Developmental Role for Endocannabinoid Signaling in Regulating Glucose Metabolism and Growth

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Treatment of *ob/ob* (*obese*) mice with a cannabinoid receptor 1 (Cnr1) antagonist reduces food intake, suggesting a role for endocannabinoid signaling in leptin action. We further evaluated the role of endocannabinoid signaling by analyzing the phenotype of Cnr1 knockout *ob/ob* mice. Double mutant animals show a more severe growth retardation than *ob/ob* mice with similar levels of adiposity and reduced IGF-I levels without alterations of growth hormone (GH) levels. The double mutant mice are also significantly more glucose intolerant than *ob/ob* mice. This is in contrast to treatment of *ob/ob* mice with a Cnr1 antagonist that had no effect on glucose metabolism, suggesting a possible requirement for endocannabinoid signaling during development for normal glucose homeostasis. Double mutant animals also showed similar leptin sensitivity as *ob/ob* mice, suggesting that there are developmental changes that compensate for the loss of Cnr1 signaling. These data establish a role for Cnr1 during development and suggest that compensatory changes during development may mitigate the requirement for Cnr1 in mediating the effects of leptin. The data also suggest a developmental role for Cnr1 to promote growth, regulate the GH/IGF-I axis, and improve β-cell function and glucose homeostasis in the setting of leptin deficiency. ***Diabetes*** **62:2359–2367, 2013**

Endocannabinoids (enCBs) modulate numerous neural systems, and cannabinoid receptor 1 (Cnr1) inhibitors have been used in a number of clinical settings. Cnr1 is broadly expressed in the brain in many different cell types (1–3). Electrophysiological studies have shown that Cnr1 functions as a presynaptic modulator of both excitatory and inhibitory synaptic transmissions and mediates short- and long-term plasticity of neurons (3,4). enCB/Cnr1 signaling has also been reported to regulate energy balance and metabolism through central and peripheral pathways (5–10).

A role for Cnr1 in mediating some of the effects of leptin has been suggested by studies showing that leptin-deficient *ob/ob* (*obese*) mice have increased enCB levels in the hypothalamus and that these increased levels can be normalized by exogenous leptin (5). In addition, Cnr1 knockout (KO) mice have been reported to be leaner than wild-type animals and resistant to diet-induced obesity (11). Although these studies provided pharmacologic evidence that enCBs may play a role in mediating some of the effects of leptin, they do not establish the relative contribution of enCBs to the phenotype of *ob/ob* mice or address a potential role for enCB signaling during development. We thus asked whether loss of Cnr1 function could ameliorate the obese phenotype of *ob/ob* mice. Toward this end, we compared the metabolic phenotype and leptin response of Cnr1 and leptin single and double mutant mice. We found that congenital Cnr1 deficiency does not suppress the *ob* phenotype but, rather, leads to an exacerbation of the growth retardation of *ob/ob* mice and a worsening of their diabetes. These effects differ from those observed after treatment of *ob/ob* mice with a Cnr1 antagonist, invoking a role for Cnr1 during development.

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RESEARCH DESIGN AND METHODS

Animals. Cnr1 KO mice were obtained from Dr. Kunos (5), which had been backcrossed to C57BL/6J background at the home institute. They were bred to heterozygous leptin mutant ($ob^{+/-}$) mice (The Jackson Laboratory stock no. 000632), and double heterozygous offspring were used to generate wild-type (C++L++), Cnr1 KO (C−−L++), *ob/ob* (C++L−−), and double KO (C−−L−−) animals. All animal usage and experimental procedures complied with guidelines from the National Institutes of Health and approved by the Rockefeller University Comparative Bioscience Center (protocol no. 11402).

Body weight, food intake, and body composition. Animals were weaned and single caged starting at 3–4 weeks of age. Body weight and food intake were measured weekly. Body composition was measured by dual-energy X-ray absorptiometry (DEXA) (Lunar PIXImus2). Body length was measured from nose to anus.

Glucose tolerance test. Mice were fasted overnight (14–16 h). Ten microliters of 10% glucose in 0.9% NaCl was injected per gram of body weight, and blood was collected by tail snipping. Blood glucose level was measured by a Breeze2 blood glucose meter (Bayer Healthcare LLC). For the purpose of data analysis, all out-of-range high values were calculated as 600 mg/dL.

Insulin tolerance test. Food was removed for 4–6 h (typically from 10 A.M.–2 P.M.) and 20.4 U (for C++L−− and C−−L−−) or 0.75 U (for C++L++ and C−−L++) recombinant human insulin (Sigma) in 0.9% NaCl was injected per kilogram of body weight.

Glucose-stimulated insulin secretion in mice. Mice were fasted overnight (14–16 h). Ten microliters of 10% glucose in 0.9% NaCl was injected per gram of body weight, and blood was collected by retro-orbital bleeding from awake animals. Insulin was measured with Mouse Insulin Ultrasensitive ELISA (ALPCO Diagnostics).

IGF-I tolerance test. Food was removed 4–6 h (typically from 10 A.M.–2 P.M.) before the test. Two micrograms (for C++L−− and C−−L−−) or 1 μg (for C++L++ and C−−L++) recombinant mouse IGF-I peptide (R&D Systems) in 0.9% NaCl was injected per gram of body weight.

Hormone measurements. Food was removed for 4–6 h (typically from 10 A.M.–2 P.M.) before retro-orbital bleeding, and blood was collected from unanesthetized animals into EDTA-coated glass capillaries (Drummond Scientific). Blood was kept on ice and centrifuged at 4,000 rpm at 4°C for 15 min. Serum was stored at −20°C. Leptin, IGF-I, and growth hormone (GH) were measured according to manufacturers' instructions (Mouse Leptin Quantikine ELISA Kit [R&D], Mouse/Rat IGF-I ELISA Kit [R&D], and Mouse/Rat GH Kit [Millipore]).

IGF-I mRNA and protein content in the liver. Liver (~10 mg, lower left lobe) was homogenized in 0.5 mL Trizol (Invitrogen) and phase separated as instructed. RNA was purified with an RNeasy Micro Kit (Qiagen). RNA quality was measured with an Agilent 2100 Bioanalyzer. Reverse transcription was carried out with a Quantitect RT Kit (Qiagen), and cDNA was purified with a Qiaquick PCR Purification Kit (Qiagen). Real-time PCR was performed using mouse IGF-I Taqman Gene Expression Assay Mm00439560_m1 (Applied Biosystems).

Liver (~10 mg, lower left lobe) was also homogenized in 0.2 mL PBS containing 1× protease inhibitor cocktail (Roche). Total protein was quantified with a BCA Protein Assay Kit (Thermo Scientific). IGF-I content was quantified with a Mouse/Rat IGF-I1 ELISA Kit (R&D).

Cnr1 antagonism. Rimonabant (SR141716A) was obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program (S-705). Intraperitoneal injections of 10 mg/kg/day were given starting at 17 or 26 weeks of age for 28 days.

Leptin treatment. Leptin 250 ng/h (Amylin Pharmaceuticals) was infused with the use of osmotic pumps (ALZET model 2004). Filled pumps were soaked in 0.9% NaCl, primed at 37°C overnight, and inserted subcutaneously at the back when animals were 12 weeks old. Body weight was measured every 2–3 days.

