CCK-8 and CCK-58 differ in their effects on nocturnal solid meal pattern in undisturbed rats

Running head: CCK-8 and CCK-58: Effect on feeding pattern


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Abstract

Various molecular forms of cholecystokinin (CCK) reduce food intake in rats. Although CCK-8 is the most studied form, we reported that CCK-58 is the only detectable endocrine peptide form in rats. We investigated the dark phase rat chow intake pattern following injection of CCK-8 and CCK-58. Ad libitum fed male Sprague-Dawley rats were intraperitoneally injected with CCK-8, CCK-58 (0.6, 1.8 and 5.2 nmol/kg), or vehicle. Food intake pattern was assessed during the dark phase using an automated weighing system allowing continuous undisturbed monitoring of physiologic eating behavior. Both CCK-8 and CCK-58 dose-dependently reduced 1-h dark phase food intake with an equimolar dose of 1.8 nmol being similarly effective (-49% and -44%). CCK-58 increased the latency to the first meal, whereas CCK-8 did not. The inter-meal interval was reduced after CCK-8 (1.8 nmol/kg, -41%) but not after CCK-58. At this dose, CCK-8 increased the satiety ratio by 80% and CCK-58 by 160%, respectively, compared to vehicle. When behavior was assessed manually, CCK-8 reduced locomotor activity (-31%), whereas grooming behavior was increased (+59%). CCK-58 affected neither grooming nor locomotor activity. In conclusion, reduction of food intake by CCK-8 and CCK-58 is achieved by differential modulation of food intake microstructure and behavior. These data highlight the importance of studying the molecular forms of peptides that exist in vivo in tissue and circulation of the animal being studied.

Keywords: dark phase, meal microstructure, satiation, satiety, satiety ratio, solid food
Introduction

Bayliss and Starling first reported in 1902 that intestinal extracts stimulated pancreatic secretion (3). Ivy and Oldenberg showed in 1928 that a different preparation of intestinal extracts caused contraction of gallbladder and termed the principle “cholecystokinin” (CCK) (27). In 1943 Harper and Raper demonstrated that pancreatic secretion could be stimulated by intestinal extracts and named the principle “pancreozymin” (24). During the purification of CCK, Mutt and Jorpes observed that a biological activity causing pancreatic enzyme secretion co-purified with the CCK activity and suggested that these two activities were exerted by one molecule, then termed cholecystokinin-pancreozymin later shortened to cholecystokinin and abbreviated CCK (29). CCK was originally described as a 33 amino acid peptide (38) expressed in small intestinal endocrine I-cells (40). Since then several forms of CCK have been characterized chemically from intestinal extracts in multiple species, namely CCK-58 (41, 44), CCK-39 (28), CCK-33 (47), CCK-22 (4, 11, 34), and CCK-8 (16, 35). In rats, endocrine forms suggested from chromatography of plasma samples and assay of fractions collected have varied from laboratory to laboratory. The major forms reported were CCK-8 (23, 35), CCK-33/39 (35) and CCK-58 (42). An important observation is that endocrine forms smaller than CCK-58 can be produced ex vivo during the processing of plasma samples. This could explain the disparity of results obtained when blood was not collected under conditions that prevented degradation of large molecular forms, because there are variable extents of degradation during processing of the blood. We recently established a novel blood processing method, the RAPID method, employing reduced temperatures, acidification, protease inhibition, isotopic exogenous controls and dilution that prevents degradation of endocrine cholecystokinin as blood is processed (54). Using this method the recovery of exogenous radiolabeled CCK-58 added to rat blood was improved from 20% to 88% when compared to standard blood processing (EDTA blood on ice and plasma formation) (54). Moreover, RAPID processing resulted in detection of the added exogenous 125I-CCK-58, whereas after standard blood processing, 100% of the labeled CCK-58 was degraded into smaller forms. This has led us to suggest that small forms such as CCK-8, CCK-22 and CCK-33 observed
by others are produced ex vivo during formation and processing of plasma and we demonstrated that there is no production of smaller forms during the RAPID method (54).

If rat blood is processed in a manner that prevents degradation of $^{125}$I-CCK-58, the only endocrine form detected is CCK-58 (42) and endogenous endocrine CCK-8 detected by others (23, 35) is most likely a product of peptide degradation during plasma formation. Importantly, CCK-58 is the major intestinal (8, 14) and endocrine (9, 13) form in dogs (8, 13), rats (46), and humans (8, 9, 14) suggesting its biological action should be evaluated and the influence of CCK-58 on feeding patterns should be studied. The importance of this suggestion is indicated by the fact that CCK-8 and CCK-58 show different actions on various physiological processes. However, most reports have used CCK-8 to study gastrointestinal functions. The difference between the most studied form (CCK-8) and the most abundant form (CCK-58) of cholecystokinin is demonstrated by patterns of pancreatic secretion elicited by the two peptides.

Intravenous (iv) infusion of CCK-58 but not CCK-8 ranging from 62.5 to 1,000 pmol/kg/h over a 3-h period strongly stimulated pancreatic fluid secretion in a dose-dependent fashion in an in vivo rat model (46, 62). CCK-58 induces a more sustained pancreatic protein secretion than CCK-8 as shown by the return to basal values at the end of the 3-h infusion period with CCK-8 whereas protein output remained significantly elevated compared with basal in CCK-58-treated rats (46, 62). In addition, iv infusion of CCK-58 at 2 or 4 nmol/kg/h for 6 h in a conscious rat model did not induce pancreatitis at a dose where CCK-8 induced several parameters indicative of pancreatitis including interstitial edema, inflammatory cell infiltration, intracellular vacuolization, increased pancreatic myeloperoxidase activity and elevated serum amylase levels (61).

