Central insulin signaling modulates hypothalamus—pituitary—adrenal axis responsiveness

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ABSTRACT

Objective: Obesity is often accompanied by hyperactivity of the neuroendocrine stress axis and has been linked to an increased risk of psychiatric disorders. Insulin is reciprocally regulated with the stress hormone corticosterone (CORT), raising the possibility that insulin normally provides inhibitory tone to the hypothalamus-adrenal-pituitary (HPA) axis. Here we examined whether disrupting signaling via the insulin receptor (InsR) in hypothalamic subpopulations impacts the neuroendocrine response to acute psychological stress.

Methods: We used Nkx2.1-Cre, Sim1-Cre and Agrp-Cre transgenic driver lines to generate conditional knockouts of InsR signaling throughout the hypothalamus, paraventricular nucleus of the hypothalamus (PVH) and in neurons expressing Agouti-related peptide (AgRP) in the arcuate nucleus of the hypothalamus (ARH), respectively. We used a combination of molecular, behavioral and neuroendocrine criteria to evaluate the consequences on HPA axis responsiveness.

Results: Endpoints related to body weight and glucose homeostasis were not altered in any of the conditional mutant lines. Consistent with observations in the neuronal InsR knockout mice (NIRKO), baseline levels of serum CORT were similar to controls in all three lines. In male mice with broad disruptions of InsR signals in Nkx2.1-expressing regions of the hypothalamus (IRNkx2.1 KO), we observed elevated arginine vasopressin (AVP) levels at baseline and heightened neuroendocrine responses to restraint stress. IRNkx2.1 KO males also exhibited increased anxiety-like behaviors in open field, marble burying, and stress-induced hyperthermia testing paradigms. HPA axis responsivity was not altered in IRSim1 KO males, in which InsR was disrupted in the PVH. In contrast to observations in the IRNkx2.1 KO males, disrupting InsR signals in ARH neurons expressing Agrp (IRAgp KO) led to reduced AVP release in the median eminence (ME).

Conclusions: We find that central InsR signals modulate HPA responsivity to restraint stress. InsR signaling in AgRP/NPY neurons appears to promote AVP release, while signaling in other hypothalamic neuron(s) likely acts in an opposing fashion. Alterations in InsR signals in neurons that integrate metabolic and psychiatric information could contribute to the high co-morbidity of obesity and mental disorders.

Keywords Insulin; Hypothalamus; AgRP; HPA axis; Stress response

1. INTRODUCTION

Epidemiological studies have identified a link between obesity and psychiatric disorders, particularly those involving stress-related depressive symptoms [1–4]. The well-documented effects of glucocorticoids to promote fuel mobilization and insulin secretion led several groups to hypothesize that dysregulation of the neuroendocrine stress axis contributes to the development of metabolic and cardiovascular diseases [5–7]. However, evidence from some prospective studies supports the idea that obesity promotes risk of depression, but not the reverse [8,9]. HPA axis hyperactivity in obese patients is likely due to alterations in central signaling pathways, as they exhibit normal pituitary sensitivity to negative feedback from glucocorticoids [10]. Progress in elucidating the molecular and neuronal players that could mediate the effects of signals of metabolic status on stress responses have been described in several recent reviews [11–13].

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Abbreviations: ACTH, adrenocorticotropic hormone; AgRP, agouti-related peptide; ARH, arcuate nucleus of the hypothalamus; AVP, arginine vasopressin; CORT, corticosterone; CRH, corticotropin-releasing hormone; FST, forced swim test; Gr, Glucocorticoid receptor; HPA axis, Hypothalamus—Pituitary—Adrenal axis; InsR, insulin receptor; IRAgp KO, knockout of InsR using Agrp-Cre; IRNkx2.1 KO, knockout of InsR using Nkx2.1-Cre; IRSim1 KO, knockout of InsR using Sim1-Cre; MB, marble burying test; MBH, mediobasal hypothalamus; ME, median eminence; NPY, neuropeptide Y; NSF, novelty suppressed feeding test; OF, open field test; POMC, pro-opiomelanocortin; SIH, stress-induced hyperthermia test