Statistical analysis. Results were plotted as mean and SE. Student *t* test, one-way ANOVA, or two-way ANOVA (in Microsoft Excel or GraphPad Prism) was used to assess the difference of the mean between different groups, which was considered significant at $P < 0.05$.

RESULTS

Cnr1 loss of function leads to smaller obese mice on leptin-deficient *ob/ob* background. Leptin-deficient Cnr1 KO double mutant mice (C−−L−−), leptin-deficient *ob/ob* mice (C++L−−), Cnr1 KO mice (C−−L++), and wild-type littermates (C++L++) were produced by crossing double heterozygotes (C+–L+–). Consistent with a previous report (12), we observed an increased mortality in both C−−L−− and C−−L++ genotypes. At weaning, 24.1 and 16.5% of all pups ($n = 79$) were C−−L−− and C−−L++, respectively, and by 6 months of age, this dropped to 15.9 and 11.1%, respectively. After weaning at 3–4 weeks, body weight, food intake, body length, percent fat, fat mass, and lean mass of single-caged animals were measured (Fig. 1 for C−−L−− and C++L−− mice and Supplementary Fig. 1 for C−−L++ and C++L++ male mice). Body weight was significantly lower in C−−L−− mice versus C++L−− mice at all time points (4 weeks 11.85 ± 0.85 vs. 15.00 ± 0.81 g, $P < 0.001$; 4 months 59.12 ± 1.50 vs. 64.00 ± 1.08 g, $P < 0.05$) (Fig. 1*A*). Their body weight remained significantly different at 12 months of age (53.23 ± 3.04 vs. 68.01 ± 2.33 g, $P < 0.01$) (Fig. 1*A* inset). The accumulated daily food intake averaged 6.11 ± 0.43 and 8.05 ± 0.51 g ($P < 0.0001$) for C−−L−− and C++L−− mice between 7 and 21 weeks of age (Fig. 1*B*). The C−−L−− mice weighed significantly more than the C++L++ mice at all time points (compare Fig. 1*A* and Supplementary Fig. 1*A*). At 6 weeks of age, C−−L−− mice weighed 22.27 ± 0.72 g, whereas C++L++ mice weighed 18.24 ± 0.49 g (both sexes included, $P < 0.0001$).

Despite weighing less than C++L−− mice, adult C−−L−− mice still showed increased adiposity of similar magnitude to that of C++L−− mice. Although DEXA scans of juvenile animals showed that the percentage of fat of C−−L−− mice was lower than that of C++L−− mice (6 weeks 31.79 ± 0.77 vs. 35.36 ± 0.62%, $P < 0.001$; 8 weeks 41.41 ± 1.08% vs. 43.31 ± 0.78%, $P < 0.05$), the adiposity of C−−L−− mice was equivalent to that of the C++L−− mice at all time points after 8 weeks (9 weeks 44.30 ± 1.47 vs. 46.27 ± 0.38%, $P > 0.1$; 17 weeks 52.16 ± 1.83 vs. 53.83 ± 1.85%, $P > 0.2$) (Fig. 1*D*). Thus, despite weighing less, fully grown C−−L−− mice are equivalently obese to C++L−− mice. DEXA scans also showed that the reduced body weight of C−−L−− mice resulted from decreases in both fat mass (6 weeks 7.20 ± 0.43 vs. 10.51 ± 0.38 g, $P < 0.01$; 17 weeks 25.56 ± 2.42 vs. 31.60 ± 1.32 g, $P < 0.05$) and lean mass (6 weeks 15.14 ± 0.50 vs. 19.08 ± 0.48 g, $P < 0.01$; 17 weeks 23.06 ± 097 vs. 27.00 ± 0.67 g, $P < 0.01$) compared with C++L−− mice (Fig. 1*E* and *F*).

Consistent with the reduced adipose and lean mass, we noted that C−−L−− double mutant mice were significantly shorter than C++L−− mice (6 weeks 8.17 ± 0.09 vs. 8.76 ± 0.08 cm, $P < 0.001$; 17 weeks 9.27 ± 0.16 vs. 9.79 ± 0.10 cm, $P < 0.01$) (Fig. 1*C*). C−−L++ mice were similar in length to C++L++ mice (6 weeks 9.08 ± 0.09 vs. 9.20 ± 0.07 cm, $P > 0.1$; 12 weeks 9.60 ± 0.08 vs. 9.68 ± 0.07 cm, $P > 0.2$) (Supplementary Fig. 1*C*). Although a previous report also showed that C−−L++ mice weighed less, ate less, and had less fat mass than C++L++ mice (6), the present data revealed that C−−L++ mice had significantly lower lean mass than C++L++ mice (12 weeks 19.95 ± 0.40 vs. 21.84 ± 0.43 g, $P < 0.01$) and that the difference in percentage of fat was indistinguishable (12 weeks 11.85 ± 0.35 vs. 12.47 ± 0.47%, $P > 0.1$) (Supplementary Fig. 1*D* and *F*). These data show that Cnr1 deficiency leads to a reduced body size (with decreased lean and fat mass) in wild-type and *ob/ob* mice.

Cnr1 loss of function leads to exacerbated glucose intolerance and impaired insulin secretion in leptin-deficient animals. We next monitored glucose metabolism and insulin sensitivity at 6 weeks and 6 months of age (Fig. 2). Fasting blood glucose levels in 6-week-old C−−L−− mice were indistinguishable from C−−L++ or C++L++ animals (141 ± 15 vs. 118 ± 5 or 113 ± 5 mg/dL, $P > 0.05$ for either pair of comparisons), and this level was significantly lower than that in C++L−− mice (202 ± 21 mg/dL, $P < 0.001$) (Fig. 2*A*). Note that as previously mentioned, the body fat of the double mutant mice at this age was lower than that of C++L−− mice as both a percentage and an absolute mass. However, despite the lower fasting glucose levels, the C−−L−− mice were significantly more glucose intolerant than the C++L−− mice as assessed by glucose tolerance test (GTT) (Fig. 2*B*). At 45, 60, and 90 min after a single bolus of subcutaneous glucose challenge, plasma glucose levels in C−−L−− animals were 426 ± 28, 411 ± 32, and 336 ± 30 mg/dL, whereas in C++L−− animals, levels were 314 ± 28, 286 ± 24, and 236 ± 26 mg/dL at the same time point ($P < 0.01$ for paired comparisons at all three time points). The C−−L−− mice also had significantly worse GTTs than C++L++ mice ($P < 0.01$ or 0.001 for all paired comparisons at 15, 30, 45, 60, 90, and 120 min postinjection). A worsening of the GTT was also seen in wild-type mice lacking Cnr1. At 6 weeks of age, a time when fasting glucose levels between the two groups were indistinguishable, C−−L++ mice showed a small, but significant impairment of GTTs compared with C++L++ animals (15 min postinjection 270 ± 18 vs. 231 ± 11, $P < 0.05$; 45 min 230 ± 16 vs. 194 ± 10, $P < 0.05$; 60 min 194 ± 14 vs. 163 ± 8, $P < 0.05$; 90 min 145 ± 9 vs. 121 ± 5, $P < 0.05$; 120 min 126 ± 6 vs. 108 ± 5 mg/dL, $P < 0.01$) (Fig. 2*B*).

