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Short communication

Co-localization of cart peptide immunoreactivity and nitric oxide synthase activity in rat hypothalamus

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Abstract

Because of the reported presence of both CART peptide and NOS activity in the same hypothalamic nuclei, their colocalization was examined. Eighteen percent of the neurons in the supraoptic nuclei, and 16% of the neurons in the paraventricular nucleus contained both CART immunoreactivity and NOS activity. Many other neurons in these regions stained for only one marker although they were often close by. Thus, CART peptides and NO may interact in these regions.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Regional localization of receptors and transmitters

Keywords: CART peptides; Nitric oxide synthase; Hypothalamus

CART (cocaine and amphetamine regulated transcript) refers to an mRNA [9] whose peptide products have been proposed to be neurotransmitters involved in various physiological processes [18]. CART mRNA and peptides are abundant and localized to specific brain regions and peripheral areas [5,16,14,11,28,13]. Particularly, CART peptides are found in brain areas associated with feeding and they indeed appear to have an inhibitory effect on food intake [5,16,14,28,17,19,10,4]. Also CART peptides are found in the HPA axis suggesting a role in stress [16].

There is also evidence that nitric oxide (NO) has an inhibitory effect on food intake, perhaps partly through hypothalamic mechanisms [22,23,29]. Nitric oxide synthase (NOS), identified using NADPH-diaphorase histochemistry, is abundant in different hypothalamic areas including the paraventricular nucleus [3]. NO stimulates the activity of neurons in the paraventricular nucleus of the hypothalamus (PVN; [20]), a brain location where CART is abundant [9,16,10], and is inhibited by glucocorticoids [27,21]. Because of the apparent similar effects and distribution of NOS and CART peptides, we compared their co-localization in the same neuronal populations in hypothalamic nuclei using a histochemical–immunohistochemical double labeling.

Abbreviations: AHN, anterior hypothalamic nucleus; ARH, arcuate nucleus of hypothalamus; BST, bed nuclei stria terminalis; Cir, circumventricular nucleus; dp, dorsal parvicellular part of paraventricular nucleus; f, forniceal part of paraventricular nucleus; fx, fornix; LHA, lateral hypothalamic area; MEex, external layer of median eminence; mpd, posterior magnocellular part dorsal zone of paraventricular nucleus; MPO, medial preoptic area; opt, optic tract; ox, optic chiasm; pmm, posterior magnocellular part medial zone of paraventricular nucleus; pv, periventricular part of paraventricular nucleus; PVa, anterior periventricular nucleus; PVHap, anterior parvicellular part of paraventricular nucleus; PVHlp, lateral parvicellular part of the paraventricular nucleus; PVHpv, periventricular part of paraventricular nucleus; PVpo, preoptic periventricular nucleus; SO, supraoptic nucleus; SOr, retrochiasmatic part of supraoptic nucleus; VMH, ventromedial nucleus of the hypothalamus

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Six sexually mature male Sprague–Dawley rats (3 months, 220–250 g) were used in the experiments. The rats were kept under standard colony conditions (3–4 per cage, 20–22°C, 12-h light/dark cycle) with food and water provided ad libitum.

The protocol used in the present study has been approved by the 'Animal Ethics Committee' of Ege University and the treatment of animals is in compliance with European Communities Council Directive (86/609/EEC) for the care and use of laboratory animals.

The rats were perfused intracardially initially with phosphate-buffered saline and then with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde. Brains were dissected out and cut in blocks. Each block was postfixed in the same fixative solution and then cryoprotected with 30% sucrose (12 h at 4°C) and stored using tissue cryoprotecting solution at −70°C. Tissue blocks were sectioned at 25-μm thickness in six series on a cryostat, along coronal planes.

Antiserum to CART peptide fragment 79–102 was prepared by Cocalico (Reamstown, PA) in rabbits. Specificity of this antiserum has been previously demonstrated [16].