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The neuroendocrine stress response axis is strongly influenced by the availability of food and energy stores. Basal activity is lowest and stress responsivity highest in the energy replete state [5]. Conversely, fasting is associated with elevated basal levels of CORT and decreased responsivity to stressors [5,14]. ARH neurons sense nutrient and hormone signals of energy status [15] and project to critical nodes of the HPA axis [16], and thus are well-positioned to serve as the conduit for metabolic influences on stress responses. Inhibition of the activity of the mediobasal hypothalamus (MBH) produces a fasting-like pattern of HPA axis activity in fed animals, with no effect on stress responses in the fasted state [14], consistent with idea that the MBH provides signals of positive energy balance to the HPA axis.

There are several hormone signals of positive energy balance that are sensed by MBH neurons and are also reported to modulate stress responsiveness. There are several hormone signals of positive energy balance that are sensed by MBH neurons and are also reported to modulate stress responsiveness. There are several hormone signals of positive energy balance that are sensed by MBH neurons and are also reported to modulate stress responsiveness. There are several hormone signals of positive energy balance that are sensed by MBH neurons and are also reported to modulate stress responsiveness. There are several hormone signals of positive energy balance that are sensed by MBH neurons and are also reported to modulate stress responsiveness. There are several hormone signals of positive energy balance that are sensed by MBH neurons and are also reported to modulate stress responsiveness. There are several hormone signals of positive energy balance that are sensed by MBH neurons and are also reported to modulate stress responsiveness. There are several hormone signals of positive energy balance that are sensed by MBH neurons and are also reported to modulate stress responsiveness.

2.2. Generation of IR\(^{Nkx2.1}\), IR\(^{Sim1}\), and IR\(^{Agrp}\) KO mice

To generate conditional knockouts of Insr in the hypothalamus, we crossed the Nkx2.1-Cre driver line (C57BL/6J-Tg[Nkx2.1-Cre]2Sand/J, provided by S. Anderson, Well Cornell Medical College) [27] to mice homozygous for a floxed allele of Insr (B6.129S4-Fvb(Insr)) [30] (IR\(^{Nkx2.1}\) KO) and Agrp-Cre/Insr\(^{tm}\) (IR\(^{Agrp}\) KO) mice were generated by a similar mating scheme using Sim1-Cre (B6.FVB(129X1)-Tg(Sim1-cre)1Lowl/J) [30] and Agrp-Cre (Agrp\(^{tm}\)[cre/Lox]) [31] drivers, respectively (provided by Brad Lowell, Beth Israel Deaconness Medical Center). Insr\(^{tm}\) littermates served as controls in all studies. To avoid confounding effects of estrus cyclicity on the HPA axis [32], we performed all analyses in males. Male genotypes were assessed by PCR on genomic DNA from tail tips using the following primers: Cre: 5’ GCGTCTGACTGAGTAAGAACTATC 3’ (forward), 5’ GTGAACAGAAGGTCGACACTT 3’ (reverse); Insr: 5’ TGCAACCATGCTGGACACCC 3’ (forward), 5’ GCTCTGGAATGCTGACGCC 3’ (reverse).

2.3. Measurement of serum corticosterone levels

We collected serum from tail bleeds on minimally-stressed animals between 10am and noon (AM samples) or 6–7pm (PM samples), or at 10am after an overnight (14–16 h) fast with ad libitum access to drinking water. Blood was clotted at room temperature for one hour and centrifuged to isolate serum. Serum was stored at −20°C until assayed by CORT radioimmunoassay (MP Biomedicals) or Rat/Mouse CORT ELISA (Alpco).

2.4. Restraint stress response and dexamethasone suppression test

We restrained mice individually in a 50-ml falcon tube for 30 min. We collected tail blood samples at 0, 30 (end of restraint), and 60 min (30 min after restraint) after the beginning of a restraint. For the dexamethasone (DEX) suppression test, we injected mice with saline or DEX (0.1 mg/kg, i.p.) 60 min before a 60 min-long restraint session. We collected tail blood at baseline (before the saline/DEX injections), immediately before restraint (60 min after the saline/DEX injections), at the end of the 60 min restraint period, and 30 min after the end of restraint. We collected and analyzed CORT levels in serum samples as described above.

