Involvement of Cholecystokinin 2 Receptor in Food Intake Regulation: Hyperphagia and Increased Fat Deposition in Cholecystokinin 2 Receptor-Deficient Mice

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The role of cholecystokinin (CCK) as a satiety factor has been extensively documented. Although most work implies that CCK1 receptor mediates the control of food intake, a contributing role for CCK2 receptor (CCK2R) in the CCK-induced satiety cannot be totally excluded. The hypothesis that CCK2R invalidation disrupts regulatory pathways with impact on feeding behavior was examined in CCK2R−/− mice. CCK2R−/− mice developed obesity that was associated with hyperphagia. Obesity was related with increased fat deposition resulting from adipocyte hypertrophy. Expression of several adipokines was dysregulated consistently with obesity. Moreover, obesity was associated with disturbed glucose homeostasis as revealed by increased fasting glycemia and insulinemia, impaired glucose tolerance, and hepatic insulin resistance in CCK2R−/− mice. In vitro analysis of isolated adipocytes metabolism was consistent with increased storage but preserved insulin sensitivity. Suppression of feeding and concomitant increased expression of hypothalamic proopiomelanocortin after intracerebroventricular injection of gastrin into control mice demonstrates that hypothalamic CCK2 receptors mediate inhibition of food intake. Comparative analysis of hypothalamic mediator gene expression in fed knockout and control mice demonstrated overexpression of ghrelin receptors in CCK2R−/− mice, indicating up-regulation of orexigenic pathways. This effect was also observed after body weight normalization, indicating a causative role in the development of hyperphagia and obesity of CCK2R−/− mice. Our results give evidence that CCK2 receptor activity plays a contributing regulatory role in the control of food intake. (Endocrinology 148: 1039–1049, 2007)
paminergic, and melanocortin systems that are involved themselves in the regulation of appetite or in behavioral responses with potential impact on food intake (13). Furthermore, mice without CCK2R display enhanced gastric emptying that could also support an inhibitory role of CCK2R for feeding behavior because increased gastric distension contributes to the CCK-satiety response (14, 15).

Despite the potential contributory role of CCK2R to the action of CCK, there was, up to now, no complete characterization of feeding behavior and energy homeostasis of CCK2R knockout mice. Indeed, prior studies mostly focused on gastric abnormalities in CCK2R−/−, and investigations in the field of energy homeostasis were scarce, yielding incomplete or equivocal results. It appears from one report that CCK2 receptors do not participate in short-term satiety responses to CCK administration (7). Interestingly, two other studies concluded to an alteration of energy homeostasis in CCK2R knockout mice. In the first one, energy metabolism of a few 9-month-old mice were followed during 3 d. Increased energy intake was documented and associated with increased energy expenditure, enhanced basal metabolism rates, and thus similar body weight gain compared with wild-type mice (16). In the other study, mice aged 3–4 months were tested during 4 d. Significantly increased body weight and food intake were associated with decreased scotophase locomotor activity of CCK2R knockout mice (17). Long-term impact of these alterations has not been investigated, and underlying mechanisms were not explored. Although recent evidence linked several behavioral effects to altered function of several neurotransmitters in CCK2R-deficient mice, feeding behavior was not analyzed in these studies (18–20). This aspect was examined in mice deleted for gastrin gene expression. The observed increase in obesity in these mice was related to decreased locomotor activity and increased anxiety, but unchanged food intake (21, 22). Mechanisms mediating these changes are still unknown. Whether they include variations of expression and/or activation of CCK2R or other receptor subtypes has not been determined.

We made the hypothesis that CCK2 receptor invalidation should reveal regulatory disruptions with potential impact on feeding behavior. We examined this possibility by performing phenotypic characterization of CCK2R−/− mice with a special attention to body weight gain, food intake, fat deposition, and glucose disposal. Moreover, mRNA expression levels of hypothalamic genes regulating feeding behavior were investigated.

**Materials and Methods**

**Mice**

Generation of CCK2R−/− mice used in this study has been previously reported (23). Genotyping was carried out by means of PCR on genomic DNA extracted as in Ref. 24. Mutant CCK2R was detected by amplification of the neomycin gene using sense primer 5′-CTGCCGCCCAGCGCAACTGTTTCG-3′ and antisense primer 5′-CCGTTAAAGCAGCAGGGAGGAAGGCTTC-3′. Homozygous mutation was then screened on positive DNAs using the following pair of primers amplifying the CCK2R: sense primer 5′-TGGGCTATGAGCTCATCTCCCGCAA-3′ and antisense primer 5′-CGGCTGACTACGCGCGACACACGCGCA-3′. Mice positive for the first PCR and negative for the second PCR were considered homozygous for CCK2R deletion.

Male mice were studied. Mice were kept in a pathogen-free environment on a 12-h light, 12-h dark schedule in a temperature- and humidity-controlled animal facility. Mice were weaned at 4 wk of age and housed two to three per cage. They were given water and chow (Teklad global 16% protein rodent diet; Harlan, Blackthorn, Bicester, UK) ad libitum. Mice were backcrossed to C57BL/6J mice for five generations. Control mice used were age-matched C57BL/6J. The animal care committee of the Institut Fédératif de Recherche 31 (Toulouse, France) approved all procedures.

