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Peripheral injected cholecystokinin-8S modulates the concentration of serotonin in nerve fibers of the rat brainstem

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ABSTRACT

Serotonin and cholecystokinin (CCK) play a role in the short-term inhibition of food intake. It is known that peripheral injection of CCK increases c-Fos-immunoreactivity (Fos-IR) in the nucleus of the solitary tract (NTS) in rats, and injection of the serotonin antagonist ondansetron decreases the number of c-Fos-IR cells in the NTS. This supports the idea of serotonin contributing to the effects of CCK. The aim of the present study was to elucidate whether peripherally injected CCK-8S modulates the concentration of serotonin in brain feeding-regulatory nuclei. Ad libitum fed male Sprague-Dawley rats received 5.2 and 8.7 nmol/kg CCK-8S ($n = 3$/group) or 0.15 M NaCl ($n = 3$–5/group) injected intraperitoneally (ip). The number of c-Fos-IR neurons, and the fluorescence intensity of serotonin in nerve fibers were assessed in the paraventricular nucleus (PVN), arcuate nucleus (ARC), NTS and dorsal motor nucleus of the vagus (DMV). CCK-8S increased the number of c-Fos-ir neurons in the NTS (mean ± SEM: 72 ± 4, and 112 ± 5 neurons/section, respectively) compared to vehicle-treated rats (7 ± 2 neurons/section, $P < 0.05$), but did not modulate c-Fos expression in the DMV or ARC. Additionally, CCK-8S dose-dependently increased the number of c-Fos-positive neurons in the PVN (218 ± 15 and 128 ± 14, respectively vs. 19 ± 5, $P < 0.05$). In the NTS and DMV we observed a decrease of serotonin-immunoreactivity 90 min after injection of CCK-8S (46 ± 2 and 49 ± 8 pixel/section, respectively) compared to vehicle (81 ± 8 pixel/section, $P < 0.05$). No changes of serotonin-immunoreactivity were observed in the PVN and ARC. Our results suggest that serotonin is involved in the mediation of CCK-8's effects in the brainstem.

Introduction

Hunger and satiety, food intake and body weight are regulated by various hormonal and neural signals [51]. Cholecystokinin (CCK) is synthesized by a population of endocrine cells in the small intestine [27], and the release of the peptide is stimulated by food intake [16,30]. Gibbs et al. were the first researchers who demonstrated that CCK terminates the intake of solid and fluid food [11,12]. The endogenous form of CCK, i.e. CCK-58, decreases food intake without a compensatory reduction of the subsequent inter-meal interval [14], while after intraperitoneal (ip) injection of the most studied form, the fragment CCK-8, shortens the inter-meal interval indicating an effect of CCK-8 on satiation only [34]. CCK is likely to reduce food intake via its CCK_1 receptors localized on afferent vagal nerve terminals localized in the gastrointestinal mucosa [44,50]. The vagus nerve relays signals to the hindbrain, especially to the nucleus of the solitary tract (NTS), where noradrenergic projections from the A2-cell group are activated [43]. Consecutively, both catecholaminergic and non-catecholaminergic neurons of the NTS project to several hypothalamic brain areas [42,46,47].

The mapping of brain neuronal circuits recruited by hormone signals has been widely obtained by assessing changes of the proto-oncogene c-Fos, which allows identification of activated neurons at the cellular level [45]. Several studies have revealed that peripherally injected CCK induces c-Fos-immunoreactivity (c-Fos-IR) in different autonomic brain nuclei, namely in the paraventricular nucleus of the hypothalamus (PVN), locus coeruleus (LC), nucleus of the solitary tract (NTS), and the area postrema (AP), indicating that a complex neural network in the brain is activated by CCK [4,7,29,33,43].

The neurotransmitter serotonin could be involved in the mediation of CCK's brainstem effects as it shows similarities with the activation pattern of CCK. Serotonin is produced centrally in the

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raphe nuclei of the brainstem and peripherally in enterochromaffin cells scattered in the small intestinal and colonic mucosa [10,22]. As described for CCK, the peripheral release of serotonin is stimulated by the presence of food [49]. Serotonin binds to receptor terminals of vagal afferent fibers localized in the mucosa of the gastrointestinal tract [35] and delays gastric emptying [41]. As established for CCK, serotonin is known to reduce food intake [38]. High densities of the serotonin receptor were found on neurons in the NTS, AP and the dorsal motor nucleus of the vagus (DMV) [35].

Taken together, CCK and serotonin not only have similar effects on food intake but also both act via the vagus nerve and partly influence the same brain nuclei. Previous investigations have therefore examined a possible interaction between CCK and serotonin. Hayes et al. observed an increased intake of sucrose solution in rats after microinjection of the serotonin antagonist ondansetron into the medial NTS [18]. Interestingly, they also showed a reduced intake of sucrose after injection of CCK, an effect that was blunted by the serotonin type-3 (5-HT3) receptor antagonist, ondansetron [18]. Moreover, Helm et al. injected CCK and serotonin unilaterally into the PVN and observed a significant inhibition of food intake in the early dark period which was associated with a greater increase of dopamine than injection of each transmitter alone [20]. According to the results given above, both the NTS and the PVN are likely to play a role in the combined mediation of cholecytokinergic and serotonergic effects on food intake inhibition. However, more detailed neurophysiological studies are needed to corroborate this hypothesis.