2.5. Preservation of hypothalamus and pituitary

At the time of sacrifice, animals were anesthetized with 2.5% Avertin, 0.02 ml/kg i.p., before cervical dislocation. Hypothalami (whole hypothalamus, PVH, or median eminence) and pituitaries were harvested within 5 min of the avertin injection, and snap-frozen in liquid nitrogen. PVH was dissected out bilaterally from the anterior part (1 mm-thick coronal section) of the hypothalamus and median eminence was dissected from the posterior part (2 mm-thick coronal section) of the basal hypothalamus.

2.6. Quantitative RT-PCR

For IR\(^{Nkx2.1}\) KO and IR\(^{Agrp}\) KO mice, we isolated total RNA from fresh-frozen hypothalamus and pituitary using the RNasy Plus Universal Kit (Qiagen) and synthesized cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche). We used a LightCycler 480 SYBR Green I Master System (Roche) in quantitative PCR experiments. We normalized the expression of target genes against β-actin. For IR\(^{Sim1}\) KO mice, we extracted RNA using the ARCTurus Picorpure RNA Isolation
Kit and performed quantitative PCR in an ABI-PRISM 7900 HT Sequence Detection system using fluorogenic Taqman probes specific for the target genes (Applied Biosystems).

Primer Sequences:

**B. actin (β-actin):**
- 5′ CGCCACCTGCGCATGCAGCTG 3′ (forward),
- 5′ TTTGACATGCGCGAGC 3′ (reverse);
- Corticotropin-releasing hormone (Crt); 5′ AGGAGGCATCCCTGAAGAAGT 3′ (forward),
- 5′ CATGTAGGGGCGCTTCCTC 3′ (reverse);
- Arginine vasopressin (Avp);
- 5′ ACTACCTGCCCTGCCTGC 3′ (forward),
- 5′ GCCAGCGAGCTCCTGGC 3′ (reverse);
- Glucocorticoid receptor (Gr);
- 5′ ACTTCGCAGGCGGCTCTG 3′ (forward),
- 5′ TGGTCCCGTTGCTGTGGAGC 3′ (reverse);
- Neuropeptide Y (Npy);
- 5′ GGAAGGGTCTTCAAGCCTTGTT 3′ (forward),
- 5′ ACTACCTGCCCTGCCTGC 3′ (reverse);
- Corticotropin Releasing Factor Chemiluminescent EIA (Phoenix Pharmaceuticals)

Vasopressin [Arg8] Chemiluminescent EIA (Phoenix Pharmaceuticals) and Corticotropin Releasing Factor Chemiluminescent EIA (Phoenix Pharmaceuticals) kits, respectively. ACTH levels were measured by RIA in the lab of Sharon Wardlaw, as previously described [33].

2.7. Hypothalamic peptide levels

Hypothalamic and median eminence AVP and corticotropin releasing hormone (CRH, also known as CRF) levels were measured using Chemiluminescent EIA (Phoenix Pharmaceuticals) kits, respectively. ACTH levels were measured by RIA in the lab of Sharon Wardlaw, as previously described [33].

2.8. Behavioral tests

All behavioral tests were performed between 10am and 2pm.

Open Field Test (OF): After one-hour acclimation to the testing room, we recorded the activity of each mouse in the open field arena for 30 min. We calculated the total distance traveled, and ratio of time spent in the periphery and center of the field; reduced central activity is considered to be an indicator of increased anxiety [34].

Marble Burying (MB): We placed each mouse in a cage filled with 4–5 cm of wood chip bedding, and placed 12 marbles, evenly spaced, on top of the bedding. We recorded the number of marbles buried in the bedding within a 20-minute period. An increase in the number of marbles buried is used as an indicator of anxiety- and obsessive-compulsive-disorder-like behavior [35]. We compared marble burying behavior at baseline and one hour after an i.p. injection with 50 μl saline (stress condition).

Stress-Induced Hyperthermia Test (SIH): Stress associated with handling and insertion of a rectal temperature probe thermometer (Thermoworks) causes an increase in core body temperature, an anxiety response that is suggested to be mediated in part by the effects of CORT to increase sympathetic tone [36]. We measured body temperature at baseline and 60 min after stress.