Abnormalities in glucose metabolism were still evident at 6 months of age (Fig. 2*A* and *C*), with C−−L−− animals showing a more abnormal GTT than C++L−− animals (30 min postinjection 409 ± 39 vs. 301 ± 32; 45 min 384 ± 32 vs. 280 ± 27; 60 min 368 ± 48 vs. 256 ± 29; 90 min 341 ± 52 vs. 238 ± 26; 120 min 326 ± 61 vs. 209 ± 20 mg/dL; $P < 0.05$ at all time points), even though their fasting glucose levels had become similar (169 ± 9 vs. 170 ± 10 mg/dL, $P > 0.5$). In contrast to the effect of the Cnr1 KO on *ob/ob* mice, the abnormal GTT in young C−−L++ animals normalized to wild-type levels at 6 months of age ($P > 0.2$ at all time points between C−−L++ and C++L++). Insulin tolerance tests were performed, and C−−L−− and C++L−− mice showed similar responses to insulin infusions (measured at age 6 months; $P > 0.5$ at all time points) (Fig. 2*D*). Fasting blood insulin levels were indistinguishable at 8 weeks of age (5.95 ± 2.11 vs. 7.96 ± 1.02 ng/mL, $P > 0.05$) (Fig. 2*E*) but were significantly different at age 5 months (7.55 ± 1.38 vs. 15.25 ± 3.16 ng/mL, $P < 0.01$).

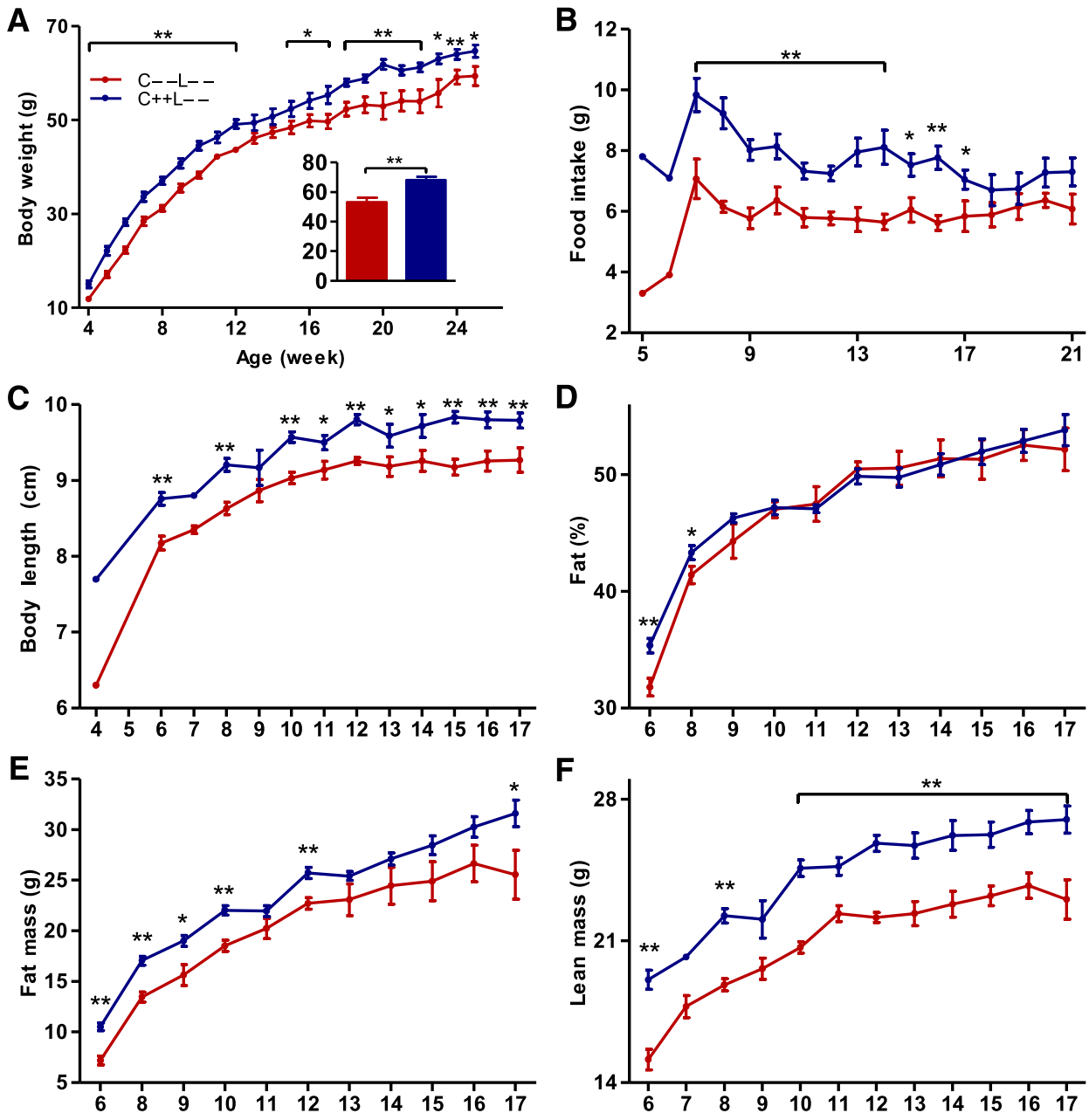

FIG. 1. Growth curves of C−−L−− and C++L−− mice. The body weight, food intake, and body composition of C−−L−− and C++L−− mice were determined and plotted over time. *A*: Body weight. C−−L−− mice had significantly lower body weight than C++L−− mice. *$P < 0.05$; **$P < 0.01$. The inset shows a comparison of the body weight between C−−L−− and C++L−− mice at 12 months of age. **$P < 0.01$. *B*: Food intake. C−−L−− mice consumed less food than C++L−− mice. *$P < 0.05$; **$P < 0.01$ for paired comparisons at indicated time points. *C*: Body length. C−−L−− mice were significantly shorter than C++L−− mice. *$P < 0.05$; **$P < 0.01$. *D*: Percent fat. Percentage of fat relative to total body weight was lower in young (6 and 8 weeks old) C−−L−− mice than in C++L−− mice. *$P < 0.05$; **$P < 0.01$. *E*: Fat mass. C−−L−− mice had a significantly lower amount of fat than C++L−− mice. *$P < 0.05$; **$P < 0.01$. *F*: Lean mass. C−−L−− mice had significantly less lean mass than C++L−− mice. **$P < 0.01$.