In 1973, Smith and Gibbs originally reported that synthetic CCK-8 and natural CCK-33 reduce food intake (19) opening the path to a multitude of investigations on the importance of this hormone in feeding regulation. Most of these studies were performed using CCK-8 because of its availability and the concept that all forms of CCK that contain a sulfated tyrosine would have the same biological activity (26). However, as described above we have reported that with proper blood processing CCK-58 is the only detectable circulating form of rat CCK (42).
CCK-58 exert differential effects on food intake although this has not been characterized in detail. One report suggested that although CCK-8 and CCK-58 at the doses of 3.5 and 7 nmol/kg equally reduced food intake at 30 min post intraperitoneal (ip) injection at the onset of the dark phase in rats that were fasted for 4 hours, only the reduction of food intake induced by CCK-58 at an ip dose of 7 nmol/kg was preserved over a period of 210 min (20). However, in their study, Glatzle et al. used ground powder rat chow that does not resemble normal rat diet and did not evaluate changes in feeding behavior to assess whether it was related to changes in inter-meal interval (IMI) or other effects on the pattern of feeding behavior. In another study duodenal fat infusions reduced meal size and prolonged IMI which could be attenuated by the specific CCK₁ antagonist devazepide (5, 59). However, exogenous CCK-58 has not been evaluated for its influence on IMI.

In the present study we first compared the effects of CCK-8 and CCK-58 injected ip on dark phase food intake, the photoperiod where rats normally eat (48). Since the analysis of the feeding microstructure is essential to assess the mechanisms regulating feeding behavior (18), we investigated the feeding pattern using a newly developed automated episodic food intake monitoring device which allows the undisturbed continuous monitoring during the dark phase with minimal human interference and has been used in rats before (15, 31, 39, 49) and was established recently for the use in mice (21, 52). To investigate the specificity of the observed effects on feeding behavior, other behavioral parameters such as grooming and locomotor activity were assessed as established originally by Antin and co-workers (1) and in our previous studies (50, 53).
Materials and Methods

Animals

Adult male Sprague-Dawley rats (Harlan, San Diego, CA) weighing 280–350g were group housed under controlled illumination (0600-1800h) and temperature (21–23°C) until the start of experiments. Animals had free access to standard rodent chow (Prolab RMH 2500; LabDiet, PMI Nutrition, Brentwood, MO) and tap water. All experiments were started at the onset of the dark phase. Rats had resting periods of at least 3 days between experiments. Protocols were approved by the Institutional Animal Care and Use Committee of the Veterans Administration (#99059-04 and #11045-09).

Substances

Sulfated CCK-8 (CCK-8S; Bachem, Torrance, CA) and rat CCK-58 (43) were dissolved in 1 ml 0.1% trifluoroacetate, concentrations determined by their absorbance at 280 nm, diluted 50:50 with 0.2% bovine serum albumin (BSA, Sigma–Aldrich, St. Louis, MO, USA), divided to 10 pmole aliquots and dried using vacuum centrifugation. Peptide powder was stored at -80°C and dissolved in saline containing 0.1% BSA for a final ip injection volume of 0.5 ml/rat immediately before use.

Food intake microstructure

Food intake pattern was assessed as established in our previous studies (21, 52) using an automated episodic food intake monitoring system for regular pellet rodent food adapted for rats (BioDAQ, Research Diets, Inc., New Brunswick, NJ). Water was provided ad libitum from regular water bottles. Rats were housed singly and habituated to the system for one week. Animals adapted to the system quickly as indicated by normal food intake and body weight gain already at the second day of the habituation period. During that time, rats were also handled mimicking ip injection including practice of the upside down position.
The food intake monitoring system weighs the hopper with food (± 0.01 g) second by second and detects 'not eating' as weight stable and 'eating' as weight unstable. Feeding bouts (changes in stable weight before and after bout) are recorded with a start time, duration, and amount consumed. Bouts are separated by an inter-bout interval (IBI), and meals consist of one or more bouts separated by an inter-meal interval (IMI). The minimum IMI was defined as 15 min, the minimum meal amount as 0.1 g. Therefore, food intake was considered as one meal when the feeding bouts occurred within 15 min of the previous response and their sum was equal to or greater than 0.1 g. When bouts of feeding were longer than 15 min apart, they were considered as a new meal. The software continuously records every action related to rats touching the food hopper. Meal parameters extracted from the software (BioDAQ Monitoring Software 2.2.02) for these studies included latency to the first meal, duration of first meal, meal size of first and second meal, IMI between first and second meal and rate of ingestion of first meal. Amounts of food consumed for each photo period and for the entire 24-h period for the day of the study were also obtained. The satiety ratio (IMI in minutes between first and second meal / first meal size in grams) was calculated.

On the day of the experiment gates were closed to prevent pre-dark phase food intake 90 min before the onset of the dark phase and for maintenance (cleaning hoppers, refilling food, body weight monitoring). Immediately before lights off, rats were injected ip with CCK-8 or CCK-58 (0.6, 1.8 and 5.2 nmol/kg, n= 6-12 animals/group), or vehicle (saline containing 0.1% BSA, n=9-11 animals/group), placed back in their home cage, the gates opened, recording started and lights turned off. The time needed to inject rats was ~ 30 sec/rat. The dose of CCK was based on previous studies showing a robust reduction of food intake following injection of 2nmol/kg CCK-8 (17, 56). Experiments were repeated in a crossover design in the same batch of rats for each treatment.

