Synergistic effect of CART (cocaine- and amphetamine-regulated transcript) peptide and cholecystokinin on food intake regulation in lean mice
Lenka Maletínská¹, Jana Maixnerová¹, Resha Matyšková¹, Renata Haugvicová², Zdeno Pirník³, Alexander Kiss³ and Blanka Železná*¹

Address: ¹Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 16610 Prague 6, Czech Republic, ²Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14200 Prague 4, Czech Republic and ³Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 83306 Bratislava, Slovak Republic

Email: Lenka Maletínská - maletin@uochb.cas.cz; Jana Maixnerová - maixnerova@uochb.cas.cz; Resha Matyšková - matyskova@uochb.cas.cz; Renata Haugvicová - haugvic@biomed.cas.cz; Zdeno Pirník - ueenpirn@savba.sk; Alexander Kiss - ueenkiss@savba.sk; Blanka Železná* - zelezna@uochb.cas.cz

* Corresponding author

Abstract

Background: CART (cocaine- and amphetamine-regulated transcript) peptide and cholecystokinin (CCK) are neuromodulators involved in feeding behavior. This study is based on previously found synergistic effect of leptin and CCK on food intake and our hypothesis on a co-operation of the CART peptide and CCK in food intake regulation and Fos activation in their common targets, the nucleus tractus solitarii of the brainstem (NTS), the paraventricular nucleus (PVN), and the dorsomedial nucleus (DMH) of the hypothalamus.

Results: In fasted C57BL/6 mice, the anorexigenic effect of CART(61-102) in the doses of 0.1 or 0.5 μg/mouse was significantly enhanced by low doses of CCK-8 of 0.4 or 4 μg/kg, while 1 mg/kg dose of CCK-A receptor antagonist devazepide blocked the effect of CART(61-102) on food intake. After simultaneous administration of 0.1 μg/mouse CART(61-102) and of 4 μg/kg of CCK-8, the number of Fos-positive neurons in NTS, PVN, and DMH was significantly higher than after administration of each particular peptide. Besides, CART(61-102) and CCK-8 showed an additive effect on inhibition of the locomotor activity of mice in an open field test.

Conclusion: The synergistic and long-lasting effect of the CART peptide and CCK on food intake and their additive effect on Fos immunoreactivity in their common targets suggest a co-operative action of CART peptide and CCK which could be related to synergistic effect of leptin on CCK satiety.

Background

Information on the metabolic status of the organism enters and is processed in the hypothalamus and in the nucleus tractus solitarii (NTS) of the brainstem (hind-brain). In the hypothalamic arcuate nucleus (ARC), adiposity signal leptin influences expression of peptides affecting food intake such as anorexigenic cocaine- and amphetamine-regulated transcript (CART). ARC neurons
project to other hypothalamic areas such as the paraven-
tricular nucleus (PVN) and the lateral hypothalamic area
(LHA) [for reviews, see [1-5]]. Both PVN and LHA convey
neuronal signals to the brainstem where they are inte-
grated with afferent input of cholecystokinin (CCK) [2],
satiety peptide of gut origin. For the satiety effect of CCK,
leptin signaling in ARC was found necessary [6]. Recently,
CCK was shown to facilitate access of leptin to hypo-
thalamic areas and modulate body weight [7].

Satiety effect of CCK is mediated by cholecystokinin A
(CCK-A or CCK-1) receptors [8] expressed abundantly not
only in the brainstem but also in the hypothalamus
[9,10]. Unlike CCK receptors, receptors of CART peptide
have not been found yet despite of a well-known anorex-
igenic effect of CART [3,11,12] and its stimulating effect
on anxiety-like reactions [13] or analgesia [14]. Analog-
gously, CART receptor antagonists have not been designed
yet.

After its peripheral administration, CCK affected neuronal
activity particularly in NTS, the area postrema, the locus
coeuruleus, PVN, and the dorsomedial nucleus (DMH)
[5,15-18]. Similarly, injection of the CART peptide either
into the third, fourth, or the lateral ventricle suppressed
food intake [11,12,14,19,20] and stimulated expression
of c-Fos in NTS, the parabranchial nucleus, PVN and
DMH [21,22].