Sections were washed in Tris-buffered saline (0.05 M, pH 7.4; TBS) 3×10 min, and incubated with 4% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.4% Triton X-100. The sections were then incubated with primary antibody overnight at 4°C in a buffer containing 0.4% TritonX-100, 1% NGS and 1% BSA. The next day, the sections were brought to room temperature for 2.5 h in the primary antibody incubation mixture. The sections were then washed 10×10 min in mixtures of TBS, 0.25% BSA and 0.02% Triton X-100. Thereafter, tissue was incubated with biotinylated anti-rabbit IgG (Vector Labs, Burlingame, CA, USA) at a concentration of 1.33 μg/ml for 60 min at room temperature in TBS containing 1% BSA and 0.02% Triton X-100. The sections were rinsed 4×15 min in TBS containing 0.25% BSA and incubated with the ABC complex for 1 h at room temperature (Vectastain™, 1:200 in 1% BSA; Vector Labs). The sections were washed in 175 mM imidazole acetate buffer pH 7.2, 2×10 min and developed in DAB solution (3,3′-diaminobenzidine, containing 2% nickel, Vector Labs) at room temperature under microscope control. Following development, the sections were washed in imidazole acetate buffer twice for 10 min at room temperature, washed in TBS twice for 10 min at room temperature, one set of sections mounted onto slides and the remaining sections were subjected to NADPH-diaphorase histochemistry. After air drying, the mounted sections were washed in distilled water, dehydrated through graded ethanol, cleared with xylene and coverslipped with Entellan.

Sections were washed in phosphate buffer (PB) two times for 10 min each time and then incubated at 37°C in a medium containing 0.08% Triton X-100 (Riedel-de Haen, 56029), 0.8 mM nitroblue tetrazolium (Sigma, N-6876) 1 mM β-NADPH (Sigma, N-7505) and 0.1 M Tris–HCl (pH 8.0) for 60 min.

The course of the reaction was controlled under the microscope in order to obtain the maximum contrast. To terminate the reaction, the incubation medium was removed and sections were rinsed 3×10 min in PB. Then, they were mounted onto gelatin-coated slides, dried overnight at 37°C, dehydrated through ethanol series, cleared with xylene, and coverslipped with Entellan (Merck). The sections were stored in the dark until analysed. Controls for specificity of the histochemical reaction were carried out: (1) omission of β-NADPH, (2) omission of nitroblue tetrazolium, (3) substitution of β-NADPH by β-NADP (Sigma, N-0505), and (4) denaturation of the enzyme by heating the tissue at 84°C for 5 min. No reaction was observed in these control experiments.

Using both described protocols, some sections previously processed for NADPH-d were immunostained for CART. Tests showed that when the histochemistry reaction was carried out in sections previously processed for immunostaining, NADPH-d staining was very faint. When the double staining was carried out beginning with the NADPH-d technique, both activities were clearly observed.

Co-localisation studies were carried out as described elsewhere [6]. Double-stained sections were examined and photographed, and then rinsed in formamide for 2 h. Fading of dark blue staining was checked under microscope and another set of pictures from the same fields were taken. The sections were then rinsed in formamide overnight to completely remove the blue formazan reaction product. A third set of pictures were taken and compared to the previous ones. Neurons showing both blue NADPH-d labeling and brown CART labeling at the same focusing plane, were marked as double labeled.

The localisation of CART peptide immunoreactivity and NOS (NADPH-d) activity is shown both in microphotographs (Fig. 1) and also in camera lucida drawings (Fig. 2). Many neurons contained either CART or NOS. CART peptide immunoreactivity and NOS activity were often found in separate cells that showed proximity which was compatible with the notion of an interaction of these cells, since nitric oxide freely diffuses across membranes. However, we also observed a significant colocalization of both CART and NOS within the same neurons.

Regions where colocalisation was observed included the PVN, SON, circumventricular nucleus, medial preoptic area and lateral hypothalamic area. The colocalization was clearly demonstrated using formamide which selectively washes out the blue staining of NOS activity (Fig. 1) facilitating the comparison of both stains. The percentage of neurons that contained both CART and NOS activity averaged 18% in the SO and 16% in the PVN at the level shown in Fig. 2.

