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Research paper

Fourth ventricular CART peptide induces c-fos in the area postrema and nucleus of the solitary tract via a CRF-receptor dependent mechanism

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HIGHLIGHTS

- Rats were given 4th icv injections with CART peptide in a dose that inhibits food intake and gastric emptying.
- CARTp treatment induced c-fos in the area postrema and NTS but not in the hypothalamus. In animals pre-treated with a non-selective, competitive CRF antagonist, no c-fos was seen.
- The findings indicate that CARTp activates neurones in the NTS and AP via a CRF-receptor dependent pathway.

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ABSTRACT

Cocaine-and amphetamine-regulated transcript peptides (CARTp) suppress gastric emptying and nutritional intake following 4th icv administration. Whereas, the CARTp inhibition of gastric emptying was blocked by pre-treatment with a non-selective corticotropin releasing factor (CRF) antagonist, sucrose drinking was not, suggesting that CARTp- and CRF controls for food intake and gastric emptying are operated through separable dorsal hindbrain mechanisms. The aim of the study was to explore CARTp–CRF brainstem interactions on patterns of neuronal activation in areas of the brainstem and midbrain relevant to gastrointestinal control and feeding regulation. Rats received 4th icv injections of combinations of vehicle, CARTp (1 μg), or the nonselective CRF antagonist, α-helical CRF9-41 (αCRF), in a randomized order. Brain sections were processed for c-fos by immunohistochemistry followed by image analysis at defined levels of the brain. CARTp (1 μg, 4th icv) induced a robust c-fos response in the nucleus of the solitary tract (NTS) and area postrema (AP), whereas, no c-fos could be detected in the parabrachial nucleus (PBN), the paraventricular nucleus of the hypothalamus (PVN) or the arcuate nucleus of the hypothalamus (ARC). The c-fos expression in the structures of the dorsal vagal complex (DVC) was completely blocked by pre-treatment with the CRF antagonist, which did not by itself induce c-fos at any examined level. After CARTp and αCRF in combination, there was a tendency towards an increased c-fos response in the ARC. We conclude that CARTp activates cells of the area postrema and NTS via a downstream, CRF-dependent mechanism.

1. Introduction

Cocaine-and amphetamine-regulated transcript derived peptides (CARTp) have been suggested to be endogenous ligands that act in the brain and contribute to a range of physiological and behavioural effects including food intake, gastrointestinal and neuroendocrine regulation [5,11,14,15,17,19,21]. The specific neuronal targets for CARTp to exert its central effects are not fully known since the receptors for CARTp are yet anatomically undefined. The proposed site(-s) of action of CARTp are thus derived mainly from functional placement studies as well histological studies on specific brain nuclei where expression of CART and CARTp, or of occurrence of immediate-early oncogenes as markers for neuronal activation in response to CARTp, have been detected.

Although, a putative CARTp-receptor structure is yet to be demonstrated, CARTp is considered to act in the hypothalamus as

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well as at a hindbrain level to control gastrointestinal functions and feeding. Fourth icv application of CARTp suppresses solid and liquid food intake as well as gastric emptying [3,9,14,15]. A study by Aja et al. highlights the role of the dorsal hindbrain for CARTp in feeding control by demonstrating that injection of CARTp into the third ventricle inhibits food intake, and this effect was lessened when the aqueduct was closed by a grease plug, preventing the peptide from reaching the fourth ventricle and hindbrain structures [3]. CART induces expression of c-fos in the dorsal vagal complex following lateral icv and fourth icv administration [22,23]. Since CARTp is present in the vagus nerve and CART and CARTp are found in vagal sensory and motor nuclei of the dorsal hindbrain, attempts have been made to link the food intake suppressive effect of CARTp to these areas. However, although 4th icv administration of CARTp inhibits food intake and activates neurons of the dorsal vagal complex (DVC), local microinjection of CARTp into the DVC had no effect on food intake [24] or on gastric acid secretion [14], although it did suppress gastric emptying in the rat [14], providing support for separable controls as well as separable brainstem target sites of action.