The aim of the present study was to investigate whether peripherally injected CCK modulates the concentration of serotonin in nerve fibers of the NTS, DMV, arcuate nucleus (ARC) and the PVN. CCK-8S injection is associated with a well-established distinct neuronal activation pattern in the PVN and NTS. Consequently, we also investigated the influence of CCK injected ip on the density of c-Fos neurons in the PVN and NTS, as well as DMV and ARC which served as control nuclei. In addition, serotonin-immunoreactivity in the NTS was determined to assess the distribution of serotonin nerve fibers in relation to activated neurons colocalized with tyrosine hydroxylase a marker for catecholaminergic cells.

Materials and methods

Animals

Male Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) weighing 250–300 g were housed under conditions of controlled illumination (12:12 h light/dark cycle, lights switched on at 6 a.m.), humidity, and temperature (22 ± 2 °C) for at least 21 days prior to the experiments. Animals were fed with a standard rat diet (Altromin®, Lage, Germany) and tap water ad libitum. All animals were accustomed to the experimental conditions for a period of 14 days by handling them daily and putting them in the position to mimic the procedure of ip injection. Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the state authority for animal research conduct (protocol 0053/06).

Peptide

In this study we used sulphated CCK-8 (CCK-8S) which is the most commonly used form of CCK in behavioral and pharmacological studies on food intake. CCK-8S (Bachem AG, Heidelberg, Germany) was dissolved in water with 1% (v/v) 1 N NH_4OH, aliquoted and stored at −20 °C. Immediately before starting the experiments, the peptide was diluted in vehicle solution consisting of sterile 0.15 M NaCl (Braun, Melsungen, Germany) to reach the final concentration of 5.2 and 8.7 nmol/kg body weight (b wt; 6 and 10 μg/kg b wt). Peptide solutions were kept on ice for the duration of the experiments. The doses of CCK-8S were selected based on our previous studies [26,32,37].

Experimental protocol

Experiments were started at the same time of day (between 10:00 a.m. to 10:30 a.m.), i.e. 3.5–4 h after the start of the light phase to achieve maximum consistency. Ad libitum fed rats received an ip injection (final volume: 500 μl) of 5.2 and 8.7 nmol/kg CCK-8S ($n = 3$/group) or vehicle solution (0.15 M NaCl; $n = 3$–5). At 90 min after CCK-8S injection, rats were deeply anesthetized with ip 100 mg/kg ketamine (Ketanest®, Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun® 2%, Bayer, Leverkusen, Germany) and heparinized with 2500 IU heparin injected ip (Liquemin®, Hoffmann-La Roche, Grenzach-Whylen, Germany). Transcardial perfusion was performed as described in detail before [23].

Immunohistochemistry

Single staining for c-Fos detection in the arcuate nucleus, in the paraventricular nucleus, in the nucleus of the solitary tract, and in the dorsal motor nucleus of the vagus in the brainstem

Free-floating 25 μm brain sections were pre-treated with 1% (w/v) sodium borohydride in phosphate buffered saline (PBS) for 15 min. Subsequently, sections were incubated in a solution containing 1% (w/v) bovine serum albumin (BSA) and 0.3% (v/v) Triton X-100, and 0.05% (v/v) phenylhydrazine in PBS for 60 min to block unspecific antibody binding. Thereafter, the diluted primary antibody solution (rabbit anti-c-Fos, Oncogene Research Products, Boston, MA, USA; 1:3000 in a solution of 1% (w/v) BSA, and 0.3% (v/v) Triton X-100 in PBS) was applied for 24 h at room temperature. After washing in PBS, sections were incubated with the secondary antibody solution (goat biotin-SP-conjugated anti rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:1000 in 1% (w/v) BSA in PBS) for 12 h at room temperature. After rinsing in PBS three times, sections were incubated in avidin-biotin peroxidase complex (ABC; 1:200; Vector Laboratories, UK) in PBS for 6 h. Subsequently, sections were rinsed in PBS three times again, and then incubated in TSA™ tetramethyl rhodamine tyramide in amplification solution (PerkinElmer, Waltham, MA, USA) for 10 min at room temperature. After washing in PBS, sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) for 15 min to counterstain cell chromatin. Brain sections were finally embedded in 8 μl anti-fading solution (100 mg/ml 1,4-diazabicyclo [2.2.2] octane, Sigma, St. Louis, USA; in 90% (v/v) glycerine, 10% (v/v) PBS, pH 7.4) and analyzed using confocal laser scanning microscopy (cLSM 760, Carl Zeiss, Germany).

Double staining for c-Fos and serotonin in the arcuate nucleus, in the paraventricular nucleus of the hypothalamus, and in the nucleus of the solitary tract, and in the dorsal motor nucleus of the vagus of the brainstem

Staining for c-Fos followed the protocol described above (see section 'Single staining for c-Fos detection in the arcuate nucleus, in the paraventricular nucleus, in the nucleus of the solitary tract, and in the dorsal motor nucleus of the vagus in the brainstem'). Sections were then incubated with the second primary antibody solution (rabbit anti-serotonin; Novus Biologicals; Littleton, CO, USA; 1:1000 in PBS containing 1% (w/v) BSA) for 24 h at room temperature. After rinsing in PBS three times, fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma–Aldrich) in PBS was applied for 12 h at room temperature. After washing in PBS three times, sections were stained with DAPI for 15 min. Finally, sections were rinsed in PBS three times and embedded in 8 μl