Co-localization of CART peptide and CCK-A receptor in
vagal afferent neurons suggested that CART peptide might
take part in mediating satiety effects of cholecystokinin
[23]. Interestingly, a lowered leptin level after 48- hour
food deprivation affected expression neither of CCK-A
receptor [23] nor CART in nodose ganglion neurons [24].
However, recently, CART expression in rat vagal afferent
neurons was found negligible after 24-hour fasting, up-
regulated by CCK, and restored after re-feeding. The
action of CCK on CART expression was shown to be medi-
atied by activation of protein kinase C and cAMP response
element binding protein (CREB) and was inhibited by
orexigenic ghrelin [25].

Relationship between CCK and the CART peptide was
documented also at pancreatic exocrine secretion of amyl-
ase where the stimulating effect of CART peptide was
inhibited in vivo but not in vitro by CCK-A receptor antag-
onist devazepide [26].

Finally, a synergistic anorexigenic effect of CCK and CART
peptide was suggested in goldfish [27], but no experimen-
tal details were given.

Data on interaction of CART peptide with other peptides
regulating food intake have been scarce up to now.

Besides the well-known suppression of the orexigenic
effect of NPY by CART peptide [11,28], CART peptide-
induced hypophagia and brain c-Fos expression was pre-
vented by blocking central receptors for glucagon-like
peptide 1 (GLP-1) [29].

The previously described findings point to a neurochemi-
cal link between CART peptide and CCK with regard to a
previously found synergistic effect of leptin and CCK on
food intake [15]. Therefore, in the present study, the idea
is proposed that a co-operative action of the CART peptide
and CCK might be involved in communication between
ARC and NTS. Potential cooperation between central
cART peptide and peripheral CCK in the short-term regu-
lation of food intake in lean mice was investigated. To
compare neuronal activation after administration of
CART peptide, CCK, or simultaneous administration of
CART peptide and CCK, c-Fos activation in three impor-
tant brain areas involved in food intake regulation, PVN,
DMH, and NTS, was also determined. In addition, the
exploratory behavior of mice after administration of the
above-mentioned compounds is described, which is an
important element complementing food intake data.

Methods
Materials
Cholecystokinin octapeptide (CCK-8, Asp-Tyr(SO 3H)-
Met-Gly-Trp-Met-Asp-Phe-NH2, NeoMPS, Strasbourg,
France), CCK-A receptor antagonist devazepide
(L364,718) or CCK-B receptor antagonist L365,260 (gift
from ML Laboratories, Liverpool, UK) and CART(61-102)
(Bachem, Bubendorf, Switzerland) were used in the exper-
iments.

The Fos (No 94012) antiserum was kindly provided by
Dr. J.D. Mikkelsen (NeuroSearch A/S Ballerup, Denmark).
The specificity and sensitivity of Fos antiserum have
already been tested previously [30].

Experimental animals
Male C57BL/6 mice obtained from the Institute of Molec-
ular Genetics (Prague, Czech Republic) were housed in
standard conditions (temperature of 23°C, daily cycle of
12 h light and dark (light from 6:00)). They were given ad
libitum water and standard chow diet (St-1, Velaz, Koleè,
Czech Republic). They were 14–16 weeks old (25–30 g)
when cannulation and following experiments were per-
formed. All experiments followed the ethical guidelines
for animal experiments and the Act of the Czech Republic
Nr. 246/1992 and were approved by the Committee for
experiments with laboratory animals of the Academy of
Sciences of the Czech Republic.
Cannula placement
Mice were implanted with cannulas (Plastics One, Roanoke, USA) into the third ventricle (AP 2 mm, V 3 mm) as described earlier [31]. Thereafter, the animals were placed into separate cages with free access to food and water and allowed to recover from surgery at least seven days before starting the experiment.

Food intake experiments
Before starting the food intake experiment, the mice were randomly divided into groups of 6–8 mice and were fasted overnight (17 h) with free access to water.

On the day of the experiment, the individual groups of mice underwent the following treatments before being given weighed food (at t = 0 around 8:30) and the registration of food intake started.

Experiment 1 Individual and combined administration of CCK-8 and CART(61-102)
Doses, route of administration and time schedule are described in Table 1.

Experiment 2 Individual and combined administration of CCK receptor antagonists and CART(61-102)
Doses, route of administration and time schedule are described in Table 1.

The volume of i.p. injected solutions was 0.2 ml/mouse, i.c.v. injected solutions were of 5 μl/mouse and were infused in 20 s using an infusion pump; the infusion cannula was left in place for a further 20 s to prevent reflux. Each animal was used only once, the experiment was repeated with a new set of mice.