Novelty Suppressed Feeding Test (NSF): After an overnight fast, we placed each mouse in a brightly lit box (30 cm × 40 cm) with bedding on the floor and an accessible food pellet tied down to a circle of Whatman paper in the center of the box. We recorded the time it took an animal to enter the brightly lit center of the arena and to bite the food pellet. Increased latency to feed in a novel environment is considered to be an indicator of anxiety-like behavior [37].

Forced swimming test (FST): We placed mice individually in beakers (46 cm tall × 32 cm in diameter) containing 23–25 °C water 30 cm deep.
Figure 3: Altered gene expression of HPA axis components at baseline and during restraint test in IRNkx2.1 KOs. (A) Key components of HPA axis (black text) that release hormones/neuropeptides (red text) and express genes (green text) assessed in our studies. (B) CRH and (C) AVP protein levels in the median eminence. (D) ACTH protein levels in the pituitary. (E) Crh and (F) Avp mRNA expression levels in the hypothalamus at baseline, at the end of restraint (30 min), and 30 min post-restraint; n ≥ 5 for all groups at all time points. (G) Pomc mRNA in the pituitary; n = 4 for all groups at all time points. (H) Hypothalamic and (I) pituitary Gr mRNA; n ≥ 4 for all groups at all time points. (J) Baseline mRNA levels of Pomc, Npy, and AgRP in the hypothalamus of male IRNkx2.1 KO and controls; n ≥ 4 for all groups. All data are mean ± SEM of control and IRNkx2.1 KO males. *P < 0.05 control versus IRNkx2.1 KO.
deep. We conducted two swim sessions between 12:00 and 18:00 h: an initial 6-minute pretest followed 24 h later by a 6-minute test. We monitored and videotaped mice while swimming and recorded the amount of time immobile. Increased immobility is considered to be an indicator of depressive behavior [38].

2.9. Glucose homeostasis
Baseline glucose levels measurements and glucose tolerance test were performed as previously described [39].

2.10. Statistics
Data are presented as group mean ± SEM. We performed statistical comparisons between groups using a one-way ANOVA, followed by a post-hoc Bonferroni correction. We considered a P value of 0.05 or less to be statistically significant.

3. RESULTS

3.1. Diminished InsR signals in Nkx2.1-expressing neurons leads to HPA axis hyperactivity
To explore the possibility that central insulin signals modulate the HPA axis, we assessed the consequences of disrupting InsR signals in Nkx2.1-expressing neurons in the forebrain on CORT levels at baseline and in response to psychological and physiological stress. We did not observe a difference between IRNkx2.1 KO and control males in the morning (Figure 1A), at the nadir of circadian fluctuations in serum CORT levels [40]. CORT levels were approximately 31% and 56% higher in IRNkx2.1 KO males in the evening and in response to fasting, respectively; however, these differences did not reach significance (Figure 1A). Control and IRNkx2.1 KO males exhibited similar CORT levels at the end of a 30-minute restraint period, but CORT levels 30 min after release from restraint were ~17% higher in IRNkx2.1 KOs (Figure 1B). Since mice carrying the Nkx2.1-Cre transgene alone did not show an overall change in response to restraint when compared to wild-type animals (data not shown), the altered stress response in IRNkx2.1 KOs can be attributed to the loss of InsR signals. As the neuroendocrine response to restraint was elevated in IRNkx2.1 KOs, we next performed a dexamethasone (DEX)-suppression test to determine whether the deficit in negative feedback occurs at the level of the pituitary. Saline-injected IRNkx2.1 KO males exhibited a 51% increase in CORT levels as compared to controls at the end of the 60 min restraint period (Figure 2A) and a 14% increase in stress responsivity (Figure 2C). IRNkx2.1 KO males were sensitive to DEX suppression (Figure 2B), supporting the idea that negative feedback to the HPA axis via the pituitary is not impaired.