**Weight measurements and food intake**

Weight and food intake were measured every week between 4–52 wk of age. Food was monitored for spillage which was negligible. Mean daily intake was determined by averaging a 7-d total. Food restriction experiments were performed with 18-wk-old knockout mice that received the same amount of food as consumed by age-matched control mice.

**Fat histology**

Perigonadal fat pads were fixed in Bouin and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. Fat cell diameters were determined by measuring all the intact cells at >100 magnification and using the longest diameter as the final measurement. An average of 20 fields was examined per slide, and two sections were examined in each strain.

**Circulating blood hormone level**

Blood samples were obtained in the morning from overnight fasted animals. Plasma insulin concentration was determined by ELISA (Merckodia, Uppsala, Sweden) or RIA (Linco, St Charles, MO) from plasma sampled from the tail vein during the oral glucose tolerance tests. Plasma active and leptin concentrations were determined by RIAs (Linco).

**Lipolysis and glucose uptake measurement**

Visceral white adipose tissue (WAT) (perigonadal plus perirenal fat pads) were removed from CCK2R−/− and control mice and subjected to collagenase digestion (1–1.5 mg/ml) in a Krebs-Ringer solution buffered with 10 mM HEPES plus 15 mM bicarbonate (pH 7.4) and containing 3.5% (wt/vol) BSA and 6 mM glucose, (KRBHA). Adipocytes were then isolated and washed three times in KRBHA as previously reported (25). Isolated fat cells were distributed in plastic tubes containing 14–20 mg of cellular lipids in 0.4 ml KRBHA and incubated at 37°C under gentle shaking. After 90 min of incubation, the tubes were placed in an ice bath and 200-μl aliquots of the infranatant were taken for enzymatic determination of glycerol, used as lipolysis index as previously reported (25). Deoxyglucose uptake was measured during 10 min after preincubation of 45 min with the indicated drugs as already described (25). Final concentration of 2-deoxyglucose was 0.1 mM, and KRBHA buffer was supplemented with 2 mM pyruvate instead of 6 mM glucose present in lipolytic assays.

**RNA preparation and RT-PCR experiments**

RNA was extracted from isolated adipocytes using the RNeasy mini kit (Qiagen SA, Courtaboeuf, France) and from individual hypothalami as described in (26). Total RNAs were treated with DNase I and then reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). The same reaction was performed without Superscript II (RT-) to estimate residual DNA contamination.

Expression of murine CCK2 receptors in adipocytes was assessed by performing two different sets of primers either separately or successively in nested PCR. Sense primer 5′-ATGGATCTGCTCAAAGCTGAAAAAGG-3′ and antisense primer 5′-GTCCCCAGGGTGTGTTGTA-3′ were used to amplify the entire coding sequence by performing 45 cycles at 62°C. Amplification using sense primer 5′-CTGGTATATCCCGGCGAAGC-3′ and antisense primer 5′-ATGACACAGTCAAAGG-3′ were used to amplify the entire coding sequence by performing 45 cycles at 70°C. Amplification using sense primer 5′-CGTGTATCAGCAGCA-3′ and antisense primer 5′-TATACCAATACGAGGAGGAGTACAGGCA-3′ were used to amplify the entire coding sequence by performing 45 cycles at 70°C. Negative control, where PCR was performed without CDNA, was included in all experiments.
Expression of adipokines and of hypothalamic peptides was assessed by real-time PCR using 12.5 ng cDNA from isolated adipocytes or 100 ng from hypothalami and both sense and antisense oligonucleotides (for sequences of primers see Table 1) in a final volume of 25 μl using the SYBR green TaqMan Universal PCR Master Mix (Applied Biosystems, Applera France SA, Courtaboeuf, France). Fluorescence was monitored and analyzed in a GeneAmp 7000 detection system instrument (Applied Biosystems, Applera France SA). Analysis of the 18 S ribosomal RNA was performed in parallel using the ribosomal RNA control TaqMan Assay Kit (Applied Biosystems) to normalize gene expression. Results are expressed as 2^(-ΔΔCt) (29), where Ct corresponds to the number of cycles needed to generate a fluorescent signal above a predefined threshold. Comparative expression of hypothalamic neuropeptides was determined, after normalization in each sample for 185 ribosomal RNA, relatively to their expression in standard mouse brain (mouse brain total RNA; Clontech, Mountain View, CA). Oligonucleotide primers were designed using the Primer Express software (Applied Biosystems). All primers used were validated for PCR efficiency.

**Metabolic assays**

For glucose tolerance tests, mice of 12 wk of age were fasted overnight. Blood was withdrawn at indicated times from the tail vein, and glucose was measured using a One Touch II glucose monitor (LifeScan, Milpitas, CA). Surgical procedures for implantation of catheter devoted to infusion of insulin or [3H]-[3H]-glucose (PerkinElmer Life Sciences, Courtaboeuf, France) as well as experimental conditions procedures for hyperinsulinemic-euglycemic clamps have been previously described (27). Briefly, an intraperitoneal catheter was indwelled under anaesthesia and externalized on the top of the skull 3 d before the infusions. The day of the infusions, the mice were fasted for 6 h and connected to the infusing system. Saline or insulin was infused at a rate of 4 or 18 mU/kg/min for 3 h in the basal and insulin-stimulated states, respectively. Simultaneously, a [3H]-glucose infusion was performed to assess hepatic glucose production, whole body glycolysis, glycogen synthesis, and utilization rates. To determine the individual tissue glucose utilization rate, an injection of 1 μCi/g of mouse of 2-[3H]deoxyglucose (Perkin Elmer Life Sciences) through the femoral vein was performed in parallel using the ribosomal RNA control TaqMan Assay Kit (Applied Biosystems) and analyzed in a GeneAmp 7000 detection system instrument (Applied Biosystems). Fluorescence was monitored and analyzed in a GeneAmp 7000 detection system instrument (Applied Biosystems). All primers used were validated for PCR efficiency.