At t = 0 min, i.e. 15 min after i.c.v. injection of CART(61-102) or 20 min after i.p. injected CCK-8 and 45 min after i.p. injection of devazepide or L365,260, mice were given weighed food pellets. The pellets were replaced with fresh ones every 30 min and weighed. Food intake was followed for 5 h. Animals had free access to water during the experiment. The results are expressed in grams of food consumed per mouse. The placement of the cannula was verified histologically.

Fos Immunohistochemistry

Tissue processing
For Fos immunohistochemical processing, overnight fasted mice (n = 5 mice/group) were treated: a/i.c.v. with saline, b/i.p. with CCK-8 (0.4 μg/kg), c/i.c.v. with CART(61-102) (0.1 μg/mouse) or d/i.p. with CCK-8 followed by i.c.v. injected CART(61-102) (doses as described above). The time schedule of injections was identical as in the food intake study (Table 1). Sixty minutes after i.c.v. injection, the mice were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 0.1 M phosphate buffer (PB, pH 7.4) containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 10% picric acid (w/w). Then the brains were removed, postfixed in the same fixative overnight at 4°C, and infiltrated with 30%

Table 1: Administration of compounds in mice, in food intake Experiment 1 and 2

| Experiment | Compound | Dose i.p. | Time of application | Compound | Dose i.c.v. | Time of application | No. animals |
|------------|----------|-----------|---------------------|----------|-------------|---------------------|-------------|
| Experiment 1 | saline | -20 min | | | | | 8 |
| CCK-8 | 0.4 μg/kg | -20 min | | | | | 6 |
| | 4 | -20 min | | | | | 6 |
| CART | 0.1 μg/mouse | -15 min | | | | | 8 |
| | 0.5 | -15 min | | | | | 6 |
| Experiment 2 | devazepide | 1 mg/kg | -45 min | | | | 6 |
| | 1 | -45 min | CART | 0.1 μg/mouse | -15 min | | 6 |
| | 1 | -45 min | | 0.5 | -15 min | | 6 |
| L365,260 | 1 mg/kg | -45 min | | | | | 6 |
| | 1 | -45 min | | 0.5 | -15 min | | 7 |

At time = 0 min, mice were given weighed food pellets.
sucrose in 0.1 M PB for 48 h at 4°C. Before sectioning, the brains were rapidly (20 sec) frozen in cold isopentane (-30/-40°C) and placed into a Reichert cryocut device adjusted to -16°C for 1 h. Location of i.c.v. cannulas in the third ventricle was verified during sectioning and 30 μm coronal sections were cut from the brains and collected as free floating in cold (4°C) PB.

**Immunohistochemistry**

Free floating sections were repeatedly washed in cold PB followed by preincubation in 3% H₂O₂ for 40 min at room temperature. They were incubated with polyclonal Fos protein antiserum (1:2000), diluted in 0.1 M PB containing 4% normal goat serum (Gibco, Grand Island, NY, USA), 0.5% Triton X-100 (Koch-Light Lab. Ltd., Colnbrook, Berks, England), and 0.1% sodium azide for 48 h at 4°C. After several rinses in PB, the sections were incubated with biotinylated goat-anti-rabbit IgG (1:500, Vector Stain Elite ABC, Vector Lab., Burlingame, CA, USA) for 90 min at room temperature. Next PB rinses were followed by incubation with biotinylated goat-anti-rabbit IgG (1:500, Vector Stain Elite ABC, Vector Lab., Burlingame, CA, USA), 0.5% Triton X-100 (Koch-Light Lab. Ltd., Colnbrook, Berks, England), and 0.1% sodium azide for 48 h at 4°C. After several rinses in PB, the sections were incubated with avidin-biotin peroxidase complex (1:250) for 90 min at room temperature. PB washing was followed by washing in 0.05 M sodium acetate buffer (SAB, pH 6.0). The Fos antigenic sites were visualized with 0.0266% 3,3′-diaminobenzidine tetrahydrochloride (DAB) dissolved in SAB containing 0.0042% H₂O₂ and 2.5% nickel ammonium sulfate, for 7 min. The metal-intensification of DAB produced black staining in the labeled nuclei. Finally, the sections were rinsed in 0.05 M SAB, mounted onto 0.1% of gelatine dissolved in 0.0125 M SAB, air-dried and coverslipped with Permount (Sigma, St. Louis, MO, USA). Immunostaining of negative control, which did not show any antiserum immunolabeling, included substitution of the primary antiserum with normal rabbit serum, and sequential elimination of the primary or secondary antibody from the staining series.