3.2. Altered expression of HPA axis components in IRNkx2.1 KOs
To identify the sites of diminished InsR signals that contribute to HPA axis hyperactivity in IRNkx2.1 KO mice, we evaluated gene expression and peptide levels of several major HPA axis components in the hypothalamus, median eminence (ME) and pituitary at baseline, the end of the 30-minute restraint period, and 30 min after restraint (Figure 3A). Baseline levels of Avp mRNA expression (Figure 3F) and AVP protein levels in the ME (Figure 3C) were significantly increased by 53% and 18%, respectively, in IRNkx2.1 KO males. Conversely, baseline levels of pituitary POMC expression were 40% lower (Figure 3G) and adrenocorticotropic hormone (ACTH) protein levels in the pituitary trended lower (Figure 3D) in IRNkx2.1 KO males. At baseline,
hypothalamic expression of \( \text{Crh} \), \( \text{Gr} \), \( \text{Pomc} \), \( \text{Npy} \) and \( \text{Agrp} \) mRNA, CRH protein levels in the ME, and pituitary expression of \( \text{Gr} \) were similar between \( \text{IRNkx2.1 KO} \) males and controls (Figure 3). At the end of the restraint period, we found that \( \text{Gr} \) expression was 30% lower in the hypothalamus (Figure 3H), but increased in the pituitary of \( \text{IRNkx2.1 KO} \)s (Figure 3I), consistent with a centrally-mediated deficit in negative feedback to the HPA axis [41]. Expression of hypothalamic \( \text{Avp} \) and pituitary \( \text{Pomc} \) were significantly different in \( \text{IRNkx2.1 KO} \)s 30 min after release from restraint, but the changes were in the opposite direction as had been observed at baseline (Figure 3F–G).

3.3. Increased anxiety-like behaviors in \( \text{IRNkx2.1 KO} \) males following stress
In light of the observed HPA axis hyperactivity in \( \text{IRNkx2.1 KO} \) males, we evaluated their performance in a series of neuro-behavioral tests. We used open field (OF), marble burying (MB), stress-induced hyperthermia (SIH) and novelty suppressed feeding (NSF) tests to measure anxiety-like behaviors [42]. To evaluate depressive-behavior, we assessed learned helplessness in a forced swim test (FST) [38].

\( \text{IRNkx2.1 KO} \) males exhibited reduced locomotor activity after 20 min in the OF, but not at earlier timepoints (Figure 4A, left panel). The decrease in locomotor activity observed at later timepoints was not associated with fewer entries to the center of the field (Figure 4A, right panel). Marble burying behavior was the same in \( \text{IRNkx2.1 KO} \) and control males at baseline, but was roughly 50% higher in \( \text{IRNkx2.1 KO} \)s in response to stress (5 ± 1 control vs. 8 ± 1 KO, \( P < 0.05 \)) (Figure 4B). Similarly, core body temperature was not different at baseline, but was roughly 0.3 °C higher in \( \text{IRNkx2.1 KO} \) males after stress (36.6 ± 0.1 control vs. 36.9 ± 0.1 KO, \( P < 0.05 \)) (Figure 4C). The latency to feed in the NSF test tended to be higher in \( \text{IRNkx2.1 KO} \) males as compared to controls, although this difference did not reach significance (Figure 4D). Finally, time spent immobile in the FST, an indicator of depressive-like behavior in mice, was not altered in \( \text{IRNkx2.1 KO} \) males (Figure 4E). In summary, performance of \( \text{IRNkx2.1 KO} \) males was the same as controls at baseline, but was increased following exposure to stress.