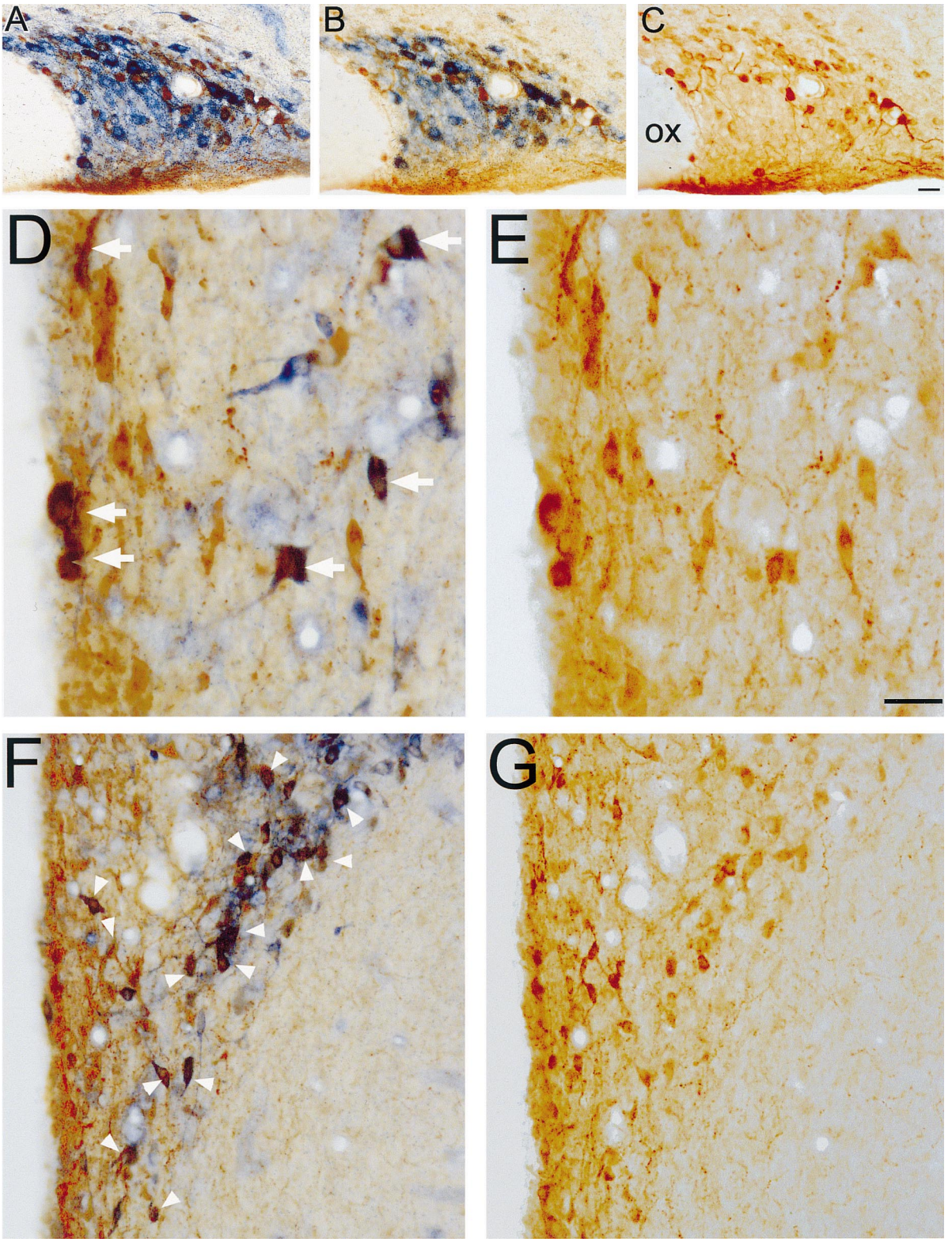

Fig. 1. Co-localisation of CART peptide immunoreactivity and NADPH-diaphorase activity in supraoptic and paraventricular nuclei of hypothalamus. Determination of colocalization was facilitated by comparing sections both before and after washing out the blue stain for NADPH-d and thus clearly revealing the brown stain for CART. SO nucleus is shown before treatment (A), after 2 h of formamide treatment (B) and after overnight formamide treatment (C). Formamide washes out the blue reaction product selectively. PVN is shown before treatment (D, F) and after overnight formamide treatment (E, G). White arrow and arrowheads indicate double-labeled neurons. Bars: A, B, C, F, G: 30 μm; D, E: 20 μm.

