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Leptin-Mediated Hypothalamic Pathway of Cholecystokinin (CCK-8) to Regulate Body Weight in Free-Feeding Rats

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Regulation of body weight (BW) results from the interplay between different hormonal systems acting at central and peripheral level. This study aims at characterizing the involvement of cholecystokinin (CCK) in BW and energy balance regulation. We have characterized, in free-feeding rats, the effect of CCK-8 on 1) food intake, BW, and adiposity; 2) skeletal muscle metabolism; 3) leptin signaling pathway within the arcuate nucleus of the hypothalamus; and 4) the permeability of brain barriers to leptin. We demonstrate here that CCK-8 acutely decreases BW by a mechanism partially dependent on central leptin pathways, based on the following results: 1) the effect of CCK was less intense in rats lacking functional leptin receptors (Zucker *fa/fa*), 2) CCK-8 facilitated the uptake of leptin from peripheral circulation to cerebrospinal fluid (CSF), 3) the concentration of leptin in CSF of rats receiving CCK was more elevated in those animals showing higher loss of BW, and 4) CCK activated leptin signaling pathways within the hypothalamus as well as phosphorylation of AMP-activated protein kinase in skeletal muscle. We also suggest that gain of BW may be linked to individual susceptibility to the effect of CCK, because we observed that in animals treated with this hormone, the increase of BW negatively correlated with leptin concentration within the CSF. Our data show that CCK has a negative impact on energy balance and suggest that CCK facilitates the access of leptin to hypothalamic areas, thus allowing leptin to act on hypothalamic targets involved in BW control. (*Endocrinology* 149: 1994–2000, 2008)

THE EFFECT OF cholecystokinin (CCK) on food intake has been extensively investigated from early studies of Gibbs in the 1970s (1) and often reviewed (2–4). CCK decreases meal size rather than overall food intake, and its effect is detected shortly after peripheral administration to food-deprived (5) or free-feeding rats (6). Moreover, circadian variations in the efficacy of CCK as a satiating agent, as well as differences due to the fed/fasted state, have been reported (6, 7). The physiological relevance of CCK in satiety is supported by a large body of research devoted to characterizing the orexigenic effect of CCK receptor antagonists (8–10) as well as by the overfeeding observed in Otsuka Long-Evans Tokushima fatty (OLETF) rats, which lack CCK_1 receptors (11).

The importance of CCK in regulating energy expenditure and body weight (BW) is suggested by numerous reports showing that CCK shares targets with leptin in the hypothalamus (12–16), the vagus nerve (17), and the nucleus of tractus solitarius (18). Morton *et al.* (18) have demonstrated that leptin-signaling integrity in the arcuate nucleus of the hypothalamus (ARN) is critical for the observance of CCK-induced satiety. On the other hand, the lack of effect of peripherally administered leptin in OLETF rats strongly suggests that pathophysiological mechanisms leading to leptin resistance involve CCK pathways (19). Because OLETF rats keep full responsiveness to intracerebroventricularly administered leptin, we have reasoned that CCK might play a key role in regulating the access of leptin to hypothalamic targets.

Leptin regulates energy balance (20) by acting on hypothalamic sites where it modulates the expression of both anorectic and orexigenic mediators (21). Leptin binds at least two receptors in the central nervous system (CNS) encoded by the Ob-R gene. The Ob-Ra receptor, also called short form, widely expressed in the choroid plexus (22, 23) and brain vessels (24), is involved in leptin transport from blood to the CNS. The Ob-Rb receptor, identified in the arcuate and other hypothalamic nuclei (25, 26), is coupled to the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathway and regulates food intake and sympathetic activity (12), which accounts for the increase of energy expenditure evoked by leptin. Nevertheless, leptin also acts directly on oxidative tissues to stimulate the oxidation of fatty acids by increasing the activity of adenosine AMP-activated protein kinase (AMPK) in skeletal muscle (27). In this context, leptin seems to contribute to the impairment of ectopic deposition of lipids in lean tissues (28, 29).

On the basis of all these antecedents, we have hypothesized that an interplay between leptin and CCK systems might be integral to physiological circuits aimed at maintaining BW and energy balance. The hypothesis to be tested was that an eventual effect of CCK-8 in decreasing BW might be linked to the activation of leptin pathways within the

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Abbreviations: ACC, Acetyl-coenzyme A-carboxylase; AMPK, AMP-activated protein kinase; ARN, arcuate nucleus of the hypothalamus; BW, body weight; CCK, cholecystokinin; CNS, central nervous system; CSF, cerebrospinal fluid; JAK, Janus kinase; OLETF, Otsuka Long-Evans Tokushima fatty; p, phospho-; SD, Sprague Dawley; STAT3, signal transducer and activator of transcription 3.

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hypothalamus as well as to the activation of energetic metabolism in peripheral tissues. To this end, we have characterized, in free-feeding rats, the effect of CCK-8 on 1) food intake, BW, and adiposity; 2) skeletal muscle metabolism; 3) activity of JAK/STAT3 signaling pathway within the ARN; and 4) permeability of brain barriers to leptin.

Materials and Methods

Animals

Eight-week-old male Sprague Dawley rats (CRIFA, Barcelona, Spain) and Zucker *fa/fa* rats (Harlan, Gannat, France) were housed in single wire hanging cages, under a 12-h light, 12-h dark cycle (lights on 0800–2000 h), in a temperature-controlled room (22 C) with food and water available *ad libitum*. Animals were handled daily, for at least 1 wk, to avoid stress by manipulation on the day of the experiment. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) for the care and use of laboratory animals. All experiments were carried out in free-feeding animals without previous fasting.

Drugs and doses

CCK-8 was from Sigma Chemical Co. (St. Louis, MO) and was administered ip (1, 10, or 100 μg/kg). Human recombinant leptin (Sigma) was administered ip at 100 μg/kg.

Treatment and experimental design

This study was organized in to four independent experiments aimed at characterizing the effect of CCK-8 on 1) food intake, body weight, and adiposity; 2) phosphorylation of both AMPK and acetyl-coenzyme A-carboxylase (ACC) in skeletal muscle, as an index of fatty acid β-oxidation; 3) c-Fos and phospho-STAT3 (pSTAT3) immunostaining within the ARN, as markers of neuronal activity and leptin responsiveness, respectively; and 4) distribution of leptin in rats receiving a tracer dose of human recombinant leptin.

