Origin of Cocaine- and Amphetamine-Regulated Transcript-Containing Axons Innervating Hypophysiotropic Corticotropin-Releasing Hormone-Synthesizing Neurons in the Rat

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Cocaine- and amphetamine-regulated transcript (CART) has stimulatory effects on the hypothalamic-pituitary-adrenal axis through direct effects on hypophysiotropic CRH neurons. Recently CART-containing axons have been demonstrated to densely innervate the hypophysiotropic CRH neurons. Based on the sources of the CART-immunoreactive (IR) innervation of the paraventricular nucleus, the putative origins of these CART-containing fibers include neurons of the hypothalamic arcuate nucleus that coexpress αMSH and medullary adrenergine-producing neurons. To determine whether these cell groups contribute to the CART innervation of the hypophysiotropic CRH neurons, we performed a quadruple-labeling immunofluorescent study using antisera against CRH, CART, αMSH, and phenylethanolamine-N-methyltransferase (PNMT), the latter as a marker for adrenaline. Consistent with previous observations, PNMT- and CART-IR axons densely innervated all CRH neurons, whereas the αMSH-IR innervation was sparse. Although approximately 60% of CART-IR varicosities in juxtaposition to CRH neurons cocontained PNMT, only approximately 18% of them were immunopositive for αMSH. All αMSH-IR boutons and approximately 90% of PNMT-containing varicosities on the surface of CRH neurons were also labeled for CART. The remaining 22% of CART axon varicosities in contact with CRH neurons contained neither αMSH nor PNMT. These results indicate that medullary adrenergic/CART neurons are the major source for the CART innervation of CRH neurons in the paraventricular nucleus; however, to a lesser extent the arcuate nucleus also contributes to the CART-IR innervation of these neurons. The observation that nearly 20% of the CART-IR afferents contain neither αMSH nor PNMT, however, suggests that additional sources also contribute to the CART-IR input of hypophysiotropic CRH neurons. (*Endocrinology* 146: 2985–2991, 2005)

CRH-SYNTHESIZING NEURONS, located in the paraventricular nucleus of the hypothalamus (PVN), are the principal regulators of the hypothalamic-pituitary-adrenal (HPA) axis (1, 2). These so-called hypophysiotropic CRH neurons project to the median eminence and secrete CRH into the portal capillary system, thereby controlling corticosterone secretion through the modulation of ACTH release by the pituitary gland (1, 2). These cells also integrate a variety of neuronal and humoral signals and form the final common pathway in the regulation of the HPA axis.

Cocaine- and amphetamine-regulated transcript (CART), a neuropeptide widely expressed in the brain (3, 4), has an important role in neuroendocrine regulation including a stimulatory effect on the HPA axis (5–7). CART-containing axons heavily innervate the PVN (3) and form synaptic specializations on the surface of hypophysiotropic CRH neurons (8). CART increases CRH mRNA levels in hypothalamic explants (7) and when injected into the PVN causes a rapid increase in plasma ACTH and corticosterone levels (6, 7, 9) through induction of CRH release (7). Furthermore, CART rapidly induces phosphorylation of the cAMP response element-binding protein (CREB) in approximately 75% of hypophysiotropic CRH neurons (8). Because the CRH gene contains a cAMP response element (10), these studies suggest a mechanism whereby CART may stimulate the synthesis of CRH.

In previous studies, we identified four different CART-containing neuron populations that contribute to the innervation of the PVN (11). These include neurons of the hypothalamic arcuate nucleus that coexpress αMSH (12), neurons of the medullary C1–3 regions that also produce adrenaline, neurons in the lateral hypothalamus/zona incerta, and neurons in the nucleus of the solitary tract (11). Because the propiomelanocortin (the precursor peptide of αMSH and other peptides) expressing neurons in the arcuate nucleus and the medullary adrenergic neurons are known to densely innervate hypophysiotropic CRH neurons (13, 14), we hypothesized that these two cell populations are major sources for the CART-immunoreactive (IR) innervation of CRH neurons in the PVN.
To test this hypothesis, we performed quadruple-labeling immunocytochemistry using antibodies raised in four different species against CART, CRH, αMSH, and phenylethanolamine-N-methyltransferase (PNMT), the enzyme synthesizing adrenaline. Furthermore, we estimated the proportion of CART-IR innervation of CRH neurons originating from the arcuate nucleus, C1–3 regions of the medulla, and yet unknown origins by determining the percentage of CART/αMSH-IR and CART/PNMT-IR double-labeled and CART-IR single-labeled axon varicosities on the surface of hypophysiotropic CRH neurons.