The study design had to be modified for the assessment of the latency to eat the first meal where gates remained open during the 90 min before the dark phase and rats were ip injected before lights off as described above. Food intake microstructure was assessed during the dark phase. In these studies maintenance was performed 6 hours before lights out.
We have previously established the automated system in mice and shown the same food intake as observed manually during light and dark phase (52). Since these data were generated in a different species we performed an additional experiment in rats to validate automated vs. manual measurement of dark phase food intake. Rats habituated to the automated feeding system were randomly divided in two groups (n=7-8) and housed in automated cages connected to the computer (feeding from hopper) or unplugged cages (feeding from cage top). Gates were closed 90 min before lights off as done in the previous experiments and similarly, food pellets were removed from the unplugged cages. Rats were ip injected with vehicle (saline containing 0.1% BSA) right before lights off and food was made available or gates were opened respectively. Food intake of standard rodent diet was monitored for 60 min by manual assessment or in an automated fashion. The experiment was repeated in a crossover design in the same batch of rats.

Behavior

Just prior to the dark phase, singly housed, freely fed animals were injected ip with CCK-8 (n=6) or CCK-58 (n=8) at the equimolar dose of 1.8 nmol/kg or vehicle (n=8) and placed in their home cage with a paper grid under the cage divided into six equal squares. Since CCK-8 and CCK-58 dose-dependently reduced 1-h dark phase food intake with an equimolar dose of 1.8 nmol being similarly effective, this dose was chosen for the behavioral monitoring. Immediately after injection lights were turned off and pre-weighed rat chow replaced the daily chow. Rats had free access to food and water before and during the experiment. Behavior was assessed manually and simultaneously in 4 rats/investigator as described in our previous studies (50, 51, 53). This method requires a time-sampling technique by two investigators who performed all experiments. Inter-investigator variability was <5 %. Briefly, there was a latency of 10 min after lights off, then behaviors including grooming (washing, licking and scratching), locomotor activity, eating and drinking were monitored manually for 30 min by the observers who sat motionless in front of the cages with a dim light allowing surveillance of the animals and a silent timer to keep track of time in seconds. Eating behavior was defined as eating as well as food approach which consists of sniffing and
licking food. Drinking behavior included drinking and water approach as defined by sniffing. Locomotor activity was defined as at least one rat paw crossing the boundary of one square. The total number of squares crossed was counted. The investigators were blinded to the animals’ treatment. Each behavior was counted again when it lasted >5 sec. In the behavior experiments food intake was assessed manually at one time point (40 min) by measuring the weight difference of rat chow before and after the 40-min period.

Statistical analysis

Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey post hoc test and two-way ANOVA followed by Holm-Sidak method. \( P<0.05 \) was considered significant. Correlations were determined by univariate linear regression. Due to the differential experimental set-up the analysis for latency to the first meal was conducted separately from analyses of the other feeding parameters.
Results

Automated and manual food intake monitoring yield similar results

No differences in amount of food intake during the first h after lights off were observed using the two different methods of examination. Rats that were monitored by the automated system ate similar amounts as rats in which food intake was assessed manually (4.41 ± 0.48 vs. 4.67 ± 0.35 g, P>0.05; data not shown).

CCK-58 is more potent than CCK-8 at prolonging latency to first meal

In the only experiments with gates open (access to food) before lights off, CCK-8 increased the latency to the first meal only at the highest dose used (5.2 nmol/kg, ip) by 4-times compared to vehicle (F(3,25) = 4.7, P<0.05; 48.8 ± 17.0 vs. 12.2 ± 4.7 min, P<0.05; 5.2 nmol/kg vs. vehicle) and by 15-times compared to the 0.6 nmol/kg dose (48.8 ± 17.0 vs. 3.4 ± 1.3 min, P<0.05; 5.2 nmol/kg vs. 0.6 nmol/kg) whereas lower doses (1.8 nmol/kg or 0.6 nmol/kg) had no effect (Fig. 1A). In contrast, CCK-58 increased the time to eat almost with significance at the lowest dose (F (3,30) = 3.0, P<0.05; 40.9 ± 18.8 min vs. vehicle: 14.8 ± 4.9 min, P>0.05), significantly by 4-times at 1.8 nmol/kg, and with a similar effect at 5.2 nmol/kg compared to vehicle (52.7 ± 14.5 and 58.5 ± 21.2 min respectively vs. 14.8 ± 4.9 min, P<0.05; Fig. 1B). Two-way ANOVA showed a significant influence of treatment (F(1,55) = 5.2, P<0.05) and dose (F(3,55) = 3.7, P<0.05) but not treatment x dose (F(3,55) = 1.0, P>0.05).