At 2200 h, rats and chow were weighed, and then CCK-8 was administered. At 2400 h, rats and remaining food were reweighed, and a sample of blood was collected in chilled tubes coated with disodium EDTA (Microvette, Sarstedt, Germany) by means of a transversal cut in the tail. For cerebrospinal fluid (CSF) sampling, rats were immediately anesthetized with ketamine/xylazine, and CSF was obtained by puncturing the cisterna magna, following the method described by Waynforth and Flecknell (30). Briefly, the head was positioned to form a 50° angle with the vertebral column, and a microsyringe was placed between the external occipital bone and the atlas. The atlanto-occipital membrane was then perforated, and CSF (~100 μl) was extracted. Blood-contaminated samples were discarded and the remaining samples centrifuged for 10 min at 4000 rpm and stored at −80 C until assay. After CSF collection, rats were decapitated, and lumbar, mesenteric, and epididymal adipose tissue as well as soleus muscle were dissected, weighed, and processed for pAMPK and pACC Western blotting. In another set of experiments, rats were administered 10 μg/kg CCK-8 (or saline) at 2200 h, and then anesthetized with urethane 120 min later and immediately perfused with 300 ml saline, followed by 300 ml 4% paraformaldehyde. After perfusion, brains were removed and postfixed overnight in 4% paraformaldehyde until pSTAT3 and c-Fos immunolabeling.

A final experiment was designed to evaluate the effect of CCK-8 on the distribution of a peripherally administered tracer dose of leptin as well as the effect of CCK-8 on leptin-evoked activity of Ob-R signaling pathways. Rats received 100 μg/kg human recombinant leptin (or saline) 30 min after CCK-8 (10 μg/kg). Two hours after CCK-8 administration, samples of blood and CSF were obtained as indicated above, and then full hypothalami were dissected for pSTAT3 immunoblotting. Animals receiving the same treatment were anesthetized and perfused as described, and brains were prepared for pSTAT3 immunohistochemistry.

Biochemical determinations

Plasma leptin concentration was determined by using a specific ELISA kit for rat leptin (Assay Designs Inc., Ann Arbor, MI). Intra- and interassay variations were 11.6 and 11%, respectively. Leptin concentration in CSF was measured by means of an ultrasensitive specific ELISA kit (ALPCO, Salem, NH). Intra- and interassay variations were 4.4 and 4.7%, respectively. Human leptin was determined in plasma and CSF by using a specific ELISA kit (Assay Designs) exhibiting low cross-reactivity (<0.1%) with rat leptin. Intra- and interassay variations were 5.6–13.9 and 9.9–11.2%. Insulin was determined by using an ELISA kit (1.8% intraassay variation, 3.8% interassay variation) for rat insulin (Mercodia, Bagsvaerd, Denmark). Glucose was measured by a spectrophotometric method (glucose Trinder method; Roche, Sant Cugat, Spain). Plasma triglyceride concentration was measured by the glycerol phosphate oxidase method (Biolabo, Maizy, France). Free fatty acids were measured by a colorimetric method (acyl-coenzyme A synthetase-acyl-coenzyme A oxidase) by means of a commercial assay kit (Wako, Neuss, Germany).

Western blotting of pSTAT3, pAMPK, and pACC

STAT3 and pSTAT3 were measured in whole-cell extracts of full hypothalami. AMPK, pAMPK, ACC, and pACC were analyzed in whole-cell extracts of soleus muscle. Briefly, tissues were homogenized in an ice-cold buffer containing 0.42 M NaCl, 20 mM HEPES (pH 7.9), 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 20% glycerol, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 20 mM sodium fluoride, 1 mM trisodium orthovanadate, and 2 mM phenylmethylsulfonyl fluoride. After 15 min centrifugation, equivalent amounts of proteins (50 μg) present in the supernatant were loaded (50 mM Tris, 10% SDS, 10% glycerol, 5% mercaptoethanol, and 2 mg/ml bromophenol blue) and size-separated in SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia, Barcelona, Spain) using a transblot apparatus (Bio-Rad, Madrid, Spain). For immunoblotting, membranes were blocked with 10% nonfat dried milk in Tween-PBS for 2 h. Primary antibodies against STAT3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1/500 final dilution) and pSTAT3 (1/100 final dilution), AMPK-α (1/1000 final dilution), pAMPK-α Thr^{172} (1/1000 final dilution), and ACC, and pACC (all from Cell Signaling Technology, Inc., Beverly, MA) were applied overnight at 4 C. After washing, appropriate secondary antibodies were applied for 1 h. Blots were washed, incubated in commercial enhanced chemiluminescence reagents (ECL; Amersham, Spain) and exposed to autoradiographic film. Films were scanned for computer analysis using Molecular Analyst Software from Bio-Rad. Values for pSTAT3, pAMPK, and pACC were normalized with STAT3, AMPK, and ACC, respectively, to account for variations in gel loading.

pSTAT3 and c-Fos immunohistochemistry

Brain sections (40 μm) containing the ARN were obtained on a Vibratome (Leica 1000 M). Free-floating sections were bathed for 30 min in 60% methanol containing 0.3% H_2O_2 to block endogenous peroxidase activity. Sections were rinsed three times for 5 min each and once for 10 min in 0.1 M PBS (pH 7.4), then once for 10 min in PBS containing 0.1% Triton X-100. Sections were preincubated once for 30 min in PBS-Triton containing 5% normal goat serum. Anti pSTAT3 (Cell Signaling) or anti c-Fos rabbit antiserum (Calbiochem, La Jolla, CA) were added at a final dilution of 1:1000 and 1:5000, respectively, and incubated overnight at 4 C. The next day, sections were washed with PBS (three times for 5 min each and once for 10 min) and incubated with goat antirabbit secondary antiserum (Vector Laboratories, Burlingame, CA) diluted in PBS 1:200 for 2 h. Sections were rinsed in PBS (three times for 5 min each and once for 10 min) and incubated with the avidin-biotin-peroxidase complex (ABC kit; Vector). After washing with 0.05 M Tris-HCl (pH 7.4), sections were developed with 3,3′-diaminobenzidine (Vector) and then mounted and coverslipped with distyrene plasticiser xylene (Aldrich, Milwaukee, WI). Counting was performed through a ×20 air objective by using a Leika DMLS microscope. For each animal, the number of pSTAT3-stained cells was an average value from two to three sections. Cell counts were made randomly by two individuals. The ARN was arbitrarily divided into three rostrocaudal levels (bregma −2.12, bregma −2.80, and bregma −3.30). Individual group comparisons were made using a one-

way ANOVA, followed by a Newman-Keuls test. Statistical significance was set at $P < 0.05$.