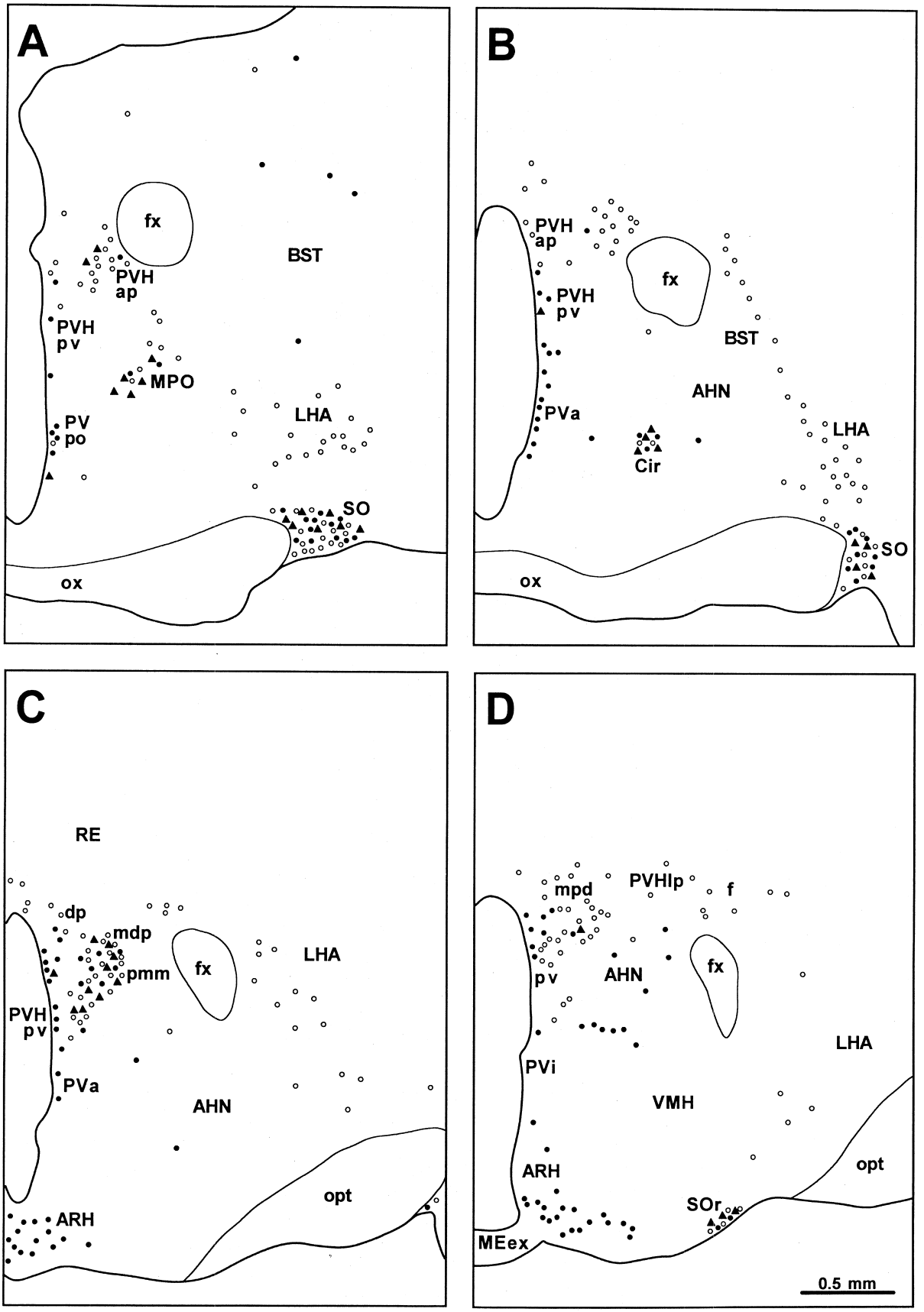

Fig. 2. Co-localisation of CART immunoreactivity and NADPH-diaphorase activity in different hypothalamic levels. Open circles: NADPH-d positive neurons; filled circles: CART positive neurons; triangles: double-labeled neurons.

Colocalisation of CART and NOS in the PVN and SO nuclei suggests that projections to the posterior pituitary also contain both CART and NOS. These findings point to a possible interaction between CART and NO, or a co-release in their modulatory effects of neuroendocrine function.

Both NO and CART involve food intake mechanisms. NO and CART decrease after fasting and it has been demonstrated that this action occurs through leptin [1,12]. These findings suggest a cotransmitter role for NO and CART on feeding.

Colocalization of CART peptides with other chemical messengers in other brain regions has been established. CART peptides colocalize with GABA [25,26], norepinephrine [15], POMC, oxytocin, and vasopressin [8,30]], somatostatin [13,30], and MCH [30]. Similarly, it has been shown that nitric oxide colocalizes with many other peptides or enzymes such as acetylcholinesterase [6,7] somatostatin [2], calretinin [3], vasopressin and oxytocin [24]. These findings, taken together with the data presented here, suggest that CART peptides are cotransmitters in many neuronal systems and that NO is colocalized and potentially interacts with CART peptide as well.

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