**Evaluation of the immunostaining**

An identical set of mice was used for determination of Fos immunoreactivity in NTS, PVN, and DMH. Counting of Fos immunoreactive cells within the NTS, from Bregma -7.48 mm to Bregma -7.32 mm, PVN, from Bregma -0.7 mm to -0.94 mm, and DMH, from Bregma -1.46 mm to –1.82 mm according to the mouse brain atlas [32], was performed separately in each side of the sections. Quantitative assessment was performed from the images captured with a Canon digital camera (PowerShot S40) and Leica DMLS light microscope in a computer screen obtained from 5–6 brain sections per animal. Representative sections were captured by the same computerized system. The counting of Fos-positive neurons was done by one of the authors under blinded conditions (the counted slides from each animal were analyzed independently and randomly and encoded by other person).

**Open field locomotor activity**

Locomotor activity was measured using the VideoMot system (TSE Systems, Bad Homburg, Germany). *Ad libitum* fed mice were placed individually in the open field (1 × 1 m) and their locomotor activity, i.e. total distance traveled, was measured for 10 min. Mice were administered with i.p. injection of CCK-8 (4 μg/kg), i.p. injection of devazepide (1 mg/kg), i.c.v. administration of CART(61-102) (0.1 and 0.5 μg/mouse) or their combination with a time schedule as described in feeding experiments in Table 1 (n = 5 mice/group).

**Statistics**

Data are presented as means ± SEM for the number of animals indicated in the Figures. They were analyzed by the non-repeated measures one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test using GraphPad Software (San Diego, CA, USA). P < 0.05 was considered statistically significant.

**Results**

**Synergistic action of CART peptide and CCK-8 in food intake of fasted lean mice**

### Experiment 1

Cumulative food intake of male C57BL/6 mice was measured for all doses of compounds described in Methods (Table 1) and is illustrated by curves in Fig. 1. The feeding response to saline injected i.p. or i.c.v. did not show any significant difference.

CART(61-102) at a dose of 0.5 μg/mouse significantly attenuated food intake up to 105 min after its i.c.v. injection with maximum between 45 and 75 min after i.c.v. injection, i.e. after the first and second measurements of food intake (Fig. 1). A dose-dependent anorexigenic effect of CART(61-102) is obvious in Fig. 2a. In C57BL/6 fasted mice, CCK-8 doses of 0.4 and 4 μg/kg i.p. did not significantly modify food intake (Fig. 2a).

Simultaneous application of CCK-8 and CART(61-102) reduced food intake more significantly than each single peptide (Fig. 1, 2a), and the effect lasted for more than five hours (Fig. 1). Doses of CCK-8 (0.4 and 4 μg/kg) increased the anorexigenic effect of CART(61-102) in a dose-related way (Fig. 2a). Table 2 summarizes values of food intake at the time of maximal effect of administered peptides (30 minutes after addition of pellets).

### Experiment 2

CCK-A receptor inactivation with its antagonist devazepide prevented the anorexigenic effect of CART(61-102) (0.1 and 0.5 μg/mouse), whereas the specific CCK-B receptor antagonist L-365,260 did not affect the CART-induced decrease in food intake (Fig. 2b, Table 2).
Fos immunoreactivity in NTS, PVN, and DMH after CART peptide and CCK administration

The doses of CCK-8 (4 μg/kg) and CART(61-102) (0.1 μg/mouse) used by us for determination of Fos immunoreactivity correspond to those used previously by others [18,19,30]. Statistical analysis by one way ANOVA documented the impact of CCK-8, CART(61-102) as well as their combined treatment on the number of Fos immunopositive cells both in NTS (F_{3,16} = 26.23, p < 0.000001), PVN (F_{3,13} = 100.35, p < 0.000001), and DMH (F_{3,12} = 58.19, p < 0.000001) in lean C57BL/6 mice (Fig. 3).

In NTS, the Fos immunoreactivity in saline-treated controls was minimal and sporadic (Fig. 3a). The number of Fos immunoreactive cells distinctly increased after i.p. application of CCK-8 (0.4 μg/kg) compared with saline-treated mice (P < 0.05) (Fig. 3a, d). A similar, but statistically insignificant increase was observed after i.c.v. infusion of CART(61-102) (0.1 μg/mouse). The post hoc test showed a significant effect (P < 0.01) of parallel administration of CCK-8 and CART peptide on the Fos activation of cells in NTS in comparison with all other groups of animals (Fig. 3a, d).