Statistical analysis

Individual group comparisons were made using a two-way ANOVA. The effect of CCK-8 within a given group was analyzed using a one-way ANOVA, followed by a Newman-Keuls' *post hoc* test. Statistical significance was set at $P < 0.05$.

Results

Effect of CCK-8 on food intake, BW, plasma parameters, and adipose tissue

The effect of CCK-8 on food intake and BW was evaluated in free-feeding rats receiving a single dose of CCK-8 (1, 10, or 100 μg/kg) or saline at 2200 h (Table 1). One-way ANOVA revealed a significant effect of CCK-8 in reducing both food intake [$F_{(3,33)} = 4076$; $P < 0.05$] and BW [$F_{(3,37)} = 4364$; $P < 0.01$]. Plasma insulin, glucose, triglycerides, free fatty acids, and leptin remained unchanged in rats treated with CCK-8 (Table 2). The effect of CCK-8 on adipose tissue was estimated by measuring the amount of lumbar, mesenteric, and epididymal fat pads. As shown in Table 2, CCK-8 did not modify mean values of these adipose tissues, although the effect of CCK-8 on the amount of lumbar adipose tissue approached the limit of statistical significance ($P = 0.06$). To evaluate the influence of decreased food intake on the loss of BW detected in CCK-treated rats, a parallel study was carried out using a pair-fed group of rats; food intake and BW were monitored between 2200 and 2400 h in a group of 12 rats weighing 255.4 ± 6.4 g. On d 1, animals received food *ad libitum* and consumed 2.9 ± 0.4 g. BW increased 2.5 ± 0.7 g. On d 2, animals received 1.45 g preweighed chow. Under these conditions, BW increase was 0.5 ± 0.5 g (see Table 1 for comparing the effect of CCK-8 on BW and food intake with the *ad libitum*-fed group). A similar assay was carried out using a matched age group of Zucker *fa/fa* rats. As already observed in Sprague Dawley (SD) rats, administration of 10 μg/kg CCK-8 reduced food intake from 5.3 ± 0.7 to 2.5 ± 0.8 g [one-way ANOVA $F_{(1,8)} = 6897$; $P < 0.05$]. In contrast, comparison of the effect of CCK-8 on BW variation between SD and Zucker *fa/fa* rats, by means of two-way ANOVA, revealed only an effect of treatment [$F_{(1,30)} = 5829$; $P < 0.05$], which was significant in SD [one-way ANOVA $F_{(1,22)} = 7836$; $P < 0.05$] but not in Zucker *fa/fa* rats (BW was reduced from 2.1 ± 0.7 to 0.1 ± 1.0 g; $F_{(1,8)} = 2792$; not significant). The effect of strain and the interaction between treatment and strain were without statistical significance. Similar results were found when BW increase was expressed as percentage of the initial BW (data not shown).

TABLE 1. Effect of CCK-8 on food intake and BW

	Vehicle	CCK-8 (μg/kg)		
		1	10	100
Food intake (g/rat)	3.1 ± 0.4	1.8 ± 0.7	1.6 ± 0.3[a]	1.2 ± 0.5[a]
ΔBW (g)	1.6 ± 0.6	0.4 ± 1.0	−1.5 ± 1.3[a]	−2.2 ± 1.1[b]

The assay was carried out between 2200 and 2400 h. Values are mean ± SEM of 7–15 animals.

[a] $P < 0.05$ compared with the vehicle-treated group (Newman-Keuls test).

[b] $P < 0.01$ compared with the vehicle-treated group (Newman-Keuls test).

TABLE 2. Effect of CCK-8 on adipose tissue weight, plasma parameters, and CSF leptin

	Vehicle	CCK-8 (10 μg/kg)
Lumbar mesenteric adipose tissue (mg)	916.6 ± 68.9	707.6 ± 58.6
Mesenteric adipose tissue (mg)	2251.1 ± 114.6	2032.2 ± 159.7
Epididymal adipose tissue (mg)	1960.1 ± 128.3	2009.1 ± 134.5
Insulin (μg/dl)	0.60 ± 0.09	0.69 ± 0.10
Glucose (mg/dl)	147.6 ± 4.4	145.8 ± 6.4
Triglycerides (mg/dl)	80.1 ± 12.3	54.9 ± 9.7
Free fatty acids (μM)	0.22 ± 0.02	0.21 ± 0.01
Plasma leptin (ng/ml)	12.5 ± 2.6	13.3 ± 1.4
CSF leptin (pg/ml)	55.0 ± 14.3	57.5 ± 18.6

The assay was carried out between 2200 and 2400 h. Values are mean ± SEM of 7–15 animals.

