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Irs2 and Irs4 synergize in non-LepRb neurons to control energy balance and glucose homeostasis

Marianna Sadagurski¹,*, X. Charlie Dong¹,*,**, Martin G. Myers Jr.²,³, Morris F. White¹,****

ABSTRACT

Insulin receptor substrates (Irs1, 2, 3 and Irs4) mediate the actions of insulin/IGF1 signaling. They have similar structure, but distinctly regulate development, growth, and metabolic homeostasis. Irs2 contributes to central metabolic sensing, partially by acting in leptin receptor (LepRb)-expressing neurons. Although Irs4 is largely restricted to the hypothalamus, its contribution to metabolic regulation is unclear because Irs4-null mice barely distinguishable from controls. We postulated that Irs2 and Irs4 synergize and complement each other in the brain. To examine this possibility, we investigated the metabolism of whole body Irs4⁻/⁰ mice that lacked Irs2 in the CNS (bIrs2⁻/⁻·Irs4⁻/⁰) or only in LepRb-neurons (Lepr⁺/⁻·Irs4⁻/⁰), bIrs2⁻/⁻·Irs4⁻/⁰ mice developed severe obesity and decreased energy expenditure, along with hyperglycemia and insulin resistance. Unexpectedly, the body weight and fed blood glucose levels of Lepr⁺/⁻·Irs4⁻/⁰ mice were not different from Lepr⁺/⁻ mice, suggesting that the functions of Irs2 and Irs4 converge upon neurons that are distinct from those expressing LepRb.

Keywords Insulin receptor substrate 2; Insulin receptor substrate 4; Leptin; Obesity; Nutrient homeostasis; Energy balance

1. INTRODUCTION

The hypothalamus integrates signals from peripheral tissues and central nodes to regulate feeding, energy balance, nutrient flux, and counter-regulatory responses that maintain nutrient homeostasis [1]. Insulin is secreted from the pancreatic beta-cells during meals to promote peripheral nutrient homeostasis [2]; however, it also modulates energy and glucose homeostasis by acting on hypothalamic and dopaminergic neurons [3]. By comparison, leptin produced by adipocytes is a dominant signal that informs the brain about peripheral energy stores [4]. Leptin binds to the long form of the leptin receptor (LepRb) found largely—but not entirely—on hypothalamic neurons [5]. LepRb neurons sense and integrate signals relevant to nutrient homeostasis to control satiety, energy balance and metabolism [4]. LepRb generates multiple downstream signals by activating JAK2 and recruiting SHP2, STAT3 and STAT5, along with SH2B, Irs1/2 and Irs4 [6,7]. The insulin receptor substrates (Irs1, 2, 3 and 4) are principle targets for the insulin and IGF1 receptor tyrosine kinases, which play a central role in somatic growth and metabolic regulation [8]. IRS-proteins are also phosphorylated by the receptors for some cytokines (IL4, IL9, IL13), growth hormone and leptin in various cells and tissues [7,9–14]. The IRS-proteins share a common structure, including an NH₂-terminal pleckstrin homology (PH) domain followed by a phosphotyrosine binding (PTB) domain, and a tail containing many Tyr and Ser/Thr phosphorylation sites. The PH and PTB domains mediate recruitment to appropriate activated receptors in the plasma membrane [15,16]. Although the COOH-terminal amino acid sequences of the various IRS-proteins diverge significantly, multiple short tyrosine phosphorylation motifs can bind and activate similar SH2-domain containing proteins, including the type 1 phosphatidylinositol 3-kinase [17]. The various IRS-proteins have distinct physiologic functions. These differences in biological specificity among the various IRS-proteins may be conferred by sequence divergence or by differences in patterns of expression. Irs1 and Irs2 are widely expressed in mammalian tissues, whereas Irs3 is largely restricted to nonhuman adipose tissue (where it promotes adipogenesis [18,19]). Although some reports describe a role...
for Ir4 in regenerating liver and hepatocellular carcinoma [20–22], Ir4 is expressed mainly in the hypothalamus in healthy animals [23–25]. Potential overlap in the expression of Ir1 and Ir2 with Ir4 appears to occur mainly in neurons of the ventral hypothalamus [24]. Based upon genetic deletion experiments, Ir1 promotes embryonic and postnatal body growth, and mediates insulin sensitivity in classical insulin target tissues [26]. By comparison, Ir2 mediates several important functions that are not shared with Ir1—including pancreatic beta-cell growth and survival. CNS/hypothalamic nutrient sensing, endothelial cell function, and sensitivity to neurodegenerative disease [27–29]. Ir1/– mice develop insulin resistance that is compensated by elevated circulating insulin and sensitivity to neurodegenerative disease [27–29]. Ir1/– mice are not shared with Ir1 and Ir2 apparently mediate some essential functions, since mice null for both genes die before weaning [8].