Materials and Methods

Animals

The experiments were carried out on adult male Wistar rats, weighing 200–500 g, housed under standard environmental conditions (light between 0600 and 1800 h, temperature 22 ± 1 °C, rat chow and water ad libitum). All experimental protocols were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine of the Hungarian Academy of Sciences and Tufts-New England Medical Center.

Tissue preparation

Because colchicine treatment is necessary to visualize the perikarya and dendrites of the hypophysiotropic CRH neurons and our preliminary studies indicated that a low dose of colchicine (40 μg) does not alter the staining pattern of CART, PNMT, or αMSH axons in the PVN, we used colchicine-treated rats for our studies. Three animals were deeply anesthetized with sodium pentobarbital (35 mg/kg body weight, ip) and stereotaxically injected intracerebroventricularly with 40 μg colchicine in 2 μl 0.9% saline. After 20 h of survival, the animals were perfused transcardially with 20 ml 0.01 M PBS (pH 7.4), followed sequentially by 100 ml of 2% paraformaldehyde/4% acrolein in 0.1 M phosphate buffer (pH 7.4) and 50 ml of 2% paraformaldehyde in the same buffer. The brains were immediately removed, and cryoprotected in 30% sucrose in 0.01 M PBS overnight at 4 °C, and quick frozen on dry ice. Serial 40-μm-thick coronal sections through the PVN were cut with a freezing microtome (Leica Microsystems, Wetzlar, Germany), collected in freezing solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer) and stored at -20°C until used.

Quadruple-labeling immunofluorescence for CART, CRH, αMSH, and PNMT

Immunocytochemistry was performed on every third section through the PVN. The sections from each brain were treated with 1% sodium borohydride in distilled water for 30 min and 0.5% Triton X-100/0.5% H2O2 in PBS for 20 min. Additional 20 min of 0.5% Triton X-100 was applied to improve antibody penetration. To reduce nonspecific antibody binding, the sections were then incubated in the following mixture of primary antibodies for 3 d at 4 °C: murine monoclonal antibody to CART (a gift from Les Thorm Claussen, Novo Nordisk, Bagsvaerd, Denmark) at a dilution of 3.34 μg/ml, guinea pig anti-CRH serum (Peninsula Laboratories Inc., San Carlos, CA) at 1:500, sheep anti-αMSH serum (a gift from Jeffrey B. Tatro, Tufts-New England Medical Center, Boston, MA) at 1:2000 and rabbit anti-PNMT serum (a gift from Martha C. Busciglio, Northwestern University Medical School, Chicago, IL) at 1:500. After rinses in PBS, the sections were incubated in the mixture of secondary antibodies for one day at 4 °C. The secondary antibodies were as follows: CY3-conjugated donkey antimouse IgG (Jackson ImmunoResearch, West Grove, PA) at 1:200, biotinylated donkey antiguinea pig IgG (Jackson ImmunoResearch) at 1:250, CY3-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch) at 1:100 and fluorescein isothiocyanate (FITC)-conjugated donkey antirabbit IgG (Jackson Immunoresearch) at 1:50. The sections were then rinsed in PBS, and incubated in 7-amin-4-methyl-coumarin-3-acetic acid (AMCA)-conjugated avidin D (Vector Laboratories, Burlingame, CA), diluted 1:200 for 1 d at 4 °C. Primary and secondary antibodies and AMCA-avidin D were diluted in PBS that contained 2% normal horse serum and 0.2% sodium azide. The sections were mounted onto glass slides and coverslipped with Vectashield (Vector) mounting medium.