Leptin concentration within the CSF negatively correlated with BW increase and adiposity in rats treated with CCK-8

We analyzed the correlation between CSF leptin concentration and BW variation, food intake, and adiposity both in controls and in CCK-treated rats (10 μg/kg) by means of simple regression. Correlations in control animals and CCK-treated rats were plotted separately. Figure 1, A and C, illustrates the correlation between CSF leptin concentration and BW increase/food intake, respectively, in control animals. Simple regression analysis revealed a positive significant correlation for both food intake [$F_{(1,9)} = 11{,}117$; $P < 0.05$], and BW [$F_{(1,7)} = 12{,}038$; $P < 0.05$]. In contrast, animals treated with CCK-8 exhibited a significant negative correlation between CSF leptin and BW increase [$F_{(1,11)} = 6819$; $P < 0.05$] (Fig. 1B). No correlation was found between CSF leptin and food intake (Fig. 1D). We analyzed the correlation between CSF leptin and the amount of lumbar and mesenteric adipose tissues. We also found that correlations between CSF leptin concentration and the amount of adipose tissue switched from positive to negative in animals treated with CCK-8. Figure 2, A and C, illustrates the positive correlations

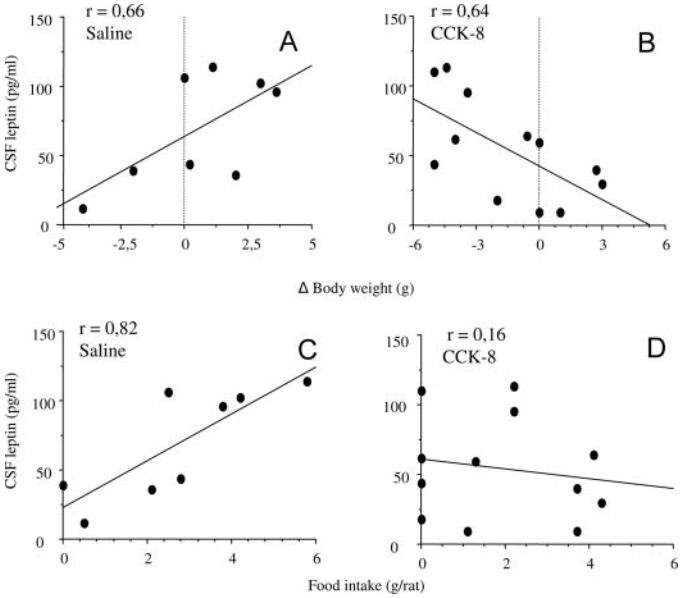

FIG. 1. Correlation between leptin concentration in the CSF and BW increase (A, saline; B, CCK-8) or food intake (C, saline; D, CCK-8) in control rats and in CCK-8-treated animals.

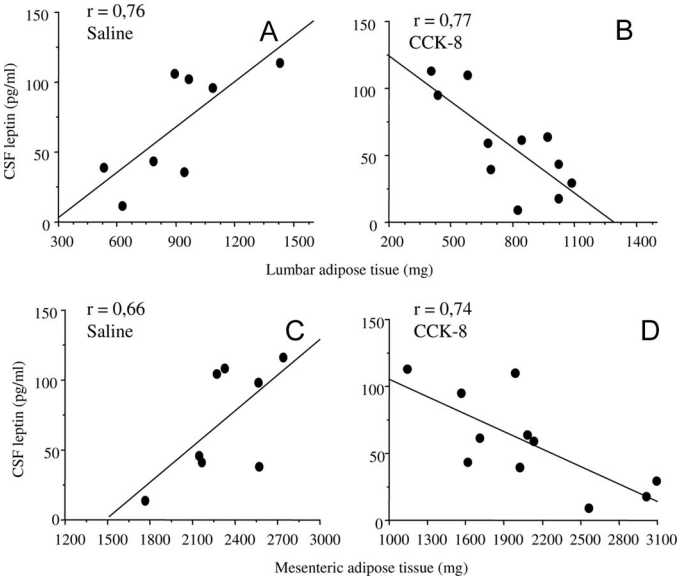

FIG. 2. Correlation between leptin concentration in the CSF and the amount of lumbar (A, saline; B, CCK-8) or mesenteric (C, saline; D, CCK-8) adipose tissue in control rats and CCK-8-treated animals.

between leptin CSF concentration and the amount of lumbar [$F_{(1,9)} = 5874$; $P < 0.05$[or mesenteric adipose tissue [$F_{(1,9)} = 10{,}980$; $P < 0.05$] in control animals. Figure 2, B and D, shows that these correlations became negative in CCK-8-treated animals [$F_{(1,11)} = 7840$; $P < 0.05$ and $F_{(1,11)} = 5184$; $P < 0.05$ for lumbar and mesenteric adipose tissues, respectively].

Treatment with CCK-8 induced pSTAT3 immunostaining in the arcuate nucleus of the hypothalamus

To test the hypothesis that CCK-8 effects were mediated by endogenous leptin, we analyzed the effect of CCK-8 (10 μg/kg) on leptin signaling pathways by measuring STAT3 phosphorylation within the hypothalamus (Fig. 3). As summarized in Table 3, administration of CCK-8 (10 μg/kg) led to a statistically significant increase of the number of pSTAT3-immunolabeled cells throughout the ARN. Under these conditions, pSTAT3 immunolabeling appears to be restricted to the ARN. No immunostaining was detected either in the paraventricular or in the ventromedial nucleus. One-way ANOVA revealed a significant effect of CCK-8 at bregma −2.12 [$F_{(1,4)} = 12{,}138$; $P < 0.05$], −2,80 [$F_{(1,4)} = 8445$; $P < 0.05$], and −3.30 [($F_{(1,4)} = 63{,}473$; $P < 0.01$].

c-Fos immunostaining appears widely distributed in the hypothalamus of CCK-8-treated rats

The effect of CCK-8 (10 μg/kg) on c-Fos expression was examined throughout the hypothalamus. The assay was carried out in free-feeding rats receiving CCK-8 at 2200 h. As illustrated in Fig. 4, c-Fos-immunopositive cells appear distributed not only in the ARN (Fig. 4, C and D) but also in other hypothalamic areas, such as the paraventricular nucleus (Fig. 4, A and B) or the ventromedial nucleus (data not shown).

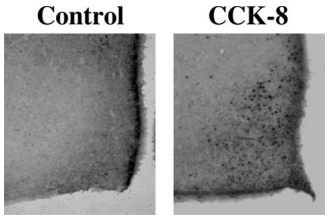

FIG. 3. Effect of CCK-8 (10 μg/kg) on pSTAT3 immunostaining within the ARN.

