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Diet-Genotype Interactions in the Development of the Obese, Insulin-Resistant Phenotype of C57BL/6J Mice Lacking Melanocortin-3 or -4 Receptors

Gregory M. Sutton[*], **James L. Trevaskis**[*], **Matthew W. Hulver**, **Ryan P. McMillan**, **Nathan J. Markward**, **M. Josephine Babin**, **Emily A. Meyer**, and **Andrew A. Butler**
Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana 70808

Abstract

Loss of brain melanocortin receptors (*Mc3rKO* and *Mc4rKO*) causes increased adiposity and exacerbates diet-induced obesity (DIO). Little is known about how Mc3r or Mc4r genotype, diet, and obesity affect insulin sensitivity. Insulin resistance, assessed by insulin and glucose tolerance tests, Ser^{307} phosphorylation of insulin receptor substrate 1, and activation of protein kinase B, was examined in control and DIO wild-type (WT), *Mc3rKO* and *Mc4rKO* C57BL/6J mice. *Mc4rKO* mice were hyperphagic and had increased metabolic efficiency (weight gain per kilojoule consumed) relative to WT; both parameters increased further on high-fat diet. Obesity of *Mc3rKO* was more dependent on fat intake, involving increased metabolic efficiency. Fat mass of DIO *Mc3rKO* and *Mc4rKO* was similar, although *Mc4rKO* gained weight more rapidly. *Mc4rKO* develop hepatic insulin resistance and severe hepatic steatosis with obesity, independent of diet. DIO caused further deterioration of insulin action in *Mc4rKO* of either sex and, in male *Mc3rKO*, compared with controls, associated with increased fasting insulin, severe glucose intolerance, and reduced insulin signaling in muscle and adipose tissue. DIO female *Mc3rKO* exhibited very modest perturbations in glucose metabolism and insulin sensitivity. Consistent with previous data suggesting impaired fat oxidation, both *Mc3rKO* and *Mc4rKO* had reduced muscle oxidative metabolism, a risk factor for weight gain and insulin resistance. Energy expenditure was, however, increased in *Mc4rKO* compared with *Mc3rKO* and controls, perhaps due to hyperphagia and metabolic costs associated with rapid growth. In summary, DIO affects insulin sensitivity more severely in *Mc4rKO* compared with *Mc3rKO*, perhaps due to a more positive energy balance.

Ventromedial hypothalamic lesions cause hyperphagia, obesity, and hyperinsulinemia, indicating that hypothalamic neurons are critical for energy homeostasis (1–3). The best-characterized hypothalamic neurons involved in energy homeostasis express proopiomelanocortin (POMC), a propeptide processed into α-, β-, and γ-MSH; agouti-related peptide (AgRP), or melanocortin receptors (Mc3r and Mc4r) (2,4). POMC and AgRP fibers project from the arcuate nucleus of the hypothalamus to hypothalamic and extra-hypothalamic centers involved in energy homeostasis (2,5). These neurons release agonists (MSH) or an Mc3r/Mc4r antagonist (AgRP), respectively, in response to orexigenic and anorectic inputs from sources in the periphery and brain (2,5). A small population of POMC-positive neurons in the nucleus tractus solitarius in the brain stem also regulates feeding behavior (6).

Address all correspondence and requests for reprints to: Andrew A. Butler, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, Louisiana 70808. butleraa@pbrc.edu.
[*]G.M.S. and J.L.T. contributed equally to this work.

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The prevailing model of the regulation of energy homeostasis by melanocortins proposes that increased POMC neuronal activity inhibits food intake and increases energy expenditure (2, 3). Conversely, stimulation of AgRP neurons has the opposite effect by inhibiting MSH action and, through the release of the inhibitory neurotransmitter γ-aminobutyric acid, reducing the activity of POMC neurons (2,7). Neuropeptide Y (NPY) release from AgRP neurons may also inhibit POMC neuronal activity (8). In humans, *Pomc* or *Mc4r* gene mutations are associated with childhood hyperphagia and obesity (9). Mutations reducing efficacy of Mc3r activation by MSH may also cause childhood obesity (10). The phenotypes of transgenic mutant mice are generally consistent with the prevailing model, with loss of POMC synthesis (11,12) or melanocortin receptor function (13–16) resulting in obesity, and ablation of AgRP neurons in adults results in hypophagia and weight loss (17–19).

Mc3r and *Mc4r* knockout (*Mc3rKO* and *Mc4rKO*) mice have been used to investigate the role of each receptor in regulating energy homeostasis. Comparison of single and double knockouts suggests that Mc3r and Mc4r function independently to regulate energy homeostasis (13–15). Analysis of conditional *Mc4r* mutant mice, where expression is limited to neurons, suggests that most, if not all, of Mc4r regulation of energy balance occurs in the brain (16). The acute effects of nonselective melanocortin receptor agonists on feeding behavior and energy expenditure require Mc4r (13,15,16,20). Mc4r are required for stimulation of sympathetic nervous activity (21,22) and thyroid function (23) by nonselective melanocortin receptor agonists. Stimulation of sympathetic nervous activity and the thyroid are mechanisms by which Mc4r activation could increase energy expenditure. Inhibition of food intake by the satiety factor cholecystokinin involves stimulation of POMC neurons in the brain stem and also requires Mc4r (6).

The regulation of energy homeostasis by the Mc3r has not been extensively studied. *Mc3rKO* initially exhibited a very modest obesity syndrome, involving modest increases in fat mass (FM) and reduced lean mass without hyperphagia and with a normal anorectic response to melanocortin agonists (13,15). However, a recent study indicated that Mc3r and Mc4r may be of approximately equal importance in preventing weight gain on moderately high-fat chow and demonstrated that *Mc3rKO* also exhibit aberrant regulation of feeding behavior by leptin (22). Moreover, male *Mc3rKO* backcrossed onto the C57BL/6J (B6) background exhibit a modest hyperphagia on high-fat diets (24). Intracerebroventricular administration of AgRP increases food intake of *Mc4rKO*, suggesting that inhibition of Mc3r may increase food intake (25). Other factors that may contribute to increased adiposity of *Mc3rKO* include low fatty acid oxidation (FAO), indicated by a high respiratory exchange ratio, and reduced physical activity (13,15,24).

Diet and genetic background profoundly affect obesity and diabetes (“diabesity”) in mice (26,27). Studies using mice on mixed genetic backgrounds and various diets indicate that diet-induced obesity (DIO) is more severe in *Mc3rKO*, *Mc4rKO*, *Pomc*−/−, or *Pomc*+/− mice (11,15,28). However, very little is known about how genotype and diet affect insulin sensitivity of melanocortin mutant mice at the tissue level. Central administration of nonselective melanocortin receptor agonists or antagonist in rats improves, or worsens, insulin sensitivity independently of changes in adiposity, which is thought to primarily involve Mc4r (29).

The current study further investigated the effects of Mc3r and Mc4r deficiency on DIO, metabolism, and insulin resistance of B6 mice. Insulin receptor signaling was examined in liver, muscle, and retroperitoneal adipose tissue of knockout and wild-type (WT) B6 mice fed low- or high-fat diets. Insulin resistance was assessed by measuring the phosphorylation of insulin receptor substrate 1 (IRS1) on Ser^{307}, which inhibits association of IRS1 with the insulin receptor tyrosine kinase (30). Activation of protein kinase B (PKB), which is a target of phosphatidyl-3 kinase (PI3K) and involved in the regulation of glucose uptake (31), and

phosphorylation of downstream targets of PKB [Forkhead Box O1 (FoxO1) and glycogen synthase kinase-3 (GSK3)], were also measured in liver, skeletal muscle (quadriceps), and retroperitoneal adipose tissue to assess insulin responsiveness. The original hypothesis, based on analysis of *Mc3rKO* and *Mc4rKO* on outbred backgrounds (13–15,20,28), was that diabesity would be most severe in *Mc4rKO* and mild in *Mc3rKO*. However, our data suggest a more complex interaction between Mc3r and Mc4r deficiency and dietary fat on the development of obesity and insulin resistance in B6 mice.

Materials and Methods

Experimental animals

The Pennington Biomedical Research Center Institutional Animal Care and Use Committee approved all experiments. *Mc3rKO*, *Mc4rKO*, and WT B6 littermates were obtained from mating heterozygote parents, as described previously (13,32). Mice were housed on a 12-h light, 12-h dark period (lights on 0600–1800 h). Adiposity (percent body fat) was estimated by measuring FM and fat-free mass (FFM) by nuclear magnetic resonance (NMR) (Bruker Mice Minispec NMR Analyzer; Bruker Optics Inc., Billerica, MA), validated against dual x-ray absorptiometry and standard chemical composition analysis (33). Low-fat (Research Diets no. 12450B), moderately high-fat (no. 12451), and high-fat (no. 12492) diet, with 10, 45, or 60% kJ from fat, respectively, were purchased from Research Diets, Inc (New Brunswick, NJ).

Food intake and energy balance

Food intake (kilojoules per day) and gain in body mass energy (kilojoules per day) were measured over 8 wk (*black line* in Fig. 1, A and B) using mice housed in wire-mesh caging; spillage was collected in trays beneath the cage floor and subtracted from the reduction of food weight in the feeder to provide an accurate measure of actual intake. Mice were placed in the wire mesh caging at 7–8 wk of age and allowed 10 d to acclimate to the new conditions. Gain in body mass energy was calculated using NMR measurements of FM and FFM, using a value of 37.7 kJ/g FM and 4.2 kJ/g FFM (assumes 25% of fat-free mass is protein, with the remainder being water). For estimating basal daily energy expenditure in a weight-stable state (bEE_{est}), the energy cost of depositing FM and protein was estimated using a value of 55.3 (FM) and 9.2 (FFM) kJ/g (34). Metabolic efficiency ($ME_{\%}$) is the gain in body mass energy as a percentage of total energy intake (35).

Energy expenditure, fatty acid oxidation, and mitochondrial function

Total and resting energy expenditure (TEE_{cal} and REE_{cal}) were also measured by indirect calorimetry, using a 16-chamber Oxymax system (Columbus Instruments, Columbus, OH), as previously described (24,36). Ambulatory activity in the x and z axes were evaluated using an OPTO-M3 sensor system (Columbus Instruments) (24).

Calorimetry data collected over several days provides a more accurate assessment of energy expenditure, compared with short-term measurements (*i.e.* 3–4 h). Calorimetry data were collected over a 7- to 10-d period, with chambers disassembled for maintenance every 3 d. Energy expenditure of mice housed in this system is stable over a 14-d period (Refs. 24 and 36 and data not shown). Some of the data presented are from studies published previously (24,32,36). Nonresting energy expenditure ($NREE_{cal}$), due to ambulation and the thermic effects of feeding (37), was calculated by subtracting REE_{cal} from TEE_{cal}. Energy expenditure data are presented as kilojoules per animal (kilojoules per day), adjusted for body weight (kilojoules per gram body weight per day), or fat-free mass determined using NMR (kilojoules per gram FFM per day). REE_{cal} from TEE_{cal} were also analyzed by linear regression against body weight and FFM (37).

FAO was assessed by measuring production of CO_2 and acid-soluble metabolites after incubation of fresh liver and muscle extracts with [^{14}C]palmitate (38,39). Citrate synthase activity was measured as previously described (40).