Glucose-stimulated insulin secretion (GSIS) assays were performed to establish the functionality of the β-cells (Fig. 2*F–H*). C−−L−− mice showed significant impairment of insulin secretion when compared with C++L−− mice (at 8 weeks of age, 90 min postinjection 1.89 ± 0.31 vs. 5.79 ± 0.74, $P < 0.01$; 120 min 1.90 ± 0.15 vs. 5.74 ± 0.91 ng/mL, $P < 0.01$; at 5 months old, 20 min postinjection 4.35 ± 0.13 vs. 7.99 ± 1.51, $P < 0.05$; 40 min 4.14 ± 0.23 vs. 6.93 ± 1.32, $P < 0.05$; 60 min 4.02 ± 0.19 vs. 6.28 ± 0.85, $P < 0.05$; 120 min 7.04 ± 1.35 vs. 20.17 ± 5.08 ng/mL, $P < 0.001$). Insulin secretion was also reduced in 8-week-old C−−L++ mice compared with C++L++ mice (30 min postinjection 0.29 ± 0.02 vs. 0.57 ± 0.08 ng/mL, $P < 0.01$). The area under the curve was calculated during the entire time course of glucose stimulation (Fig. 2*H*), and C−−L−− mice showed a significant reduction compared with C++L−− mice (8 weeks 330.58 ± 70.13 vs. 689.12 ± 73.49, $P < 0.05$; 5 months 654.49 ± 58.78 vs. 1,307.02 ± 237.40, $P < 0.05$). Young but not adult C−−L++ mice also showed a significant reduction in insulin response compared with C++L++ mice as calculated (8 weeks 23.19 ± 1.61 vs. 36.02 ± 4.24, $P < 0.05$; 5 months 31.27 ± 4.43 vs. 43.91 ± 7.72, $P > 0.05$). Finally, GSIS in isolated

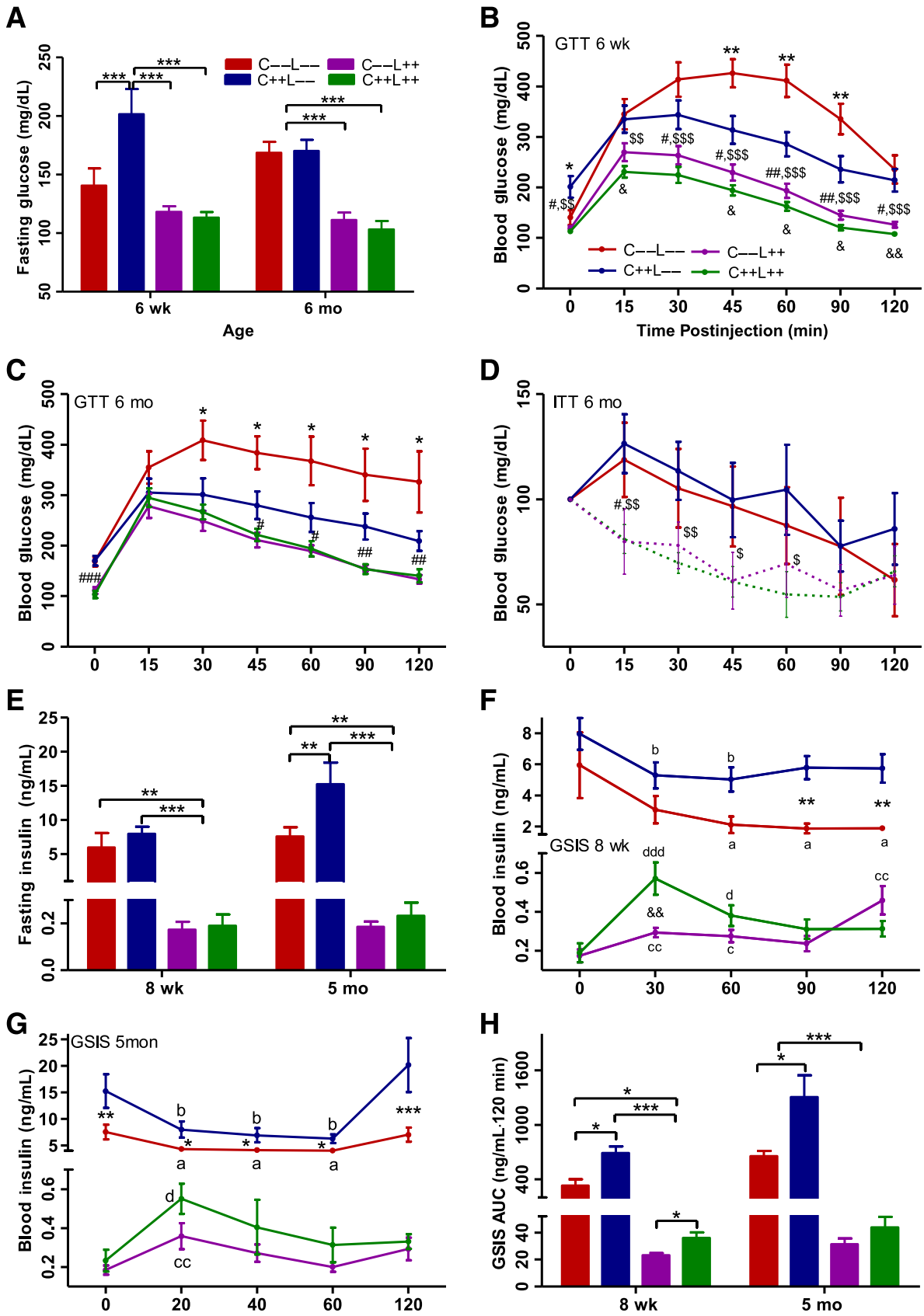

FIG. 2. GTT, insulin sensitivity, and GSIS in C−−L−−, C++L−−, C−−L++, and C++L++ mice. A set of assays monitoring glucose metabolism was performed on the four genotypes of mice as indicated. *A*: Fasting blood glucose at 6 weeks and 6 months of age. Fasting blood glucose levels were significantly higher in C++L−− mice than in all the other three genotypes at 6 weeks of age. At 6 months of age, both C−−L−− and C++L−− had significantly elevated fasting glucose levels vs. C−−L++ and C++L++. ***$P < 0.001$ for all paired comparisons. *B*: GTT at 6 weeks of age. C−−L−− mice were significantly more glucose intolerant than C++L−− mice. *$P < 0.05$; **$P < 0.01$. C−−L−− mice also had significantly worse GTTs than C++L++ mice. \$\$$P < 0.01$; \$\$\$$P < 0.001$. C++L−− mice showed significantly worse GTT than C++L++ mice. #$P < 0.05$;

pancreatic islets showed a similar trend (Supplementary Fig. 2*B* and *C*), although the difference was not statistically significant (see DISCUSSION).

C−−L−− mice show severe dysregulation of the GH/IGF-I axis. Because the C−−L−− mice showed concomitant glucose intolerance and a reduced body size, we considered the possibility that there might be abnormalities of the GH/IGF-I axis, which regulates both growth and glucose metabolism. Serum GH levels were significantly reduced in C−−L−− and C++L−− mice at young and adult ages compared with C−−L++ and C++L++ mice (at 6 weeks, C−−L−− 0.78 ± 0.15, C++L−− 2.31 ± 1.38, C−−L++ 5.66 ± 2.49, C++L++ 9.50 ± 2.52 ng/mL, $P < 0.05$ for C−−L−− vs. C−−L++, $P < 0.01$ for C−−L−− vs. C++L++ and C++L−− vs. C++L++; at 6 months, 1.53 ± 0.67, 1.05 ± 0.27, 3.77 ± 1.49, 4.63 ± 1.67 ng/mL, $P < 0.05$ for C−−L−− vs. C++L++ and C++L−− vs. C++L++) (Fig. 3*A*). These data are consistent with a previous report that leptin deficiency leads to downregulation of GH (13), indicating that a lower blood GH level was secondary to leptin deficiency and did not account for the growth retardation of C−−L−− vs. C++L−− mice.