Image analysis

The quadruple-labeled sections were examined using a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK). From each brain, at least three sections were analyzed from different rostrocaudal levels of the medial parvocellular subdivision of the PVN in which hypophysiotropic CRH neurons are located. The atlas by Paxinos and Watson (15) was used to identify the subdivisions of the PVN.

With 60× oil lens, 180 × 180 μm areas were recorded of the entire medial parvocellular subdivision of the PVN in each section. Two consecutive scans were recorded from each area. The first scan was for FITC, CY3, and CY5 (laser excitation lines 488 nm for FITC, 543 nm for CY3, and 673 nm for CY5); dichroic emission filters, 560/500–530 nm for FITC, 650/570–590 nm for CY3, and a 660-nm-long-pass filter for CY5. The second scan was for AMCA (laser excitation line 405 nm and emission filter 420–480 nm). Pinhole sizes were set to obtain optical slices less than 0.7 μm thick, and the series of optical slices were recorded with a 0.6 μm step. The series of optical sections were merged and displayed with LaserVox and Image Pro Plus software (BioRad Laboratories) and an IBM compatible personal computer. Tracing individual CRH-IR neurons and their dendrites through the series of optical sections, the number of axon varicosities containing only CART, only PNMT, both CART and PNMT, and both CART and αMSH in juxtaposition to CRH neurons were counted. A varicosity was considered in contact with or juxtaposition to CRH neurons if a gap could not be recognized between the two profiles by confocal microscopy. Whereas it is recognized that this analysis does not prove synaptic association between the profiles, studies from our laboratories have previously demonstrated the presence of synaptic associations between axon terminals containing CART, PNMT, and ACTH, a marker of αMSH-synthesizing neurons, with hypophysiotropic CRH neurons (8, 13, 14). CRH neurons were included in the analyses only if the entire cell body of the neuron was contained within the stack of the optical sections. Data are presented as mean ± se.

To illustrate quadruple labeling, we used the three basic colors (red, green, and blue) and show pairs of triple-colored images of the same field and magnification in adjacent figures. Thus, CART, CRH, and PNMT immunoreactivity was displayed in one image, whereas CART, CRH, and αMSH immunoreactivity was shown in the second image. Accordingly, CART and CRH immunoreactivities were displayed in red and blue, respectively, in both images, whereas the green color labeled either PNMT or αMSH immunoreactivity. Therefore, CART/PNMT and CART/αMSH-IR double-labeled axons appeared yellow. All presented images represent single optical slices. Images captured through ×20 objective (Fig. 1, A1 and A2) are less than 2.1 μm thick, whereas images captured through ×60 oil lens (Figs. 1, B1 and B2, and 2) are less than 0.7 μm thick.

Control experiments

Specificity of anti-CART, anti-αMSH, and anti-PNMT sera have been reported previously (12, 16, 17). The specificity of guinea pig anti-CRH serum was determined by double immunostaining with rabbit anti-CRH serum (Peninsula Laboratories), the specificity of which has been reported elsewhere (18). Double-labeling immunocytochemistry showed that guinea pig anti-CRH serum labeled exactly the same neurons in the medial parvocellular subdivision of PVN as the rabbit anti-CRH serum did.

To exclude the possibility that CY3-conjugated antirabbit IgG binds to PNMT or that the FITC signal denoting PNMT immunofluorescence is seen in the red channel causing false PNMT and CART double labeling, we performed several control experiments. After the incubation in rabbit anti-PNMT serum, the sections were transferred into the mixture of FITC-conjugated antirabbit IgG and CY5-conjugated antirabbit IgG without incubation in mouse CART antibody. Scanning the sections with confocal laser microscope using the same...
scanning method described in Image analysis and the same settings, we found a dense PNMT-IR fiber network, but no signal was detected between 570 and 590 nm wavelengths, corresponding to the emission of CY3. We also performed sequential double immunostainings for CART and PNMT, staining first with anti-CART serum, followed by anti-PNMT serum and vice versa. Both resulted in the same degree of colocalization, excluding the possibility that the high level of co-localization was the result of any cross-reactivity.