Histology and serum assays

Hematoxylin and eosin staining was performed on 15-μm sections of liver tissue collected in 10% paraformaldehyde, dehydrated, and mounted in paraffin. Hepatic lipid and triglyceride content was measured as previously described (32). Total plasma cholesterol and triglycerides were measured after an overnight fast using a Beckman Synchron CX7. Insulin ELISAs were performed in duplicate using kits from Crystal Chem (Downer's Grove, IL).

Gene expression

Total RNA from liver was isolated using TRI Reagent (Molecular Research Centre Inc., Cincinnati, OH) and reverse transcribed using Superscript III RT system (Invitrogen, Carlsbad, CA). Oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Quantitation of target gene mRNA using cyclophilin B as a reference was performed in 384-well plates using SYBR Green, or TaqMan Universal PCR Master Mix (Applied Biosystems) and an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Sequences of primers and probes used for expression analyses were as follows: cyclophilin B sense 5′-ggtggagagcaccaagacaga-3′ and antisense 5′-gccggagtcgacaatgatg-3′; apolipoprotein A IV (*Apoa4*) sense 5′-tggtgcccttt-gtcgtacag-3′, antisense 5′-catcatgcggtcacgtaggt-3′, and probe 5′-tgagtgggcatctagccaaggaaactga-3′; stearoyl CoA desaturase 1 (*Scd1*) sense 5′-caacaccatggcgttcca-3′ and antisense 5′-ggtgggcgcggtgat-3′. 6′-Carboxyfluorescein (FAM)-labeled probes were synthesized by Applied Biosystems (Foster City, CA).

Insulin sensitivity and insulin receptor signaling

Whole-body insulin sensitivity and glucose tolerance were estimated using insulin tolerance tests (ITT) (1 U/kg Humulin; Eli Lilly, Indianapolis, IN) or ip glucose tolerance test (IPGTT) (1 g/kg glucose), as previously described (32). Blood glucose was measured using a Glucometer Elite XL (Bayer Corp., Elkhart, IN).

Insulin receptor signaling was analyzed using muscle, liver, and retroperitoneal adipose tissue extracts from mice killed 10 min after receiving a single ip injection of 1 U/kg insulin or saline after overnight fast. Tissues were homogenized in ice-cold buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EDTA, 1% Triton X-100, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin], and 20 μg protein was diluted in sample buffer [50 mM Tris HCl (pH 6.7), 4% wt/vol glycerol, 4% SDS, 1% 2-mercaptoethanol, and 0.02 mg/ml bromphenol blue] and separated on a 10% SDS-polyacrylamide gel. Protein was transferred to Immobilon-P polyvinylidene difluoride membranes in Towbin-transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.01% SDS).

Membranes were incubated with antibodies for phospho-PKB, phospho-FoxO1, or phospho-GSK3 α/β or antibodies against total PKB, FoxO1, or GSK3 α/β (Cell Signaling Technology, Beverly, MA). Antigen-antibody-peroxidase complexes were detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Phosphorylation of IRS1 on Ser^{307} was determined by immunoprecipitation using a polyclonal antibody against the C terminus, followed by blotting using a polyclonal antibody against Ser307 of IRS1 (Cell Signaling Technology).

PKB kinase activity was measured using a nonradioactive assay kit (Cell Signaling Technology). PKB was immunoprecipitated from 200 μg lysate using a mouse monoclonal

antibody (1G1). PKB-bound beads were then incubated at 37 C for 30 min with ATP (10 mM) and 1 μg of a fusion protein containing the phosphorylation sites of GSK-3 α/β (Ser^{21} for GSK-3α and Ser^{9} for GSK-3β). The GSK-3 fusion protein was solubilized in Laemmli sample buffer and separated on 10% SDS-PAGE gels, and GSK-3 phosphorylation was detected using an antibody recognizing both Ser^{21} and Ser^{9} of GSK-3α and -β. Signal on autoradiograms was quantified by densitometry using QuantityOne (Bio-Rad, Hercules, CA). Data are presented as arbitrary units, relative to the mean of insulin-treated WT mice. Phosphospecific antibody data were also normalized for total protein.

Statistics

Data presented are mean ± SE. Sigmastat software (SPSS Inc., Chicago, IL) was used for one-way and two-way ANOVA. The strength of association between energy expenditure and body mass was assessed using the cor and lm functions in R (R Development Core Team, http://cran.r-project.org/). Tukey or Student-Newman-Keuls *post hoc* tests were performed for data exhibiting homogeneous or nonhomogeneous variance, respectively. Significance was assumed for P values < 0.05.

Results

Effect of diet-genotype interactions on obesity and energy balance

Weight gain and adiposity—The effect of a high-fat diet (60% kJ from fat) on weight gain and adiposity of male and female *Mc3rKO* (n = 4 males and 3 females), *Mc4rKO* (n = 4 males and 6 females), and WT mice (n = 8 males and 11 females) is shown in Fig. 1. The increase in weight gain associated with the high-fat diet was significantly affected by gender and genotype (Fig. 1, A–D, and Table 1). In descending order, the increase gain in body mass energy per week on the high-fat diet, and the net increase on the high-fat diet compared with the low-fat diet [Δ (high-fat − low-fat)] was greatest for male and female *Mc4rKO* (15.2 or 19.3 kJ/d, an increase of 8.6–9.3 kJ/d over low-fat), followed by male *Mc3rKO* (11.3 kJ/d, + 9.0 kJ), followed by female *Mc3rKO* and male WT (4.4 or 5.1 kJ/d, + 3.9 or + 3.6 kJ), whereas female WT mice were most resistant to DIO (1.5 kJ/d, + 1.1 kJ).

Weight gain and adiposity of 6-month-old female *Mc3rKO*, *Mc4rKO*, and WT mice (n = 4–6 per group) was also compared after 3 months on either a low-fat or high-fat diet (Fig. 1, E–I). Significantly increased body mass of *Mc3rKO* relative to WT was observed only on the high-fat diet (Fig. 1E), although *Mc3rKO* fed a low-fat diet exhibited a modest increase in adiposity. The high-fat diet resulted in a doubling of body mass of *Mc3rKO* and *Mc4rKO* (Fig. 1F). On the high-fat diet, adiposity and FM of female *Mc3rKO* were not significantly different from *Mc4rKO* (Fig. 1, D, G, and H). Data shown in Fig. 1 and Table 1 are representative of several experiments. Similar results were also observed using a moderately high-fat diet (45% kJ from fat) (data not shown and Refs. 24, 32, and 36).

Food intake and metabolic efficiency—*Mc4rKO* were hyperphagic compared with *Mc3rKO* and WT mice, irrespective of sex and diet (Table 1 and Fig. 2, A and B). The high-fat diet further increased energy intake of male and female *Mc4rKO* [diet effect within genotype for males and females, $P < 0.01$; comparison of Δ (high-fat − low-fat) intake between WT and *Mc4rKO*, $P < 0.01$]. Diet effects on energy intake of WT and *Mc3rKO* were gender specific (Table 1). Male *Mc3rKO* increased energy consumption on the high-fat diet by +6.9 kJ/d, compared with the low-fat diet (effect of diet, $P < 0.05$). There was a modest, and not statistically significant, increase in kilojoules intake of male WT on a high-fat diet (+3.8 kJ/d). Diet did not significantly affect daily kilojoules consumed of female *Mc3rKO* (+ 1.6 kJ/d). However, female WT mice exhibited a small increase comparable to that observed in males that was statistically significant (+3.9 kJ/d, $P < 0.01$) (Table 1).

With the exception of female WT, the high-fat diet significantly increased $ME_{\%}$ (Table 1). $ME_{\%}$ of *Mc4rKO* of either sex was always higher compared with WT. For *Mc3rKO*, $ME_{\%}$ was significantly increased compared with WT only on the high-fat diet, with the high-fat diet having a greater impact in males (Table 1).

Energy expenditure—For this study, bEE_{est} is assumed to be energy available for basal metabolism and physical activity once the metabolic cost of protein and fat deposition is taken into account. The bEE_{est} was lower in *Mc3rKO* compared with WT and *Mc4rKO*, with the high-fat diet increasing the difference (Table 1). For female *Mc3rKO*, bEE_{est} was 5% lower compared with WT on the low-fat diet, the difference increasing to 15% on the high-fat diet ($P < 0.05$ on high-fat diet). A similar difference was observed in male *Mc3rKO*, with a strong tendency ($P = 0.058$) for bEE_{est} of male *Mc3rKO* to be lower compared with WT. Moreover, the decline in bEE_{est} of male *Mc3rKO* from 53.5 to 47.1 kJ/d was statistically significant ($P < 0.05$). The bEE_{est} of male *Mc4rKO* was not statistically different from WT irrespective of diet. The bEE_{est} of female *Mc4rKO* was modestly increased and was also not affected by diet. These data suggest that energy available for physical activity and basal metabolism, once metabolic costs of fat deposition have been subtracted from the energy budget, is significantly reduced in *Mc3rKO* relative to WT and *Mc4rKO*.

The efficiency of deposition of fat and protein is estimated at 70 and 50%, respectively (34). It is worth noting that the metabolic costs estimated for fat and protein deposition were high for *Mc4rKO* of either sex (8–10 kJ/d on the high-fat diet) relative to WT controls (1.0–2.5 kJ/d). *Mc3rKO* on the high-fat diet also exhibited a significantly higher value compared with WT, particularly in males (5.5 kJ/d). Metabolic costs of fat deposition in mice rapidly gaining weight, such as *Mc4rKO* (Fig. 1, A and B), may therefore represent a significant proportion of the energy expenditure budget.

Indirect calorimetry data from a large cohort of mice, whose body mass data are shown in Fig. 3A, also indicated that *Mc4rKO* of either sex had increased TEE_{cal}, due to increases in REE_{cal} and $NREE_{cal}$ relative to WT (Fig. 3B). There was an intermediate increase in TEE_{cal}, REE_{cal}, and $NREE_{cal}$ of male, but not female, *Mc3rKO*. The increase in TEE_{cal} of *Mc4rKO* appeared most severe for females. TEE_{cal} adjusted for FFM or body mass of female *Mc4rKO* was still increased relative to WT and *Mc3rKO*. However, in males, energy expenditure was not significantly different between genotype when the data were adjusted for FFM or body mass.

Multiple regression analysis of the combined groups indicated a positive association of TEE_{cal} and REE_{cal} with FM (R = 0.58 and 0.60), FFM (R = 0.57 and 0.53), and total body mass (R = 0.62) (Fig. 4, A and B, and data not shown). Linear regression with genotype indicated that the association between body mass and either TEE_{cal} or REE_{cal} of *Mc4rKO* was significantly higher relative to the regression line for *Mc3rKO* (Fig. 4, A and B). No association was evident in WT mice, perhaps due to the narrow range of body weights. These data suggest that metabolic rate of *Mc4rKO* is higher compared with *Mc3rKO* as a function of body mass.