In contrast to GH, serum IGF-I levels were significantly lower in C−−L−− mice than in all the other three genotypes, including C++L−−, at all ages (at 6 weeks, C−−L−− 176.94 ± 13.25, C++L−− 272.90 ± 13.10, C−−L++ 295.27 ± 13.9, C++L++ 325.08 ± 14.12 ng/mL, $P < 0.001$ for C−−L−− vs. any of the other genotypes; at 6 months old, 223.83 ± 13.61, 311.62 ± 21.73, 270.50 ± 14.26, 321.06 ± 11.77 ng/mL, $P < 0.05$ for C−−L−− vs. C++L−− or C−−L++, $P < 0.0001$ for C−−L−− vs. C++L++) (Fig. 3*B*). These data indicate that the small phenotype of the C−−L−− mice is associated with lower levels of IGF-I and suggest that Cnr1 signaling influences the circulating levels of plasma IGF-I.

We next analyzed the effect of Cnr1 KO on the production of IGF-I in the liver. Taqman real-time PCR showed that IGF-I mRNA (normalized to HPRT [hypoxanthine-guanine phosphoribosyltransferase] mRNA) was significantly reduced in C−−L−− mice compared with C++L−− mice (0.252 ± 0.018 vs. 0.328 ± 0.026, $P < 0.05$). There was no difference between C−−L++ and C++L++ mice (0.720 ± 0.060 vs. 0.763 ± 0.043, $P > 0.05$). Both obese groups also showed significant reduction compared with either nonobese group ($P < 0.001$ for each paired comparison). Similarly, IGF-I peptide content in the liver (normalized to total protein extracted) was significantly reduced in the two obese groups (0.239 ± 0.015 vs. 0.267 ± 0.019 ng for C−−L−− and C++L−−, respectively) compared with the two nonobese groups (0.425 ± 0.025 vs. 0.493 ± 0.090 ng for C−−L++ and C++L++, respectively, $P < 0.001$ for each paired comparison). The difference between the two obese groups or between the two nonobese groups showed a similar trend to plasma IGF-I but were not statistically significant ($P > 0.05$ in both cases).

To test whether this reduced IGF-I is also associated with IGF-I insensitivity, we performed an IGF-I tolerance test in 6-week-old animals (Fig. 3*C*). We found that C−−L−− and C++L−− mice responded with similar magnitudes of resistance to exogenous IGF-I ($P > 0.05$ at all time points), whereas a twofold lower dose of IGF-I effectively reduced plasma glucose in both C−−L++ and C++L++ animals ($P > 0.05$ at all time points). This finding suggests that a Cnr1 KO does not cause a defect in IGF-I responsiveness and that the reduced level of plasma IGF-I may contribute to the glucose intolerance of C−−L−− and C++L−− mice.

Leptin sensitivity in C−−L−− mice. We next tested whether loss of function of Cnr1 leads to an impairment in leptin action by treating 12-week-old mice with exogenous leptin (delivered through subcutaneous osmotic pumps at 250 ng/h). As shown in Fig. 4*A*, this dose of leptin led to equivalent weight loss in C−−L−− and C++L−− mice. The slopes of body weight loss were statistically indistinguishable (−1.006 ± 0.02615 and −1.067 ± 0.02670, respectively, $P > 0.1$). Note that the C++L−− mice remained significantly heavier than the C−−L−− mice throughout the time course as a result of their larger initial size. A GTT was performed after 17 days of leptin infusion. Leptin treatment fully corrected glucose intolerance in both C−−L−− and C++L−− animals (Fig. 4*B*). C−−L−− mice and C++L−− animals responded similarly ($P > 0.1$ at all time points), and both genotypes normalized relative to C++L++ mice ($P > 0.05$ at all time points). In addition, this dose of leptin fully normalized the slight glucose intolerance in C−−L++ mice compared with C++L++ mice ($P > 0.1$ at all time points). Thus, the potency of leptin was not abrogated by a loss of Cnr1.

Pharmacologic studies of Cnr1 in *ob/ob* mice. The worsening of the GTT seen in C−−L−− mice differs from the previously reported effect of Cnr1 antagonists (8,14–16). We thus tested whether the effect was recapitulated by pharmacologic blockade of Cnr1 in *ob/ob* mice. Adult (4 months of age) *ob/ob* mice were treated with the Cnr1-specific antagonist SR141716A (rimonabant) 10 mg/kg/day i.p. for 28 days. This treatment led to a significant reduction in body weight and food intake ($P < 0.05$) (Fig. 5*A* and *B*). These data show that long-term Cnr1 blockade is effective in reducing body weight in *ob/ob* mice fed ad libitum and are consistent with previous results showing that short-term Cnr1 blockade in adult animals is capable of reducing food intake in animals with monogenic forms of obesity (5). However, in contrast to the effect of a Cnr1 mutation, this treatment did not lead to exacerbated glucose intolerance in *ob/ob* mice ($P > 0.05$ at all time points by Student *t* test) (Fig. 5*C*) or to changes in blood IGF-I

##$P < 0.01$. Finally, C−−L++ mice showed a small, but significant impairment of GTT compared with C++L++ animals. &$P < 0.05$; &&$P < 0.01$. *C*: GTT at 6 months of age. C−−L−− mice remained more glucose intolerant than C++L−− mice at this age. *$P < 0.05$. C−−L−− and C++L−− mice also remained significantly more glucose intolerant than C++L++ mice. #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$. C−−L++ and C++L++ responded similarly ($P > 0.05$ at all time points). *D*: Insulin tolerance test at 6 months of age. C−−L−− and C++L−− mice responded similarly to a single bolus of insulin ($P > 0.05$ for paired comparisons at all time points) as did C−−L++ and C++L++ ($P > 0.05$ for paired comparisons at all time points). C−−L−− and C++L−− mice showed greater insensitivity than C++L++ mice. $$P < 0.05$; $$$P < 0.01$; #$P < 0.05$ for paired comparisons. *E*: Fasting blood insulin levels at 8 weeks and 5 months of age. C−−L−− mice showed significantly lower fasting blood insulin levels than C++L−− mice at 5 months of age. At both ages, C−−L−− and C++L−− mice showed significantly higher fasting blood insulin levels than C−−L++ and C++L++ mice. **$P < 0.01$; ***$P < 0.001$. *F, G*: GSIS at 8 weeks and 5 months of age. C−−L−− mice showed significantly reduced insulin secretion vs. C++L−− mice at both time points (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). C−−L++ mice showed significantly reduced insulin secretion than C++L++ mice at 8 weeks of age (&&$P < 0.01$). All four genotypes of mice responded to glucose stimulation with significant changes in blood insulin when compared to time 0 within each genotype ([a]$P < 0.05$ for C−−L−−; [b]$P < 0.05$ for C++L−−; [c]$P < 0.05$; [cc]$P < 0.05$, 0.01 for C−−L++; [d]$P < 0.05$; [ddd]$P < 0.05$ or 0.001 for C++L++). *H*: Area under the curve of GSIS at 8 weeks and 5 months of age. C−−L−− mice showed significantly reduced insulin secretion vs. C++L−− mice at both ages. C−−L++ mice showed significantly reduced insulin secretion vs. C++L++ mice at 8 weeks of age. *$P < 0.05$; ***$P < 0.001$.