CCK-8 and CCK-58 differentially alter food intake microstructure at the onset of the dark phase

In these experiments gates were closed 90 minutes before lights off and then opened at lights off. With regards to meal consumption the first hour dark phase food intake, first and second meal size as well as 12-h and 24-h food intake were investigated. CCK-8 injected ip (0.6, 1.8 and 5.2 nmol/kg) dose-dependently decreased the first 1-h dark phase food intake in ad libitum fed rats by 35%, 49% and 51% respectively compared to vehicle (F(3,27) = 7.7, P<0.001; 3.0 ± 0.4, 2.3 ± 0.4 and 2.2 ± 0.7 g respectively.
Similarly, CCK-58 (0.6, 1.8 and 5.2 nmol/kg, ip) injected at the onset of
the dark phase decreased first 1-h food intake by 26%, 44% and 70% respectively compared to vehicle

\( F_{(3,29)} = 6.4, P<0.01; 3.6 \pm 0.6, 2.8 \pm 0.5 \text{ and } 1.5 \pm 0.6 \text{ g respectively vs. } 4.9 \pm 0.4 \text{ g, } P<0.01; \text{CCK-58: 0.6 vs. 5.2 nmol/kg, } P<0.05; \text{Fig. 2B). Two-way ANOVA showed a significant influence of dose (} F_{(3,54)} = 13.6, P<0.001) \) but not treatment (\( F_{(1,54)} = 0.3, P>0.05 \) or treatment \( \times \) dose (\( F_{(3,54)} = 0.7, P>0.05 \)).

After establishing a similar potency on reduction of dark phase food intake using equimolar doses of
CCK-8 and CCK-58, the underlying food intake microstructure was investigated using automated episodic
food intake monitoring. Ip injection of CCK-8 reduced the duration of the first meal after 1.8 nmol/kg and
5.2 nmol/kg by 47% and 51% respectively compared to vehicle (\( F_{(3,27)} = 3.4, P<0.05; 18.9 \pm 3.1 \) and 17.2
\( \pm 2.6 \text{ min respectively vs. } 35.5 \pm 5.9 \text{ min, } P<0.05; \text{Fig. 3A) whereas with CCK-58 the reduction of the
first meal duration did not reach significance at any dose tested (} F_{(3,29)} = 1.1, P>0.05; \text{Fig. 3B). Two-way
ANOVA showed a significant effect of dose (} F_{(3,53)} = 3.5, P<0.05) \) whereas treatment (\( F_{(1,53)} = 0.1, P>0.05 \) or treatment \( \times \) dose (\( F_{(3,53)} = 0.4, P>0.05 \) had no significant effect. The meal size of the first meal
of the dark phase was reduced by 1.8 nmol/kg and 5.2 nmol/kg CCK-8 injected ip by 57% and 41%
respectively compared to vehicle (\( F_{(3,27)} = 6.2, P<0.01; 1.7 \pm 0.3 \text{ and } 2.4 \pm 0.5 \text{ respectively vs. } 4.1 \pm 0.4 \text{ g,}
\( P<0.05; \text{Fig. 4A) while for CCK-58 only 1.8 nmol/kg significantly reduced first meal size (} F_{(3,29)} = 6.9, P<0.05; -67%, 2.9 \pm 0.6 \text{ vs. } 4.4 \pm 0.4 \text{ g, } P<0.05; \text{Fig. 4B). Two-way ANOVA showed a significant
influence of dose (} F_{(3,53)} = 5.7, P<0.01) \) but not treatment (\( F_{(1,53)} = 2.8, P>0.05 \) or treatment \( \times \) dose (\( F_{(3,53)} = 0.3, P>0.05 \)). The inter-meal interval between first and second meal of the dark phase was significantly
reduced by ip injection of 1.8 nmol/kg CCK-8 compared to vehicle (-41%, \( P<0.05; \text{Fig. 4C) and non-
significantly reduced at the other doses, while there was no alteration of inter-meal interval following

CCK-58 at any dose (\( F_{(3,29)} = 0.1, P>0.05; 0.6 \text{ nmol/kg: } 60.3 \pm 5.8 \text{ min; } 1.8 \text{ nmol/kg: } 59.1 \pm 8.3 \text{ min; } 5.2
\text{ nmol/kg: } 63.9 \pm 5.6 \text{ min vs. vehicle: } 52.8 \pm 14.7 \text{ min, } P>0.05; \text{Fig. 4D). Two-way ANOVA indicated a
significant influence of treatment (} F_{(1,54)} = 4.5, P<0.05) \) but not dose (\( F_{(3,54)} = 0.3, P>0.05 \) or treatment \( \times \) dose (\( F_{(3,54)} = 0.9, P>0.05 \)). At 1.8 nmol/kg these alterations resulted in a 1.8- and 2.6-times increase of the
satiety ratio (inter-meal interval/meal size) induced by CCK-8 (\( 25.9 \pm 6.7 \text{ vs. } 14.3 \pm 1.9 \text{ min/g food eaten)
and CCK-58 (30.1 ± 6.2 vs. 11.6 ± 2.5 min/g) respectively compared to vehicle (\(P<0.05\); Fig. 4E-F). Two-way ANOVA showed a significant effect of dose (\(F(3,54) = 3.0, P<0.05\)) whereas treatment (\(F(1,54) = 0.1, \ P>0.05\)) or treatment x dose (\(F(3,54) = 0.2, P>0.05\)) had no significant effect. The linear regression analysis of meal size and IMI following injection of CCK-8 showed a significant positive correlation (\(r=0.56, P<0.01\)) which was not observed following injection of CCK-58 (\(P>0.05\)). The rate of ingestion of the first meal was not significantly altered at any dose of CCK-8 injected ip at the onset of the dark phase (\(F(3,27) = 1.2, P>0.05\); Fig. 5A) while CCK-58 (1.8 nmol/kg, ip) decreased the rate of ingestion by 48% compared to vehicle (\(F(3,29) = 3.6, P<0.05; 34.1 ± 5.2 \text{ vs. } 65.8 ± 11.1 \text{ mg/min, } P<0.01\); Fig. 5B). Two-way ANOVA indicated a significant effect of dose (\(F(3,53) = 4.6, P<0.01\)) but not treatment (\(F(1,53) = 0.1, P>0.05\)) or treatment x dose (\(F(3,53) = 0.5, P>0.05\)).