FAO and mitochondrial function in red muscle—FAO ($[^{14}C]$palmitate oxidation) and citrate synthase activity were measured in red oxidative muscle isolated from the gastrocnemius of female WT, *Mc3rKO*, and *Mc4rKO* fed a low-fat or high-fat diet (Fig. 4, C and D). Body mass data for this cohort were shown in Fig. 1, E–I. On the low-fat diet, FAO was significantly reduced in *Mc3rKO* compared with WT, with a tendency for a reduction in *Mc4rKO* (Fig. 4C). On the high-fat diet, FAO was not significantly different between genotype groups. This was due to FAO in the melanocortin receptor knockout being higher on the high-fat diet, a change that was statistically significant for *Mc3rKO* ($P < 0.05$). Citrate synthase activity was significantly reduced in *Mc3rKO* and *Mc4rKO* compared with WT mice irrespective of diet

(Fig. 4 D). The high-fat diet also significantly reduced citrate synthase activity in female WT mice ($P < 0.01$) but had no effect on citrate synthase activity of either *Mc3rKO* or *Mc4rKO*.

Spontaneous locomotor behavior—Male and female *Mc3rKO* (n = 5) and *Mc4rKO* (n = 4 females and 3 males) mice fed a high-fat diet were hypoactive compared with WT (n = 5) (least squares mean for total beam breaks per day was 50,286 ± 4,457 for WT; 29,334 ± 4,457 for *Mc3rKO;* and 29,220 ± 5,382 for *Mc4rKO;* WT *vs. Mc3rKO* and *Mc4rKO, P* < 0.05).

Effect of diet-genotype interactions on insulin resistance

Fasting insulin and glucose—Fasting insulin and glucose were measured in male and female *Mc3rKO, Mc4rKO,* and WT mice at 6 months of age after 3 months on diets with variable fat content (10, 45, or 60% kJ from fat) (Fig. 5, A–D). In males, Mc3r and Mc4r deficiency exacerbated fasting hyperinsulinemia associated with consumption of the 60% high-fat diet (Fig. 5, A and C, and data not shown). For males on the 45% fat diet, a significant increase in fasting insulin was observed only in *Mc4rKO*. In females, *Mc4rKO* exhibited a more marked response to both the 45% and 60% high-fat diet, with *Mc3rKO* having insulin levels comparable to WT irrespective of diet.

ITT and GTT results—Fasting insulin data suggest that *Mc4rKO* are more sensitive, relative to *Mc3rKO,* to the effects of dietary fat on insulin resistance. In particular, female *Mc3rKO* on high-fat diets appeared to develop a syndrome of obesity not associated with severe insulin resistance. Further investigation of insulin sensitivity used mice fed a moderately high-fat diet (45% kJ from fat) to avoid type 2 diabetes (fasting blood glucose > 200 mg/dl) observed on the high-fat diet (60% kJ from fat) (Fig. 5, C and D).

ITT and GTT results from 6-month-old WT, *Mc3rKO,* and *Mc4rKO* on the low-fat diet were not significantly different (Fig. 5, E and G). The moderately high-fat diet caused reduced glucose tolerance that was particularly severe for *Mc4rKO* (Fig. 5H). The glucose-lowering action of insulin was also significantly compromised in *Mc4rKO*, compared with *Mc3rKO* and WT mice, on the moderately high-fat diet (Fig. 5F).

Effect of genotype and diet on insulin receptor signaling—Phosphorylation of PKB, a downstream mediator of PI3K action, is involved in mediating intracellular effects of insulin in target organs, including stimulating glucose uptake (31). Phosphorylation of PKB at Ser^{473} and Thr^{308} is an indicator of kinase activation (41–43). In *Mc4rKO,* insulin-stimulated PKB activity was significantly reduced compared with both WT and *Mc3rKO* irrespective of diet (Fig. 6, A and B). Phosphorylation of two downstream targets of PKB, GSK3β and FoxO1, was also reduced in liver of *Mc4rKO* compared with both WT and *Mc3rKO*.

Insulin stimulation of PKB activity in muscle and adipose was not significantly affected by genotype in mice fed the low-fat diet (Fig. 7, A and B, and Fig. 8, A and B). Phosphorylation of GSK3β in muscle, and FoxO1 in adipose tissue, was also not affected by genotype in mice on the low-fat diet. However, on the moderately high-fat diet, there was a marked deterioration of insulin-stimulated PKB activity in *Mc4rKO* in skeletal muscle (Fig. 7, A and B) and modest reduction of PKB activity in adipose (Fig. 8, A and B). This correlated with a significant reduction in the phosphorylation of GSK3β in muscle, and FoxO1 in adipose tissue, after insulin treatment (Figs. 7C and 8C).

Ser^{307} phosphorylation of IRS1 reduces the efficiency of association with the insulin receptor tyrosine kinase, reducing insulin activation of PI3K and MAPK cascades (30). Insulin resistance of knockout and WT mice fed the moderately high-fat diet was further compared by measuring Ser^{307} phosphorylation of IRS1 in liver, muscle, and adipose (Fig. 9). Ser^{307} phosphorylation of IRS1 was significantly increased in liver and muscle of insulin-treated

Mc4rKO compared with WT (Fig. 9, A and B). In muscle, there was a strong tendency ($P = 0.06$) for Ser^{307} phosphorylation of IRS1 to be significantly increased in *Mc4rKO* compared with *Mc3rKO*. A significant increase in Ser^{307} phosphorylation of IRS1 was also observed in liver of *Mc3rKO* although not as severe as that observed in *Mc4rKO*. Genotype did not significantly affect Ser^{307} phosphorylation of IRS1 in adipose tissue (Fig. 9C).

Liver metabolism—Severe insulin resistance associated with leptin deficiency is associated with hepatomegaly, steatosis, and abnormal hepatic expression of genes involved in lipid metabolism (41,44,45). Liver weight and lipid content and hepatic *ApoA4* and *Scd1* mRNA expression were measured in male and female mice after 3 months on the 60% high-fat or 45% moderately high-fat diet (Fig. 10 and data not shown). Compared with WT and *Mc3rKO*, *Mc4rKO* were hepatomegalic and had more severe steatosis (Fig. 10, A and C). Analysis of liver weight as a function of adiposity indicated a positive linear association in *Mc4rKO*, with no effect of increasing adiposity in WT or *Mc3rKO* (Fig. 10B). Measurement of hepatic lipid content indicated mild steatosis in *Mc3rKO* compared with *Mc4rKO* (lipid content in mg/g: WT, 39.1 ± 6.4; *Mc3rKO*, 103.5 ± 19.7; and *Mc4rKO*, 184.9 ± 18.6; all $P < 0.05$). In females, the expression of *ApoA4* and *Scd1* mRNA was significantly increased in *Mc4rKO* compared with WT, with normal expression in *Mc3rKO*. In males, *Mc3rKO* exhibited an intermediate increase in *ApoA4* and *Scd1* mRNA compared with *Mc4rKO* (Fig. 10, D and E).

Discussion

Melanocortins have an essential role in regulating energy homeostasis in mice and humans, acting through two receptors that are expressed in regions of the central nervous system involved in regulating energy homeostasis (2,9). These experiments demonstrate the complexity, and importance, of the interaction between the two melanocortin receptors and dietary fat in the development of obesity and insulin resistance. A strength of these experiments is that both knockout strains have been backcrossed onto C57BL/6J, removing the confounding effect of genetic background on obesity and diabetes. Overall, these results suggest that the Mc4r has a critical role in preventing hepatic insulin resistance, with the high-fat diet associated with a broader insulin resistance.

The results from mice fed a low-fat diet are consistent with previous results demonstrating a modest obese phenotype of *Mc3rKO* compared with *Mc4rKO*. On a high-fat diet, the phenotype of the two knockout strains is far less distinct. Surprisingly, DIO male *Mc3rKO* mice are very similar to male *Mc4rKO* in terms of weight gain, metabolic efficiency, and fasting hyperinsulinemia. Additional experiments comparing the insulin-resistant phenotype of DIO male *Mc3rKO* and *Mc4rKO*, and their response to compounds stimulating melanocortin receptor activity, are currently in progress. The interaction of the Mc3r with dietary fat is gender specific. The less rapid weight gain of female *Mc3rKO* on a high-fat diet may be a factor in the modest perturbations of glucose homeostasis compared with *Mc4rKO*.

DIO of Mc3rKO and Mc4rKO

Reduced MSH synthesis (11,12), or loss of MSH action through Mc3r (15) or Mc4r (28,32), is associated with weight gain and an exacerbation of DIO. The results of these experiments confirm these findings and demonstrate that obesity associated with Mc3r deficiency is more dependent on dietary fat. On a low-fat diet, *Mc3rKO* exhibit a modest increase in FM, and normal body weight, which was previously reported (13,15). On a high-fat diet, FM of *Mc3rKO* is comparable to that of *Mc4rKO*. *Mc4rKO* also exhibit a marked response to a high-fat diet, consistent with previous results using outbred mice and standard or moderately high-fat chows (22,28). However, *Mc4rKO* exhibit a robust obesity that is independent of dietary fat content.

Food intake is clearly a factor in the different sensitivity of *Mc3rKO* and *Mc4rKO* to a high-fat diet. Previous data indicate that hyperphagia contributes to the obese phenotype of *Mc4rKO* on a low-fat diet (14,46,47). In this study, on the low-fat diet, *Mc4rKO* were massively hyperphagic compared with *Mc3rKO* and WT, a difference further compounded by the high-fat diet. As previously reported, hyperphagia was not a factor in the obese phenotype of *Mc3rKO* on a low-fat diet (13,15). However, on the high-fat diet, male *Mc3rKO* were modestly hyperphagic. We have previously observed a modest hyperphagia, restricted to the lights-on period, in male *Mc3rKO* after introduction of a high-fat diet (24). Loss of Mc3r in the ventromedial hypothalamus could be involved in obesity of *Mc3rKO*. Lesions in the ventromedial hypothalamus of rats are associated with obesity (48,49), at least partially due to hyperphagia during the lights-on period (49).

Although activation of Mc3r does not appear to inhibit feeding behavior (20,50,51), these are not the first data to indicate that inhibition or loss of Mc3r modestly increases food intake. Intracerebroventricular infusion of the Mc3r/Mc4r antagonist AgRP modestly increases food intake in *Mc4rKO* (23,25). Loss of Mc3r also attenuates the inhibition of food intake by leptin (22). However, female *Mc3rKO* were not hyperphagic on the high-fat diet, which may be a factor in the less rapid gain of body mass energy observed in female *Mc3rKO* (5.1 kJ/d) compared with male *Mc3rKO* (11.3 kJ/d). The regulation of feeding behavior by Mc3r thus appears to be gender specific, having a modest role in males to prevent hyperphagia on a high-fat diet.

Energy metabolism of Mc3rKO and Mc4rKO

Obesity results from an imbalance of energy intake and energy expenditure. Mc3r and Mc4r are expressed in regions of the hypothalamus and hind brain involved in the regulation of energy expenditure (5,52). However, examination of the response of knockout mice to nonselective melanocortin receptor agonists suggest a primary role for the Mc4r in the acute regulation of energy expenditure (16,20–22,47). The Mc4r has a crucial role in the regulation of pathways known to regulate energy expenditure, including the hypothalamo-pituitary-thyroid axis (23), sympathetic nervous activity (21), and stimulation of brown adipose tissue thermogenesis (22,47), with no compensatory role for the Mc3r. Diet-induced thermogenesis, an adaptive metabolic response to hyperphagia that is dependent on β-adrenergic receptor signaling (53), requires a functional Mc4r but is normal in Mc3r-deficient mice (13,28).