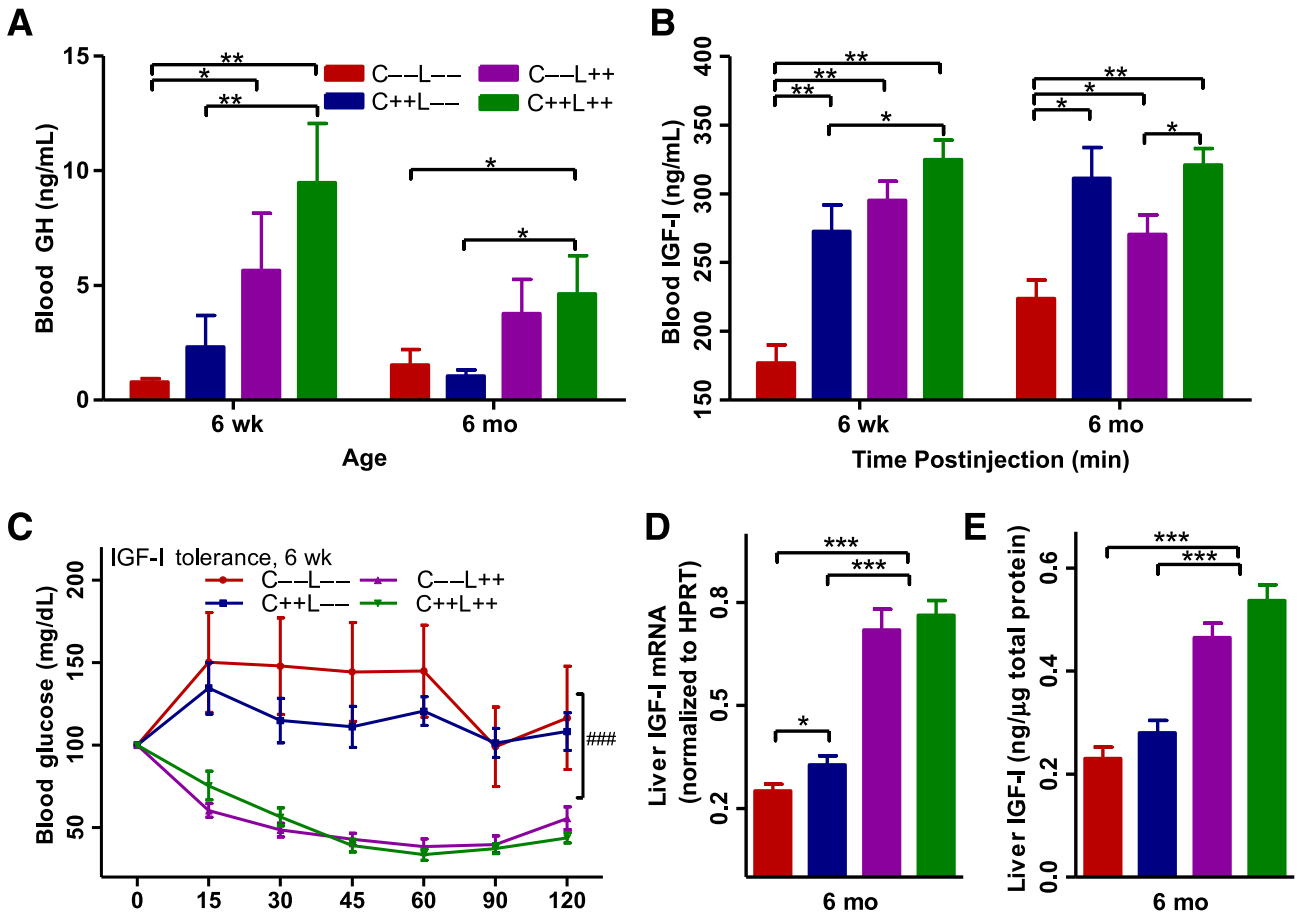

FIG. 3. GH and IGF-I levels in C−−L−−, C++L−−, C−−L++, and C++L++ mice. GH and IGF-I levels were measured at 6 weeks and 6 months of age. *A*: Plasma GH levels were significantly lower in C−−L−− and C++L−− mice than in C−−L++ or C++L++ mice at both ages. *$P < 0.05$; **$P < 0.01$. There was no significant difference in GH levels between C−−L−− and C++L−− animals. *B*: Plasma IGF-I was significantly lower in C−−L−− mice than in C++L−− mice at both ages. Plasma IGF-I was also significantly lower in C−−L−− mice than in both C−−L++ and C++L++ mice at both ages. C−−L++ mice showed significantly reduced plasma IGF-I levels than C++L++ mice at 6 months of age. *$P < 0.05$; **$P < 0.01$ for comparisons. *C*: IGF-I tolerance tests were performed at 6 weeks of age. The *ob/ob* mice with and without a Cnr1 mutation were significantly intolerant to exogenous IGF-I vs. wild-type mice with and without a Cnr1 mutation. ###$P < 0.001$ for C−−L−− or C++L−− vs. C−−L++ or C++L++ by one-way ANOVA. There was no significant difference in the IGF-I tolerance test between C−−L−− and C++L−− animals. *D*: IGF-I mRNA in the liver was measured by real-time PCR (Taqman assays) in 6-month-old mice. C−−L−− mice showed significantly reduced IGF-I mRNA vs. C++L−− mice. *$P < 0.05$. Both C−−L−− and C++L−− mice showed significantly reduced IGF-I mRNA than C−−L++ or C++L++ animals. ***$P < 0.001$. *E*: IGF-I protein levels in the liver from 6-month-old mice were measured by ELISA. Both C−−L−− and C++L−− mice showed significantly reduced IGF-I protein content than did C−−L++ or C++L++ mice. C−−L−− and C−−L++ mice showed lower IGF-I protein content than C++L−− and C++L++ mice, respectively, but neither difference was statistically significant. ***$P < 0.001$.

levels ($P > 0.05$) (Fig. 5*D*). Thus, although it reduced weight, the Cnr1 antagonist did not improve the GTTs of *ob/ob* mice. The differences between the effects of a mutation of Cnr1 and a small molecule antagonist provide further support that Cnr1 plays a role in growth and metabolism during development that is not recapitulated by pharmacologic blockade of the receptor in adults.

DISCUSSION

A link between enCBs and leptin signaling has been reported in prior studies (5). To further probe this relationship, we performed an epistasis experiment by generating mice with mutations in leptin and Cnr1 to test whether a defect in enCB signaling in mice with a Cnr1 KO would suppress some or all of the metabolic abnormalities of *ob/ob* mice and alter the response to leptin. However, rather than ameliorating the *ob* phenotype, we found that a deficiency of enCB signaling worsened the abnormal glucose metabolism and the growth retardation of *ob/ob* mice without reducing adiposity or blunting the response to leptin. These data suggest that the role of Cnr1 in mediating the effects of leptin is complex and inconsistent with a simple epistatic relationship. However, because we and others (5) have found that pharmacologic inhibition of Cnr1 reduces food intake and body weight, the data suggest that Cnr1 signaling may interact with leptin to regulate growth and metabolism during development.