The deletion of Ir2 specifically in the CNS can extend life span while producing early-onset obesity, insulin resistance and glucose intolerance [31,32]. The metabolic phenotypes resulting from Ir2 ablation are at least in part attributable to Ir2 function in a relatively small subset of LepRb-expressing neurons in the brain [29]. In contrast, while Ir4 is highly conserved and displays a very specific and restricted pattern of expression that overlaps with LepRb cells in the ventral hypothalamus, the deletion of Ir4 alone has very mild or no effects upon energy balance or glucose metabolism in mice [33]. To reveal a potential physiological function for Ir4 in the CNS, we intercrossed mice without Ir2 in the brain [29]. In contrast, while Ir4 is highly conserved and displays a very specific and restricted pattern of expression that overlaps with LepRb cells in the ventral hypothalamus, the deletion of Ir4 alone has very mild or no effects upon energy balance or glucose metabolism in mice [33]. To reveal a potential physiological function for Ir4 in the CNS, we intercrossed mice without Ir2 in the brain [29]. In contrast, while Ir4 is highly conserved and displays a very specific and restricted pattern of expression that overlaps with LepRb cells in the ventral hypothalamus, the deletion of Ir4 alone has very mild or no effects upon energy balance or glucose metabolism in mice [33]. To reveal a potential physiological function for Ir4 in the CNS, we intercrossed mice without Ir2 in the brain [29].
Figure 1: blrs2\(^{-/-}\) - Irs4\(^{-/-}\) mice are obese. (A) Average body weights of male blrs2\(^{-/-}\) - Irs4\(^{-/-}\) mice (open squares), Irs4\(^{-/-}\) mice (closed squares), blrs2\(^{-/-}\) mice (open circles) and control Irs2\(^{L/L}\) mice (closed circles) on regular chow diet was determined in each age group and compared by generalized linear regression (SPSS, v19). The number of mice in each group is indicated in parentheses (mean ± SD; *, Bonferroni p < 0.001). (B) Representative image of 3-months-old male mice. (C) Body length and (D) percent body fat was determined by DEXA using 12-week-old mice (mean ± SEM; n=8–10 genotype, *, p<0.05 vs. controls).

Figure 2: Representative H&E staining of (A) white adipose tissue (WAT) and (C) liver sections of 6-month-old blrs2\(^{-/-}\) - Irs4\(^{-/-}\) mice, Irs4\(^{-/-}\) mice, blrs2\(^{-/-}\) or Irs2\(^{L/L}\) mice. Liver sections of 9-month-old ob/ob mice are also shown. (B) Morphometric analysis of adipocyte cell size in epididymal adipose tissue (n=5 animals per genotype; *, p<0.05 vs. control).
heterozygous females from this breeding were mated to obtain female heterozygous and Irs4<sup>+/y</sup> mice, as well as male wild-type and Irs4<sup>+/y</sup> mice. To focus our study on the interaction between Irs2 and Irs4 in the CNS, we utilized previously published nestin-cre transgenic mice intercrossed to loxP-flanked alleles to delete Irs2<sup>L/L</sup> specifically in neurons (bIrs2<sup>L/L</sup>) [31]. Mice with a combined deficiency of Irs2 and Irs4 were generated by intercrossing female bIrs2<sup>+/+</sup>/Irs4<sup>+/y</sup> mice with male bIrs2<sup>+/+</sup>/Irs4<sup>+/y</sup> mice, as well as male bIrs2<sup>+/+</sup>/Irs4<sup>+/+</sup> and female bIrs2<sup>+/+</sup>/Irs4<sup>+/+</sup> mice, as well as control genotypes. There was no detectable embryonic lethality or early death associated with the Irs4 null phenotype, and the male Irs4<sup>+/y</sup> and female Irs4<sup>+/y</sup> mice were fertile and displayed normal size and a healthy appearance.