Despite a lack of experimental evidence supporting a role for the Mc3r in regulating energy expenditure, the results from these experiments and previous studies (13,15) demonstrating obesity of *Mc3rKO* without hyperphagia strongly suggests an important role in regulating peripheral metabolism and energy balance. Indeed, the comparable increase in metabolic efficiency of male *Mc3rKO* and *Mc4rKO* fed a high-fat diet suggests an equal role for the receptors in promoting a thrifty phenotype. It has, however, been difficult to demonstrate a reduction in energy expenditure of *Mc3rKO* (13,15) or *Mc4rKO* (20,32,47). The problems associated with interpreting energy expenditure data from lean and obese subjects of any species have been discussed by other investigators (37,54). This study measured energy expenditure using two methods, first using food intake and estimates of gain in body mass energy (34) and second by indirect calorimetry. Mitochondrial activity in skeletal muscle was also measured to obtain another estimate of potential metabolic rate.

The energy expenditure data from the current study suggest that energy expenditure of *Mc4rKO* is significantly increased compared with WT, with no significant difference in *Mc3rKO*. The seemingly paradoxical finding that energy expenditure was highest in the strain with the fastest rate of fat deposition may be explained by two observations. The metabolic costs of digestion will be higher for *Mc4rKO* consuming an extra 21–36 kJ/d compared with *Mc3rKO* and WT. In addition, the metabolic cost of fat and protein deposition is estimated to

be increased for *Mc4rKO*. This variable was estimated at 3–5 kJ/d for *Mc4rKO* on the low-fat diet, increasing to 7–10 kJ/d on the high-fat diet, or 5- to 10-fold greater than that estimated for WT mice. Such a large metabolic cost of weight gain in *Mc4rKO* is likely transient and may not occur once a new set-point in body weight has been achieved. Clearly, taking into account the growth rate of *Mc4rKO* and other models where obesity is associated with rapid gains in body mass is important when analyzing and interpreting energy expenditure data. The metabolic cost of fat deposition was also significant for male *Mc3rKO* (5.5 kJ/d) but not for female *Mc3rKO* depositing FM at a slower rate.

Preferential nutrient partitioning to adipose remains an attractive hypothesis explaining the obese phenotype in *Mc3rKO*. Indeed, removing the metabolic costs of fat deposition from the daily energy budget reveals a significant reduction in the amount of energy available for other aspects of basal metabolism of *Mc3rKO* compared with WT and *Mc4rKO*. Preferential nutrient partitioning to fat is also probably a factor in *Mc4rKO* (47). However, for a reason that is not clear, but may involve the metabolic cost of digestion, a comparable reduction in bEE_{est} was not observed for *Mc4rKO*.

The mechanisms involved in the nutrient partitioning defect of *Mc3rKO* and *Mc4rKO* are not known. However, on the B6 background, both knockout mice have a high respiratory exchange ratio, indicating reduced oxidation of fatty acids that is associated with a positive imbalance of fat intake (24,32). In this study, skeletal muscle oxidative metabolism, as determined by oxidation of [^{14}C]palmitate and citrate synthase activity, was reduced in both *Mc3rKO* and *Mc4rKO* when compared with controls. Citrate synthase activity has been suggested to be an indicator of mitochondrial content and function (40,55). Reduced capacity of FAO in muscle may therefore be a factor contributing to the high respiratory exchange ratio of *Mc3rKO* and *Mc4rKO*. The mechanisms by which Mc3r and Mc4r regulate oxidative metabolism in muscle are not clear at this time. Both receptors are expressed in the hypothalamus, which has been shown to be an important site for the regulation of FAO in muscle by leptin, likely through the regulation of the autonomic nervous system (56).

Insulin resistance of Mc3rKO and Mc4rKO is largely secondary to obesity

The melanocortin system is thought to be a promising target for the treatment of obesity and insulin resistance. Treatment of DIO C57BL/6J with nonselective melanocortin agonists reduces body weight and improves insulin sensitivity (57,58). Moreover, infusion of SHU9119 intracerebroventricularly in rats is associated with the development of hepatic insulin resistance (29). *Mc4rKO* exhibit marked hyperinsulinemia and hyperglycemia, which can be attenuated but not completely reversed by pair-feeding with WT controls (14,47). Type 2 diabetes and glucose intolerance are also observed in 6- to 8-month-old *viable yellow* (A^{vy}) and *lethal yellow* (A^{y}/a) mice, which ectopically express an antagonist of the Mc1r and Mc4r (59). In contrast, *Mc3rKO* exhibit a very mild insulin-resistance phenotype (13,15). Taken together, these observations suggest that the Mc4r has a more important role in preventing insulin resistance.

A goal of these experiments was to compare insulin action in age- and weight-matched *Mc3rKO* and *Mc4rKO*. The results of this study, and those of a previous investigation (32), suggest the development of a comparatively mild insulin resistance of *Mc4rKO* fed a low-fat diet. Obese *Mc4rKO* fed a low-fat diet exhibit a modest increase in fasting insulin and a deterioration of hepatic insulin sensitivity. This result is surprising, when the diabetic phenotype of *viable yellow* (A^{vy}) and *lethal yellow* (A^{y}/a) mice is taken into consideration (59). It is tempting to speculate that ectopically expressed agouti may affect insulin sensitivity or β-cell function independently of Mc4r, as recently demonstrated for adipocyte metabolism (60,61). However, carefully controlled studies using *Mc4rKO*, A^{y}/a and A^{vy} mice on the same B6 background are required to test this hypothesis.

The current study extended previous investigations to include insulin receptor signaling in tissues of female knockout and WT mice, which exhibited the clearest distinction between genotype in terms of fasting hyperinsulinemia, glucose tolerance, and response to insulin treatment. The stimulation of PKB activity and the phosphorylation of targets of PKB, FoxO1, and GSK3β after insulin treatment was reduced in liver of 6-month-old female *Mc4rKO*. Insulin stimulation of PKB kinase activity or Ser^{473} phosphorylation is normal in liver, and also in muscle, of 6-wk-old *Mc4rKO* (Sutton, G. M., and A. A. Butler, unpublished data).

The development of severe insulin resistance in skeletal muscle of *Mc4rKO*, associated with a marked decline in the stimulation of PKB activity and GSK3β phosphorylation by insulin, was observed only on the high-fat diet. This was associated with a marked deterioration of glucose tolerance and insulin-stimulated glucose disposal, consistent with the importance of muscle as a site of glucose disposal. The mechanisms involved in the development of severe insulin resistance in *Mc4rKO* on a high-fat diet were not determined in this study. However, several factors could be involved in the development of severe muscle insulin resistance of *Mc4rKO* fed a high-fat diet, including but not limited to the intramyocellular accumulation of fatty acids (62) and inflammation (63). At the level of neuroendocrine systems, the progressive development of hypothalamic leptin resistance might also be involved in the deterioration of insulin action in *Mc4rKO*, independently of diet (36,44,50). Alternatively, leptin-independent regulation of Mc4r activity may be able to compensate for hypothalamic leptin resistance in WT mice, preventing severe hepatic insulin resistance and steatosis.

Reduced mitochondrial function has also been proposed as a risk factor for type 2 diabetes (64). Interestingly, reduced mitochondrial function does not appear to explain the increased sensitivity of *Mc4rKO* to muscle insulin resistance induced by a high-fat diet. Although female *Mc3rKO* and *Mc4rKO* exhibiting a comparable reduction in citrate synthase activity, insulin stimulation of PKB activity was severely compromised in *Mc4rKO*. These data may suggest that other factors, such as hyperphagia and high-fat diet, may interact with reduced mitochondrial function to severely impair insulin signaling in muscle.

C57BL/6J mice are a commonly used model of DIO (65) and rapidly develop insulin resistance in liver, muscle, and brown and white adipose tissue on a high-fat diet (66). The interaction of diet with melanocortin receptor genotype thus represents an additive effect, over and above what is occurring in WT controls. It may also be premature to state, based on the data presented here, that the stimulation of glucose uptake in muscle and adipose are not lower, relative to controls, in *Mc4rKO* fed a low-fat diet. However, if present, any deficits are likely to be modest and were undetectable using glucose clearance after insulin or glucose injections.

Gender has a marked effect on the insulin-resistant state of DIO *Mc3rKO*. Irrespective of the high-fat diet used (45 or 60% kJ from fat), DIO female *Mc3rKO* exhibit modest perturbations of glucose metabolism. DIO female *Mc3rKO* exhibited a modest glucose intolerance compared with DIO WT, perhaps related to the development of mild hepatic insulin resistance indicated by the increase in Ser^{307} phosphorylation of IRS1. A deterioration of pancreatic β-cell function may also be a factor in glucose intolerance observed in DIO mice, irrespective of genotype, although this was not examined in these experiments. However, the fact that glucose intolerance of female DIO *Mc3rKO* is very mild, with glucose returning to normal levels within 90 min, is not consistent with a severe decompensation of β-cell function.

Male *Mc3rKO* on a very-high-fat diet may also develop severe insulin resistance, with fasting hyperinsulinemia not significantly different from *Mc4rKO*. Compared with female *Mc4rKO* with similar body fat content, female *Mc3rKO* fed high-fat diets exhibited only a very modest deterioration of glucose clearance. The mechanisms explaining the gender difference in DIO

insulin resistance of *Mc3rKO* are not known. One possible factor may be the mild perturbations of energy balance causing weight gain, and the absence of hyperphagia, in female *Mc3rKO*.

To summarize, these data suggest that reduced energy expenditure is likely an important factor contributing to obesity of *Mc3rKO*. A very modest hyperphagia may also contribute to DIO of male *Mc3rKO* relative to WT mice. Because energy expenditure of *Mc4rKO* is increased relative to WT controls, hyperphagia coupled with nutrient partitioning to adipose is likely a primary mechanism driving obesity. Reduced capacity for FAO in red muscle may also be a factor contributing to the lower energy expenditure of *Mc3rKO* and may also be involved in low whole-body fat oxidation of *Mc3rKO* and *Mc4rKO*. The metabolic costs associated with digestion and fat and protein deposition may also account for the higher energy expenditure of *Mc4rKO* compared with *Mc3rKO*. Finally, *Mc3rKO* on the B6 background may be a useful model for investigating the development of insulin resistance in the obese state.