A notable effect of a Cnr1 KO in *ob/ob* mice is profound growth retardation. It has been previously observed that *ob/ob* mice are shorter than wild-type mice (17). We found that Cnr1 KO exacerbates this growth phenotype in *ob/ob* and to a lesser extent in wild-type mice. Consistent with this, both C−−L++ and C−−L−− mice (compared with C++L++ and C++L−− animals, respectively) showed decreased lean and fat mass, which argues against a specific role of Cnr1 signaling on fat accumulation or lipid metabolism as previously suggested (6). In addition, Cnr1 loss of function led to significant dysregulation of the GH/IGF-I axis. The level of plasma IGF-I was reduced in both young and adult Cnr1 KO and C−−L−− mice. IGF-I secretion from liver contributes to plasma levels of IGF-I and is

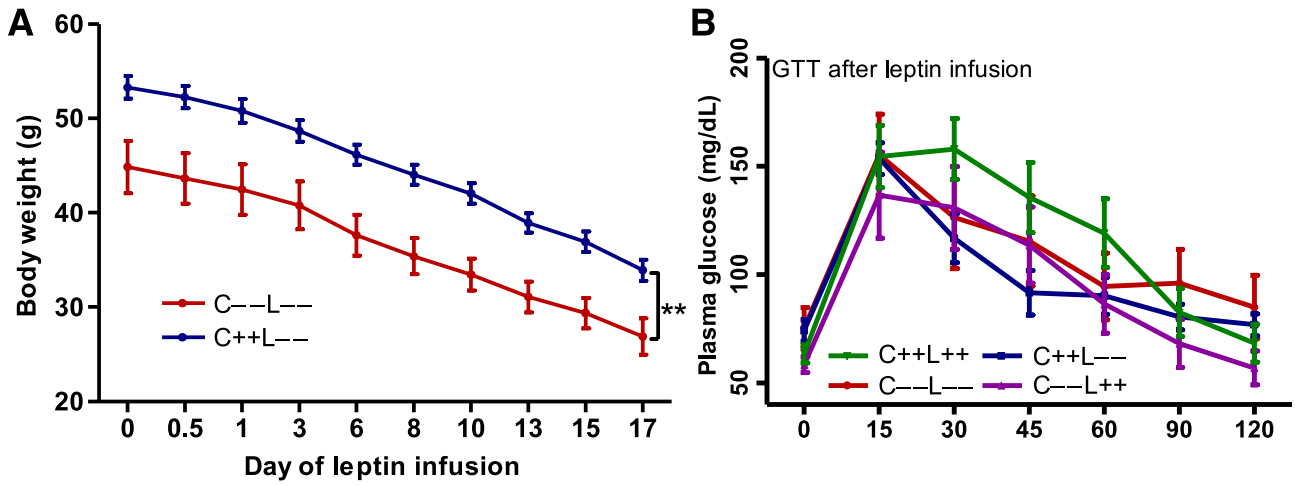

FIG. 4. Leptin sensitivity and GTT after leptin treatment in C−−L−−, C++L−−, C−−L++, and C++L++ mice. The effects of leptin on body weight and glucose metabolism were assayed in 12-week-old mice of all four genotypes. *A*: Body weight is shown for C−−L−− and C++L−− mice at indicated time points during leptin treatment. Both genotypes lost a significant amount of body weight by the end of the treatment ($P < 0.001$ for either genotype). The body weight of C−−L−− mice remained significantly lower than that of C++L−− mice during the course of the leptin treatment. **$P < 0.01$ by one-way ANOVA. *B*: GTTs were performed at the end of the treatment. Leptin treatment fully normalized the glucose intolerance in both C−−L−− and C++L−− mice, and both genotypes responded indistinguishably from C++L++ mice ($P > 0.05$ for paired comparisons at all time points).

normally controlled by GH secreted from the pituitary gland after the activation of GH releasing hormone–expressing neurons in the hypothalamus (18). The present data on liver IGF-I mRNA and peptide content suggest that congenital Cnr1 deficiency has an adverse effect on IGF-I production. Further studies will be required to determine whether extrahepatic effects of Cnr1 on IGF-I turnover are also responsible (9,10). The present data on the IGF-I tolerance test show that Cnr1-deficient mice respond indistinguishably from wild-type mice, suggesting that Cnr1 is unlikely to have a major effect on IGF-I turnover.

We also noted that both young and adult *ob/ob* mice showed reduced GH levels compared with C++L++ mice but that the lower IGF-I levels of young *ob/ob* animals returned to normal among adult *ob/ob* mice. This rebound of IGF-I levels with age in adult *ob/ob* mice might explain the observation that the maximal growth of *ob/ob* animals occurs between 3 and 6 months of age, during which time their bone length and density increase significantly. The mechanism underlying the complex relationship between GH and IGF-I in *ob/ob* mice is unknown, and it is possible that Cnr1 deficiency prevents the improvement in IGF-I production that normally occurs as *ob/ob* mice age. It is also noteworthy that the diabetes of *ob/ob* mice has been reported to improve as animals age, and a partial recovery of IGF-I levels could contribute to this. Detailed assays on pulsatile GH release and GH receptor signal transduction in Cnr1 KO mice might allow a further delineation of the role of Cnr1 in the control of the GH/IGF-I axis and growth (and possibly glucose metabolism), although the small plasma volume of mice makes frequent sampling impractical. Nonetheless, it is worth pointing out that one study has shown that pharmacologic blockade of peripheral Cnr1 signaling inhibited pituitary GH pulsatile secretion (19). Consistent with this, we observed a reduction in plasma GH levels in 6-week-old Cnr1 KO mice, although this was not statistically significant.

In addition to a worsening of the growth retardation of *ob/ob* mice, we found that Cnr1 KO significantly worsened the already abnormal glucose metabolism of *ob/ob* mice. This finding was unexpected because previous reports showed that a Cnr1 antagonist can improve glucose homeostasis in mouse and human (8,14–16,20) and that Cnr1 KO provides a beneficial effect on glucose homeostasis in mice fed a high-fat diet (10). These previous data differ from those in the present study, which show that young Cnr1 KO mice that are not leptin deficient are also mildly glucose intolerant, although this phenotype normalizes by adulthood without the development of diabetes. In addition, C−−L−− mice are significantly more glucose intolerant than *ob/ob* mice. The glucose intolerance in both C−−L−− and C−−L++ mice develops early and is independent of body fat content. Thus, the protective effect of Cnr1 KO on glucose metabolism in mice fed a high-fat diet is likely secondary to its effect to restrain body weight gain rather than an independent effect on glucose metabolism.

In contrast to the genetic studies, when treated with rimonabant (the same Cnr1-specific antagonist as used previously) for 28 days, adult *ob/ob* mice lost body weight and had reduced food intake, with neither worsening nor improvement of their glucose intolerance. This confirms and extends a previous study that showed that short-term (10 min) Cnr1 blockade leads to reduced food intake (5). It also points out that congenital Cnr1 deficiency and pharmacologic blockade of Cnr1 (even as long as 28 days) have distinct effects, at least in the context of leptin deficiency, suggesting a developmental role of Cnr1 signaling in linear growth, muscle and fat growth, and glucose metabolism.

