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Research report

Medullary adrenergic neurons contribute to the cocaine- and amphetamine-regulated transcript-immunoreactive innervation of thyrotropin-releasing hormone synthesizing neurons in the hypothalamic paraventricular nucleus ☆

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Abstract

Cocaine- and amphetamine-regulated transcript (CART)-IR axons densely innervate the thyrotropin-releasing hormone (TRH) neurons in the hypothalamic paraventricular nucleus (PVN), partly arising from neuronal perikarya in the hypothalamic arcuate nucleus. The source of the remaining CART innervation, however, is unknown. We have recently demonstrated that neurons co-containing adrenaline and CART in the C1–3 areas of the medulla project to the PVN. Since adrenergic neurons densely innervate the hypophysiotropic TRH neurons, we raised the possibility that adrenergic neurons contribute to the CART-IR innervation of hypophysiotropic TRH neurons. Combined in situ hybridization and immunocytochemistry was performed to study the colocalization of CART and phenylethanolamine *N*-methyltransferase (PNMT), the synthesizing enzyme of adrenaline, in axons innervating the hypophysiotropic TRH neurons. PNMT was observed in 44% of CART-IR axons in juxtaposition to the hypophysiotropic TRH neurons and CART-IR was observed in approximately 50% of all PNMT axons in contact with proTRH perikarya in the PVN. We conclude that adrenergic neurons of the medulla give rise to approximately half of the CART-IR axons innervating hypophysiotropic TRH neurons in the PVN, and propose that CART may play important role in the modulation of adrenergic input to the hypothalamic–pituitary–thyroid axis.

Keywords: Hypothalamic paraventricular nucleus; Thyrotropin-releasing hormone; Cocaine- and amphetamine-regulated transcript; Phenylethanolamine *N*-methyltransferase; Adrenergic neuron; Colocalization

1. Introduction

Cocaine- and amphetamine-regulated transcript (CART) has important role in the regulation of the energy homeostasis [19,20]. Central administration of CART inhibits food intake, whereas inhibition of endogenous CART by the intracerebroventricular administration of CART antiserum increases food intake [19,20]. More recently, it has been shown that CART can exert its catabolic effects not only by reducing food intake, but also by increasing energy expenditure [25]. CART has a widespread distribution in the brain [7,8,17,18] including hypothalamic and brainstem nuclei involved in the regulation of energy homeostasis [7,8,17,18]. In the arcuate nucleus CART colocalizes with the anorexic peptide, α-melanocyte-stimulating hormone (α-MSH) [10,12], and similar to α-MSH, is upregulated by leptin [2,19].

Another important component of the regulatory mechanism involved in the control of energy expenditure is the hypothalamic–pituitary–thyroid (HPT) axis, regulated by thyrotropin-releasing hormone (TRH)-synthesizing neurons residing in the hypothalamic paraventricular nucleus (PVN) [21]. These neurons not only are heavily innervated by axon

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terminals containing CART [12], but also show a marked increase in TRH mRNA following the exogenous administration of CART to fasting animals [12]. Two different kinds of CART-IR axons in contact with hypophysiotropic TRH neurons have been identified [12]. The first type establishes large varicosities on the surface of TRH neurons and co-contain α-MSH [12]. These axons arise exclusively from the arcuate nucleus [12]. The second type of contact has smaller varicosities, do not contain α-MSH, and are more numerous on the surface of hypophysiotropic TRH neurons than those containing α-MSH [12]. These features indicate that the second type of CART-containing axon terminal in contact with TRH neurons originates outside of the arcuate nucleus. Possible sources for these terminals include the zona incerta, lateral hypothalamus, perifornical region, C1–3 areas in the medulla, parabrachial nucleus and nucleus tractus solitarius, based on the accumulation of retrogradely transported material in CART-producing neurons in these regions following its injection into the PVN [14]. Within the C1–3 areas, the majority of CART neurons projecting to the PVN also produce the adrenaline-synthesizing enzyme, phenylethanolamine *N*-methyltransferase (PNMT) [14]. Since TRH neurons in the PVN are heavily inundated by axon terminals producing both epinephrine and norepinephrine [23], we raised the possibility that C1–3 neurons also contribute to the CART innervation of these neurons. Therefore, we performed a triple fluorescence immunolabeling study, using PNMT as a marker for CART containing axons originating from the C1–3 regions, to determine whether these axons innervate hypophysiotrophic TRH neurons.

2. Materials and methods

2.1. Animals

The experiments were carried out on six adult, male Wistar rats, weighing 280–320 g. The animals were 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.

2.2. Tissue preparation and triple-labeling fluorescent in situ hybridization and immunofluorescence

Since, in preliminary experiments, colchicine was necessary to increase the number of the detected TRH-IR neurons by immunofluorescence but markedly decreased the number of CART-IR axons in the PVN, CART- and PNMT-containing axons were detected by immunocytochemistry and proTRH-synthesizing neurons by in situ hybridization histochemistry in intact animals. Under sodium pentobarbital anesthesia (35 mg/kg BW, i.p.), animals were perfused by intracardiac perfusion with 20 ml 0.01 M phosphate buffer saline (PBS), pH 7.4, followed by 150 ml 4% paraformaldehyde in PBS. The brains were removed and postfixed by immersion in the same fixative for 2 h at room temperature. Tissue blocks containing the hypothalamus were cryoprotected in 20% sucrose in PBS at 4 °C overnight, then frozen on dry ice. Serial 20 μm thick coronal sections through the rostrocaudal extent of the PVN were cut with a freezing microtome and collected in freezing solution (30% ethylene glycol; 25% glycerol; 0.05 M phosphate buffer) and stored at −20 °C until used.