Radioligand binding assay

(Thr,Nle)-CCK-9 was conjugated with Bolton-Hunter reagent and radioliodinated as described previously (28), and crude membrane was prepared from isolated adipocytes as reported previously (29), except that centrifugation was performed at 39°C. Binding to both CCK1 and CCK2 receptors was tested using with the nonselective agonist [125I]-BH-(Thr,Nle)-CCK-9. Binding to CCK1 receptors was tested using the CCK1 receptor-selective antagonist [1H]SR-27,897 (30). Nonspecific binding was measured in the presence of 1 μM CCK or SR-27,897.

**Intracerebroventricular (icv) injection**

An icv catheter was indwelled and secured on the top of the skull under anaesthesia using a stereotactic device. A cannula was placed into the lateral ventricle as described (31). Mice were allowed 1 wk of postoperative recovery during which they were handled daily and acclimated to individual metabolic cages. After injections of saline, gastrin, and/or antagonists, mice were placed in individual metabolic cages and acute food intake during a 4-h period was monitored. Mice were then euthanized, hypothalami were dissected, and RNA was extracted. One nanogram of human sulfated gastrin 17 (generous gift from L. Moroder, Max-Planck-Institut für Biochemie, Martinsried, Germany), 100 ng SR-27,897, a specific CCK1 receptor antagonist (30), or 100 ng RPR-101048, a specific CCK2 receptor antagonist (32), were used. Gastrin and antagonists were freshly dissolved and injected alone or combined as indicated. The ivc volume of drugs and vehicle was limited to a total volume of 6 μl. For these studies, nine CCK2R/−/− and control mice of 12 wk of age were used in each group.

**Results**

**Increased body weight and fat content**

CCK2R−/− mice were viable and fertile. They were weighed weekly from weaning to 52 wk of age and compared with control mice. Growth curves show a significant increase in body weight by 8 wk of age that reached 28% at 20 wk of age and 45% at 1 yr of age (Fig. 1A). A trend toward increased proportion of visceral and sc WAT was evident by 4 wk of age (Table 2). The difference between control and knockout mice was significant at 10 wk of age and persisted throughout adulthood (Table 2). To determine whether increased fat deposition resulted from hyperplasia or hypertrophy we performed an histological morphometric analysis (Fig. 1B and C). Cell sizing revealed that, in epididymal fat pads, mean adipocyte diameter was 1.7-fold larger in CCK2R−/− mice than in control (Fig. 1D). These data demonstrate that increased weight of CCK2R−/− mice reflects an increased adiposity resulting from adipocyte hypertrophy.
knockout compared with control mice (Table 3). It was, respectively, 1.7- to 1.9-fold higher at 10 and 30 wk of age. Expression of adiponectin exhibited a trend toward lower values in CCK2R$^{-/-}$ mice that did not reach statistical significance. Conversely, resistin expression was significantly decreased by 3.5- and 5-fold at 10 and 30 wk of age, respectively, in transgenic adipocytes compared with controls. Secreted protein acidic and rich in cysteine (SPARC) gene expression was markedly increased (more than 3-fold) in CCK2R$^{-/-}$ adipocytes at 30 wk of age. Apelin, a newly identified adipokine, was significantly up-regulated by 3-fold in CCK2R knockout mice.

As expected, changes of leptin mRNA expression were associated with a parallel variation of protein secretion as CCK2R$^{-/-}$ mice exhibited significantly increased leptin plasma levels (Table 4). Changes of plasma ghrelin were inversely related to those of leptin and significantly decreased in transgenic mice compared with control (Table 4).

Because CCK2 receptor activation has been shown to regulate leptin expression and secretion in rat adipocytes we asked whether changes in adipocyte gene expression were directly related to the deficiency of CCK2 receptor in fat cells (33). However, in contrast with rat adipocytes, RT-PCR experiments demonstrated the lack of CCK2 receptor in adipocytes isolated from control mice, although we performed up to 45 cycles for amplification as well as nested PCR (data not shown). Binding experiments on membrane from isolated adipocytes from CCK2R$^{-/-}$ or control mice using radiolabeled CCK in the presence or not of specific CCK1 or CCK2 receptor antagonists confirmed the absence of either CCK2 or CCK1 receptor on this tissue (data not shown).

Taken together these results show that expression of several adipocyte-secreted proteins is modified in isolated adipocytes from CCK2R$^{-/-}$ in relation with adipocyte hyper trophy and obesity.

