaning male (A) and female (B) BWs were observed, but female VgatIRKO mice weighed significantly more at 30 weeks of age (C) due to increased abdominal adiposity (D). E and F, No differences in glucose AUC (inset) in adult male (E) and female (F) mice were observed, but male VgatIRKOs exhibited significantly elevated glucose vs CONs at the 60-minute time point. G and H, Nonparametric analysis revealed that females exhibited significantly increased fasting plasma insulin (n = 4 per group) (G) and leptin (n = 4–6 per group) (H) concentrations. I and J, No differences in HOMA-IR (I) or HOMA-%β (J) were observed between VgatIRKO and CON mice (n = 4–6 per group). Unless otherwise specified, n = 7–10 animals per group. Values are mean ± SEM. *, P < .05 vs CON mice.
Similarly, the female VgatIRKO mice exhibited a BW phenotype that could be suggestive of a hypoestrogenic state. However, unlike the male VgatIRKO mice, they did not exhibit altered LH levels or uterine weights. Thus, any putative impairments in gonadal function were too subtle to cause any reproductive deficits. It should also be noted that the trend toward reduced seminal vesicle weight was not matched by reduced circulating testosterone concentrations in VgatIRKO males. VgatIRKO females also exhibited normal testosterone levels in contrast to the hyperandrogenic, polycystic ovary syndrome-like pro-opiomelanocortin neuronal InsR- and LepR-KO mice described by Hill et al (6).

Interestingly, VgatIRKO females but not males exhibited a mild but significant obese phenotype, which closely recapitulates the metabolic phenotype of the brain-specific InsR-KO mice (4). However, unlike the brain-specific InsR-KO females, which exhibited a significant BW phenotype from a young age (4), the VgatIRKO females exhibited normal postweaning BW gain, and it was not until adulthood that a BW phenotype was identified. In addition to displaying significantly increased adulthood BW and adiposity, VgatIRKO females exhibited fasting hyperinsulinemia and hyperleptinemia. Comparison of hormone regression slopes (plotted against BW) revealed that the difference in insulin concentration, but not leptin concentration ($P < .005$), between VgatIRKO and CON females can be explained by the difference in BW.

However, despite the mild obesogenic phenotype and hyperinsulinemia observed in the VgatIRKO females, no differences were observed in their fasting blood glucose levels, glucose tolerance, or HOMA scores. Unfortunately, insulin tolerance was not tested, and it can therefore only be speculated that the VgatIRKO females were insulin-resistant. Male VgatIRKO mice, on the other hand, exhibited mild glucose intolerance compared with CON mice, despite the fact they exhibited normal fasting glucose concentration, insulin concentration, and HOMA scores. It has previously been shown that insulin signaling in agouti-related peptide neurons, which are GABAergic, play a role in hepatic insulin sensitivity and endogenous glucose production (5, 30). Although speculative, it is therefore possible that, in the absence of compensatory hyperinsulinemia, the male VgatIRKO mice exhibited reduced hepatic insulin sensitivity and/or a failure to reduce endogenous glucose production during the GTT. Nevertheless, we can conclude that insulin signaling via GABAergic neurons plays an important role in the central regulation of energy homeostasis. It remains to be determined whether VgatIRKO mice, like NIRKO mice, exhibit increased sensitivity to diet-induced perturbations, such as

![Figure 6](https://academic.oup.com/endo/article-abstract/155/11/4368/2422615)
high-fat feeding, and whether VgatIRKO mice are more likely to experience reproductive deficits when exposed to a metabolic challenge.

Although GABAergic neurons are widespread, the absence of insulin actions on GABA neurons in the ARC may be partially responsible for the metabolic phenotype observed in the VgatIRKO female mice (31, 32). We showed significantly reduced insulin signaling in the ARC of VgatIRKO vs CON females, which exhibited increased BW and adiposity. This suggests insulin signaling in ARC GABAergic neurons may stimulate efferent anorexigenic pathways and/or inhibit efferent orexigenic pathways to modulate appetite and energy expenditure. Although only a trend (P = .08) toward reduced Insr mRNA expression in the MBH micropunches of VgatIRKO mice was observed, this is likely due to the fact that much of the tissue in these punches includes the ventromedial hypothalamic nucleus, which is almost entirely glutamatergic (18). Interestingly, insulin signaling via tyrosine hydroxylase (TH)-expressing neurons (ie, dopamine neurons) in the ventral tegmental area (VTA) has been shown to play a critical role in the long-term regulation of food intake and fat storage (33), and some of these midbrain VTA dopaminergic neurons have likewise been shown to be GABAergic, as reviewed by Lammel et al (34). It is therefore possible that the metabolic phenotype observed in the VgatIRKO mice is partly due to a loss of insulin signaling in Vgat-expressing VTA dopamine neurons. TH-expressing neurons are also found in the ARC (35); however, it remains unknown whether insulin signaling via ARC TH neurons is involved in the regulation of energy balance. Other nonhypothalamic sites of insulin’s metabolic actions include the brainstem dorsal vagal complex and the amygdala (36), although it is unknown whether the target neurons are GABAergic.

Evidence suggests brain InsR signaling plays a key role in regulating synaptic plasticity (37) and neuronal survival as early as embryonic day 15.5 in mice (38), and a possibility thus remains that the loss of InsR signaling from GABAergic neurons may have caused impairments in GABAergic and/or other brain circuitry that could explain the observed VgatIRKO phenotypes. However, it was recently demonstrated that leptin treatment can engage hypothalamic neurocircuitry to permit survival in the complete absence of insulin (39), demonstrating there are likely

Figure 7. Glutamate-specific Insr-KO (VglutIRKO) male (n = 8) and female (n = 5) mice have normal metabolic control in comparison with CON male (n = 4) and female (n = 10) mice. A–D, No differences in adulthood BW (A and B) or fasting glucose and glucose tolerance (C and D) were observed between VglutIRKO and CON mice. E–H, Fasting insulin (E) and leptin (F) concentrations, and HOMA-IR and HOMA-%β (G and H) were likewise not different between VglutIRKO and CON males (n = 5–6 per group) or females (n = 3–5 per group). Values are mean ± SEM.
compensatory pathways in place to preserve critical brain circuitry. Furthermore, the VglutIRKO mice likewise exhibited a widespread loss of InsR signaling in the brain without exhibiting a significant phenotype. These data highlight one of the key limitations of the conditional KO models used in this study, namely, the strong possibility that a meaningful degree of developmental compensation occurred to preserve critical function in both the VgatIRKO and VglutIRKO mice.

In summary, this study tested the hypothesis that insulin signaling via GABAergic and/or glutamatergic neurons plays a critical role in the metabolic regulation of fertility in mice. The data demonstrate that reproductive maturation and function is not impaired in the absence of InsR signaling in either GABAergic or glutamatergic neurons. However, insulin signaling via GABAergic neurons appears to play a critical role in the regulation of energy homeostasis in female, but not male, mice. These findings highlight the likelihood that the metabolic control of fertility by insulin involves numerous redundant neuronal pathways to the reproductive axis.

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