Fig. 1. A1 and A2, Low-magnification confocal images of the same field demonstrate the distribution of CART (red), CRH (blue), and PNMT-IR (green) (A1) and CART (red), CRH (blue), and aMSH-IR (green) (A2) elements in the PVN. A1, CART immunoreactivity is present in the vast majority of PNMT-IR axons in the PVN, resulting in the yellow color. Note the high density of CART/PNMT-IR fibers surrounding CRH neurons. A2, CART immunoreactivity is present in all aMSH-IR axons in the PVN. CART/aMSH-IR fibers are more sparse in the region in which CRH neurons are located but are more abundant in the dorsal, periventricular, and ventral parvocellular subdivisions and in the ventral part of the medial parvocellular subdivision. B1 and B2, Medium-power confocal images of the same field illustrate the different CART-IR varicosities in juxtaposition to CRH neurons. B1, Color matching: CART, red; PNMT, green; CRH, blue. CRH neurons are embedded in a dense network of CART/PNMT-IR fibers (yellow due to color mixing). Numerous CART/PNMT-IR varicosities (indicated by arrows) are in tight apposition to CRH neurons. B2, Color matching: CART, red; aMSH, green; CRH, blue. In the same optical section, CART/aMSH-IR axons (yellow due to color mixing) are much less abundant. Arrows point to CART/aMSH-IR boutons in contact with CRH neurons. Arrowheads indicate single-labeled CART-IR boutons (containing neither PNMT nor aMSH) closely apposed to CRH neurons. In both B1 and B2, asterisk labels a CRH neuron contacted by both CART/PNMT-IR and CART/aMSH-IR boutons; x, a CRH neuron contacted by CART/PNMT-IR and single-labeled CART-IR boutons; a, a CART-IR CRH neuron. Scale bars, 100 μm (shown in A2) for A1 and A2, and 20 μm (shown in B2) for B1 and B2. III, Third ventricle.
CART-IR axons densely innervated all parvocellular subdivisions of the PVN. In addition, numerous cell bodies in the parvocellular as well as the magnocellular subdivisions of the PVN were immunofluorescent for CART. For the most part, CART- and CRH-IR neurons formed two distinct neuron populations in the PVN, with only occasional CRH neurons containing CART-immunoreactivity (Fig. 1). Whereas only a subpopulation of CART-IR fibers contained αMSH in the PVN, all αMSH-containing axons cocontained CART in this nucleus, as we have previously described (5). Similarly, the vast majority of PNMT-IR fibers contained CART in the PVN. In the parvo cellular subdivisions of the PVN, the distribution of αMSH- or PNMT-containing CART-IR axons showed major differences. CART/αMSH-IR axons were mainly concentrated in the periventricular, dorsal, and ventral parvocellular subdivisions, whereas the occurrence of these fibers was less frequent in the medial parvocellular subdivision (Fig. 1, A1), except for the ventral part and the most caudal region of this subdivision. In contrast, all parvocellular subdivisions were densely innervated by CART/PNMT-IR axons, and the most abundant network of these fibers was observed in the median parvocellular subdivision (Fig. 1, A2) in which the main cluster of CRH neurons was located.

CART/PNMT-IR axon varicosities were found in juxtaposition to nearly all CRH neurons (95.00 ± 1.53%) (Figs. 1 and 2). An average of 5.45 ± 0.14 CART/PNMT boutons per CRH cell were observed. CART/αMSH-containing boutons were also found in close proximity to CRH neurons but not as frequently as CART/PNMT boutons (Figs. 1 and 2). CART/αMSH fibers were in juxtaposition to 58.67 ± 3.71% of CRH neurons, and an average of 2.73 ± 0.13 CART/αMSH boutons were found on the surface of the innervated cells. CRH neurons were more frequently contacted by CART/αMSH varicosities in the ventral part of the medial parvocellular subdivision. In these regions, more CART/αMSH boutons were juxtaposed to the CRH neurons than in the dorsal part of the subdivision. Both CART/PNMT-IR and CART/αMSH-IR varicosities were observed in juxtaposition to the soma as well as to the dendrites of CRH neurons.