**Hyperphagia**

We then assessed whether obesity was related to hyperphagia by measuring food consumption of the CCK2R$^{-/-}$ mice. As shown in Fig. 1E, cumulative weekly food intake was increased in CCK2R$^{-/-}$ mice after weaning throughout the first year. Indeed, CCK2R$^{-/-}$ mice consumed 12, 24, 34, and 40% more than control mice at 5, 15, 20, and 52 wk of age, respectively. Thus, these results link the increased weight gain of CCK2R$^{-/-}$ mice to an increased food intake.

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**Adipocyte gene expression and circulating blood hormones levels**

Dysregulated expression of genes encoding adipocyte-produced proteins, or adipokines, has been associated with obesity in obese mice and humans. Therefore, we compared the expression of genes of several adipokines in adipocytes isolated from visceral white fat tissue of CCK2R$^{-/-}$ mice with that found in control mice. Consistent with increased fat cell size, leptin gene expression was increased in CCK2R$^{-/-}$ mice (Table 3). It was, respectively, 1.7- to 1.9-fold higher at 10 and 30 wk of age. Expression of adiponectin exhibited a trend toward lower values in CCK2R$^{-/-}$ mice that did not reach statistical significance. Conversely, resistin expression was significantly decreased by 3.5- and 5-fold at 10 and 30 wk of age, respectively, in transgenic adipocytes compared with controls. Secreted protein acidic and rich in cysteine (SPARC) gene expression was markedly increased (more than 3-fold) in CCK2R$^{-/-}$ adipocytes at 30 wk of age. Apelin, a newly identified adipokine, was significantly up-regulated by 3-fold in CCK2R knockout mice.

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**TABLE 2.** Body and adipose tissue weights in CCK2R$^{-/-}$ and control mice

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Control</th>
<th>RCCK2$^{-/-}$</th>
<th>Control</th>
<th>RCCK2$^{-/-}$</th>
<th>Control</th>
<th>RCCK2$^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (g)</td>
<td>17.1 ± 0.2</td>
<td>16.0 ± 0.5</td>
<td>24.9 ± 0.3</td>
<td>29.6 ± 1.2a</td>
<td>27.6 ± 0.5</td>
<td>39.2 ± 1.8b</td>
</tr>
<tr>
<td>SCWAT</td>
<td>2.2 ± 0.1</td>
<td>2.6 ± 0.4</td>
<td>2.1 ± 0.3</td>
<td>3.7 ± 0.5b</td>
<td>3.6 ± 0.3</td>
<td>5.0 ± 0.1c</td>
</tr>
<tr>
<td>VWAT</td>
<td>1.5 ± 0.1</td>
<td>1.9 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>4.8 ± 0.4a</td>
<td>4.5 ± 0.2</td>
<td>5.9 ± 0.1c</td>
</tr>
</tbody>
</table>

Age of mice is expressed in weeks. Organ and tissue weights are expressed as percentage of body weight. All values are means ± SEM, n = 7–18 mice for body weight, three to five mice for tissue weights measurements. SCWAT, sc WAT; VWAT, visceral WAT; nd, not determined.

a Significance at P < 0.001 comparing CCK2R$^{-/-}$ values vs. control values.

b Significance at 0.001 < P < 0.01 comparing CCK2R$^{-/-}$ values vs. control values.

c Significance at 0.01 < P < 0.05 comparing CCK2R$^{-/-}$ values vs. control values.
Glucose tolerance tests

There is a large body of evidence that suggests a tight link between obesity and non-insulin-dependent diabetes mellitus. To determine whether the increased adiposity was related to an impairment of glucose metabolism in CCK2R knockout mice, we first measured fasted blood glucose and insulin concentrations. It is noteworthy that no difference was observed between knockout and control mice younger than 10 wk of age (data not shown). At later stages, glycemia was significantly higher in fasted CCK2R−/− mice than in controls (Table 4). Plasma insulin levels of fasted CCK2R−/− mice were also significantly increased by about 2-fold compared with controls (Table 4). To assess the response to glucose administration, we then performed oral glucose tolerance tests. CCK2R−/− mice showed glucose intolerance as revealed by their higher hyperglycemic responses at 15 and 30 min after glucose load (Fig. 2A). However, glyceremia returned to the basal level 60 min after the glucose challenge. Plasma levels of insulin exhibited an increase of about 4-fold in control animals 15 min after glucose load (Fig. 2B). In contrast, no variation was observed in CCK2R−/− mice that maintained a high insulinemia during glucose excursion. These results indicate that genetic deficiency of the CCK2 receptor disturbs glucose homeostasis by inducing glucose intolerance in relation with impaired insulin action.

Glucose turnover

Hyperinsulinemic euglycemic clamp experiments were performed to further investigate whether glucose intolerance and elevated basal plasma insulin level could be associated with peripheral insulin resistance in CCK2R−/− mice. Whole body glucose turnover was determined in the basal state and during insulin infusion at either supraphysiological (18 mU/kg min) or physiological (4 mU/kg min) rate.