In PVN and DMH, Fos immunoreactivity followed a similar trend as in the NTS. In control animals, a weak Fos signal was found (Fig. 3b,c). A significant increase in Fos immunoreactivity was registered after application of CCK-8 (p < 0.01), CART(61-102) (p < 0.01), and parallel administration of CCK-8 and CART(61-102) (p < 0.01) in comparison with saline-treated mice (Fig. 3b, d). The post hoc test revealed a significantly higher increase in the number of Fos-immunopositive cells in PVN and DMH after CART(61-102) application compared with application of CCK-8 (Fig. 3b, c); simultaneous application of the above-mentioned peptides (p < 0.01) activated Fos in PVN and DMH more significantly than each particular peptide alone (p < 0.01).
Dose-related inhibition of food intake in 17-h fasted mice after administration of: A/ CCK-8 (0.4 and 4 μg/kg, i.p.) or i.c.v. saline, CART(61-102) (0.1 and 0.5 μg/mouse, i.c.v.), CCK-8 (0.4 and 4 μg/kg, i.p.) plus CART(61-102) (0.1 and 0.5 μg/mouse, i.c.v.), or B/ i.p. devazepide (1 mg/kg) alone or followed by i.c.v. CART(61-102) (0.1 and 0.5 μg/mouse) and i.p. L365,260 (1 mg/kg) alone or followed by i.c.v. CART(61-102) (0.5 μg/mouse). Food intake is expressed in grams of food consumed in the first 30 min after presentation of food (n = 6–8 mice per group). ** P < 0.01, *** P < 0.001 vs. saline-treated group, + P < 0.05 vs. CART(61-102) 0.1 μg/mouse, ++ P < 0.05 vs. CART(61-102) 0.5 μg/mouse [ANOVA, F_{12,78} = 13.88]. CART – CART(61-102), dev – devazepide, L365 – L365,260.

Figure 2
Dose-related inhibition of food intake in 17-h fasted mice after administration of: A/ CCK-8 (0.4 and 4 μg/kg, i.p.) or i.c.v. saline, CART(61-102) (0.1 and 0.5 μg/mouse, i.c.v.), CCK-8 (0.4 and 4 μg/kg, i.p.) plus CART(61-102) (0.1 and 0.5 μg/mouse, i.c.v.), or B/ i.p. devazepide (1 mg/kg) alone or followed by i.c.v. CART(61-102) (0.1 and 0.5 μg/mouse) and i.p. L365,260 (1 mg/kg) alone or followed by i.c.v. CART(61-102) (0.5 μg/mouse). Food intake is expressed in grams of food consumed in the first 30 min after presentation of food (n = 6–8 mice per group). ** P < 0.01, *** P < 0.001 vs. saline-treated group, + P < 0.05 vs. CART(61-102) 0.1 μg/mouse, ++ P < 0.05 vs. CART(61-102) 0.5 μg/mouse [ANOVA, F_{12,78} = 13.88]. CART – CART(61-102), dev – devazepide, L365 – L365,260.
Behavioral effect after parallel CART peptide and CCK-8 injection

Open field locomotor activity of fed lean mice was measured for 10 min after i.c.v. administration of CART(61-102), i.p. administration of CCK-8 and devazepide or their combination (see Methods). The goal was to find out whether in behavioral tests, the additive effect of CART peptide and CCK-8 occurred similarly as in the food intake experiments. The results clearly showed (Fig. 4) that the CART peptide (0.5 μg/mouse) alone or in combination with CCK-8 (4 μg/kg) significantly shortened the distance traveled in the open field compared to that of the saline-treated group. The decrease of locomotor activity after CART peptide treatment was reversed by CCK-A antagonist devazepide (Fig. 4).

Discussion

In this study, the effect of simultaneous administration of CART peptide and CCK on food intake, Fos immunoreactivity in NTS, PVN, and DMH and locomotor activity points to a positive cooperation between the CART peptide and CCK.