Of all CART-containing axon varicosities located on the surface of CRH neurons, 59.60 ± 2.10% contained PNMT, whereas only 18.47 ± 1.55% contained αMSH. An additional 21.93 ± 1.98% of CART-IR varicosities were exclusively single labeled, suggesting that these axons originate from loci other than the arcuate nucleus and the C1–3 areas. These single-labeled CART-IR varicosities were juxtaposed to 68.33 ± 4.91% of CRH neurons. No regional preference was found in the distribution of neurons innervated by single labeled CART-IR varicosities. Table 1 shows the results of the quantitative analysis.

The majority of CRH neurons were contacted by more than one type of CART-IR axons. More than half (56.67 ± 3.71%) of the CRH neurons were contacted by both CART/PNMT and CART/αMSH-varicosities. The ratio of CRH neurons contacted by both single-labeled and CART/PNMT-IR boutons were 64.33 ± 5.84%, whereas 38.67 ± 3.38% of CRH neurons were innervated by the three different types of CART-IR axons.

The vast majority (88.26 ± 1.61%) of PNMT-IR boutons and all αMSH-IR boutons in juxtaposition to CRH neurons contained CART-immunoreactivity.

**Discussion**

In the present study, we demonstrate that the CART-IR innervation of hypophysiotropic CRH neurons is heterogeneous and originates from at least three different sources: 1) adrenergic CART neurons in the medullary C1–3 areas, 2) CART/αMSH neurons in the arcuate nucleus, and 3) an additional population of CART-IR neurons of yet unknown origin(s). The majority, approximately 60%, of the CART-IR innervation of hypophysiotropic CRH neurons originates from the adrenergic CART neurons of the brain stem. CART/PNMT-IR varicosities were found in juxtaposition to nearly all CRH neurons (95%). Because CART and PNMT are coexpressed in the majority of neurons in the cranial part of the C1 area and throughout the C2 and C3 cell groups (11, 19, 20), we presume that CART/PNMT axons in contact with hypophysiotropic CRH neurons originate from one or more of these regions. The minority (12%) of adrenergic axon varicosities in contact with CRH neurons in the PVN that did not cocontain CART presumably originate from the caudal portion of the C1 region (11).

Little is known about the physiological role of CART synthesized in the adrenergic neurons. Stimulation of adrenergic receptors, however, has activating effects on CRH neurons, increasing CRH mRNA and CRH hnRNA content in the medial parvocellular subdivision of the PVN (21–23) and also increasing pituitary POMC mRNA and ACTH secretion (24). The coexistence of adrenaline and CART in the same varicosities therefore raises the possibility that these substances act independently or potentiate each other’s effect in the regulation of...
CRH neurons. Along these lines, α1-adrenergic receptors are expressed in virtually all CRH neurons (25). When activated, these receptors can induce the phosphorylation of CREB (26) and therefore may stimulate the CRH promoter by binding of phospho-CREB to the cAMP response element in the CRH gene (10). As previously shown in our laboratories, CART also induces the phosphorylation of CREB in hypophysiotropic CRH neurons (8), suggesting either direct effects of CART (the CART receptor is yet unknown) or indirect effects potentially mediated by the release of catecholamines.

One of the best described physiological effects of medullary C1–3 neurons in the regulation of the HPA axis is their role in mediating increased CRH release in response to glucoprivation. If all noradrenergic and adrenergic neurons projecting to the PVN are ablated, the corticosterone response to glucoprivation is severely impaired and the induction of CRH heteronuclear RNA in the PVN totally abolished (27). C1–3 adrenergic neurons are implicated in this response because glucoprivation activates these neurons, whereas it does not activate noradrenergic neurons in A2 and A6 regions, which are also known to project to the PVN (28, 29).