TABLE 3. Adipocyte gene expression

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Control</th>
<th>RCCK2−/−</th>
<th>Control</th>
<th>RCCK2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Leptin</td>
<td>22.2 ± 2.6</td>
<td>39.5 ± 4.6a</td>
<td>53.2 ± 10.9</td>
<td>102.3 ± 8.7b</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>30.2 ± 4.4</td>
<td>26.2 ± 1.1</td>
<td>40.9 ± 5.9</td>
<td>31.5 ± 3.9</td>
</tr>
<tr>
<td>Resistin</td>
<td>63.8 ± 16.2</td>
<td>17.9 ± 1.9a</td>
<td>32.4 ± 6.6</td>
<td>6.1 ± 2.7b</td>
</tr>
<tr>
<td>SPARC</td>
<td>21.9 ± 1.8</td>
<td>37.0 ± 6.5</td>
<td>21.7 ± 3.1</td>
<td>72.5 ± 20.6b</td>
</tr>
<tr>
<td>Apelin</td>
<td>0.22 ± 0.03</td>
<td>0.67 ± 0.18b</td>
<td>0.13 ± 0.03</td>
<td>0.31 ± 0.04a</td>
</tr>
</tbody>
</table>

mRNA were quantitated using real-time RT-PCR. Values are expressed as normalized expression relative to 18S RNA. Means ± sem were obtained from three to six independent determinations, each assayed in triplicate.
a Significance at 0.01 < P < 0.05 comparing CCK2R−/− values vs. control values.
b Significance at 0.001 < P < 0.01 comparing CCK2R−/− values vs. control values.

The glucose turnover rate in the basal state was reduced by 35% and associated with a significantly decreased glyco-
gen synthesis but a normal glycolytic rate in the CCK2R−/− mice when compared with control mice (Fig. 3, A, C, and D). Glucose turnover increased in a similar manner in CCK2R−/− and control mice in response to high and low infusion rates of insulin (Fig. 3A). Insulin-increased glyco-
gen synthesis and glycolysis rates were also identical in CCK2R−/− and control mice at high and low infusion rates (Fig. 3, C and D). However, hepatic glucose production was not fully inhibited in response to low insulin infusion in the CCK2R−/− mice, thus indicating hepatic insulin resistance at physiological insulin infusion rate (Fig. 3B).

Despite a normal whole body glucose utilization rate, the glucose distribution could be altered. The rate of insulin-
stimulated glucose uptake in peripheral tissues was then estimated using the method of 2-deoxy-D-glucose injection during the hyperinsulinemic euglycemic clamp (18 mU). The glucose utilization was similar in muscles of CCK2R−/− mice and controls (Fig. 3E). Conversely, it was significantly reduced in adipose tissue in the CCK2R−/− mice when compared with controls.

In vitro adipocyte glucose uptake and lipolysis

Because in vivo glucose utilization was reduced in WAT tissue of CCK2R−/− under insulin stimulation, insulin re-
sponsiveness was explored in vitro by investigating hexose transport and lipolytic activity in adipocytes isolated from visceral WAT at two different ages. Basal glucose uptake was significantly lower in adipocytes from 10- and 30-wk-old CCK2R−/− mice than in their respective control, when expressed as nanomoles of hexose incorporated per 100 mg lipid (Fig. 4, A and B). The maximal responses to insulin were reached at 10 and 100 nM of the hormone in all the studied

TABLE 4. Blood parameters of CCK2R−/− and control mice

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Control</th>
<th>RCCK2−/−</th>
<th>Control</th>
<th>RCCK2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Glucose (fasted) (mmol/liter)</td>
<td>4.34 ± 0.09</td>
<td>5.21 ± 0.12a</td>
<td>3.68 ± 0.18</td>
<td>5.99 ± 0.44b</td>
</tr>
<tr>
<td>Insulin (fasted) (ng/ml)</td>
<td>0.81 ± 0.08</td>
<td>1.48 ± 0.14a</td>
<td>0.62 ± 0.04</td>
<td>0.80 ± 0.07b</td>
</tr>
<tr>
<td>Leptin (fasted) (ng/ml)</td>
<td>3.8 ± 0.2</td>
<td>5.8 ± 0.4a</td>
<td>3.2 ± 0.6</td>
<td>16.7 ± 5.7b</td>
</tr>
<tr>
<td>Leptin (fed) (ng/ml)</td>
<td>4.8 ± 0.4</td>
<td>12.9 ± 1.8a</td>
<td>2.9 ± 1.3</td>
<td>33.6 ± 10.9b</td>
</tr>
<tr>
<td>Ghrelin (fasted) (ng/ml)</td>
<td>14.2 ± 0.7</td>
<td>6.6 ± 0.3a</td>
<td>8.3 ± 0.3</td>
<td>4.6 ± 0.9b</td>
</tr>
</tbody>
</table>

Mean ± sem values from 10–20 (glyceremia) or five to 10 (other parameters) independent experiments, each assayed in duplicate.
a Significance at P < 0.001 comparing CCK2R−/− values vs. control values.
b Significance at 0.001 < P < 0.01 comparing CCK2R−/− values vs. control values.
c Significance at 0.01 < P < 0.05 comparing CCK2R−/− values vs. control values.
Fig. 2. Oral glucose tolerance tests. A, Blood glucose in control (black symbols) and CCK2R−/− (white symbols) mice of 12 wk of age after oral administration of glucose, n = 6. B, Plasma insulin immediately before and at 15 min after oral administration of glucose to control mice (black bars) and CCK2R−/− mice (white bars), n = 6. * and ***, Significance at 0.01 < P < 0.05 and P < 0.001, respectively, comparing CCK2R−/− values vs. control values; #, 0.01 < P < 0.05 comparing values at 0 and 15 min.

groups. The insulin-stimulated transport exhibited a tendency to be lower in CCK2R−/− mice, but the difference remained insignificant (Fig. 4, A and B). Moreover, the range of insulin-dependent activation of hexose transport was 4- to 6-fold the basal uptake in both genotypes, i.e. in agreement with a normoresponsive profile of murine adipocytes (Fig. 4, A and B). Thus the enlarged fat cell size of CCK2R−/− mice and the higher lipid content of adipose tissue were probably more responsible for the lower in vivo hexose uptake than a decreased insulin responsiveness.