Intracerebroventricular injections of CART(61-102) into the third ventricle of fasted C57BL/6 mice attenuated food intake (Fig. 1, 2) in a dose-dependent manner similarly as in the studies of Thim et al. [12], Bannon et al. [14] and Vrang et al. [22]. At the doses of 0.4 and 4 μg/kg, CCK-8 did not show any significant effect on food intake in fasted C57BL/6 mice (Fig. 2a) (with EC50 about 15 μg/kg determined in a preliminary study), unlike fasted outbred NMRI mice that were sensitive to CCK-8 dose of 4 μg/kg (with EC50 about 9 μg/kg [31]). Serum leptin after 17 h fasting was 50% higher in the robust NMRI mice compared to the subtle C57BL/6 mice (0.45 ± 0.14 ng/ml in NMRI [33] and 0.30 ± 0.06 ng/ml in C57BL/6 male mice, our non-published data), which was probably the reason why the threshold dose for CCK-8 was higher in C57BL/6 than in NMRI mice (4 versus 0.4 μg/kg, respectively).

The 48-hour long fasting was shown to attenuate the satiety response to CCK because of a lowered leptin level [34]. Barrachina et al. [15] demonstrated that leptin cooperated synergistically with satiety effect of CCK in 24-hour fasted C57BL/6 mice [15]. Therefore in this study, it is supposed that synergistic co-operation of leptin and CCK was preserved in 17-hour fasted C57BL/6 mice despite lowered leptin level after fasting.

In this study, low doses of CCK enhanced the anorexigenic effect of CART peptide in a dose-dependent way and prolonged the time of the CART peptide effect (Fig. 1) that was in agreement with our hypothesis on a synergistic effect of CART(61-102) and CCK-8 on food intake (Fig. 2a). A direct involvement of CCK-A receptor in the anorexigenic effect of CART peptide was demonstrated in this study, because selective CCK-A receptor antagonist devazepide [8] was found to block the effect of CART peptide on food intake (Fig. 2b). Similarly, the anorexigenic action of CART peptide in rats was prevented by GLP-1 receptor antagonist [29]. The fact that L365,260, selective CCK-B receptor antagonist, which is not involved in food...
**Figure 3**

**Fos immunoreactivity:** Fos-immunostained cells in coronal section of A/ NTS, B/ PVN, C/ DMH, D/ Number of Fos-immunopositive cells in NTS, PVN and DMH 60 minutes after i.c.v. application of saline (SAL), CCK-8 (4 μg/kg, i.p.), CART(61-102) (0.1 μg/mouse, i.c.v.), and their combination expressed unilaterally per animal (n = 4–5) and section (n = 5–6). *P < 0.01 vs. SAL, CCK and CART, **P < 0.05, ***P < 0.01 vs. SAL [ANOVA, for NTS F3,16 = 26.23, for PVN F3,13 = 100.35, for DMH F3,12 = 58.19]. SAL – saline, NTS – solitary tract nucleus, CC – central canal, AP – area postrema, PVN – paraventricular nucleus. Bar = 100 μm.
intake regulation [8], did not alter the decrease in food intake induced by the CART peptide (Fig. 2b) supported the idea of the involvement of exclusively CCK-A receptor type in its co-operation with the CART peptide.

Co-administration leptin with CCK-8 in a dose of 3.5 μg/kg i.p. that was considered subthreshold potentiated satiety and activation of brainstem neurons in lean C57BL/6 mice and also enhanced the number of Fos-positive neurons in PVN [15]. In this study, the Fos immunoreactivity after injection of the CART peptide or/and CCK-8 was determined in NTS, PVN, and DMH, common targets of CART peptide [21, 22] and CCK [5, 15-18], in the identical set of mice. A strong increase in the number of Fos-positive cells was found (Fig. 3) when the dose of CART peptide that significantly attenuated food intake was co-administered with a dose of CCK-8 that did not influence food intake; this response was more significant than the response to each particular peptide. This result confirmed the co-operation between the CART peptide and CCK-8 in the brainstem and hypothalamic PVN and DMH, where satiety signals originating in the periphery as well as in the forebrain are processed. PVN is highly innervated byafferent projections from DMH. It was shown that CCK might activate corticotropin-releasing factor (CRF) neurons in DMH [18] via noradrenergic projections from NTS [35]. This pathway could contribute to the additive effect of CCK and CART peptide in the DMH.

Finally, behavioral data followed the trend of the feeding tests. CART peptides are known to influence locomotor activity and analgesia [13, 14]. However, the significance of the effects differed in different studies. In this study, a dose of CCK-8 that did not attenuate food intake (4 μg/kg i.p.) did not also significantly lower the locomotor activity of C57BL/6 mice in the open field test (Fig. 4), while CART(61-102) at a dose of 0.5 μg/mouse and its combination with i.p. CCK-8 or i.c.v. CART(61-102) significantly suppressed the distance traveled in open field test. Analogously, devazepide alone did not significantly affect the locomotor activity of mice compared to the saline-treated group but blocked the effect of CART peptide (Fig. 4). These findings again suggest that the effect of CART peptide was pronounced through activation of CCK-A receptor and point to satiety-related sedation. Analgesic hot-plate test was also performed, but none of the above-mentioned compounds
significantly changed the paw-licking latency compared to the saline-treated group (data not shown).