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Abbreviations

AgRP
Agouti-related peptide

bEE_{est}
basal daily energy expenditure in a weight-stable state

DIO
diet-induced obesity

FAO
fatty acid oxidation

FFM
fat-fee mass

FM
fat mass

FoxO1
forkhead box O1

GSK3
glycogen synthase kinase-3

IPGTT
ip glucose tolerance test

IRS1
insulin receptor substrate 1

ITT
insulin-tolerance test

Mc3r
melanocortin-3 receptor

Mc4r
melanocortin-4 receptor

$ME_{\%}$
metabolic efficiency

NMR
nuclear magnetic resonance

NPY
neuropeptide Y

$NREE_{cal}$
nonresting energy expenditure

PI3K
phosphatidyl-3 kinase

PKB
protein kinase B

POMC
proopiomelanocortin

REE_{cal}
resting energy expenditure

TEE_{cal}
total energy expenditure

WT
wild type

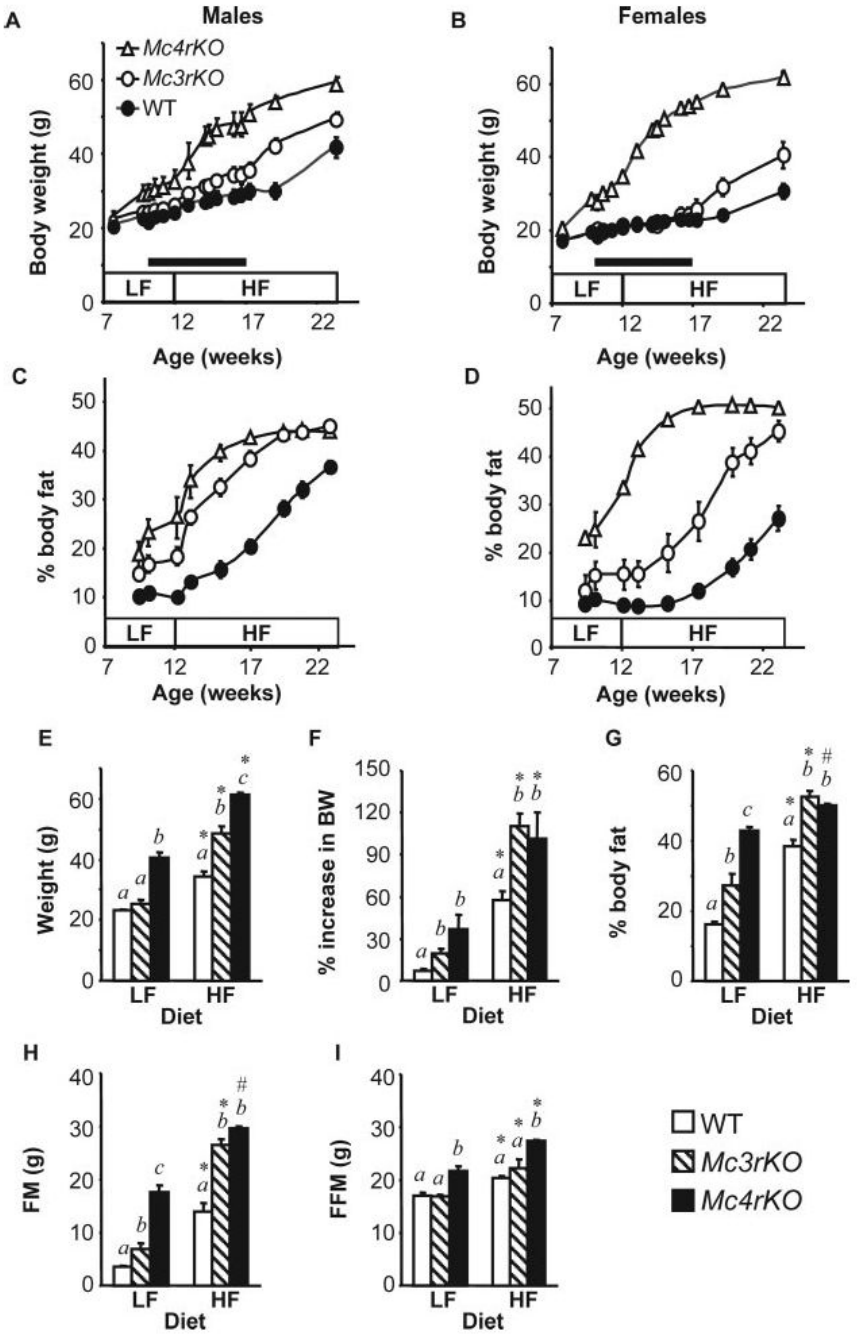

Fig. 1.

Genotype-specific effects of dietary fat content on weight gain of *Mc3rKO* and *Mc4rKO*. Data shown are for individually housed male (A and C) and female (B and D) mice that were fed low-fat diet (LF, 10% kJ from fat) from weaning to 12 wk, followed by high-fat diet (HF, 60% kJ from fat) for 12 wk. Growth curves show body mass (A and B) and adiposity (percent body fat) (C and D) of male *Mc4rKO* (n = 4), *Mc3rKO* (n = 4), and WT (n = 8) and female *Mc4rKO* (n = 6), *Mc3rKO* (n = 3), and WT (n = 11) mice. Introduction of the high-fat diet accelerated weight gain and adiposity irrespective of sex and genotype. The *black bars* in A and B indicate the period when mice were housed in wire-mesh-bottom cages to allow for collection of spillage for accurate measurements of food intake. E–I, Comparison of body mass

measurements from 6-month-old females (n = 8–9 per group for WT; n = 5 per group for *Mc3rKO* and *Mc4rKO*) group housed and fed either low-fat or high-fat diet for 12 wk. Increasing dietary fat content had equal effects on body mass (E), the increase in weight over 12 wk as a percentage of baseline or starting weight (F), and FM (H) of *Mc3rKO* relative to *Mc4rKO*. G, On the low-fat diet, adiposity of *Mc3rKO* was intermediate between lean WT and obese *Mc4rKO*. Adiposity of *Mc3rKO* and *Mc4rKO* were increased to the same extent, relative to WT controls, on the high-fat diet. I, FFM of *Mc4rKO* was significantly increased relative to WT and *Mc3rKO*, irrespective of diet. Two-way ANOVA (independent variables were diet and genotype) indicated significant effects of diet and genotype for all parameters. Significant effect of diet within genotype are indicated as follows: *, $P < 0.001$; #, $P < 0.05$. Significant effects of genotype within diet are indicated using *letters*; groups not sharing the same *letter* (a, b, or c) are significantly different ($P < 0.05$).

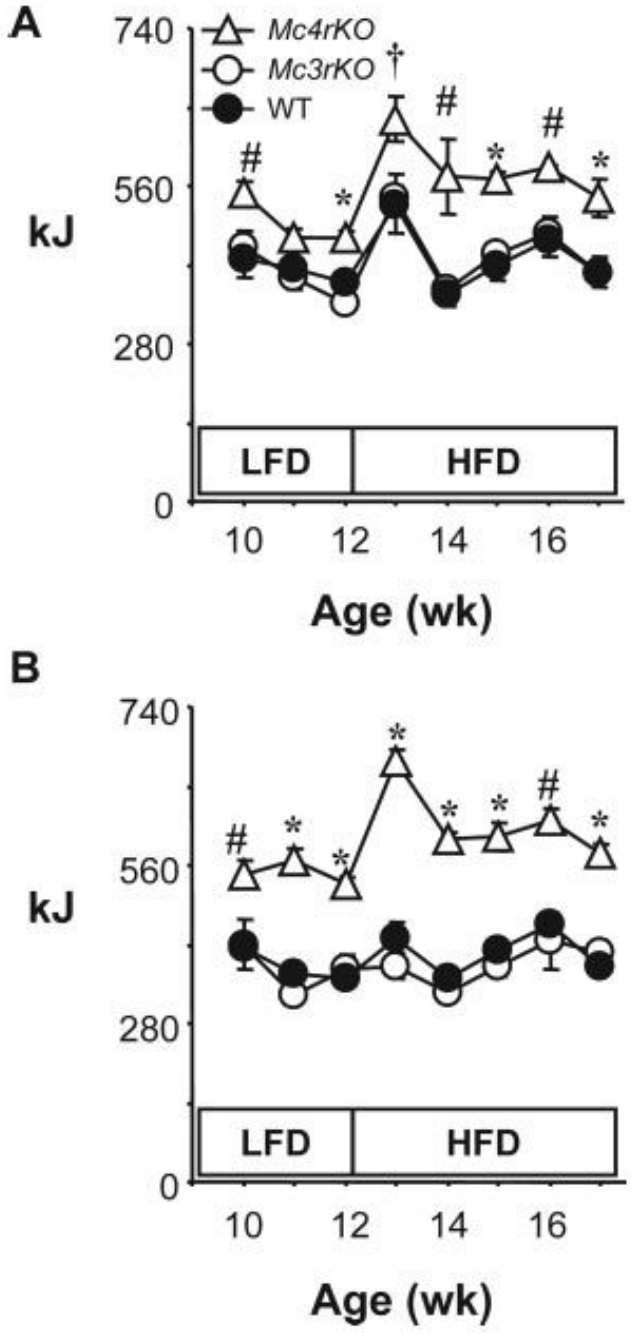

Fig. 2.

Genotype-specific effects of dietary fat content on food intake of *Mc3rKO* and *Mc4rKO*. Weekly food intake (kJ) for male (A) and female (B) WT, *Mc3rKO*, and *Mc4rKO*. The legend for this figure is the same as that for Fig. 1, A–D. Food intake was measured during the period indicated by a *black bar* in Fig. 1, A and B. For each week, significant differences ($P < 0.05$) are indicated as follows: *, *Mc4rKO vs. Mc3rKO*, WT; #, *Mc4rKO vs.* WT; †, *Mc4rKO vs. Mc3rKO*. Results of two-way ANOVA with repeated-measures analysis examining the effects of diet and genotype for mean weekly intake on low-fat diet (LFD, 10% kJ from fat) and high-fat diet (HFD, 60% kj from fat) are reported in Table 1. Food intake in kilojoules of *Mc4rKO*, but not *Mc3rKO*, is increased compared with WT controls, irrespective of diet. Male

and female *Mc4rKO* also increased kilojoules of food consumed when exposed to the high-fat diet. Male WT and *Mc3rKO* exhibited a transient hyperphagia on the high-fat diet, whereas for female WT and *Mc3rKO*, kilojoules consumed was not affected by diet.