Antilipolytic effect of insulin was tested on isoproterenol-induced lipolysis. In adipocytes from 10-wk-old mice, basal lipolysis was not influenced by the genotype but the lipolytic response to the β-adrenergic agonist was significantly decreased in CCK2R−/− when compared with control (Table 5). However, insulin counteracted the β-adrenergic activation of lipolysis to the same extent in both CCK2R−/− and control adipocytes (about 50–60% inhibition). At 30 wk of age, basal lipolysis was lower in CCK2R−/− than in control, whereas the isoproterenol-induced lipolysis was partially inhibited by insulin which hardly reached 50% in both groups (Table 5). Consistent with the lack of CCK1 or CCK2 receptor observed in binding experiments, gastrin was unable to produce any stimulatory or inhibitory effects on the lipolytic activity of murine adipocytes, either in control or CCK2R−/− mice (data not shown). Taken together, these data indicate that adipocytes of CCK2R−/− mice did not exhibit a clear-cut insulin resistance but rather a tendency to a lower sensitivity similar to that observed with ageing/fattening in controls.

Fig. 3. Glucose turnover and activity. Glucose turnover (A), hepatic glucose production (B), glycogen synthesis (C), and glycogenesis (D) have been measured after 6 h of fasting (basal) and during an hyperinsulinenic euglycemic clamp using low (4 mU/kg/min) and high (18 mU/kg/min) rates of insulin infusion. E, Rates of hexose use (nanograms per milligram per minute of wet tissue) by gonadal WAT, heart, the vastus lateralis (VL), soleus (SL), total grinded hindlimb (HL), brown adipose tissue (BAT), and skin. Black bars, Control mice; white bars, CCK2R−/− mice (six mice per group). * and ***, Significance at 0.01 < P < 0.05 and P < 0.001, respectively, comparing CCK2R−/− values vs. control values.

icv gastrin injection

Our data are consistent with the interpretation that CCK2 receptor deficiency causes increased eating behavior. We examined whether this outcome could result via the disruption of an inhibitory signal required for the regulation of eating behavior by testing the effects of icv administration of gastrin in control mice. We use gastrin to specifically target the CCK2 receptors and avoid activation of CCK1R in control mice. As shown in Fig. 5A, food intake during the 4 h after gastrin injection decreased by almost 3-fold relative to either noninjected or vehicle-injected controls, thus demonstrating the inhibitory effect of gastrin on feeding. To determine which CCK receptor subtype mediates the central effects of gastrin, experiments were performed in the presence of specific CCK1 or CCK2 receptor antagonists. The inhibitory effect of gastrin was completely prevented by RPR-101048, the CCK2 receptor antagonist, but preserved in the presence of SR-27,897, the CCK1 receptor antagonist, thus clearly demonstrating involvement of CCK2 receptors in gastrin-induced inhibition. Neither injection of SR-27,897 or
Insulin and isoproterenol were used at 100 nM. Mean the percentage of inhibition of lipolysis induced by insulin with isoproterenol-stimulated lipolysis set at 0% and complete inhibition returning to basal set at 100% in each group. Insulin and isoproterenol were used at 100 nM.

Lipolytic activity of adipocytes isolated from CCK2R

**TABLE 5.** Lipolytic activity of adipocytes isolated from CCK2R<sup>−/−</sup> and control visceral WAT

<table>
<thead>
<tr>
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<th>Control</th>
<th>CCK2R&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (wk)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Basal</td>
<td>0.33 ± 0.08</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>5.20 ± 0.44</td>
<td>1.42 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ insulin</td>
<td>2.62 ± 0.43</td>
<td>0.58 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(52.3 ± 10.4%)</td>
<td>(68.2 ± 6.8%)</td>
</tr>
<tr>
<td></td>
<td>(48.9 ± 21.2%)</td>
<td>(41.2 ± 13.6%)</td>
</tr>
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Results are expressed as micromoles of glycerol per 100 mg lipids during the 90-min incubation time. Values in parentheses correspond to the percentage of inhibition of lipolysis induced by insulin with isoproterenol-stimulated lipolysis set at 0% and complete inhibition returning to basal set at 100% in each group. Insulin and isoproterenol were used at 100 nM. Mean ± SEM of four (10-wk-old) or three (30-wk-old) determinations.

<sup>a</sup> Significance at 0.001 < P < 0.01 comparing CCK2R<sup>−/−</sup> vs. control.

<sup>b</sup> Significance at P < 0.001 comparing CCK2R<sup>−/−</sup> vs. control.
due to food deprivation. Levels of ghrelin receptor and leptin receptor mRNAs differed significantly in ad libitum-fed CCK2R<sup>−/−</sup> and control mice (Fig. 6A). Gene expression of ghrelin receptor and leptin receptor were, respectively, 4- and 5.7-fold higher in CCK2R knockout than in control mice. POMC, NPY, and ghrelin mRNAs levels were not significantly altered in knockout mice.