The long lasting effect of synergistic action of CCK and the CART peptide in this study could be interpreted according to de Lartigue et al. [25] as a result of CCK-induced expression of CART and a consequent effect of the endogenously produced CART peptide after the exogenous CART peptide was exhausted. On the other hand, Broberger et al. described that expression of CCK-A receptor does not depend on the metabolic status [23].

The integratory role of the ARC-PVN-DVC (dorsal vagal complex in which NTS is included) was suggested for the synergistic action of leptin and CCK on the attenuation of food intake and body weight [15]. Food intake was affected by parallel administration of leptin and CCK more potently than by individual application of the hormones and lasted at least 7 h. Leptin and CCK activation pathways appeared to overlap at three discrete nuclei: the PVN, parabrachial nucleus and NTS [5].

Conclusion

In this study, the synergistic effect of CCK and CART peptide on food intake targeted hypothalamic PVN and DMH and NTS, which suggested that CART peptide might act as a mediator of the leptin anorexigenic effect both in the hypothalamus and brainstem. Identification of the CART peptide receptor and designing its antagonist is necessary for identification of further possible cooperative actions of the CART peptide.

Authors’ contributions

LM and BŽ designed the study, drafted the manuscript and performed the statistical analysis. LM, JM, RM and BŽ carried out the majority of the experiments and data collection. RH participated in implantation of cannulas. ZIP and AK performed immunohistochemistry. All authors read and approved the final manuscript.

Acknowledgements

The study was supported by grants 303/05/0614 of the Grant Agency of the Czech Republic and Z40550506 of the Academy of Sciences of the Czech Republic.

We gratefully acknowledge the excellent technical assistance of A. Vytejčková and H. Vysúšilová.

References

1. Broberger C: Cocaine- and amphetamine-regulated transcript (CART) and food intake: behavior in search of anatomiy. Drug Dev Res 2000, 51:124-142.
2. Broberger C: Brain regulation of food intake and appetite: molecules and networks. J Int Med 2005, 258:301-327.
3. Hunter RG, Philpot K, Vicentie A, Dominguez G, Hubert GW, Kuhar MJ: CART in feeding and obesity. TRENDS Endocrinol Metab 2004, 15:454-459.
4. Luckman SM, Lawrence CB: Anorectic brainstem peptides: more pieces to the puzzle. TRENDS Endocrinol Metab 2003, 14:60-65.
5. McMinn JE, Baskin DG, Schwartz MW: Neuroendocrine mechanisms regulating food intake and body weight. Obes Rev 2000, 1:37-46.
6. Morton GJ, Blevins JE, Williams DL, Niswender KD, Gelling RW, Rhodes GJ, Baskin DG, Schwartz MW: Leptin action in the forebrain regulates the hindbrain response to satiety signals. J Clin Invest 2005, 115:703-710.
7. Merino B, Cano V, Guzman R, Somoza B, Ruiz-Gayo M: Leptin-mediated hypothalamic pathway of cholecystokinin (CCK-8) to regulate body weight in free-feeding rats. Endocrinology 2008, 149:1994-2000.
8. Moran TH, Ameglio PJ, Schwartz GJ, McHugh PR: Blockade of type A, not type B, CCK receptors attenuates satiety actions of exogenous and endogenous CCK. Am J Physiol 1992, 263:R89-R95.
9. Lodge DJ, Lawrence AJ: Comparative analysis of the central CCK system in Fawn Hooded and Wistar Kyoto rats: extended localisation of CCK-A receptors throughout the rat brain using a novel radioligand. Regul Pept 2001, 99:191-201.
10. Monnikes H, Lauer G, Arnold R: Peripheral administration of cholecystokinin activates c-fos expression in the locus coeruleus/subcoeruleus nucleus, dorsal vagal complex and paraventricular nucleus via capsaicin-sensitive vagal afferents and CCK-A receptors in the rat. Brain Res 1997, 770:277-288.
11. Kobelt P, Tebbe JJ, Tjandra I, Stengel A, Bae HG, Andreassen V, Voort IR van der, Veh RW, Werner CR, Klapp BF, Wiedenmann B, Wang L, Taché Y, Monnikes H: CCK inhibits the orexigenic effect of peripheral ghrelin. Am J Physiol Regul Integr Comp Physiol 2005, 288(3):R751-R758.
12. Kobelt P, Paulitsch S, Goebel M, Stengel A, Schmidtmann M, Voort IR van der, Tebbe JJ, Veh RW, Klapp BF, Wiedenmann B, Taché Y, Monnikes H: Peripheral injection of CCK-8S induces Fos expression in the dorsomedial hypothalamic nucleus in rats. Brain Res 2006, 1117:109-117.
13. Aja S, Sahandy S, Ladenaem HE, Schwartz GJ, Moran TH: Intracerebroventricular CART peptide reduces food intake and alters motor behavior at a hindbrain site. Am J Physiol Regul Integr Comp Physiol 2001, 281(6):R1862-R1867.
14. Zheng H, Patterson C, Berthoud H-R: Fourth ventricular injection of CART peptide inhibits short-term sucrose intake in rats. Brain Res 2001, 869:135-156.
15. Zheng H, Patterson LM, Berthoud H-R: CART in the dorsal vagal complex: sources of immunoactivity and effects on Fos expression and food intake. Brain Res 2002, 957:298-310.
16. Vrang N, Tang-Christensen M, Larsen PJ, Kristensen P: Recombinant CART peptide induces c-Fos expression in central areas involved in control of feeding behaviour. Brain Res 1999, 818:499-509.
23. Broberger C, Holmberg K, Kuhar Mj, Hökfelt T. Cocaine- and amphetamine-regulated transcript in the nervus vagus nerve: A putative mediator of cholecystokinin-induced satiety. Proc Natl Acad Sci USA 1999, 96:13506-11.