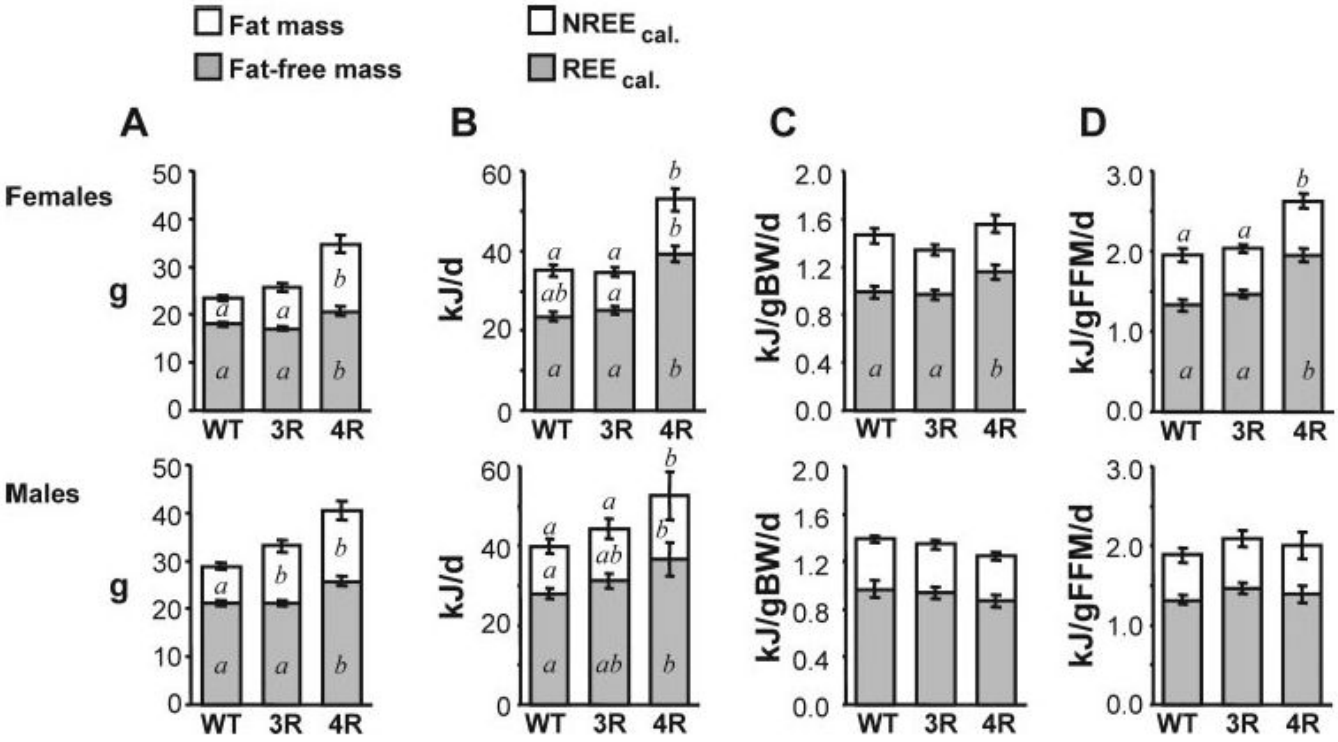

Fig. 3.

Energy expenditure of male and female *Mc3rKO*, *Mc4rKO*, and WT mice determined by indirect calorimetry. Energy expenditure (kJ/d) was determined using indirect calorimetry for WT females (n = 21) and males (n = 29), *Mc3rKO* females (n = 22) and males (n = 23), and *Mc4rKO* females (n = 16) and males (n = 10) fed a moderately high-fat diet (45% kJ from fat) for 6–8 wk. FM and FFM (A) were determined using NMR. Note that there was a strong tendency for increased FM of female *Mc3rKO* compared with WT ($P = 0.055$). REE_{cal}, $NREE_{cal}$, and TEE_{cal} are presented per animal (B) and adjusted for body weight (C) or FFM (D). Significant differences ($P < 0.05$) between groups are indicated using *letters;* groups not sharing the same *letter* are significantly different. TEE_{cal} in kilojoules per day was significantly increased in *Mc4rKO*, irrespective of sex. Female *Mc4rKO* also exhibited an increase in TEE_{cal} and REE_{cal} adjusted for FFM and for REE_{cal} adjusted for body weight. TEE_{cal} and REE_{cal} of female *Mc3rKO* was normal when expressed per animal and when adjusted for body weight and FFM. REE_{cal} and $NREE_{cal}$ of *Mc3rKO* exhibited an intermediate increase, compared with a large increase observed *Mc4rKO*. In males, energy expenditure parameters adjusted for body weight or FFM were not significantly different from WT.

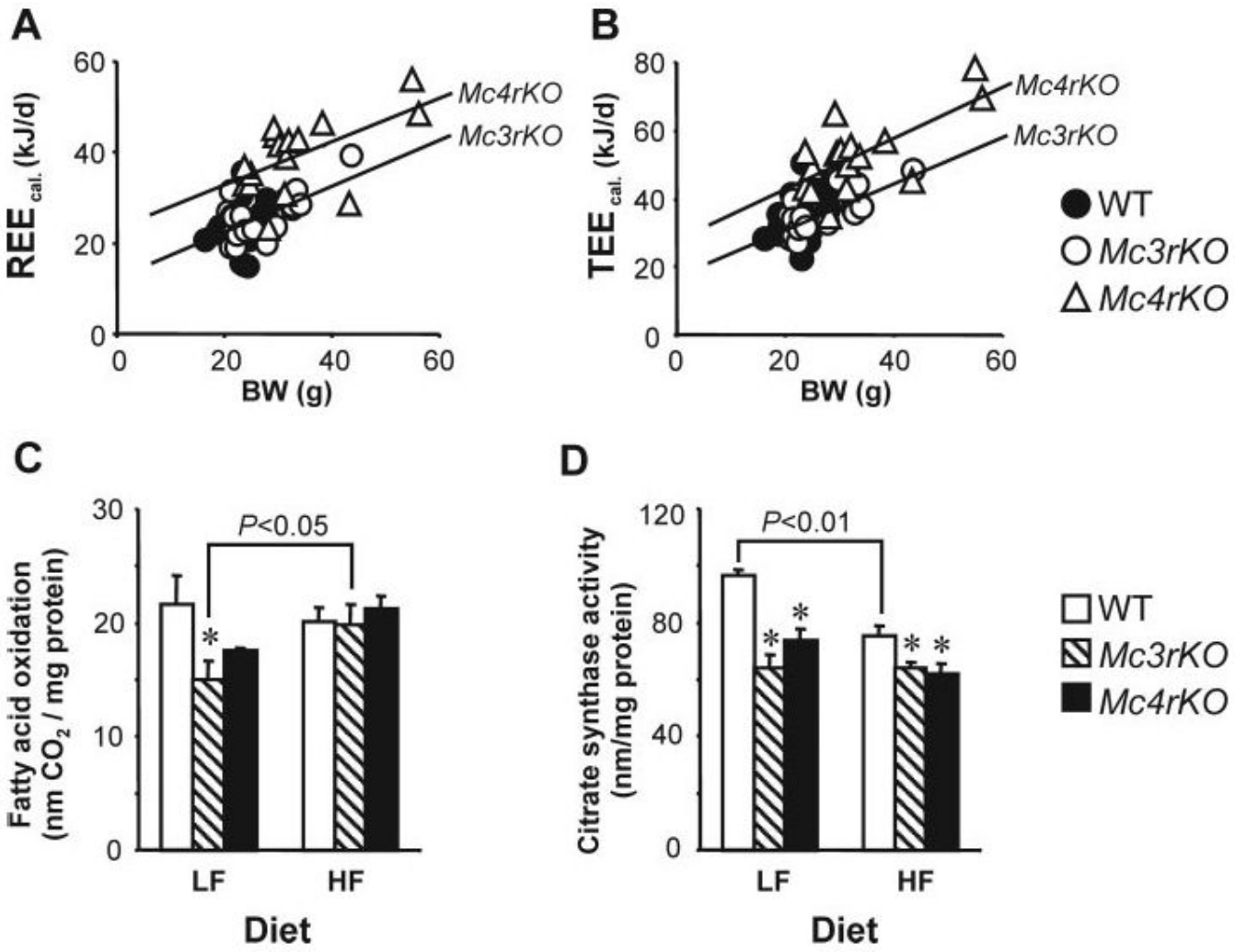

Fig. 4.

Regression analysis of energy expenditure data and muscle oxidative metabolism of female *Mc3rKO*, *Mc4rKO*, and WT mice. Regression analysis of REE_{cal} and TEE_{cal} of female mice suggest reduced energy expenditure of *Mc3rKO*, relative to *Mc4rKO*, independently of differences in body weight (A and B). The differences in energy expenditure of female *Mc3rKO* and *Mc4rKO*, determined using calorimetry, did not correlate with skeletal muscle oxidative capacity. FAO (C) was significantly reduced in *Mc3rKO* fed the low-fat (LF) diet but not the moderately high-fat (HF) diet. Citrate synthase activity in skeletal muscle of *Mc3rKO* and *Mc4rKO* was lower compared with WT controls, irrespective of diet (D). Body mass data for mice shown in C and D are shown in Fig. 1, E–I. *, $P < 0.05$ compared with WT.

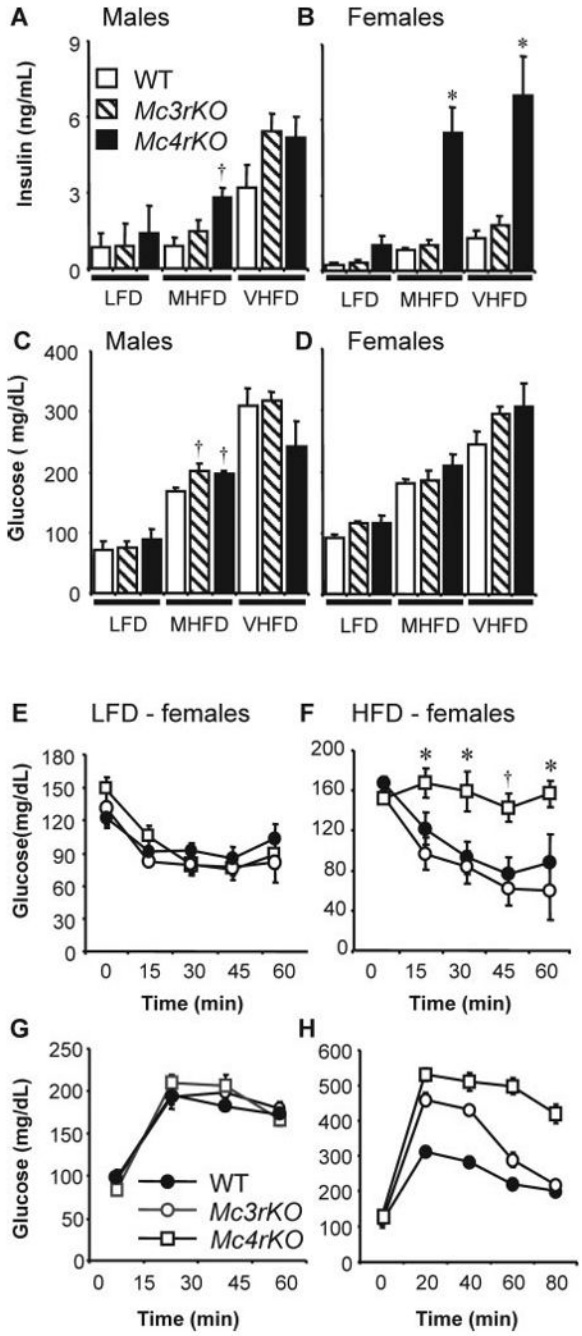

Fig. 5.

Effects of genotype and diet on fasting insulin and glucose and on ITT and IPGTT data. *Mc4rKO* are hyperinsulinemic and are glucose intolerant and insulin resistant compared with *Mc3rKO* and WT mice on a high-fat diet. A–D, Fasting serum insulin (A and B) and blood glucose (C and D) of female and male *Mc4rKO*, *Mc3rKO*, and WT mice fed a low-fat diet (LFD, 10% kJ from fat), moderately high-fat diet (MHFD, 45% kJ from fat), or very high-fat diet (VHFD, 60% kJ from fat). *, $P < 0.05$ compared with WT and *Mc3rKO;* †, $P < 0.05$ compared with WT. E–H, ITT (E and F) and IPGTT (G and H) result of obese female *Mc4rKO*, *Mc3rKO*, and WT mice fed either a low-fat diet (LFD, n = 3–5 per group) (E and G) or moderately high-fat diet (HFD, n = 5–11) (F and H).*, $P < 0.05$ *vs. Mc3rKO;* †, $P < 0.05$

vs. WT and *Mc3rKO*. For IPGTT data (H), there was no significant difference between groups at 0 min (*i.e.* baseline fasting glucose); between 20 and 60 min, all genotypes were significantly different ($P < 0.05$); at 80 min, glucose levels were significantly ($P < 0.01$) higher in *Mc4rKO* but had returned to WT levels in *Mc3rKO*.