3.2. Growth and energy balance in bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup> mice
The body weight of male control (Irs2<sup>L/L</sup>), Irs4<sup>+/y</sup>, bIrs2<sup>+/+</sup>, and Irs4<sup>+/y</sup> mice were monitored between 6 and 13 weeks of age to determine whether Irs4 interacts with neuronal Irs2 for body weight regulation. Compared to control mice (Irs2<sup>L/L</sup>), the whole body Irs4<sup>+/y</sup> mice grew to a normal size between 6 and 13 weeks of age (Figure 1A). As shown previously [31], bIrs2<sup>+/+</sup> mice were heavier and longer with significantly increased body fat compared with Irs2<sup>L/L</sup> mice (Figure 1A).

![Figure 3](image_url)

**Figure 3:** Energy expenditure in bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup> mice. (A) Serum leptin levels of 9-month-old male bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup>, bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup>, bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup>, and Irs2<sup>L/L</sup> mice (mean ± SEM; *p < 0.05 for indicated comparison). (B) Food intake of 3-month-old male bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup>, bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup>, bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup> mice fed regular chow diet (mean ± SEM; *p < 0.05 for indicated comparison). Three month-old male mice of the indicated genotype were monitored for 72 h in the CLAMS (n=10/ genotype) to assess (C) oxygen consumption (O<sub>2</sub>, l/kg/h), (D) heat production (kcal/hr/kg), and (E) RER (respiratory exchange ratio) during the light and dark phases. (F) Representative H&E staining of brown adipose tissue (BAT) of 3-month-old male bIrs2<sup>+/+</sup> and Irs4<sup>+/y</sup> mice.
and D). Through 12 weeks of age, the lack of Irs4 had no effect on body weight compared against the control mice; however, male bIrs2−/−-Irs4−/− mice were 60% heavier than Irs2−/− and 20% heavier than bIrs2−/− mice (Figure 1A and B). Dual X-ray absorptiometry (DEXA) confirmed that the obesity phenotype of bIrs2−/−-Irs4−/− mice was due to significantly increased adipose mass compared to obese bIrs2−/− mice (Figure 1D). Hematoxylin and eosin staining of 6-month-old mice confirmed that the adipocytes of bIrs2−/−-Irs4−/− mice were 20% larger ($p<0.05$) than bIrs2−/− mice (Figure 2A and B). Moreover, 9 month old bIrs2−/−-Irs4−/− mice displayed hepatic steatosis similar to that of ob/ob mice; however, steatosis was never observed in Irs4−/−, bIrs2−/− or control mice (Figure 2C).

Consistent with the graded adiposity of the bIrs2−/− and bIrs2−/−-Irs4−/− mice, leptin concentrations were elevated in adult bIrs2−/−-Irs4−/− mice and significantly greater in the bIrs2−/−-Irs4−/− mice; however, the leptin concentration was normal in the Irs4−/−-mice (Figure 3A). Food intake by chow-fed controls- and Irs4−/−-mice was indistinguishable, whereas bIrs2−/−-mice consumed more food (Figure 3B). The bIrs2−/−-Irs4−/−-mice consumed approximately 10% more food than the bIrs2−/−-mice during the 24-h test interval (Figure 3B). Thus, the lack of Irs4 augmented hyperphagia only when Irs2 was also absent from the CNS.