Neither CCK-8 (\(F(3,27) = 1.1, P>0.05\)) nor CCK-58 (\(F(3,29) = 0.5, P>0.05\)) had an effect on the size of the second meal (Fig. 6A-B). Two-way ANOVA indicated no significant effect of treatment (\(F(1,53) = 1.9, P>0.05\)), dose (\(F(3,53) = 0.5, P>0.05\)) or treatment x dose (\(F(3,53) = 1.0, P>0.05\)). Similarly, there was no change in cumulative dark phase food intake (0-12h, CCK-8: \(F(3,27) = 3.6, P>0.05\); CCK-58: \(F(3,29) = 0.6, P>0.05\)) or consecutive light phase food intake (12-24h, CCK-8: \(F(3,27) = 1.9, P>0.05\); CCK-58: \(F(3,29) = 0.1, P>0.05\)) following injection of either CCK-8 or CCK-58 (Fig. 7A-D). Two-way ANOVA indicated no significant effect of treatment (\(F(1,54) = 0.4, P>0.05\)), dose (\(F(3,54) = 1.8, P>0.05\)) or treatment x dose (\(F(3,54) = 1.4, P>0.05\)). The cumulative 24-h food intake was not changed following injection of CCK-58 (\(F(3,29) = 0.5, P>0.05\)) whereas the highest dose of CCK-8 modestly but significantly reduced the 24-h food intake compared to vehicle (\(F(3,27) = 7.4, P<0.01\); Fig. 7E-F). Two-way ANOVA showed a significant effect of dose (\(F(3,54) = 3.4, P<0.05\)) but not treatment (\(F(1,54) = 3.8, P>0.05\)) or treatment x dose (\(F(3,54) = 0.7, P>0.05\)).

**CCK-8 and CCK-58 differentially affect behavior**

Based on the food intake microstructure data described above, a dose of 1.8 nmol/kg was used for the behavioral analyses following ip injection of CCK-8 and CCK-58. To confirm the effect on feeding, food
intake was assessed as well. The 40-min dark phase food intake was significantly reduced by 64% following CCK-8 compared to vehicle (0.6 ± 0.2 vs. 1.6 ± 0.4 g, P<0.05) when food ingestion was monitored manually (Fig. 8A). Similarly, CCK-58 (1.8 nmol/kg, ip) decreased the 40-min dark phase food intake by 56% compared to vehicle injected ip (0.6 ± 0.2 vs. 1.3 ± 0.2 g, P<0.05; Fig. 9A). Two-way ANOVA indicated a significant effect of dose (F(1,26) = 9.6, P<0.01) but not treatment (F(1,26) = 0.4, P>0.05). During that time, CCK-8 reduced the eating behavior (food intake and food approach) by 59% (18.8 ± 8.8 vs. 45.8 ± 11.1, P<0.05; Fig. 8B) while drinking behavior (water intake and water approach) was not significantly altered compared to vehicle (P>0.05; Fig. 8C). Following CCK-58, eating behavior was reduced compared to vehicle (-52%, 19.9 ± 6.2 vs. 41.6 ± 7.8, P<0.05; Fig. 9B) whereas drinking behavior was not significantly altered (P>0.05; Fig. 9C). For eating behavior two-way ANOVA showed a significant effect of dose (F(1,26) = 7.6, P<0.05) but not treatment (F(1,26) = 0.1, P>0.05). CCK-8 increased grooming behavior by 59% (122.4 ± 8.9 vs. 76.8 ± 12.3, P<0.05; Fig. 8D) while locomotor activity was significantly reduced compared to vehicle (-31%, 29.7 ± 3.3 vs. 43.0 ± 4.1, P<0.05; Fig. 8E). Neither grooming behavior (Fig. 9D) nor locomotor activity (Fig. 9E) were affected by injection of CCK-58 compared to vehicle ip (P>0.05). For grooming behavior, two-way ANOVA indicated a significant interaction of treatment x dose (F(1,26) = 4.6, P<0.05) whereas for locomotor activity two-way ANOVA showed a significant effect of dose (F(1,26) = 6.8, P<0.05) but not treatment (F(1,26) = 0.1, P>0.05).
Discussion

In this study we compared and analyzed the effects of ip CCK-8 and CCK-58 on dark phase meal pattern in undisturbed rats fed a solid meal. In line with previous studies, both CCK-8 and CCK-58 dose-dependently reduced 1-h dark phase food intake and first meal size at an equimolar dose of 1.8 nmol. However, microstructure analysis suggests that this was achieved by different influences on food intake parameters and behavior. At this dose, CCK-58 but not CCK-8 increased the latency to the first meal while the inter-meal interval was reduced after CCK-8 but not after CCK-58. While CCK-8 decreased the duration of the first meal, CCK-58 decreased the rate of ingestion. In addition, CCK-8 induced fine movement and reduced locomotor activity while CCK-58 did not elicit such changes. Importantly, both, CCK-8 and CCK-58 increased satiety ratio compared to vehicle.

