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MOLECULAR METABOLISM

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 ($IR^{Nkx2.1}$ KO), we observed elevated arginine vasopressin (AVP) levels at baseline and heightened neuroendocrine responses to restraint stress. $IR^{Nkx2.1}$ 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 IR^{Sim1} KO males, in which InsR was disrupted in the PVH. In contrast to observations in the $IR^{Nkx2.1}$ KO males, disrupting InsR signals in ARH neurons expressing *Agrp* (IR^{Agrp} 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; IR^{Agrp} KO, knockout of *InsR* using *Agrp-Cre*; $IR^{Nkx2.1}$ KO, knockout of *InsR* using *Nkx2.1-Cre*; IR^{Sim1} 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 responses, including leptin, glucagon-like peptide-1 and cholecystokinin (reviewed in [13]). However, conflicting results from studies using gain- and loss-of function approaches have hindered progress toward elucidating the underlying mechanism. Whereas central administration of these hormones has been reported to activate neurons expressing c*orticotropin-releasing hormone (*CRH, also known as CRF) and promote CORT release [17–19], loss of function mutations in the corresponding receptors are associated with increased HPA tone, consistent with an inhibitory effect of these hormones on stress responses [20–22]. Differences in the type, severity and duration of the experimental stressors applied by different groups likely contribute to these apparent inconsistencies. In addition, most studies lack the temporal resolution to distinguish processes associated with the initiation of the stress response from those that are activated to provide negative feedback. Finally, it is possible that different populations in the brain and periphery that are activated by these signals could influence different aspects of the stress response.

Insulin is reciprocally regulated with CORT during stress, consistent with the idea that insulin normally provides inhibitory tone to HPA axis [5]. However, as is the case for other gain of function studies, there are studies that report effects of brain insulin to suppress [23] and enhance [24] the neuroendocrine stress response. As there is a positive correlation between HPA axis responsivity and homeostasis insulin resistance index (HOMA-IR) in obese patients [7,25], we set out to examine whether impaired central insulin signaling in lean animals impacts stress axis function. To this end, we utilized the *Nkx2.1-Cre* transgene to broadly disrupt signaling through the insulin receptor (InsR) in the hypothalamus [26–28]. After discovering that the resulting $IR^{Nkx2.1}$ KOs have impaired negative feedback to the HPA axis, we generated additional conditional InsR knockouts to assess the contribution of *Agrp-Cre*-expressing neurons in the ARH (IR^{Agrp} KOs) and *Sim1-Cre*-expressing neurons in the PVH and amygdala (IR^{Sim1} KOs) to this phenotype.

2. MATERIAL AND METHODS

2.1. Animal husbandry

Mice were maintained in a temperature- (22 ± 1 °C) and light- (12:12h light dark cycle, lights on at 7pm and off at 7pm) controlled environment. Pups were weaned on post-natal day 21. Unless otherwise noted, mice had *ad libitum* access to chow (9% calories from fat, 5058 Mouse diet 20, Labdiet) and water until the time of sacrifice. Male mice older than 8 weeks of age were used in all studies. Since female sex hormones are known to influence activity of the HPA axis [29], we limited our analysis in this study to male mice. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Columbia University Health Sciences Division.

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, Weill Cornell Medical College) [27] to mice homozygous for a floxed allele of *Insr* (B6.129S4(FVB)-*Insr*^{tm1Khn}/J) [26] to generate *Nkx2.1-cre;Insr*$^{fl/fl}$ mice ($IR^{Nkx2.1}$ KO). *Sim1-Cre;Insr*$^{fl/fl}$ (IR^{Sim1} KO) and *Agrp-Cre;Insr*$^{fl/fl}$ (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$^{tm1(cre)Lowl}$) [31] drivers, respectively (provided by Brad Lowell, Beth Israel Deaconness Medical Center). *Insr*$^{fl/fl}$ 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. Mouse genotypes were assessed by PCR on genomic DNA from tail tips using the following primers: *Cre*: 5′ GCGGTCTGGCAGTAAAAACTATC 3′ (forward), 5′ GTGAAACAGCATTGCTGTCACTT 3′ (reverse); *Insr*: 5′ TGCACCCCATGTCTGGGACCC 3′ (forward), 5′ GCCTCCTGAATAGCTGA GACC 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 assessed 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/g i.p., before cervical dislocation. Hypothalamus (whole hypothalamus, PVH, or median eminence) and pituitary 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 RNeasy 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 *Beta actin*. For IR^{Sim1} KO mice, we extracted RNA using the ARCTURUS Picopure RNA Isolation