TABLE 3. Effect of CCK-8 (10 μ/kg) on pSTAT3 immunostaining within the ARN

Level of bregma	No. of pSTAT3-immunopositive cells	
	Vehicle	CCK-8
−2.12	18.0 ± 4.2	39.0 ± 4.3[a]
−2.80	18.3 ± 5.7	39.3 ± 4.3[a]
−3.30	16.0 ± 0.6	50.7 ± 6.4[b]

Data were analyzed by one-way ANOVA followed by the Newman-Keuls test (compared with their correspondent control groups). Values are mean ± SEM from three to four animals. For each animal, two sections were counted and average values obtained.
[a] $P < 0.05$.
[b] $P < 0.01$.

pAMPK and pACC are increased in skeletal muscle of rats treated with CCK-8

AMPK and ACC account for the effect of leptin on fatty acid metabolism in skeletal muscle. Leptin-mediated phosphorylation of AMPK at Thr^{172} (pAMPK) stimulates phosphorylation of ACC at Ser^{79}, leading to the inactive form of ACC, pACC. Because ACC is a key enzyme in malonyl-coenzyme A synthesis, in *de novo* synthesis of fatty acids, inactivation of this enzyme decreases malonyl synthesis and favors β-oxidation of fatty acids (27). Early activation of AMPK by leptin is due to leptin acting directly on muscle and occurs a few minutes after leptin administration. Later activation of the enzyme is linked to the activation of the hypothalamic-sympathetic axis and is detected several hours after leptin administration. As a further confirmation of the involvement of central leptin pathways in the effect of CCK-8 on BW/energy expenditure, we explored the AMPK/ACC pathway. We analyzed the level of pAMPK-

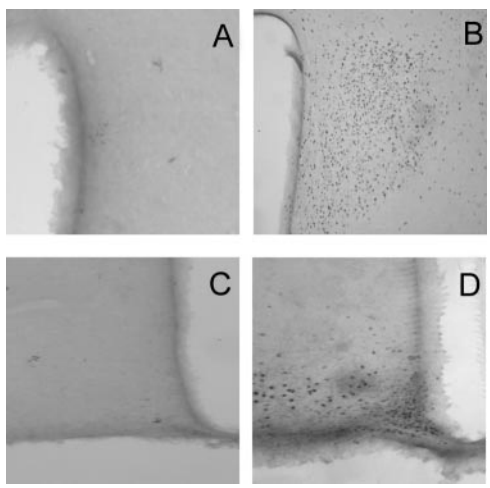

FIG. 4. Effect of CCK-8 (10 μg/kg) on c-Fos immunostaining within the hypothalamus. Microphotographs show the paraventricular nucleus of the hypothalamus in control (A) and CCK-treated (B) rats as well as a representative image of the ARN also in control (C) and CCK-treated (D) rats.

Thr[172] and pACC-Ser[79] in soleus muscle by Western blotting. As illustrated in Fig. 5, CCK-8 significantly increased both AMPK [Fig 5A; $F_{(1,24)} = 6.048$; $P < 0.05$] and ACC phosphorylation [Fig 5B; $F_{(1,10)} = 5.07$; $P < 0.05$].

CCK-8 increases leptin concentration in CSF as well as leptin signaling within the ARN of rats receiving a tracer dose of human recombinant leptin

To further confirm that the increase of leptin in CSF was due to an enhanced entry of leptin, recombinant human leptin (100 μg/kg ip) was used as a tracer. As summarized in Table 4, CCK-8 (10 μg/kg ip) decreased the concentration of human leptin in plasma [one-way ANOVA $F_{(1,16)} = 872{,}851$; $P < 0.001$]. In the CSF, human leptin was under the detection limit in rats treated with human leptin alone, but 1.1 ± 0.2 ng/ml (~0.07 nM) was found in rats treated with human leptin plus CCK-8. Immunohistochemical analysis (Fig. 6A), carried out at different rostrocaudal levels, revealed that the effect of CCK-8 on pSTAT3 immunostaining was significant throughout the ARN [Fig. 6B; bregma −2.12, one-way ANOVA $F_{(3,7)} = 4192$, $P = 0.054$; bregma −2.80, $F_{(3,8)} = 19{,}069$, $P < 0.001$; bregma −3.30, $F_{(3,7)} = 8762$, $P < 0.01$).

Discussion

We have previously reported that CCK-8 decreases plasma leptin and simultaneously increases leptin concentration in the CSF of rats challenged with an exogenous dose of leptin (31). Moreover, CCK receptor antagonists have been shown to increase plasma leptin concentration (32). These data account for a regulatory role of CCK in leptin kinetics. We have reasoned that such an effect might be endowed with functional consequences and account for the involvement of CCK in energy balance regulation. To test this hypothesis, we have designed the present study aimed at characterizing the effect of CCK-8 on hypothalamic leptin-mediated pathways regulating BW in free-feeding rats.

A major finding in this work is that treatment with CCK during periods of positive energy balance in rodents appears to activate metabolic process leading to a decrease of BW. We suggest that such an effect is leptin mediated because CCK-8 decreased food intake in obese, leptin-resistant, Zucker *fa/fa* rats but was without effect on BW. It has to be noted that, despite statistical differences, the magnitude of change between strains is rather small and probably reflects that leptin pathways account only partially for the effect of CCK on BW. The effect on BW was not linked only to the decrease of food intake, because pair-feeding to food intake of CCK-treated animals led to a modest effect on BW compared with that observed in CCK-treated animals. Under our conditions, we detected an effect of CCK only from the dose of 10 μg/kg (~2.5 μg/rat). It has been reported than CCK-elicited satiety is detected after lower doses, but most experiments are performed in the morning and in fasted animals. Closer to our conditions are those used by West *et al.* (6). These authors have described, in free-feeding rats, subjected to a scheduled treatment of five to six doses (1 μg/rat per dose) during 5 h, that CCK decreases meal size but has no effect on overall food intake. In addition, 10 μg/kg should raise plasma concentration of CCK to levels

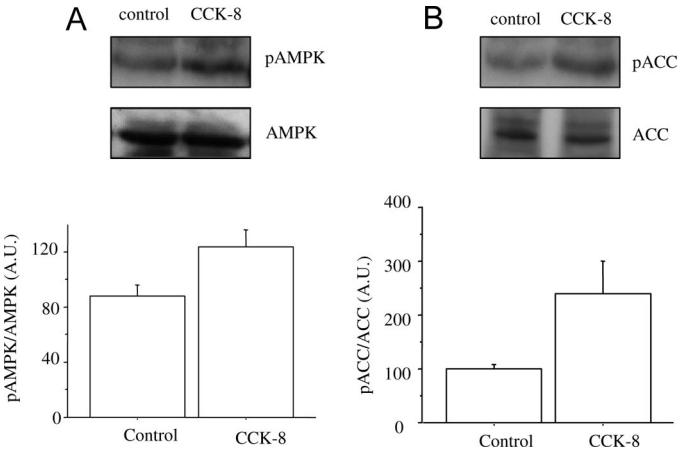

FIG. 5. Effect of CCK-8 (10 μg/kg) on phosphorylation of AMPK (A) and ACC (B) in skeletal muscle.