3.4. HPA axis function is not altered in \( \text{IRSim1 KO} \) males
Since we observed increased AVP protein levels in the ME and \( \text{Agrp} \) mRNA expression in the hypothalamus of \( \text{IRNkx2.1 KO} \) males, we explored whether InsR actions directly on AVP neurons could be responsible. To this end, we assessed neuroendocrine and molecular correlates of HPA axis responses in \( \text{IRSim1 KO} \) males, in which InsR signaling is disrupted in \( \text{Sim1} \)-expressing cells in the PVH [30]. Adult \( \text{IRSim1 KO} \) males exhibited similar body weights and fed glucose levels as control littermates (Figure 5A–B). Serum CORT levels at baseline and in response to 30 min restraint were also not altered in \( \text{IRSim1 KO} \) males (Figure 5C–D). \( \text{IRSim1 KO} \)s exhibited increased \( \text{Agrp} \) expression in the PVH and decreased \( \text{Agrp} \) expression in the ARH at baseline, although these changes did not reach significance (Figure 5E). These changes were in the same direction as those observed in \( \text{IRNkx2.1 KO} \)s (Figure 5F,J). On the other hand, \( \text{Pomc} \) expression in the hypothalamus was significantly increased at baseline in \( \text{IRSim1 KO} \)s (Figure 5E), while it was not changed in \( \text{IRNkx2.1 KO} \)s (Figure 5J). \( \text{Pomc} \) and \( \text{Gr} \) expression in the pituitary were not significantly altered in \( \text{IRSim1 KO} \)s as compared to controls (Figure 5F). In summary, our analyses are consistent with the idea that InsR signals in \( \text{Sim1} \) neurons do not exert a major influence on HPA axis responsiveness, although we cannot exclude a minor contribution from these neurons.

3.5. \( \text{IRAgRP KO} \) mice exhibit reduced AVP levels at baseline
As stress is reported to increase and insulin is reported to decrease ARH expression of \( \text{Npy} \) and \( \text{Agrp} \) [43–45], we next examined the consequences of disrupting InsR signals in \( \text{Agrp} \)-expressing neurons on HPA axis responsiveness. At 13 weeks of age, body weight, fed glucose levels, and glucose tolerance in \( \text{IRAgRP KO} \) males were similar to control littermates (Figure 6A–C). Serum CORT levels at baseline and at the end of the 30 min restraint period were also not changed in \( \text{IRAgRP KO} \) males (Figure 6D–E). In contrast to what we observed in \( \text{IRNkx2.1 KO} \) males, \( \text{IRAgRP KO} \) males exhibited a 25% decrease in CORT response 30 min after release from restraint, although this difference did not reach significance (Controls 41.5 ± 3.9 vs. \( \text{IRAgRP KO} \)s 31.2 ± 5, \( P = 0.07 \) (Figure 6E)). Consistent with decreased HPA axis reponsivity, baseline AVP protein levels in the ME were significantly lower (Figure 6F) and \( \text{Agrp} \) mRNA expression in the hypothalamus trended lower in \( \text{IRAgRP KO} \) mice (Figure 6G). In addition, baseline pituitary \( \text{Pomc} \) and \( \text{Gr} \) mRNA levels were increased in \( \text{IRAgRP KO} \) males, while \( \text{Gr} \) was not changed (Figure 6H). Together, these findings support the idea that diminished InsR signals in \( \text{Agrp} \)/\( \text{Npy} \) neurons results in decreased AVP tone and a trend toward reduced HPA axis responsiveness, the opposite of the phenotype produced by widespread disruption of InsR signals in \( \text{Nkx2.1-Cre} \)-expressing neurons in the forebrain.
4. DISCUSSION

4.1. Impaired negative feedback to HPA axis in IRNkx2.1 KO males

Our data support the hypothesis that insulin signaling in the hypothalamus modulates negative feedback to the HPA axis following stress. Nkx2.1-Cre-mediated disruption of InsR signaling in the hypothalamus, as well as ventral forebrain-derived neuronal subpopulations in the cortex and amygdala [27,28,39], did not affect plasma CORT levels at baseline or during restraint stress, but delayed the return to baseline (Figure 1B). Similarly, behaviors assessed over a short time period (i.e. NSF, FST) were not altered in IRNkx2.1 KOs, while modest but significant effects were observed in assays lasting 20 min or more (i.e. OF, MB, SIH) (Figure 4). Pituitary Gr expression and the ability of the synthetic glucocorticoid dexamethasone to suppress stress-induced activation of the HPA axis were not altered in IRNkx2.1 KOs (Figures 2C and 3I), suggesting that the pituitary response to stress is not impaired [46]. These observations are consistent with the idea that InsR signals in Nkx2.1-Cre-expressing neurons normally act to provide negative feedback to the HPA axis. This idea is also supported by reports that intranasal administration of insulin lowers stress-induced, but not basal, HPA axis responsiveness in humans [23].