Food intake and energy expenditure must be coordinately regulated to maintain energy balance and stable body weight. Irs2 was previously shown to alter energy homeostasis as young bIrs2−/− mice were less active and consumed less oxygen than age-matched control mice [31]. At 3 months of age, individually housed animals were monitored for 72 h in the Comprehensive Lab Animal Monitoring System (CLAMS). During the dark cycle, $O_2$ consumption and heat generation were reduced similarly in bIrs2−/− and bIrs2−/−-Irs4−/− mice compared to the normal parameters of Irs4−/− and control mice (Figure 3C and D). Respiratory exchange ratio (RER) was not different between the groups (Figure 3E). Nevertheless, compared against bIrs2−/− mice the hematoxylin and eosin stained brown adipose tissue (BAT) of adult 6-month-old bIrs2−/−-Irs4−/− mice contained larger lipid-filled vacuoles that resembled white adipocytes (Figure 3F). Since Irs2 signaling was previously shown to play a role in BAT thermoregulation, these findings reveal a link between central Irs4 and BAT function that was exposed when Irs2 was deleted in the CNS [29].

3.3. Hypothalamic neuropeptide expression

To test whether the hyperphagia and obesity of bIrs2−/−-Irs4−/−-mice were related to changes in hypothalamic neuropeptide expression, we measured expression of Npy, Agrp, and Pomc mRNA in the ARC by qPCR [3]. The expression of mRNA encoding orexigenic neuropeptides (Npy and Agrp) was increased in bIrs2−/− and bIrs2−/−-Irs4−/−-mice, consistent with the observed hyperphagia of these animals (Figure 4A and B). By comparison, there were no significant changes in the expression of Pomc mRNA among the various genotypes (Figure 4C). Overall, the ratio between orexigenic (Agrp) and anorexigenic (Pomc) mRNAs was similarly increased in bIrs2−/− and bIrs2−/−-Irs4−/−-mice compared to Irs4−/− and control mice (Figure 4D). Thus the mRNA expression of these hypothalamic neuropeptides was regulated by brain Irs2, but not influenced by Irs4.

3.4. Glucose homeostasis in bIrs2−/−-Irs4−/−-mice

Next, we investigated whether Irs4 synergizes with neuronal Irs2 to control systemic glucose homeostasis. Fasting glucose concentrations were normal in 3 month-old Irs4−/−-mice, but slightly increased in bIrs2−/−-mice and more dramatically increased in bIrs2−/−-Irs4−/−-mice (Figure 5A). Similarly, ad libitum-fed blood glucose values tended upward in the Irs4−/− and bIrs2−/−-mice, but only reached significance in the bIrs2−/−-Irs4−/−-mice (Figure 5B). Whereas glucose tolerance was normal in Irs4−/−-mice and only slightly impaired in bIrs2−/−-mice, bIrs2−/−-Irs4−/−-mice displayed dramatic glucose intolerance (Figure 5C). Furthermore, the bIrs2−/−-Irs4−/−-mice were resistant to the hypoglycemic effects of exogenous insulin, whereas the responses of bIrs2−/− and Irs4−/−-mice were not significantly different from controls (Figure 5D). The HOMA2-IR confirmed that insulin resistance persists in 9 month old bIrs2−/−-Irs4−/− and bIrs2−/−-mice, whereas Irs4−/−-mice displayed normal insulin sensitivity (Figure 5E). Consistent with these results, Irs4−/−-mice displayed normal insulin concentrations, whereas the bIrs2−/−-mice displayed slightly elevated circulating insulin at 9 months of age, whereas insulin concentrations in bIrs2−/−-Irs4−/−-mice were elevated approximately 5-fold (Figure 5F). The bIrs2−/− mice at 3.5 months displayed 3-fold greater β-cell mass, which was consistent with the demand for compensatory insulin secretion (Figure 6A). By comparison, the beta cell mass of 3.5 month old bIrs2−/− mice and 7.5 month old Irs4−/−-mice was indistinguishable from the controls.
3.5. Irs4 effects on obesity are independent of leptin receptor expressing neurons