TABLE 4. Effect of CCK-8 (10 μg/kg) on the distribution of human leptin (100 μg/kg)

Treatment	Plasma (ng/ml)	CSF (ng/ml)
Human leptin	11.6 ± 0.4	ND
Human leptin + CCK-8	0.5 ± 0.1[a]	1.1 ± 0.2

Recombinant human leptin was administered 30 min after CCK-8, and samples of plasma and CSF were obtained 2 h after leptin administration. Data are means ± SEM of nine determinations. ND, None detectable.

[a] $P < 0.001$, Newman-Keuls test.

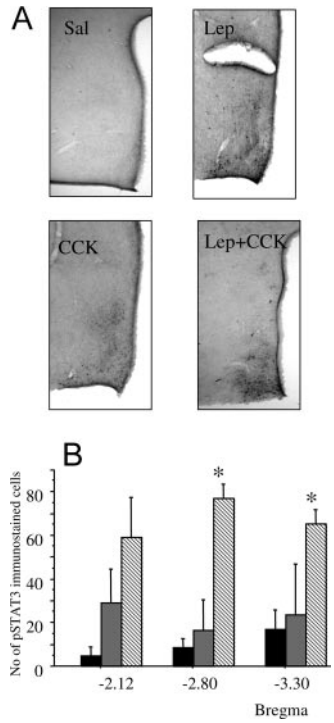

FIG. 6. Effect of CCK-8 (10 μg/kg) on phosphorylation of STAT3 in the hypothalamus of rats treated with recombinant human leptin (100 μg/kg). A, Microphotographs showing the effect of CCK-8 (10 μg/kg) on pSTAT3 immunostaining within the ARN; B, histograms summarizing the effect of leptin on pSTAT3 immunostaining within different rostrocaudal levels of the hypothalamus. *Black bars*, Human leptin; *gray bars*, CCK-8; *hatched bars*, human leptin plus CCK-8. *, $P < 0.05$, Newman-Keuls test. Lep, Leptin; Sal, saline.

(20–40 pM) comparable to those detected by some authors after feeding (33). Nevertheless, postprandial CCK concentration has been reported to be significantly lower than 20 pM by other authors (34). An eventual effect of 10 μg/kg CCK-8 in intestinal motility or in bladder tone might also account for the decrease of BW. However, urine volume and defecation were similar in CCK-treated and in control groups (data not shown).

The activation of leptin signaling pathways within the ARN observed in CCK-treated rats suggests an increase of leptin within hypothalamic areas. Obviously, the increase of pSTAT3 immunostaining evoked by CCK-8 cannot be univocally linked to leptin, because this signaling pathway is shared by many other mediators (35), but our data strongly point to the involvement of endogenous leptin in this CCK-evoked response. The absence of effect in other hypothalamic areas is striking, inasmuch as Ob-Rb receptors appear widely distributed within the hypothalamus (36). Other authors have observed that peripheral administration of elevated doses of leptin (1–4 mg/kg, *i.e.* 10- to 40-fold the dose used in this study) leads to pSTAT3 immunostaining also in ventromedial and paraventricular neurons (37–39). This discrepancy suggests that low doses of leptin mainly reach the ARN, which is the principal target for leptin in the brain (16). Alternatively, the effect of CCK on STAT3 phosphorylation might be due to a direct effect of CCK-8 within the hypothalamus. Such a possibility is unlikely, because CCK-8 does not cross the blood-brain barrier (40). In addition, CCK receptors are poorly expressed in the ARN in comparison with other hypothalamic areas (41, 42) that did not exhibit pSTAT3 immunoreactivity after CCK-8 administration. Under our conditions, CCK-8 induced c-Fos expression throughout the hypothalamus (Fig. 4), demonstrating that hypothalamic areas responsive to CCK don't exhibit increased pSTAT3 immunoreactivity after treatment with CCK-8.

The switch of the slope of individual correlations between CSF leptin and BW/adiposity induced by CCK-8 provides a further confirmation of the involvement of leptin in CCK-evoked responses. In control animals, correlations were positive (Figs. 1, A and C, and 2, A and C), indicating that, in naive rats, CSF leptin is proportional to the amount of adipose tissue (43) as well as to food intake and leptin release evoked by feeding (44). Nevertheless, in CCK-treated animals, all correlations were negative (Figs. 1, B and D, and 2, B and D). This suggests that the amount of leptin reaching the CNS as a consequence of CCK treatment might account for the effect of CCK in decreasing BW and adiposity and might indicate an individual susceptibility to CCK-8 on the activation of leptin pathways involved in regulating BW and energy expenditure. This observation doesn't necessarily mean that an acute treatment with CCK-8 provokes a loss of adipose tissue linked to the activation of lypolytic pathways or to the inhibition of lipogenic activity. We speculate that those animals exhibiting lighter adipose pads are more sensitive to CCK. In fact, fat deposits and plasma nonesterified fatty acid concentration were not significantly modified by acute CCK-8, suggesting that the metabolic rate in adipose tissue is not substantially affected by CCK-8. Finally, we detected an increase of both pAMPK and pACC in skeletal muscle, suggesting that CCK activates oxidative pathways in this tissue. Interestingly, leptin has been shown to stimulate fatty acid oxidation in skeletal muscle by increasing AMPK phosphorylation (27). This effect, linked to the activation of hypothalamic pathways, might also account for the negative effect of CCK on negative energy balance. The increase of metabolic rate in skeletal muscle elicited by CCK-8 cannot be attributed to an increase of locomotor activity because this peptide has been shown to suppress locomotion in rats (45).