Previous in vitro studies with CCK-8 and CCK-58 demonstrated that the two peptides caused similar effects on pancreatic functions as indicated by comparable increases of intracellular Ca^{2+} concentrations in murine pancreatic acinar cell preparations and similar actions on cell death (7). These actions seem to be CCK_{1} receptor mediated as both peptides bind to this receptor and drive similar actions such as Ca^{2+} response and receptor internalization (60). In contrast, in vivo studies showed differential effects of the two molecular forms on pancreatic physiology and pathology. CCK-58, but not CCK-8, strongly stimulates pancreatic fluid secretion in rats (46, 62) and CCK-8 but not CCK-58 induces pancreatitis (61). Sayegh et al. have shown that effects of endogenous CCK on meal size and inter-meal interval (33) are partly regulated by the CCK_{1} receptor (55). Since these findings suggest a differential action in vivo, although both CCK-8 and CCK-58 bind to the same receptors with different affinity (45), it is important to address what accounts for these different actions. CCK-58 showed 3-times higher potency to bind mouse pancreatic CCK_{1} receptors than CCK-8 while binding to mouse brain CCK_{2} receptors was equipotent for the two peptides (45). Differences in tertiary structure of the carboxyl terminus of CCK-8 and CCK-58 may influence receptor binding. From pharmacokinetic studies in dogs it is known that CCK-8 has ~4 times shorter circulating half life than CCK-58 (25). In their study, Hoffmann et al. determined that the half-life of CCK-58 was 4.4 ± 0.6 min compared with 1.3 ±0.1 min for CCK-8 when both forms of CCK...
were iv bolus-injected in dogs (25). Similar experiments have not been conducted in rats and it will be important to assess the half life and degradation of the two CCK forms in rats injected ip and iv. A possible explanation for different actions between CCK-8 and CCK-58 in addition of potential receptor binding is the stability to enzymatic digestion of CCK-58 after ip injection (45).

Most studies on CCK’s regulation of food intake have been done with CCK-8 (2, 22, 30), after long fasting periods or during the light phase using liquid meals (32, 33, 36, 37). So far, only one report compared the feeding microstructure in response to CCK-8 and CCK-58 using an automated system measuring the intake of powder food (20). There are several factors in our studies that help to achieve a physiological approach. The studies presented here are performed in rats with free access to solid food as compared to powder or liquid food and they are conducted without a fasting period during the dark phase when nocturnal animals have their highest food consumption. The automated feeding system provides continuous measurement and allows for dissection of food intake parameters. Our studies suggest that both, exogenous CCK-8 and CCK-58 reduce meal size. However, IMI and therefore satiety ratio are much less studied. In two studies releasers of CCK such as camostat or duodenal fat infusions released endogenous CCK in a fashion similar to a meal, and there are two studies showing in rats with duodenal cannulas that these agents caused a reduction of liquid meal size and lengthening of IMI compared to vehicle infusion (5, 59). In our studies CCK-58 tended to increase IMI but this difference was not significant. The variation between the previous studies and our study may result from the experimental set-up but more importantly the use of the endogenous form of CCK versus CCK-8. In addition, at this point, we do not know whether the longer inter-meal interval produced by CCK-58 is a paracrine or endocrine effect. This could be studied by comparing continuous iv and celiac artery infusions of the peptide as done by Cox et al. for CCK-8 (6).

An assumption about the IMI is that it is regulated by the size of the meal immediately preceding the inter-meal interval. Thus, the smaller the meal is, the shorter the IMI and vice versa. Therefore, to influence food consumption, either IMI or meal size can be modulated. An ideal satiety hormone would reduce meal size, but at the same time maintain or prolong the IMI so the total amount eaten will be
decreased. Linear regression analysis of meal size and IMI following injection of CCK-8 showed a significant positive correlation indicating that bigger meals were followed by a longer period of non-eating, whereas after smaller meals rats started to eat sooner. Whether injection of CCK-8 actively shortened the IMI or the shorter IMI reflects the anticipated concomitant shortening that follows a smaller first meal, remains to be elucidated but most likely this is a normal physiological phenomenon and not related to CCK-8. In contrast, injection of CCK-58 reduced the first meal size while not altering the inter-meal interval giving rise to a CCK-58 induced effect preventing the compensatory decrease of the IMI. These results suggest meal size and IMI are regulated at different sites or there is a different action of CCK-8 and CCK-58 at the same site. The small difference in half lives which is less than 5 min for both peptides (25) suggests that the differences in IMI do not result from varying half-lives. In addition, the increased latency to start the first meal further supports that meal size and IMI result from different mechanisms. Our results with exogenous CCK-58 are in keeping with recent findings where camostat gavage (which releases endogenous CCK) reduced food intake by decreasing meal size and prolonging IMI, whereas exogenous CCK-8 reduced food intake by reducing meal size only (33).

