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Effect of anorexinergic peptides, cholecystokinin (CCK) and cocaine and amphetamine regulated transcript (CART) peptide, on the activity of neurons in hypothalamic structures of C57Bl/6 mice involved in the food intake regulation

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ABSTRACT

The hypothalamus plays an important role in food consumption, receiving information about energy balance via hormonal, metabolic, and neural inputs. Its neurons produce neuropeptides influencing energy balance. Especially important to regulation of food consumption are certain hypothalamic structures, including the arcuate (ARC) and ventromedial (VMN) nuclei and the lateral hypothalamic area (LHA). We determined the impact of cholecystokinin (CCK) and cocaine and amphetamine regulated transcript (CART) peptide, on activity of ARC and VMN neurons and hypocretin (Hcrt) synthesizing neurons in LHA. ARC is an integrative nucleus regulating food consumption, VMN is considered to be a satiety centre, and LHA a hunger sensing centre. After overnight fasting, male C57Bl/6 mice received intraperitoneal injection of (i.p.) saline (SAL) or CCK (4 μg/kg) or intracerebroventricular injection of (i.c.v.) CART peptide (0.1 μg/mice) or CCK (i.p.) followed by CART peptide (i.c.v.) 5 min later. Sixty minutes later, the presence of Fos or Fos/Hcrt immunostaining indicated activity of ARC and VMN neurons, as well as of Hcrt cells in LHA. CCK alone did not influence neuronal activity in any of the nuclei studied. CART peptide stimulated neurons in ARC and VMN ($p < 0.01$) but decreased Hcrt neuronal activity in LHA ($p < 0.05$). Co-administration of both peptides synergistically stimulated ARC neurons ($p < 0.01$) and asynergistically inhibited LHA Hcrt neurons ($p < 0.01$). Results indicate that CCK may modify the effect of CART peptide and thus substantially influence activity of neurons in hypothalamic structures involved in regulation of food intake.

1. Introduction

The hypothalamus plays a crucial role in regulation of food consumption, and its neurons produce anorexinergic and orexinergic neuropeptides which influence energy balance of the whole body [7,21,22,24,28,30,43,49,50]. The mammalian hypothalamus contains many distinct nuclei and areas. However, regulation of food intake is associated mainly with the hypothalamic paraventricular (PVN), arcuate (ARC), ventromedial (VMN), and dorsomedial (DMN) nuclei and the lateral hypothalamic area (LHA) [4,6,25,49,50]. The ARC plays an integrative role in regulating food consumption. Axons of its anorexinergic and orexinergic neurons innervate cells in the above mentioned nuclei [8,22]. Moreover, the ARC is only partially protected by the blood–brain barrier. Thus the peripheral hormonal and metabolic signals as well as vagal nerve inputs ascending from the gastrointestinal tract (GIT) and liver may easily reach this structure [7,16].

Cholecystokinin (CCK), as an anorexinergic GIT hormone, has many biological actions [12]. It coordinates digestion and is associated with inhibition of food intake via vagal nerve stimulation [11]. As a neurotransmitter, it is produced in several brain structures [32]. CCK receptors have also been found in the vagus nerve, the nucleus of the solitary tract (NTS) or DMN [51]. On the other hand, cocaine and amphetamine regulated transcript (CART) peptide may serve as a mediator of anorexinergic signals in afferent neurons of the vagus nerve in the NTS [10]. In the hypothalamus, CART peptide producing neurons were mainly found in the ARC and LHA [46]. Axons of ARC neurons innervate not only the VMN, which is considered to be a satiety centre [22,25,50], but also the LHA, known as the centre of hunger [31,50]. On the

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other hand, hypocretin (Hcrt) neurons of the LHA send axons back to ARC, and Hcrt injected into ARC increases the food intake in rats [7,22,49,50].

The goal of this study was to determine the impact of two anorexinergic peptides, CCK and CART, on the activity of neurons in the ARC and VMN and on Hcrt neurons in the LHA of C57Bl/6 male mice by employing single Fos or dual Fos/Hcrt immunohistochemistry. This study arose from our previous dose related investigation [29] where CCK and CART peptides were applied in sub-threshold doses (4 μg/kg of CCK-8 or 0.1 μg of mouse CART (61–102)) that were selected for their inhibitory effect on food intake and locomotor activity. The combination of these two doses of CCK and CART led to a clear synergistic effect on the food intake. Moreover, the number of Fos-positive neurons in the NTS, hypothalamic paraventricular and dorsomedial nuclei were significantly higher when the peptides were combined than if administered singly. These findings convinced us to apply the same doses in the present study.

2. Materials and methods

2.1. Drugs and antisera

Cholecystokinin octapeptide (CCK-8, Asp-Tyr(SO3H)-Met-Gly-Trp-Met-Asp-Phe-NH2, NeoMPS, Strasbourg, France) and CART peptide ([CART (61–102)], Bachem, Bubendorf, Switzerland) were dissolved in isotonic saline (SAL).

The Fos (No. 94012) and Hcrt (No. 99006) antisera were kindly provided by Dr. J.D. Mikkelsen (NeuroSearch A/S Ballerup, Denmark). The specificity and sensitivity of Fos and Hcrt antisera have been tested previously [26,33,34].