An attractive hypothesis to explain our results is that CCK-8 might facilitate the uptake of peripheral leptin (46). To test this hypothesis, human leptin was administered as a tracer, and we measured both plasma and CSF human leptin concentrations by means of a specific enzyme immunoassay for human leptin. The result of this experiment (see Table 4) corroborates the hypothesis that CCK-8 increases the permeability of brain barriers to leptin. This was further confirmed by the increase of pSTAT3 detected in the hypothalamus of CCK-treated rats (Fig. 3), which demonstrates that leptin immunoreactivity corresponds to functional leptin and not to inactive fragments eventually recognized by the antiserum used in this study. In addition, the increase of pSTAT3 immunoreactivity was detected in the ARN, which is the main target for leptin in the CNS. From our data, we cannot suggest a mechanism to explain the effect of CCK, and more studies will be required to assess this question. As a possibility, activation of CCK receptors in the choroid plexus might increase leptin uptake mediated by Ob-Ra. On the other hand, it must be noted that the amount of leptin cleared from plasma in rats treated with CCK-8 largely exceeded the amount found in the CSF. It can be speculated that CCK-8 not only is involved in the entry of leptin into the CNS, but could also concern 1) the distribution of leptin in other tissues and/or 2) the metabolism/excretion of the hormone. Our results are consistent with a previous work of Niimi *et al.* (19) demonstrating that rats lacking CCK_1 receptors (OLETF) exhibit central responses to leptin only after intracerebroventricular administration. The lack of effect of CCK-8 on the concentration of endogenous leptin in the CSF may be due to the rapid distribution of leptin within the CNS after uptake (47) as well as to the elevated interindividual variability.

In summary, this work demonstrates that CCK modulates BW by a mechanism independent of its effect on food intake and involves central leptin pathways. In addition, this study shows that CCK can modulate the accessibility of leptin to its hypothalamic targets. Synergy between leptin and CCK can now be interpreted in a new context, and we hypothesize that both hormones may be integral to a homeostatic mechanism aimed at maintaining BW between narrow margins. From our data, we cannot propose a cellular mechanism to explain the effect of CCK on the permeability of the choroid plexus to leptin, and more research will be necessary to characterize the effect of CCK on mechanisms involved in leptin uptake by choroid epithelium and/or blood-brain barrier endothelium. Finally, our data must be interpreted cautiously in physiological terms, because the effect of CCK-8 on CSF leptin was observed only in animals treated with exogenous leptin. This could be due to the inter-individual variability of endogenous CSF leptin concentration. In addition, leptin concentration within the CSF mainly reflects uptake of leptin by the choroid plexus but not by the blood-brain barrier. Rapid distribution and degradation of leptin might also account for our data. In any case, these results indicate that an increase of plasma leptin may be ineffective, in terms of energy expenditure, if plasma CCK levels remain low or, alternatively, if there is a loss of functionality of CCK receptors.