Leptin, a hormone produced by adipocytes, acts upon leptin receptor (LepRb) expressing neurons to modulate energy homeostasis [4]. Our recent observations show that deletion of Irs2 in LepRb neurons (LeprΔIrs2-mice) increases body weight through the combined effects of hyperphagia and reduced energy consumption [29]. Since others have suggested that Irs4 mediates PI 3-kinase signaling in response to leptin stimulation [7], it is possible that the synergy between Irs4 and Irs2 occurs in LepRb neurons and that the deletion of Irs4 would increase the obesity observed in LeprΔIrs2-mice. However, at 3 months of age the increased body weight of the LeprΔIrs2-mice was not further increased in the LeprΔIrs2 Irs4Δy mice (Figure 7A). Furthermore, while ad libitum-fed blood glucose levels were significantly elevated in the blrs2Δ- Irs4Δy mice relative to all other groups (See Figure 5B, above), blood glucose levels remained unchanged among LeprΔIrs2, LeprΔIrs2 Irs4Δy and controls (Figure 7B). Thus, the synergy between brain Irs2 and Irs4 for body weight control occurs in neurons that are distinct from LepRb cells.

4. DISCUSSION

Compared to Irs1 and Irs2, for which genetic ablation yields substantial phenotypes, the physiologic role of Irs4 has been difficult to establish because the deletion of Irs4 alone produces minimal physiologic perturbation [33]. Since Irs1 and Irs2 are widely expressed, while the expression of Irs4 is more narrowly distributed (largely in the hypothalamus, where Irs2 plays a crucial role in metabolic homeostasis), we reasoned that Irs2 and Irs4 might play synergistic and somewhat redundant roles. Indeed, insulin continues to stimulate the
phosphorylation of AKT, a major IRS-protein-dependent insulin signaling pathway, in hypothalamic extracts from \( \text{bIrs2}^{-/-}\) animals [31], suggesting the persistent and compensatory action of a second IRS-protein in the hypothalamus.

We thus examined the possibility of functional cooperation between Irs2 and Irs4 in the brain by combining whole body Irs4 deletion (which is functionally brain-restricted) with brain-specific Irs2 deletion thereby focusing on the tissue relevance for any synergistic action and avoiding the potentially confounding phenotypes associated with deletion of Irs2 in other tissues (such as the islet). Indeed, this analysis revealed important functional synergy between brain Irs2 and Irs4 in the regulation of energy balance and glucose homeostasis: To an extent...
far greater than either deletion alone, the brain-wide ablation of Irs2 and Irs4 in combination promoted obesity and glucose intolerance. The severe metabolic phenotype in the birs2<sup>−/−</sup>−Irs4<sup>−/−</sup> mice thus reveals an important physiological role for Irs4 that is obscured by the continued presence of Irs2 in the Irs4<sup>−/−</sup>-mice [33], much as the presence of Irs4 mitigates partially the phenotype produced by CNS-specific Irs2 deletion in birs2<sup>−/−</sup> animals. The concerted action of Irs2 and Irs4 contributes not only to overall adiposity and glucose intolerance, but plays an especially crucial role in the control of insulin action and hepatic lipid accumulation, as islet size, HOMA2-IR and hepatic steatosis are dramatically elevated in birs2<sup>−/−</sup>−Irs4<sup>−/−</sup> mice, but do not differ from controls in the single mutant (birs2<sup>−/−</sup>− and Irs4<sup>−/−</sup>) mice.

Our current results, together with previous data, reveal separable functions for Irs2 and Irs4 expression in distinct neural subsets. The brain-wide ablation of Irs2 suffices to increase Agrp and Npy expression relative to Pomc, and promotes obesity and glucose intolerance. LepRb-restricted disruption of Irs2 expression similarly results in increased Agrp and Npy (and decreased Pomc) expression [29]. The Lepr<sup>fl/fl</sup>/Irs2<sup>−/−</sup> mice also display increased adiposity and decreased glucose tolerance relative to controls, although the magnitude of their metabolic phenotype is more modest than that observed in birs2<sup>−/−</sup>-mice [29]. These findings suggest that while Irs2 signaling in LepRb cells modulates Agrp, Npy and Pomc expression and contributes to the control of glucose and energy homeostasis, Irs2 acts in other (non-LepRb) neurons to mediate additional aspects of metabolic control.