To eliminate possible influence of overweight and positive energy balance state on gene expression levels, the same experiments were performed with CCK2R<sup>−/−</sup> mice that were submitted to pair-feeding. These mice normalized their body weight to that of control mice after 4 wk of food restriction (data not shown) and were then fed ad libitum to exclude effect of food deprivation. Gene expression of NPY, ghrelin, and ghrelin receptor were, respectively, 3.5-, 2.5-, and 3.3-fold higher in CCK2R knockout than in control mice (Fig. 6B). Leptin receptor gene expression was increased by 2.4-fold in CCK2R<sup>−/−</sup>(Fig. 6B). Leptin receptor gene expression was increased by 2.4-fold in CCK2R knockout compared with control mice, whereas that of POMC was not altered. Taken together these data are consistent with a potential causative role for elevated hypothalamic orexigenic pathways and dysregulation of anorexigenic signals, resulting from lack of CCK2 receptor expression, in the development of hyperphagia and obesity of CCK2R<sup>−/−</sup> mice.

**Locomotor activity and energy expenditure**

Locomotor activity and energy expenditure were monitored during 24 h. During the photophase and the scotophase, CCK2R<sup>−/−</sup> exhibited a lower activity when compared with control (Fig. 7A). The strain difference was significant during the scotophase. A circadian pattern of energy expenditure was observed for both CCK2R<sup>−/−</sup> and control mice, with greater expenditure during the scotophase (Fig. 7B). Our data show that energy expenditure of CCK2R<sup>−/−</sup> was significantly lower in both photoperiods.

**Discussion**

In the present study, we identify eating behavior abnormalities in CCK2 receptor knockout mice that demonstrate the role of CCK2 receptors in modulation of food intake and body weight regulation in mice. We report that hyperphagia occurs in the absence of the receptor and temporally precedes the development of obesity and hyperglycemia indicating a primary dysregulation of feeding with secondary consequences on body weight, fattening, and glucose metabolism. Importantly, the absence of compensatory process in CCK2 receptor-deficient mice supports a crucial inhibitory role of the CCK2 receptor in feeding behavior of mice.

CCK2 receptor knockout mice exhibit several features of both human and animal genetic and dietary models of obe-

![Fig. 6. Expression of hypothalamic neuropeptides and receptors. A, Gene expression levels of NPY, POMC, ghrelin receptor (GHSR), leptin receptor (leptin-R), and ghrelin were measured by quantitative RT-PCR in hypothalamic RNA extracts of ad libitum-fed CCK2R<sup>−/−</sup> (white bars) and control mice (black bars). B, Same experiments were performed with hypothalamic RNA of CCK2R<sup>−/−</sup> mice that normalized their body weight to that of control mice after pair-feeding food restriction. Mouse were fed ad libitum before killing. quantitative RT-PCR was performed in triplicate for four separate mice in each group. Black bars, Control mice; white bars, CCK2R<sup>−/−</sup> mice. Results are mean ± SEM.*,**, and ***, Significance at 0.01 < P < 0.05, 0.01 < P < 0.05, and P < 0.001, respectively, comparing CCK2R<sup>−/−</sup> values vs. control mice values.](https://academic.oup.com/endo/article-abstract/148/3/1039/2501624)

![Fig. 7. Locomotor activity and energy expenditure. A, Mean locomotor activity of each animal was calculated during 12-h photophase and 12-h scotophase. B, Energy expenditure was quantified during 12-h photophase or 12-h scotophase as indicated in Materials and Methods. Black bars, Control mice (n = 8); white bars, CCK2R<sup>−/−</sup> mice (n = 8). Results are mean ± SEM.* and **, Significance at 0.01 < P < 0.05 and 0.01 < P < 0.05, respectively, comparing CCK2R<sup>−/−</sup> values vs. control mice values.](https://academic.oup.com/endo/article-abstract/148/3/1039/2501624)
Hypothalamic POMC is further consistent with a satiety that hypothalamic CCK2 receptors mediate inhibition of hyperphagia that occurs at weaning. Suppression of feeding fat deposition with secondary disturbances of glucose metabolism are more likely the consequences of hyperphagia rather than events related to a failure in CCK2 receptor signaling in the fat cells.

That increased food intake is the mechanism that initiates fat deposition with secondary disturbances of glucose metabolism is further strongly suggested by the time of onset of hyperphagia that occurs at weaning. Suppression of feeding after icv injection of gastrin into control mice demonstrates that hypothalamic CCK2 receptors mediate inhibition of food intake. Moreover, concomitant increased expression of hypothalamic POMC is further consistent with a satiating role of CCK2 receptors and development of hyperphagia after disruption of this inhibitory pathway in CCK2R−/− mice. In line with this role is the differential hypothalamic gene expression of several regulators of food intake in CCK2R−/− and control mice. Indeed, results of comparative analysis show that, although circulating leptin and hypothalamic leptin receptor gene are increased in CCK2R−/− mice, POMC gene level is not up-regulated suggesting deficiency in leptin signaling pathway when CCK2 receptor is lacking (45). Importantly, our data also support a role for parallel increased orexigenic stimuli because hypothalamic ghrelin receptor, ghrelin, and NPY genes expression are increased independently of increased body weight, thus providing a causative role of these dysregulated systems in the development of hyperphagia in the absence of CCK2 receptors.