24. Broberger C, Holmberg K, Shi T-J, Dockray, Hökfelt T. Expression and regulation of cholecystokinin and cholecystokinin receptors in rat nodose and dorsal root ganglia. Brain Res 2001, 903:128-140.

25. de Lartigue G, Dimaline R, Varro A, Dockray GJ. Cocaine- and amphetamine-regulated transcript: stimulation of expression in rat vagal afferent neurons by cholecystokinin and suppression by ghrelin. J Neurosci 2007, 27:2876-2882.

26. Cowles RA, Segura BJ, Mulholland MW. Stimulation of rat pancreatic exocrine secretion by cocaine- and amphetamine-regulated transcript peptide. Regul Pept 2001, 99:61-68.

27. Volkoff H. The role of neuropeptide Y, orexins, cocaine and amphetamine-related transcript, cholecystokinin, amylin and leptin in the regulation of feeding in fish. Comp Biochem Physiol Part A 2006, 144:325-31.

28. Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y, Kuhar Mj. CART peptides in the central control of feeding and interactions with neuropeptide Y. Synapse 1998, 29:293-298.

29. Aja S, Ewing C, Lin J, Hyun J, Moran TH. Blockade of central GLP-1 receptors prevents CART-induced hypophagia and brain c-Fos expression. Peptides 2006, 27:157-164.

30. Kiss A, Palkovits M, Skirboll LR. Light microscopic-triple-colored immunohistochemical staining on the same vibratome section using the avidin-biotin-peroxidase complex technique. Histochemistry 1988, 88:353-356.

31. Maletínská L, Maixnerová J, Malýsková R, Haugvicová R, Šloncová E, Elbert T, Slaninová J, Zelezná B. Cocaine- and amphetamine-regulated transcript (CART) peptide specific binding in pheochromocyto ma cells PC12. Eur J Pharmacol 2007, 559:109-114.

32. Franklin KBj, Paxinos G. The mouse brain in stereotaxic coordinates New York: Academic Press; 1997.

33. Maletínská L, Shamas Toma R, Pirník Z, Kiss A, Slaninová J, Haluzík M, Zelezná B. Effect of cholecystokinin on feeding is attenuated in monosodium glutamate obese mice. Regul Pept 2006, 136:58-63.

34. McMinn JE, Sindelar DK, Havel Pj, Schwartz MW. Leptin deficiency induced by fasting impairs the satiety response to cholecystokinin. Endocrinology 2000, 141:4442-4448.

35. Sawcheko PE, Swanson LW. The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. Brain Res 1982, 257:275-325.