While Irs4 cooperates with Irs2 in the CNS to modulate energy balance and glucose homeostasis, Irs4 does not contribute to the control of Agrp, Npy, or Pomc expression; nor does the absence of Irs4 exacerbate the phenotype of Lepr<sup>fl/fl</sup>/Irs2<sup>−/−</sup>-mice as it does the metabolic dysfunction of birs2<sup>−/−</sup>-animals. Thus, our data are consistent with the idea that Irs4 synergizes with Irs2 in non-LepRb neurons to control energy balance and metabolism independently of LepRb neurons and the leptin-modulated ARC Pomc and AgRP/Npy neurons. In the future, it will be important to identify the relevant Irs2/Irs4-expressing non-LepRb cells to reveal their neurophysiologic function and independent roles in the control of metabolic homeostasis. Given the distribution of Irs4 and LepRb, it is tempting to speculate that the crucial Irs4-expressing neurons could lie in the paraventricular hypothalamic nucleus (PVH), which is crucial for energy balance and metabolism, but which contains little LepRb.

Importantly, while Irs4 may not synergize with Irs2 in LepRb neurons, it remains possible (especially given the distribution of Irs4 expression in the hypothalamus) that LepRb neurons contain Irs4. The lack of metabolic phenotype for Irs4<sup>−/−</sup>-mice and lack of synergy between Irs2 and Irs4 in LepRb neurons suggests that if Irs4 plays a role in LepRb neurons, however, the function of Irs4 in LepRb cells must be redundant with a protein other than Irs2, such as Irs1. Thus, it may be useful to explore potentially redundant roles for Irs1 and Irs4 in the future, including in LepRb neurons.

Previous studies suggest that Irs4 might play a role in cellular leptin action [7]. Serum leptin concentrations are normal in Irs4<sup>−/−</sup>-mice, however. Furthermore, while leptin is elevated in birs2<sup>−/−</sup>-Irs4<sup>−/−</sup> relative to birs2<sup>−/−</sup>-mice (and whole-body birs2<sup>−/−</sup>-Irs4<sup>−/−</sup> mice compared to controls [7]), the body weight of obese mice with double deletion of Irs4 and Irs2 in LepRb neurons (Lepr<sup>fl/fl</sup>/Irs2<sup>−/−</sup>/Irs4<sup>−/−</sup>) was indistinguishable from the mice lacking only Irs2 in LepRb neurons (Lepr<sup>fl/fl</sup>/Irs2<sup>−/−</sup>). These results suggest that Irs4 effect on weight gain in the brain does not involve LepRb signaling. Indeed, despite the important role of Irs2 in LepRb neurons, and the hyperleptinemia of animals null for Irs2 in LepRb cells, leptin signals normally to decrease body weight in young Lepr<sup>fl/fl</sup>/Irs2<sup>−/−</sup>-mice [29]. Thus, neither Irs2 nor Irs4 in LepRb neurons is required for leptin action, but rather presumably mediate crucial insulin signals in LepRb cells (Irs2) and non-LepRb cells (Irs2 and Irs4). Insulin action in the hypothalamus mediates important effects upon body weight and nutrient homeostasis through signaling cascades which regulate food intake, glucose and lipid metabolism, and energy expenditure [3]. Both Irs2 and Irs4 are expected to promote the PI3K→AKT→FoxO1 cascade in hypothalamic neurons, as elsewhere in the body; this pathway is known to play a crucial role in the hypothalamic control of metabolic regulation [39].

Overall, our results suggest that Irs2 and Irs4 synergize in non-LepRb neurons to mediate central insulin action, thereby controlling a variety of metabolically important phenotypes. While it contributes to the control of energy balance, Irs4 plays an especially crucial role in the control of whole-body insulin sensitivity and glucose homeostasis.

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CONFLICT OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

APPENDIX A. SUPPORTING INFORMATION

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.molmet.2013.10.004.

REFERENCES