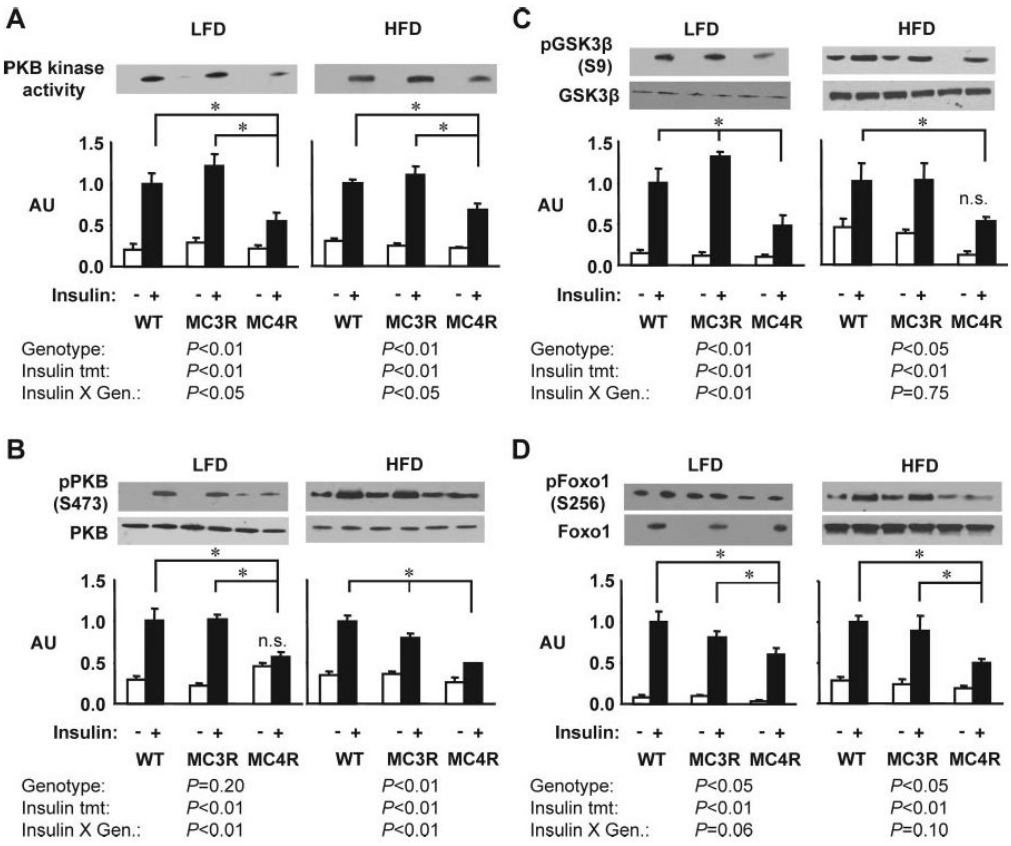

Fig. 6.

Insulin signaling in liver is impaired in female *Mc4rKO* relative to WT and *Mc3rKO*, irrespective of diet. Representative Western blots and quantified data (n = 3 per group) are shown for basal (*white bar*) or insulin-stimulated (*black bar*) PKB kinase activity (A), phosphorylation of PKB on Ser^{473} (B), phosphorylation of GSK3*β* on Ser^{9} (C), or phosphorylation of FoxO1 on Ser^{256} (D). Quantified data were analyzed within diet using two-way ANOVA (independent variables were genotype and insulin treatment). Phosphospecific antibody data (B–D) was normalized for total protein content. For most outcomes measured, insulin-stimulated activity was reduced in liver of *Mc4rKO* (MC4R), irrespective of diet, compared with *Mc3rKO* (MC3R) and WT mice. Significant differences between groups are indicated by *lines*: *, $P < 0.05$. For PKB activity assessed by kinase assay (A) or serine phosphorylation (B), two-way ANOVA indicated that the degree of insulin stimulation was dependent on genotype. The effects of insulin treatment were statistically significant ($P < 0.05$), unless indicated otherwise (n.s., not significant). AU, Arbitrary units; LFD, low-fat diet (10% kJ from fat); HFD, high-fat diet (45% kJ from fat).

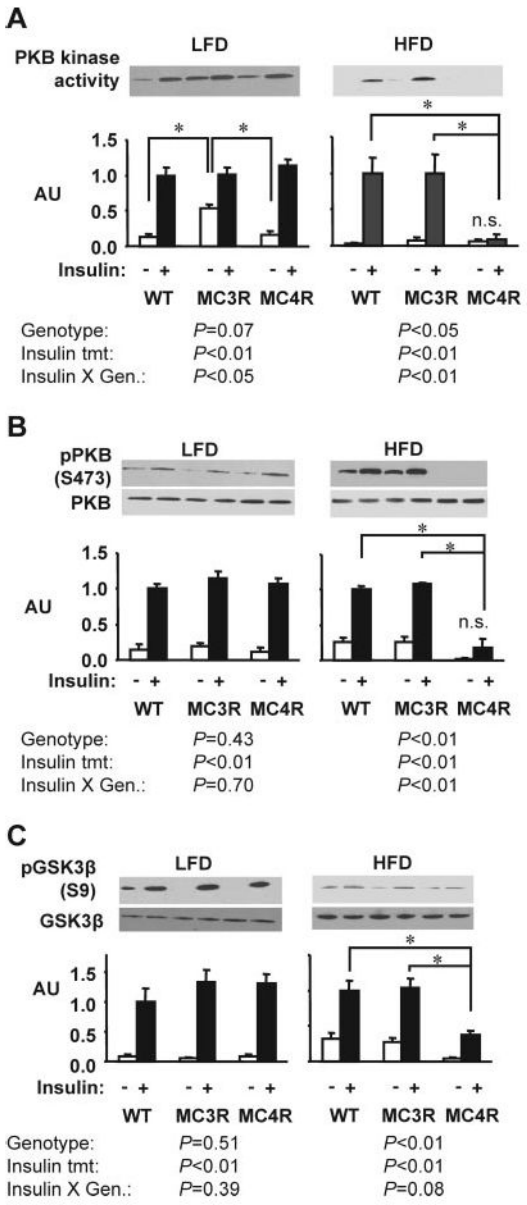

Fig. 7.

High-fat diet is associated with a marked reduction in insulin receptor signaling in skeletal muscle of female *Mc4rKO* compared with *Mc3rKO* and WT mice. Representative Western blots, with quantified data (n = 3 per group), are shown for basal and insulin-stimulated PKB kinase activity (A), phosphorylation of PKB on Ser^{473} (B), or phosphorylation of GSK3*β* on Ser^{9} (C). A significant effect of genotype was observed only for *Mc4rKO* (MC4R) fed the high-fat diet, resulting in the loss of a significant effect of insulin on PKB kinase activity, phosphorylation of PKB, and phosphorylation of GSK3*β*. Significant differences between groups are indicated by *lines*: *, $P < 0.05$. Unless indicated otherwise (n.s., not significant), treatment effects of insulin on kinase activity and phosphorylation were statistically significant ($P < 0.05$). AU, Arbitrary units; MC3R, *Mc3rKO* mice; LFD, Low-fat diet (10% kJ from fat); HFD, high-fat diet (45% kJ from fat).

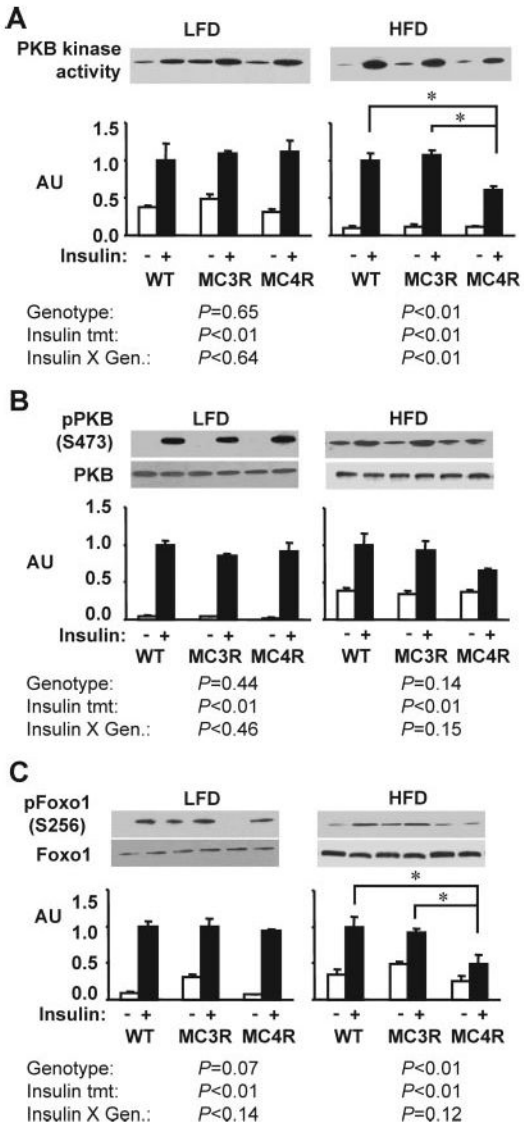

Fig. 8.

High-fat diet is associated with reduced insulin receptor signaling in adipose of *Mc4rKO* compared with *Mc3rKO* and WT mice. Representative Western blots, with quantified data (n = 3 per group), are shown for basal and insulin-stimulated PKB kinase activity (A), phosphorylation of PKB on Ser^{473} (B), or phosphorylation of FoxO1 on Ser^{256} (C). A significant effect of genotype on insulin-stimulated PKB activity and phosphorylation of FoxO1 was observed only for *Mc4rKO* (MC4R) fed the high-fat diet. Significant differences between groups are indicated by *lines*: *, $P < 0.05$. Unless indicated otherwise (n.s., not significant), treatment effects of insulin were statistically significant ($P < 0.05$). AU, Arbitrary units; MC3R, *Mc3rKO* mice; LFD, low-fat diet (10% kJ from fat); HFD, high-fat diet (45% kJ from fat).