2.2. Animals

Adult male C57Bl/6 mice obtained from the Institute of Molecular Genetics (Prague, Czech Republic) were used. The animals were housed five per cage in a room with controlled light (12 h/day), humidity (65%), and temperature (23 °C). They received regular mice chow (dry pellets) and tap water *ad libitum*. All experimental procedures followed the principles of laboratory animal care and ethical guidelines for animal experiments of 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. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

2.3. Brain ventricle cannulation

Sixteen-week-old mice (25–30 g) were implanted with cannulas (Plastics One Inc., Roanoke, USA) into the third brain ventricle under ketamine–xylazine anesthesia (100 mg/kg ketamine, 16 mg/kg xylazine, i.p., Spofa, Prague, Czech Republic). Briefly, the head of the mouse was fixed in a stereotaxic frame and a single guide cannula was implanted just above the third ventricle. The coordinates (AP 2 mm, V 3 mm) were selected based on the mouse brain atlas of Frankin and Paxinos [17]. A stainless steel screw was attached to the skull and the cannula was fixed by Duracrol resin (Spofa-Dental, Prague, Czech Republic). An appropriate dummy cannula (Plastics One Inc.) was inserted into the guide cannula to prevent its blockage. Thereafter, the animals were placed into individual cages with free access to food and water and remained undisturbed for 7 days to recover from the surgery. The location of i.c.v. implanted cannulas was verified in the brain sections at the end of the experiment.

2.4. Design of the study

To avoid the effect of diurnal variations on Fos-expression, the experiment was performed between 9:00 and 11:00 a.m. After overnight fasting, the mice (n = 5 per group) received an injection of SAL (i.c.v./i.p.), CCK (4 μg/kg, i.p.), CART peptide (0.1 μg/mice, i.c.v.) or CCK (4 μg/kg, i.p.) followed by CART peptide (0.1 μg/mice, i.c.v.) 5 min later. The volume of the applied solutions was 0.2 ml (i.p.) and 5 μl (i.c.v.) [1] respectively. I.c.v. injection was completed within 2 min. All doses of the applied peptides were selected on the basis of our previous study [29].

2.5. Transcardial perfusion

Sixty minutes after the drug application the mice were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and rapidly perfused transcardially with 10 ml of 0.1 M phosphate buffer (PB, pH 7.4) followed by 50 ml of 0.1 M PB (pH 7.4) containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 10% saturated picric acid. The brains were removed, post-fixed in the same fixative overnight at 4 °C, and infiltrated with 30% sucrose in 0.1 M PB for 48 h at 4 °C. Before sectioning, the brains were rapidly (20 s) frozen in cold isopentane (−40 °C) followed by an additional cooling (1 min) in dry ice and placed into a Reichert cryocut device for 1 h at −16 °C. The hypothalami were cut into coronal sections (30 μm thick) between the optic chiasm and the median eminence (coordinates from Bregma 0.02 mm to −2.3 mm) according to the mouse brain atlas [16] and collected in cold (4 °C) PB as free-floating sections.

2.6. Fos and Fos-Hcrt immunohistochemistry

Free-floating sections were repeatedly washed in cold 0.1 M PB followed by a preincubation with 3% H_2O_2 in 0.1 M PB (Sigma–Aldrich, Germany) for 60 min at room temperature. After several washings in 0.1 M PB the sections were incubated with a polyclonal Fos protein antiserum (1:2000) diluted in 0.1 M PB containing 4% normal goat serum (NGS, Gibco, Grand Island, NY, USA), 0.5% Triton X-100 (Sigma–Aldrich, Germany) and 0.1% sodium azide (Sigma Chemical Ltd., St. Louis, MO, USA) for 48 h at 4 °C. After several rinsings in 0.1 M PB, the sections were incubated with biotinylated goat antirabbit IgG (1:500, VectorStain Elite ABC Kit, Vector Lab, Burlingame, CA, USA) diluted in 0.1 M PB containing 4% NGS and 1% Triton X-100 for 90 min at room temperature. After additional rinsings in PB, the sections were incubated with the avidin–biotin peroxidase complex (1:250, VectorStain Elite ABC, Vector Lab., Burlingame, CA) diluted in 0.1 M PB containing 1% Triton X-100 for 90 min at room temperature. Consecutive PB washing was followed by washing in 0.05 M sodium acetate buffer (SAB, pH 6.0). Fos antigenic sites were visualized with 0.0266% 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) dissolved in 0.05 M SAB containing 2.5% nickel ammonium sulfate (Sigma) and 0.0042% H_2O_2 for 7 min. The metal intensification of DAB produced black staining in the labeled nuclei.

After Fos immunostaining, the brain sections of LHA were selected and repeatedly washed in 0.1 M PB followed by incubation with Hcrt antiserum (1:2000) diluted in 0.1 M PB containing 4% normal goat serum (NGS, Gibco, Grand Island, NY, USA), 0.5% Triton X-100 (Sigma–Aldrich, Germany) and 0.1% sodium azide (Sigma Chemical Ltd., St. Louis, MO, USA) for 48 h at 4 °C. After several rinsings in 0.1 M PB, the LHA sections were incubated with biotinylated goat anti-rabbit IgG and ABC using the same procedure as described above. PB washing was followed by washing in 0.05 M Tris buffer (pH 7.4). Hcrt antigenic sites were visualized with 0.0125% DAB dissolved in 0.05 M Tris buffer (pH