Since the satiety ratio is calculated by inter-meal interval (min) divided by the first meal (g), an extended IMI and small meal size results in a high satiety ratio. Therefore, a negative correlation of these two parameters would be indicative of the highest satiety ratio. However, the alteration of only one factor can still significantly affect the satiety ratio. The satiety ratio was increased after CCK-58 due to the decrease in meal size and unaltered inter-meal interval. The stronger decrease of meal size observed for one dose of CCK-8 also caused an increase in satiety ratio although the inter-meal interval was reduced. In addition, only CCK-58 significantly reduced the rate of ingestion of the first meal compared to vehicle. Although dark phase food intake was reduced for both, CCK-8 and CCK-58 distinctly altered feeding microstructure parameters pointing towards the hypothesis that different regulatory mechanisms control the feeding patterns induced by the two peptides.
Reduction of food intake can concur with behavioral alterations. Therefore, behavioral analyses were performed as established in our previous studies (50, 53). Both forms of CCK reduced food intake during the early dark phase due to a reduction in eating behavior (eating as well as food approach and sniffing the food). Whereas CCK-8 increased grooming and reduced locomotor activity compared to vehicle, the parameters were not changed after CCK-58. The changes observed after CCK-8 could be signs of postprandial fullness. Furthermore, the highest dose of CCK-8 modestly reduced the 24-h food intake possibly due to induction of malaise whereas cumulative 24-h food intake was not changed following injection of CCK-58. However, other studies that also evaluated different doses of CCK-8 (4-16 μg/kg, ~3.6-16.2 nmol/kg) (10) or infused CCK-8 continuously over 8 days (57) concluded the peptide specifically reduced food intake without causing malaise (10, 57). In their study, West et al. proposed a reduction in meal frequency as a determinant for malaise and taste aversion as produced by lithium chloride (57). In another study by West et al. inter-meal infusion of CCK-8 failed to prolong the IMI but initially prevented the compensatory decrease in IMI and increased feeding frequency expected after meal size was reduced (58). Failure of CCK-8 to affect feeding behavior similar to lithium chloride is indirect evidence that the reduction of food intake by CCK-8 is not simply a result of aversive behavior but is a specific effect (12). Thus, compared to taste aversive agents like lithium chloride and with the lower doses used it can be speculated that in the present study malaise or taste aversion are unlikely to contribute to the observed CCK-8 induced reduction of food intake.

**Perspectives and Significance**

CCK-8 and CCK-58 injected at equimolar doses reduce food intake to a similar extent although the underlying food intake microstructure differs. Moreover, only CCK-8 induces fine movement (grooming) while reducing locomotor activity. At equimolar doses CCK-8 significantly reduces IMI while CCK-58 does not. This suggests that meal size and IMI are regulated at different sites or they have different actions at the same site and the common idea that all molecular forms of CCK have the same physiological actions needs reconsideration highlighting the importance of studying the actual endogenous molecular
form of peptides especially considering the finding that CCK-58 is the only circulating form of cholecystokinin (9, 13, 42).
Acknowledgements

We thank Ms. Sara Bassilian for her technical support.

Grant support

Supported by National Institute of Health center grant DK-41301 (Animal Core, Peptidomic RIA Proteomic Core, Y.T., J.R.R., Jr.), R01 grant 5R01DK083449 (J.R.R., Jr.) and Veterans Administration Research Career Scientist Award and Merit Award (Y.T.).
References


**Figures**

**FIG. 1.** CCK-58 at a dose of 1.8 nmol/kg increases the latency to eat whereas CCK-8 does not. Rats housed under *ad libitum* feeding conditions with continuously open gates were injected ip with CCK-8 (0.6, 1.8 and 5.2 nmol/kg, A), CCK-58 (0.6, 1.8 and 5.2 nmol/kg, B) or vehicle (saline containing 0.1% BSA), placed back in the cage, recording started and lights turned off. The latency to the first meal was extracted and shown for CCK-8 (A) and CCK-58 (B). Bars represent mean ± SEM of number of rats indicated at the bottom. *P*<0.05 vs. vehicle. #P<0.05 vs. 0.6 nmol/kg.

**FIG. 2.** CCK-8 and CCK-58 injected ip dose-dependently decrease dark phase food intake in rats. Gates were closed at 90 min before the onset of the dark phase. Directly before lights off, rats were ip injected with CCK-8 (0.6, 1.8 and 5.2 nmol/kg, A), CCK-58 (0.6, 1.8 and 5.2 nmol/kg, B) or vehicle (saline containing 0.1% BSA), placed back in their home cage, the gates opened, recording started and lights turned off. Food intake was extracted for the first h post injection. Bars represent mean ± SEM of number of rats indicated at the bottom. **P*<0.01 and ***P*<0.001 vs. vehicle and #P<0.05 vs. 0.6 nmol/kg.

**FIG. 3.** CCK-8 reduces the duration of the first meal whereas CCK-58 does not. Gates were closed at 90 min before the onset of the dark phase. Directly before lights off, rats were ip injected with CCK-8 (0.6, 1.8 and 5.2 nmol/kg), CCK-58 (0.6, 1.8 and 5.2 nmol/kg) or vehicle (saline containing 0.1% BSA), placed back in their home cage, the gates opened, recording started and lights turned off. The duration of the first meal was analyzed and shown for CCK-8 (A) and CCK-58 (B). Bars represent mean ± SEM of number of rats indicated at the bottom. *P*<0.05 vs. vehicle.