The most prominent change in central components of the HPA axis in IRNkx2.1 KOs was increased Avp expression in the hypothalamus and AVP protein released into the median eminence at baseline (Figure 3). Elevated basal AVP levels are also observed in rodents exposed to chronic stress [47,48] and in patients with major depression [49,50]. It has been proposed that this adaptation maintains HPA axis responsiveness in the face of high circulating glucocorticoid levels during chronic stress [51]. However, it is not clear whether increased AVP in IRNkx2.1 KOs is a direct consequence of diminished hypothalamic InsR signaling, or a secondary response to chronic perturbations in the stress axis. In either case, the failure to observe increased AVP to a similar degree in IRSim1 KOs (Figure 5) argues against a major role for direct effects of insulin on CRH/AVP neurons in the PVH, although a minor contribution cannot be ruled out.

4.2. HPA axis phenotypes of IRAgrp KO males are in opposite direction as those observed in IRNkx2.1 KOs

NPY and AgRP have been reported to activate CRH neurons in vivo and in vitro [52,53], while insulin blunts stress-induced increases in NPY levels [43,54] and inhibits NPY/AgRP neuronal activity [55]. Based on these reports, we predicted that a reduction in inhibitory InsR-mediated
inputs to AgRP/NPY neurons would lead to heightened stress responses in IR^{App} KOs. Thus, our finding that IR^{App} KOs exhibit decreased AVP and a trend toward a suppressed HPA axis response was the opposite of the expected result (Figure 6). Consistent with our observations, mice overexpressing an Agrp transgene and global knockouts of Melanocortin receptor 4 also exhibit normal CORT at baseline, but a blunted response to restraint stress [56–58]. The possibility that activation of AgRP neurons can act to dampen HPA responses to psychological stress is also supported by the observation that stress-induced increases in Npy/Agrp occur after the rise in CORT levels and Pomc expression [45,59]. Differences in the timing and/or concentration of exposure to AgRP/NPY following restraint tests in genetic models versus central injections of AgRP/NPY compounds could underlie discordant effects on HPA axis activity. If our hypothesis is correct, activation of AgRP/NPY neurons under conditions of limited nutrient availability could promote survival by limiting stress-induced mobilization of fuels [60].

4.3. Possible sources of impaired InsR signals that contribute to HPA axis hyperactivity in IR^{Nkx2.1} KOs
As Agrp-Cre- and Nkx2.1-Cre-mediated disruption of InsR signaling produce opposite effects on HPA-axis related endpoints, the loss of InsR signals in AgRP/NPY neurons cannot explain the phenotype observed in IR^{Nkx2.1} KOs. Neurons in the dorsomedial hypothalamus are marked by an Nkx2.1-Cre lineage trace [39], send heavy projections to the PVH [16] and modulate HPA axis activity [61,62] (Figure 7). However, the lack of information about electrophysiological responses of DMH neurons to insulin and conflicting reports that DMH neurons provide excitatory [61,63] and inhibitory [62] inputs to the HPA axis, hinder the formulation of a hypothesis about the precise mechanism of action. In theory, InsR signaling in ARH POMC neurons could also modulate HPA responses, although projections from these neurons to the neuroendocrine PVH are much sparser than AgRP projections [64,65]. Unfortunately, efforts to explore the possible contribution of InsR in POMC neurons using Pomc-Cre-mediated recombination are complicated by the fact that signaling in pituitary corticotrophs would also be impacted.

5. CONCLUSIONS
There is a high degree of comorbidity of metabolic and psychological disorders, and the severity of depressive symptoms is correlated with insulin resistance [1,3,4]. Our findings support the idea that InsR signaling in hypothalamic neurons provides an important source of crosstalk between energy status and stress reactivity. InsR signaling in AgRP/NPY neurons appears to promote AVP release, while signaling in an unidentified population likely acts in an opposing fashion. Identification of the latter cell type is an important area for future research, because elevated AVP is thought to contribute to depressive disorders [66].

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CONFLICTS OF INTEREST
The authors do not have any conflicts of interest.

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