The relative importance of CCK1 and CCK2 receptors in appetite regulation is a matter of debate, and our results were unexpected in light of extensive literature providing support for the exclusive role of central CCK1 receptors in mediation of the CCK satieting effect. However, one must point out that most prior studies with CCK receptors agonists and/or antagonists were done in rats, and that important species differences in the hypothalamic distribution of CCK receptors exist. Particularly, it was recently demonstrated that dorso-medial hypothalamic CCK1R are absent in mice, whereas they are expressed in rats where they regulate NPY expression. Therefore, CCK1R are differentially involved in the regulation of energy balance in rats and mice (46). The possibility that a differential distribution of CCK2R in rat and mouse brains exists cannot be ruled out and could provide an explanation for prior negative results with respect to a feeding inhibitory action.

To date, expression pattern of CCK2 receptors in hypothalamic satiety centers that are the paraventricular (47), the ventromedial nuclei (47, 48), and the arcuate nuclei (10) is consistent with a regulatory role in feeding. Particularly, a subset of arcuate nucleus neurons were demonstrated to be the targets of CCK through the activation of postsynaptic CCK2R, strongly suggesting that CCK2 receptors are involved in the regulation of appetite by this important brain region (10). Pain signals were found to activate neurons of the ventromedial nuclei that express CCK2 receptor and to induce a decrease in food intake (48). This role is also supported by previous data showing the strong satieting effect of gastrin when given centrally in chicks (49).

To our knowledge, most of studies of CCK2 receptor knock-out mice examined effects related to gastric mucosa or behavioral consequences of gene invalidation. Because expression of CCK2 receptors in the brain is involved in several biological activities, therefore, it is possible that disturbances of other physiological systems can also indirectly contribute to the phenotype observed in the CCK2R−/− mice. Specifically the role of CCK2 receptors in the pathogenesis of anxiety is now accepted and was recently confirmed using an inducible transgenic approach (50). In good agreement with this, CCK2 receptor mice were shown to display reduced anxiety (18, 51). It is noteworthy that these studies also reported modifications of opioid, dopaminergic, and melanocortin systems, which have importance in feeding behavior and maintenance of body weight and, therefore, may contribute to the phenotype that we described.

The fact that other previous studies did not observe any or small weight gain in CCK2 receptor knockout mice surely relies on differences in the design of the experiments, i.e. brief observations, examination of short-term responses, few mice in examined cohorts. In particular, variations of body weight were investigated during short periods of 3 or 4 d (16, 17). However, these works reported increased energy intake and decreased locomotor activity that may favor the development of obesity. Interestingly, our observation of decreased scotophase locomotor activity in agreement with results of
previous studies examining this parameter across the entire diurnal cycle (17). The possibility that our report of decreased energy expenditure was due to decreased locomotor activity cannot be excluded.

Finally, results of the present study raise the question of whether peripheral gastrin may influence eating behavior and/or control of body weight. Indeed, the role of gastrin in the control of appetite is suggested by the demonstration that circulating gastrin activates area postrema neurons that bear CCK2 receptors and project to the NTS (52) as well as neurons in several brain regions involved in food behavior (53). It is noteworthy that mouse brain stem NTS POMC neurons are involved in the feeding-induced satiety and are activated by CCK (54). Thus, it is tempting to speculate that they could also indirectly respond to gastrin activating area postrema neurons. Experimental data showing lower concentrations of gastrin in obese diabetic mice (55) also sustain the hypothesis of a contributing role of circulating gastrin in feeding behavior. These literature data together with our results may have physiological relevance in humans in light of increased plasma levels of gastrin observed in anorexia nervosa (56). Interestingly, two recent reports of an obese phenotype of gastrin-deficient mice further suggest that gastrin may play a role in maintaining normal weight homeostasis (21, 22). However, mechanisms are not fully elucidated, and it is not known at the present time whether this phenotype, which exhibits some differences with that of CCK2R knockout mice, only results from the absence of amided gastrin and/or from that of gastrin precursors.

In conclusion, CCK2 receptor inactivation in mice alters the regulation of body weight and food intake and leads to obesity with insulin and leptin resistance that are known to predispose to diabetic state. Our data, which demonstrate a causative interaction with the hypothalamic ghrelin system and orexigenic pathways, provide an advance in understanding how CCK2 receptor play a role in the regulation of food intake. An important issue of our results to consider is that genetic variants of CCK2 receptor could contribute to obesity susceptibility and pathogenesis of this complex disease. Our data give also strong support to a contribution of CCK2 receptors to the antiobesity satieting drugs activating the CCK system. Indeed, they raise the hypothesis that activation of both CCK1 and CCK2 receptors, rather than specific targeting of CCK1 receptor may be necessary to achieve a full satiety effect.

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References

11. Noble F, Roques BP 2002 Phenotypes of mice with invalidation of cholecys- tokinin (CCK(1) or CCK(2)) receptors. Neuropeptides 36:170–177
15. Weiland TJ, Voudouris NJ, Kent S 2004 The role of CCK2 receptors in energy homeostatic insights from the CCK2 receptor-deficient mouse. Physiol Behav 82:471–476

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