Earlier studies suggest that CARTp interacts centrally with several other neuropeptides, including corticotropin-releasing factor (CRF). For example, within the dorsal hindbrain, CART and CARTp are found within the NTS [4,8], where CRF- and CRF receptor-expressing neurons [13,20] are present as well. CRF and CARTp display many similar effects at a brainstem level; both inhibit gastric acid secretion and gastric emptying after intracisternal or 4th icv delivery [11,14–16] and delay gastric emptying after local injection in the DVC [14]. Furthermore, 4th icv CARTp attenuation of gastric emptying [15] as well as inhibition of gastric acid secretion are blocked by a CRF antagonist. In contrast, αCRF failed to block the effect of 4th icv CARTp-induced suppression of sucrose intake [15]. Although, there is support for a CARTp–CRF interaction at a brainstem level, it is not fully clear whether such an interaction directly involves vagal structures of the dorsal hindbrain. In the present study, we test the hypothesis that CARTp-induction of c-fos in vagal structures of the dorsal hindbrain after 4th icv administration is CRF-dependent, thus paralleling our previous finding that CARTp modulates gastric emptying [15]. We used a dose of CARTp previously shown by us and others to induce a robust inhibition of feeding and gastric emptying after 4th icv application under similar experimental conditions [2,3,15]. Patterns of neuronal activation in response to CARTp with or without CRF receptor antagonism were explored in discrete areas of the brainstem and hypothalamus related to feeding regulation and gastrointestinal function.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Charles River Laboratories) weighing between 400 and 450 g were used. The animals were housed singly with free access to chow and tap water under conditions of controlled temperature (20 ± 1 °C) and humidity, on a 12:12 h light cycle (lights off 13.00–01.00).

2.2. Surgery

Prior to surgery, the rats were anesthetized with a 3:4 mixture of xylazine (20 mg/ml) and ketamine (100 mg/ml), injected intramuscularly. The surgeries were performed under aseptic conditions. The animals were placed in a stereotaxic frame and the skull was exposed. A chronic guide cannula (10.0 mm × 27 G) aimed at the fourth ventricle was implanted as previously described [16]. During a postoperative recovery period of 7–10 days, the rats were weighed and gently handled daily. After this, the cannula placements were assessed by a functional test to verify the fourth ventricle as the target. The animals were injected with 210 μg 5-thio-D-glucose (5TG) 4th icv, and an increase in blood glucose which exceeded 100% from baseline was the criterion for correct placement [6]. One animal did not respond to 5TG, and was therefore excluded from the study. The animals were handled daily for one week following cannula placement testing, but did not undergo any other procedures.

2.3. Drugs

Synthetic CART (55-102) peptide (rat; American Peptide, Sunnyvale, CA) and α-Helical CRF (9-41) (Sigma–Aldrich) were dissolved in saline and distilled water, respectively. The respective solvents were also used as the corresponding vehicles. The dissolved drugs were aliquoted and frozen (−20 °C). Fresh aliquots were thawed on each experimental day and the excess was discarded.

2.4. Experimental design

The animals were gently restrained by hand, and a 32-G injection needle was inserted in the guide and into the fourth ventricle. The injection needle was attached to a Gilmont micro injector via PE20 tubing, and 1.5 μl of vehicle or drug was injected into the fourth ventricle over 1 min, such that a total volume of 3 μl was administered. The needle was left in place for another 45 s to avoid the risk of back flush, after which it was removed and replaced with an obturator. The animal was then returned to its home cage.

In order to mimic the experimental conditions during previous studies on CARTp effects on food intake [2,3,15] as closely as possible, the experiment was carried out near the end of the light period, with 4th icv injections administered between 8 am and 11 am. We thus strived to reduce any potential stress for the rats, which absence of anticipated food access at lights off might have caused. The food hoppers were removed and the rats were divided into 4 experimental groups, each receiving 2 subsequent injections of vehicle and/or drug into the 4th ventricle; vehicle–vehicle ($n = 6$), vehicle–CARTp ($n = 7$), αCRF–vehicle ($n = 7$) and αCRF–CARTp ($n = 7$). The first injection was administered 90 min prior to sacrifice, and the second injection was administered 75 min prior to sacrifice. In between and after the injections, the rats were returned to their respective home cage. Immediately before sacrifice, the animals were anesthetized with a intraperitoneal injection of Euthasol (sodium pentobarbital and phenytoin; Delmarva Laboratories) and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were rapidly removed and postfixed overnight in 4% paraformaldehyde with 25% sucrose. After this, the brains were frozen, and 40 μm sections were cut on a cryostat at the rostrocaudal level of the NTS, the pons, and hypothalamus. Four free-floating sections were incubated in each well, containing 2 ml of PBS (pH 7.3). The PBS was removed and the sections were incubated for 1 h in 0.3% H_2O_2 before washing (PBS). The sections were then incubated in normal goat serum in PBS and 0.3% Triton X-100. After this, the sections were incubated with c-fos primary antibody (Oncogene, rabbit polyclonal, cat no PC38) in 4 °C for 42 h. After rinsing three times in PBS, the sections were incubated with a biotinylated secondary antibody (1:1000), 1.5% normal goat serum and 0.3% triton X-100 for 45 min before rinsing three times in PBS. The sections were subsequently incubated for 45 min with Vectastain ABC reagent (Dimention Laboratory), washed three times with PBS and incubated in nickel diaminobenzidine tetrahydrochloride (Ni-Dab) for 8–10 min. Finally, the staining reaction was inhibited by repeated PBS rinsing. The sections were dried and mounted on gelatin-coated slides and placed under coverslips and inspected