Medullary adrenergic neurons may also mediate activation of the HPA axis in response to infectious and inflammatory stimuli. Adrenergic neurons in C1 and C2 areas that project to the PVN express c-fos after systemic administration of IL-1β (30, 31), a proinflammatory cytokine, whereas lesions in the C1 or C2 area reduce the number of IL-1β-activated CRH neurons in the PVN (31). Furthermore, transection of ascending brain stem pathways, including the adrenergic fibers from the medulla, significantly reduces the IL-1β or bacterial lipopolysaccharide-induced increase in CRH mRNA levels in the PVN (30, 32). Thus, the presence of CART in the majority of axon terminals originating from C1–3 regions raises the possibility that increased CART signaling to CRH neurons might also contribute to glucoprivic- and cytokine-induced activation of hypophysiotropic CRH neurons.

The second major group that contributes to the CART-IR innervation of hypophysiotropic CRH neurons is the CART/αMSH-expressing neurons in the arcuate nucleus. This innervation is less robust than the adrenergic CART input because CART/αMSH-IR varicosities comprise only 18% of all CART-containing boutons in juxtaposition to CRH neurons. In addition, only, approximately half of the CRH neurons are contacted by CART/αMSH axons. αMSH has an activating effect on CRH synthesis in hypophysiotropic neurons (33), and central administration of melanocortin agonists induces the expression of CRH hnRNA in the PVN and increases circulating corticosterone levels in a CRH-dependent way (34, 35). It has been suggested that transcriptional activation of the CRH gene by αMSH is mediated in part by CREB phosphorylation through the activation of cAMP-protein kinase A pathway by melanocortin-3 and melanocortin-4 receptors (36). Therefore, as proposed for CART-adrenaline interactions, CART may either have an independent action on hypophysiotropic CRH neurons to induce CREB phosphorylation or potentiate the action of αMSH.

CART/αMSH neurons participate in the mechanism by which leptin signals to the central nervous system to regulate body weight. The fall in circulating levels of leptin caused by fasting reduces the synthesis of both CART and αMSH in arcuate neurons (37–39) and may be responsible for fasting-induced reduction in CRH mRNA in the PVN (33, 40). In contrast, the leptin-induced increase of CART and αMSH synthesis in arcuate nucleus neurons may contribute the recovery of CRH synthesis after refeeding (33). Because CART mRNA is increased by endotoxin in neurons of the arcuate nucleus (41), this neuron population may also contribute to the lipopolysaccharide-induced activation of the hypophysiotropic CRH neurons described above.

A final group of CART-IR axons that contain neither PNMT nor αMSH but also contributes to the CART innervation of hypophysiotropic CRH neurons contacts approximately 68% of CRH neurons in the PVN and comprises approximately 22% of the CART-IR varicosities contacting the surface of the CRH neurons. Based on retrograde tract-tracing studies (11), we presume that one or more of the CART cell groups in the lateral hypothalamus/zona incerta complex and medial subnucleus of the nucleus tractus solitarius contribute to this innervation, although CART-IR neurons residing in the PVN may also participate. These cell groups may be involved in the regulation of energy homeostasis (42), conveying visceral information to the hypothalamus/zona incerta and medial subnucleus of the nucleus tractus solitarius (43) and control of prolactin secretion (44), respectively. We cannot exclude the possibility, however, that some of the singly labeled CART-IR varicosities in association with CRH neurons actually derive from C1–3 adrenergic populations or αMSH-producing neurons in the arcuate nucleus but that the concentration of these substances was below the detection limit of the immunocytochemical procedure. Furthermore, because it was not possible to assess the association of CART-IR varicosities with the most distal portions of the CRH neuronal dendritic tree, the innervation of CRH neurons by CART-containing axon terminals could be even more varied.

We conclude that hypophysiotropic CRH neurons receive their CART-IR innervation from at least three different sources. The majority of CART-IR fibers originate from adrenergic CART neurons in the medulla, approximately 20% derives from the arcuate nucleus and an additional 20% from a yet unidentified origin. Because each region responds specifically to different physiological stimuli, CART may be used as a common messenger in the brain involved in the mediation of diverse signals that activate the HPA axis.

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