**FIG. 4.** CCK-8 and CCK-58 both reduce the meal size of the first meal and increase satiety ratio but only CCK-8 reduces the inter-meal interval. Gates were closed at 90 min before the onset of the dark phase. Directly before lights off, animals were injected ip with CCK-8 or CCK-58 (0.6, 1.8 and 5.2 nmol/kg) or...
vehicle (saline containing 0.1% BSA), placed back in their home cage, the gates opened, recording started and lights turned off. The meal size of the first meal (A, B), the inter-meal interval between first and second meal of the dark phase (C, D) was extracted and the satiety ratio (inter-meal interval/meal size) calculated (E, F). Bars represent mean ± SEM of number of rats indicated at the bottom. *$P<0.05$ and ***$P<0.001$ vs. vehicle.

FIG. 5. CCK-58 but not CCK-8 decreases the rate of ingestion of the first meal. Gates were closed at 90 min before the onset of the dark phase. Directly before lights off, rats were ip injected with CCK-8 (0.6, 1.8 and 5.2 nmol/kg), CCK-58 (0.6, 1.8 and 5.2 nmol/kg) or vehicle (saline containing 0.1% BSA), placed back in their home cage, the gates opened, recording started and lights turned off. The rate of ingestion for the first meal was analyzed and shown for CCK-8 (A) and CCK-58 (B). Bars represent mean ± SEM of number of rats indicated at the bottom. **$P<0.01$ vs. vehicle.

FIG. 6. CCK-8 and CCK-58 do not reduce the meal size of the second meal. Gates were closed at 90 min before the onset of the dark phase. Directly before lights off, animals were injected ip with CCK-8 or CCK-58 (0.6, 1.8 and 5.2 nmol/kg) or vehicle (saline containing 0.1% BSA), placed back in their home cage, the gates opened, recording started and lights turned off. The meal size of the second meal was extracted. Bars represent mean ± SEM of number of rats indicated at the bottom.

FIG. 7. CCK-8 and CCK-58 do not induce a compensatory increase of food intake within 24 hours after peptide injection. Data were analyzed over the period of 24 hours following injection. There was no change in cumulative dark phase food intake (0-12h, A, B) or consecutive light phase food intake (12-24h, C, D) following injection of either CCK-8 or CCK-58 giving no indication of a compensatory increase in food intake. The cumulative 24-h food intake was not changed following injection of CCK-58 (F) whereas the highest dose of CCK-8 (E) modestly but significantly reduced the 24-h food intake. Bars represent mean ± SEM of number of rats indicated at the bottom. *$P<0.05$ vs. vehicle and ***$P<0.01$ vs. 0.6 nmol/kg.
FIG. 8. CCK-8 increases grooming behavior and decreases locomotor activity. Rats were injected ip with CCK-8 (1.8 nmol/kg) or vehicle (saline containing 0.1% BSA) and placed back in their home cage with paper under the cage divided into six equal squares with free access to food and water. Food intake was assessed for 40 min (A). At 10 min after injection behaviors including eating (including food approach, B), drinking (including water approach, C) and grooming behavior (washing, licking and scratching, D) as well as locomotor activity (total number of squares crossed, E) were monitored manually for 30 min by an observer blinded to the animals’ treatment. Each behavior was counted again when lasting >5 sec. Bars indicate mean ± SEM of 6-8 rats/group. *$P<0.05$ vs. vehicle.

FIG. 9. CCK-58 selectively decreases eating behavior while not altering grooming and locomotor activity. Animals were ip injected with CCK-58 (1.8 nmol/kg) or vehicle (saline containing 0.1% BSA) and placed back in their home cage with paper under the cage divided into six equal squares with free access to food and water. Food intake was assessed for 40 min (A). At 10 min after injection behaviors including eating (including food approach, B), drinking (including water approach, C) and grooming behavior (washing, licking and scratching, D) as well as locomotor activity (total number of squares crossed, E) were monitored manually for 30 min by an observer blinded to the animals’ treatment. Each behavior was counted again when lasting >5 sec. Bars indicate mean ± SEM of 8 rats/group. *$P<0.05$ vs. vehicle.
Figure 1

A

Latency to first meal (min)

CCK-8

Latency to first meal (min)

B

CCK-58

nmol/kg

0

0.6

1.8

5.2

nmol/kg

0

0.6

1.8

5.2

9

6

8

6

10

6

12

6

* * 

# 

*
Figure 2

A

CCK-8

Food intake (g/lh)

Food intake (g/lh)

B

CCK-58

Food intake (g/lh)

Food intake (g/lh)
Figure 3

A

CCK-8

Duration first meal (min)

Duration first meal (min)

B

CCK-58

nmol/kg

nmol/kg

0 0.6 1.8 5.2 nmol/kg

0 0.6 1.8 5.2 nmol/kg

11 6 8 6

9 6 12 6

* *
Figure 4

A

CCK-8

Meal size first meal (g)

<table>
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B

CCK-58

Meal size first meal (g)

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Inter-meal interval (min)

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Inter-meal interval (min)

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E

Satiety ratio (min/g food eaten)

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Satiety ratio (min/g food eaten)

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Figure 5

A

Rate of ingestion first meal (mg/min)

CCK-8

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B

Rate of ingestion first meal (mg/min)

CCK-58

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Figure 8

A. Food intake

- Vehicle (ip, n=8)
- CCK-8 (1.8 nmol/kg ip, n=6)

B. Eating behavior

C. Drinking behavior

D. Grooming

E. Locomotion
Figure 9

A. Food intake

- Vehicle (ip, n=8)
- CCK-58 (1.8 nmol/kg ip, n=8)

B. Eating behavior

C. Drinking behavior

D. Grooming

E. Locomotion

Note: The y-axis represents the total number of occurrences.