Additional studies have suggested that enCB/Cnr1 signaling plays an important role in β-cell function and insulin action. For example, Cnr1 is found to be expressed by mouse pancreatic β-cells, and its activation leads to potentiation of GSIS. In β-cells, activation of Cnr1 has been shown to modulate insulin receptor substrate and AKT phosphorylation (8,14–16,21). Other studies have also suggested that Cnr1 activation leads to increased glucose uptake into adipocytes through GLUT4 (21,22). However, these effects are not uniformly observed because other studies have reported that Cnr1 agonists lead to reduced glucose clearance after a glucose challenge (14,15) and that Cnr1 antagonists lead to upregulated glucose uptake

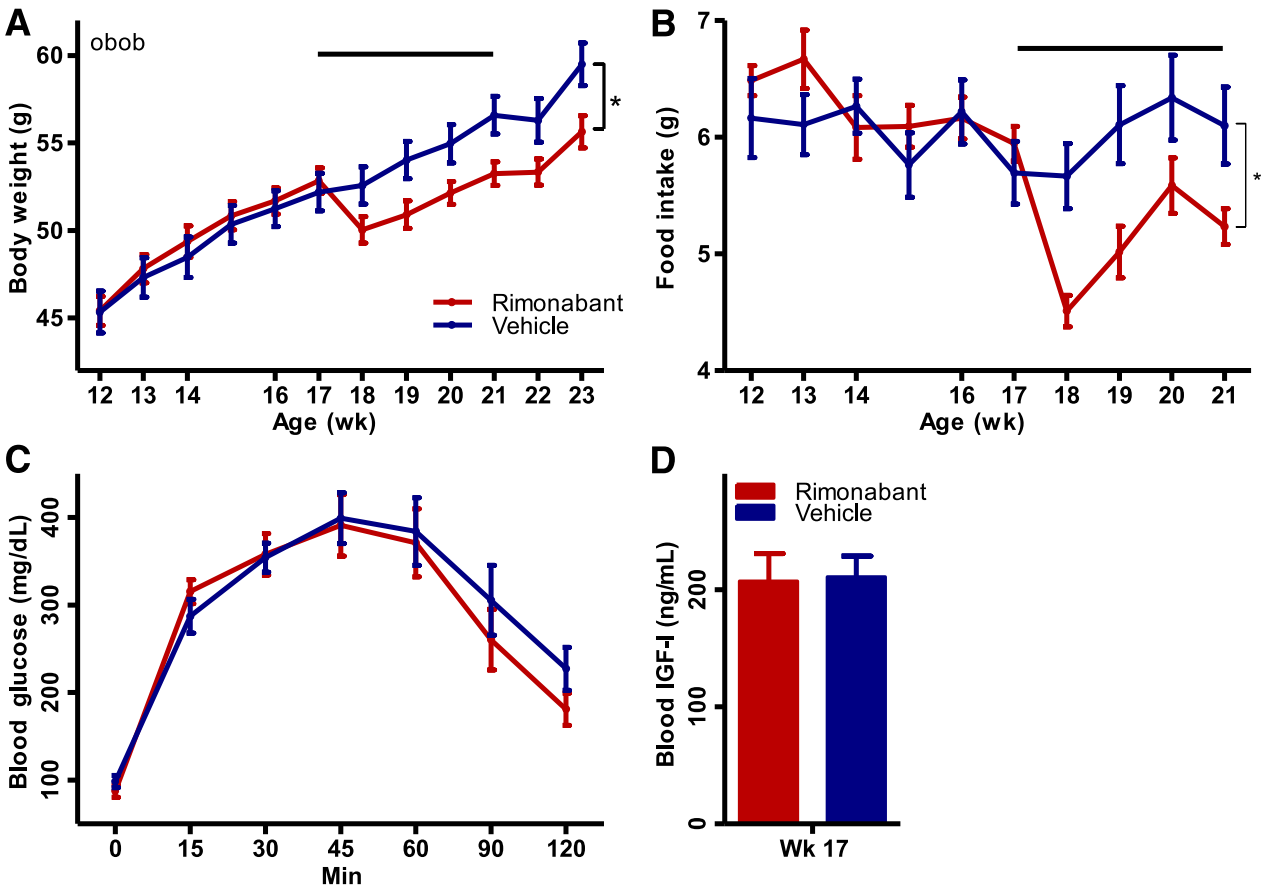

FIG. 5. Effect of long-term Cnr1 blockade on body weight, food intake, and glucose tolerance in adult *ob/ob* mice. The effect of a Cnr1 inhibitor was assayed in 17-week-old *ob/ob* mice treated with rimonabant 10 mg/kg/day i.p. or vehicle for 28 days. The black bar represents the duration of treatment. *A*: Body weight of rimonabant- and vehicle-treated *ob/ob* mice. Rimonabant treatment led to a significant reduction of body weight in *ob/ob* mice over the course of the treatment. **P* < 0.05 by one-way ANOVA. *B*: Food intake. Food consumption was significantly reduced in rimonabant-treated mice. **P* < 0.05 by one-way ANOVA. *C*: A GTT was performed at the end of the treatment. Rimonabant treatment did not improve the abnormal GTT of *ob/ob* mice because rimonabant-treated animals showed a similar GTT to vehicle-treated mice (*P* > 0.05 at all time points). *D*: IGF-I. Plasma IGF-I was measured at the end of the treatment. Rimonabant- and vehicle-treated mice showed similar levels of plasma IGF-I (*P* > 0.05).

by fat or muscle (8,16). The present data show that congenital Cnr1 deficiency impairs insulin secretion in response to increased glucose levels and support the conclusion that Cnr1 signaling positively regulates β-cell function and facilitates glucose clearance.

In conclusion, we tested the possible role of Cnr1 in leptin signaling by assaying the phenotype and leptin responsiveness of double mutant Cnr1 KO *ob/ob* mice. In contrast to data from previous studies that used Cnr1 antagonists, a Cnr1 loss of function did not suppress the *ob* phenotype or alter leptin sensitivity. Rather, Cnr1 signaling appears to be required for normal growth in part as a consequence of a reduction in IGF-I levels in Cnr1 KO mice. A loss of Cnr1 signaling also had adverse effects on glucose metabolism as a result of impaired β-cell activity and GSIS. Thus, the interaction between leptin and Cnr1 signaling is complex, and the discrepancies between the present data and that of previous studies may reflect differences between the effects of short-term modulation of Cnr1 with the use of pharmacologic agents and the effect of a congenital deficiency of Cnr1 signaling with activation of compensatory mechanisms in KO mice. Tissue-specific and inducible Cnr1 KO could provide further valuable insights into the role of Cnr1 in different tissues and metabolic pathways. These findings suggest that monitoring the effects of pharmacologic reagents on Cnr1 over longer periods of time may be necessary to fully establish its long-term efficacy and safety.

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Z.L. researched the data and wrote the initial draft of the manuscript. S.F.S. researched the data. J.M.F. reviewed, commented on, and edited the manuscript. Z.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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