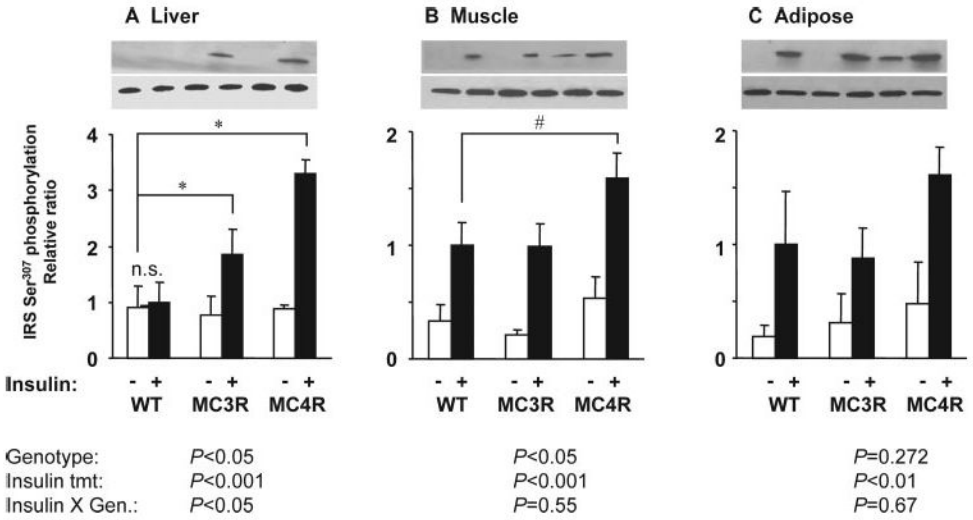

Fig. 9.

Increased Ser^{307} phosphorylation of IRS1 in liver and skeletal muscle of *Mc4rKO* compared with WT fed a moderately high-fat diet. Representative Western blots, with quantified data (n = 3 per group), are shown for basal and insulin-stimulated IRS1 phosphorylation on Ser^{307} in liver (A), skeletal muscle (B), and retroperitoneal adipose tissue (C). These samples were from the high-fat diet groups shown in Figs. 6–8. A significant effect of genotype on insulin-stimulated IRS1 Ser^{307} phosphorylation was observed for *Mc4rKO* (MC4R) and *Mc3rKO* (MC3R) compared with WT in liver, and for *Mc4rKO* compared with WT in skeletal muscle. Significant difference between groups is indicated by *lines*: *, $P < 0.05$. Unless indicated otherwise (n.s., not significant), treatment effects of insulin were statistically significant ($P < 0.05$). Mice were maintained on moderately high-fat diet (45% kJ from fat).

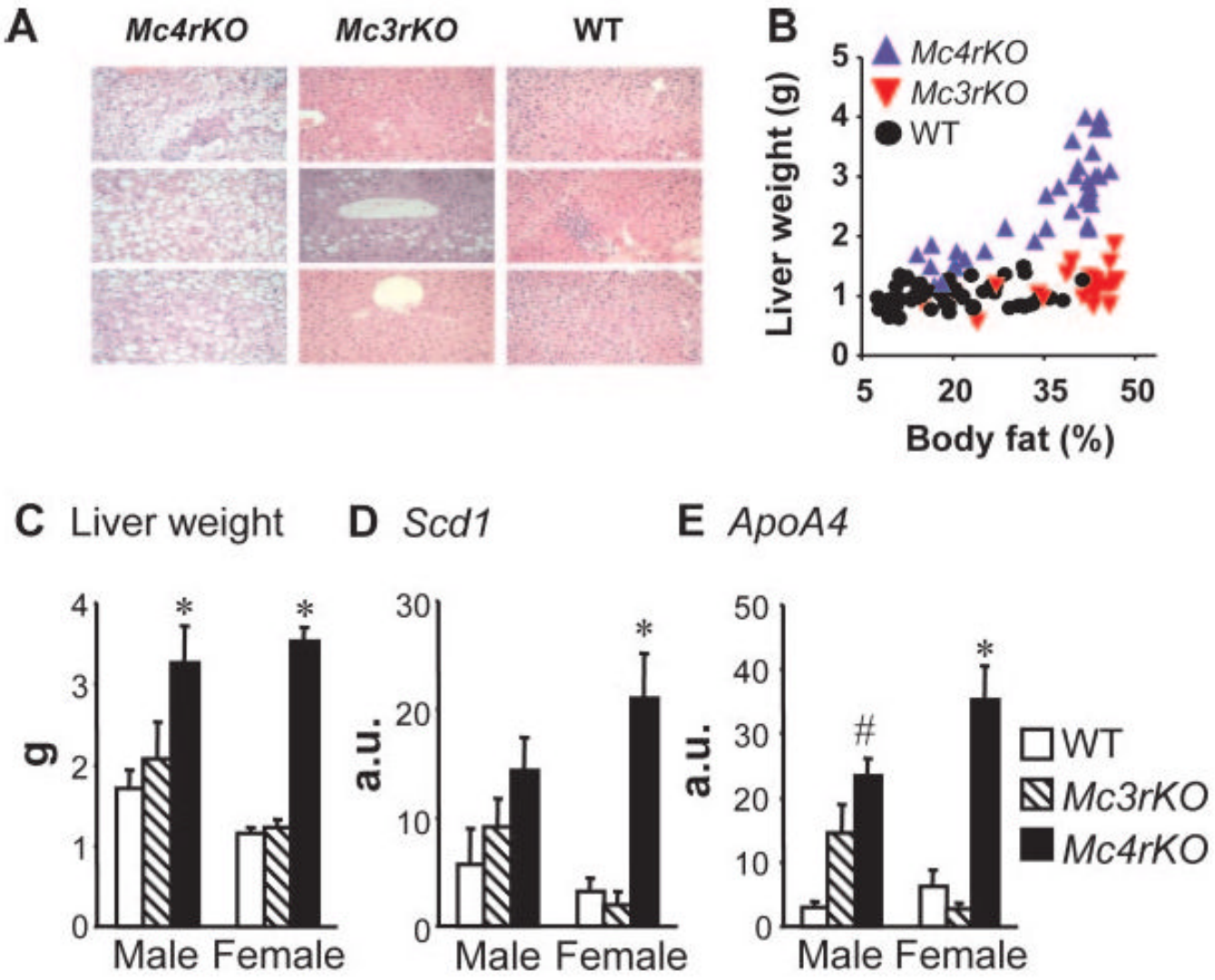

Fig. 10.

Hepatomegaly and liver gene expression data suggest hepatic insulin resistance is most severe for *Mc4rKO*. *Mc4rKO* demonstrate marked hepatic steatosis and hepatomegaly compared with Mc3rKO and WT. A, Hematoxylin and eosin-stained cross-sections of liver tissue from female *Mc4rKO*, *Mc3rKO*, and WT mice fed a high-fat diet. B, Plot of liver weight *vs.* adiposity from several studies where mice were fed low- or high-fat diets. C, Mean liver weight of female *Mc4rKO*, *Mc3rKO*, and WT mice fed a moderately high-fat diet. *Mc4rKO* also exhibited generally increased lipogenic gene expression compared with both *Mc3rKO* and WT mice (n = 5–6 per group). D and E, Expression of stearoyl-CoA desaturase 1 (*Scd1*) (D), and apolipoprotein A4 (*Apoa4*) (E) in the liver of WT, *Mc3rKO*, and *Mc4rKO* fed the high-fat diet. Data are expressed as arbitrary units (a.u.). *, $P < 0.05$ *vs.* WT and *Mc3rKO;* #, $P < 0.05$ *vs.* WT mice, within gender.

TABLE 1

Effect of dietary fat content on energy balance of male and female WT, *Mc3rKO*, and *Mc4rKO* mice

Genotype	Low-fat diet			High-fat diet			Δ (High-fat − low-fat)		
	WT	***Mc3rKO***	***Mc4rKO***	**WT**	***Mc3rKO***	***Mc4rKO***	**WT**	***Mc3rKO***	***Mc4rKO***
Gain in body mass energy (kJ/d)									
Male	0.5 ± 0.2^{a}	2.3 ± 0.7^{a}	6.6 ± 1.8^{b}	$4.4 \pm 0.6^{a\#}$	$11.3 \pm 1.1^{b*}$	$15.2 \pm 1.4^{c*}$	3.9 ± 0.6^{a}	9.0 ± 0.7^{b}	8.6 ± 2.9^{b}
Female	0.4 ± 0.2^{a}	1.7 ± 0.4^{a}	10.0 ± 1.1^{b}	1.5 ± 0.5^{a}	$5.1 \pm 1.3^{b\#}$	$19.3 \pm 0.6^{c*}$	1.1 ± 5^{a}	$3.6 \pm 1.4^{a*}$	9.3 ± 1.4^{b}
Energy intake (kJ/d)									
Male	58.6 ± 2.3^{a}	57.0 ± 2.1^{a}	70.6 ± 2.2^{b}	62.4 ± 2.8^{a}	$63.9 \pm 1.3^{a\#}$	$84.6 \pm 2.4^{b*}$	3.8 ± 1.6^{a}	6.9 ± 1.9^{a}	14.0 ± 4.0^{b}
Female	54.3 ± 1.6^{a}	53.5 ± 2.5^{a}	78.0 ± 0.5^{b}	$58.2 \pm 1.8^{a*}$	55.1 ± 2.6^{a}	$90.8 \pm 1.7^{b*}$	3.9 ± 1.4^{a}	1.6 ± 0.9^{a}	12.8 ± 1.9^{b}
bEE_{est} (kJ/d)									
Male	57.6 ± 2.3	53.5 ± 1.6	60.7 ± 2.1	55.7 ± 2.8^{a}	$47.1 \pm 1.1^{a\#}$	$61.8 \pm 2.0^{a,b}$	-1.9 ± 1.1	-6.4 ± 2.5	$+1.1 \pm 3.9$
Female	53.4 ± 1.5^{a}	50.9 ± 2.5^{a}	63.0 ± 1.6^{b}	55.8 ± 1.7^{a}	47.3 ± 0.9^{b}	62.0 ± 1.6^{c}	$+2.4 \pm 1.3$	-3.6 ± 2.0	-0.9 ± 3.1
$ME_{\%}$									
Male	0.9 ± 0.3^{a}	4.0 ± 1.1^{a}	9.2 ± 2.4^{b}	$7.1 \pm 0.9^{a*}$	$17.6 \pm 1.4^{b*}$	$17.9 \pm 1.5^{b*}$	6.2 ± 1.1^{a}	13.7 ± 1.0^{b}	$8.7 \pm 3.7^{a,b}$
Female	0.7 ± 0.4^{a}	3.1 ± 0.8^{a}	12.9 ± 1.4^{b}	2.5 ± 0.8^{a}	$9.1 \pm 1.9^{b\#}$	$21.3 \pm 0.7^{c*}$	$1.7 \pm 0.7^{a*}$	$6.0 \pm 2.1^{a,b*}$	8.4 ± 1.9^{b}

Data are from the mice whose growth curves are shown in Fig. 1, A–D, and represent weekly means collected during 3 wk on a low-fat and 5 wk on a high-fat diet. bEE_{est} represents the proportion of kilojoules consumed less the metabolic cost of fat and protein deposition and is an estimate of the kilojoules available for non-growth-related basal metabolism and physical activity. The effect of genotype and diet on each of the parameters shown was analyzed using two-way ANOVA with repeated measures (low-fat and high-fat diet). Values not sharing a *superscript letter (a, b,* and *c*) within rows and within diets are statistically different from each other at $P < 0.05$; for the effects of diet within sex and within genotype:

$^{\#}P < 0.05$;

$^{*}P < 0.01$.

The Δ (high-fat − low-fat) data were analyzed using two-way ANOVA with sex and genotype as independent variables. Values not sharing *superscripts* within rows *(i.e.* genotype) are statistically different from each other at $P < 0.05$. *, Significant effect of sex (within column) at $P < 0.05$.