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References

1. **Gibbs J, Young RC, Smith GP** 1973 Cholecystokinin elicits satiety in rats with open gastric fistulas. Nature 245:323–325
2. **Crawley JN, Corwin RL** 1994 Biological actions of cholecystokinin. Peptides 15:731–755
3. **Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, Roques BP** 1999 International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. Pharmacol Rev [Erratum (2000) 52:177] 51:745–781
4. **Moran TH, Ameglio PJ, Schwaerz GJ, McHugh PR** 1992 Blockade of type A, not type B, CCK receptors attenuates satiety actions of exogenous and endogenous CCK. Am J Physiol 262:R46–R50
5. **Smith GP, Greenberg, Falasco JD, Avilion AA, Gibbs J, Liddle RA, Williams JA** 1989 Endogenous cholecystokinin does not decrease food intake or gastric emptying in fasted rats. Am J Physiol 257:R1462–R1466
6. **West DB, Fey D, Woods SC** 1984 Cholecystokinin persistently suppresses meal size but not food intake in free-feeding rats. Am J Physiol Regul Integr Comp Physiol 246:R776–R787
7. **Kraly FS** 1981 A diurnal variation in satiating potency of cholecystokinin in the rat. Appetite 2:177–191
8. **Crawley JN, Stivers JA, Hommer DW, Skirboll LR, Paul SM** 1986 Antagonists of central and peripheral behavioral actions of cholecystokinin octapeptide. J Pharmacol Exp Ther 236:320–330
9. **Dourish CT, Rycroft W, Iversen SD** 1989 Postponement of satiety by blockade of brain cholecystokinin (CCK-B) receptors. Science 245:1509–1511
10. **Moran TH, Katz LF, Plata-Salaman CR, Schwartz GJ** 1998 Disordered food intake and obesity in rats lacking cholecystokinin A receptors. Am J Physiol 274:R618–R625
11. **Moran TH, Kinzig KP** 2004 Gastrointestinal satiety signals. II. Cholecystokinin Am J Physiol Gastrointest Liver Physiol 286:G183–G188
12. **Elias CF, Kelly JF, Lee CE, Ahima RA, Drucker DJ, Saper CB, Elmquist JK** 2000 Chemical characterization of leptin-activated neurons in the rat brain. J Comp Neurol 423:261–281
13. **Barrachina MD, Martínez V, Wang L, Wei JY, Taché Y** 1997 Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. Proc Natl Acad Sci USA 94:10455–10460
14. **Matson CA, Reid DF, Cannon TA, Ritter RC** 2000 Cholecystokinin and leptin act synergistically to reduce body weight. Am J Physiol 278:R882–R890
15. **Matson CA, Reid DF, Ritter RC** 2002 Daily CCK injection enhances reduction of body weight by chronic intracerebroventricular leptin infusion. Am J Physiol 282:R1368–R1373
16. **Morton GJ, Blevins JE, Williams DL, Niswender KD, Gelling RW, Rhodes CJ, Baskin DG, Schwartz MW** 2005 Leptin action in the forebrain regulates the hindbrain response to satiety signals. J Clin Invest 115:703–710
17. **Peters JH, McKay BM, Simasko SM, Ritter RC** 2005 Leptin-induced satiation mediated by abdominal vagal afferents. Am J Physiol Regul Integr Comp Physiol 288:R879–R884
18. **Morton GJ, Niswender KD, Rhodes CJ, Myers MG, Blevins JE, Baskin DG, Schwartz MW** 2003 Arcuate nucleus-specific leptin receptor gene therapy attenuates the obesity phenotype of koletsky (fa^k/fa^k) rats. Endocrinology 144:2016–2024
19. **Niimi M, Sato M, Yokote R, Tada S, Takahara J** 1999 Effects of central and peripheral injection of leptin on food intake and on brain Fos expression in the Otsuka Long-Evans Tokushima Fatty rat with hyperleptinaemia. J Neuroendocrinol 11:605–611
20. **Campfield AL, Smith FJ, Guisez Y, Devos R, Burn P** 1995 Recombinant mouse OB protein: evidence for peripheral signal linking adiposity and central neural networks. Science 269:546–549
21. **Korner J, Aronne LJ** 2003 The emerging science of body weight regulation and its impact on obesity treatment. J Clin Invest 111:565–570
22. **Tartaglia LA, Dembski M, Weng S, Deng N, Culperer J, Devos R, Grayson JR, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore EA, Smutko JS, Mays GG, Woolf EA, Monroe CA, Tepper RI** 1995 Identification and expression cloning of a leptin receptor, OB-R. Cell 83:1263–1271
23. **Devos R, Richards JG, Campfield LA, Tartaglia LA, Guisez Y, Van der Heyden J, Travernir J, Plaetinck G, Burn P** 1996 OB protein binds specifically to the choroid plexus of mice and rats. Proc Natl Acad Sci USA 93:5668–5673
24. **Golden PL, Maccagnan TJ, Pardridge WM** 1997 Human blood-brain barrier leptin receptor. J Clin Invest 99:14–18
25. **Fei H, Okano HJ, Li C, Lee GH, Zhaos C, Darnell R, Friedman J** 1997 Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proc Natl Acad Sci USA 94:7001–7005
26. **Håkansson ML, Brown H, Ghilardi N, Soda RC, Meister B** 1998 Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neuroscience 18:559–572
27. **Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Müller C, Carling D, Kahn BB** 2002 Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature 415:339–343
28. **Unger RH** 2003 Weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. Endocrinology 144:5159–5165
29. **Somoza B, Guzman R, Cano V, Merino B, Ramos P, Diez-Fernandez C, Fernández-Alfonso MS, Ruiz-Gayo M** 2007 Induction of cardiac uncoupling protein-2 expression and adenosine 5′-monophosphate-activated protein kinase phosphorylation during early states of diet-induced obesity in mice. Endocrinology 148:924–931
30. **Waynforth HB, Flecknell PA** 1992 Experimental and surgical technique in the rat. New York: Academic Press
31. **Cano MV, Ezquerra L, Ramos MP, Ruiz-Gayo M** 2003 Regulation of leptin distribution between plasma and cerebrospinal fluid by cholecystokinin receptors. Br J Pharmacol 140:647–652
32. **Cano MV, Ezquerra L, Ruiz-Gayo M** 2003 Involvement of cholecystokinin (CCK) in the access of leptin to the CNS. Int J Obesity P11:27 (Abstract)
33. **Linden A, Södersten P** 1990 Relationship between the concentration of cholecystokinin-like immunoreactivity in plasma and food intake in male rats. Physiol Behav 48:859–863
34. **Li Y, Hao Y, Owyang C** 2000 Diazepam-binding inhibitor mediates feedback regulation of pancreatic secretion and postprandial release of cholecystokinin. J Clin Invest 105:351–359
35. **Darnell JJ, Kerr I, Stark G** 1994 JAK-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1421
36. **Elmquist JK, Bjorbaek C, Ahima RS, Flier JS, Saper CB** 1998 Distribution of leptin receptor mRNA isoforms in the rat brain. J Comp Neurol 395:535–547
37. **Van Heek M, Compton DS, France DF, Tedesco RP, Fawzi AB, Graziano MP, Sybertz EJ, Strader CD, Davis HR** 1997 Diet-induced obese mice develop peripheral, but not central, resistance to leptin. J Clin Invest 99:385–390
38. **McCowen KC, Chow JC, Smith RJ** 1998 Leptin signalling in the hypothalamus of normal rats *in vivo*. Endocrinology 139:4442–4447
39. **Münzberg H, Flier JS, Bjorbaek C** 2004 Region-specific resistance within the hypothalamus of diet-induced obese mice. Endocrinology 145:4880–4889
40. **Oldendorf WH** 1981 Blood-brain barrier permeability to peptides: pitfalls in measurement. Peptides 2:109–111
41. **Mercer LD, Beart PM** 1997 Histochemistry in rat brain and spinal cord with an antibody directed at the cholecystokinin A receptor. Neurosci Lett 225:97–100
42. **Mercer LD, Le VQ, Ninan J, Jones NM, Beart PM** 2000 Direct visualization of cholecystokinin subtype 2 receptors in rat central nervous system using anti-peptide antibodies. Neurosci Lett 293:167–170
43. **Schwartz MW, Peskind E, Raskind M, Boyko EJ, Porte D** 1996 Cerebrospinal fluid leptin levels: plasma levels and adiposity in humans. Nat Med 2:589–593
44. **Sobhani I, Buyse M, Goiot H, Weber N, Laigneau JP, Henin D, Soul JC, Bado A** 2002 Vagal stimulation rapidly increases leptin secretion in human stomach. Gastroenterology 122:259–263
45. **Ritter RC, Kalivas P, Bernier S** 1986 Cholecystokinin-induced suppression of locomotion is attenuated in capsaicin pretreated rats. Peptides 7:587–590
46. **Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM** 1996 Leptin enters the brain by a saturable system independent of insulin. Peptides 17:305–311
47. **Fliedner S, Schultz C, Lehnert H** 2006 Brain uptake of intranasally applied radioiodinated leptin in Wistar rats. Endocrinology 147:2086–2094

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