Serial sections through the PVN were washed in 2-fold concentration of standard sodium citrate (2 × SSC), acetylated with 0.25% acetic anhydride in 0.9% triethanolamine for 10 min and then treated in 50, 70 and 50% acetone, for 5, 10 and 5 min, respectively. After further washes in 2 × SSC for 2 × 5 min, the sections were hybridized with digoxigenin-labeled cRNA probe for proTRH. Digoxigenin-labeled antisense proTRH cRNA was synthesized using a 1241-base pair cDNA template corresponding to the coding sequence of proTRH mRNA and portions of its 5′ and 3′ untranslated sequences [9,16]. The hybridization was performed in 300 μl polypropylene tubes in hybridization buffer (50% formamide, 2 × SSC, 10% dextran sulfate, 0.5% sodium dodecyl sulfate, 250 μg/ml denatured salmon sperm DNA) containing the digoxigenin labeled probe, diluted at 1:50, for 16 h at 56 °C. The sections were washed in 1 × SSC for 15 min and then treated with RNase (100 μg/ml) for 1 h, at 37 °C. After additional washes in 1 × SSC (15 min), 0.5 × SSC (15 min) and 0.1 × SSC (2 × 30 min) at 65 °C, sections were washed in PBS, treated with the mixture of 0.5% Triton X-100 and 0.5% H_2O_2 for 15 min and then with 1% bovine serum albumin in PBS for 20 min to reduce the nonspecific antibody binding. The sections were incubated in sheep anti-digoxigenin-peroxidase Fab fragments (1:100, Boehringer Mannheim) in 1% BSA in PBS overnight at 4 °C. The sections were then rinsed in PBS and then incubated in 0.1% biotinylated tyramid and 0.01% H_2O_2 in PBS for 10 min to intensify the hybridization signal [1]. After further washes, the sections were incubated in AMCA Avidin D (1:250, Vector Labs, Burlingame, CA) for 1 h. After rinsing in PBS, sections were transferred to the mixture of mouse monoclonal anti-CART (41–89) antibody (1:1000, gift of Jes Thorn Clausen, Novo Nordisk, Bagsvaerd, Denmark) and rabbit anti-PNMT (1:1,000, gift of Martha C. Bohn, Northwestern University Medical School, Chicago, IL) for 2 days at 4 °C, rinsed in PBS, and then incubated in a mixture of CY3-conjugated donkey anti-mouse IgG (1:100; Jackson ImmunoResearch) and FITC-conjugated donkey anti-rabbit IgG (1:50; Jackson ImmunoResearch) for 2 h. The sections were mounted onto glass slides, coverslipped with Vectashield mounting medium (Vector Laboratories), and analyzed under a Zeiss Axiophot epifluorescent microscope using the following

filter sets: for AMCA, excitation of 320–400 nm, bandpass of 400 nm, and emission of 430–490 nm; for CY3, excitation of 540–590 nm, bandpass of 595 nm, and emission of 600–660 nm; and for FITC, excitation of 460–500 nm, bandpass of 505 nm and emission of 510–560 nm or a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK) using the following laser excitation lines: 405 nm for AMCA, 488 nm for FITC, 543 nm for CY3 and Dichroic/emission filters 500 nm/420–480 nm AMCA, 560 nm/500–530 nm for FITC and 560–610 for CY3. Thus, pro-TRH mRNA-containing perikarya were labeled in blue, CART-containing fibers were labeled in red, and PNMT-containing fibers were labeled in green under their respective filter sets. Characterization of CART and PNMT antisera have been described elsewhere [5,29]. Images were captured with a RT Spot digital camera (Diagnostic Instrument, Sterling Heights, MI), the same field triple exposed while switching the filter sets for each fluorochrome and superimposed using Adobe Photoshop 5.5 and an IBM compatible personal computer to create a composite image for analysis. Varicosities co-containing both CART and PNMT appeared yellow in the composite images. The number of axon varicosities containing only CART, only PNMT or both CART and PNMT in juxtaposition to proTRH neurons were counted on superimposed images of three animals. The ratio of all CART- vs. the CART/PNMT-varicosities and all PNMT- vs. CART/PNMT-varicosities were calculated in each brain. Data are presented as mean ± S.E.M.

3. Results

ProTRH mRNA-containing neurons were symmetrically distributed in the parvocellular division of the PVN and readily visualized by blue fluorescence of the AMCA fluorochrome (Figs. 1 and 2). The hybridization signal filled the perikarya and in some neurons, its proximal dendrites (Fig. 2). As previously reported [6,11,12], the majority of the TRH mRNA-expressing neurons in the periventricular and medial parvocellular subdivisions co-contained CART-immunoreactivity (Figs. 1 and 2B,C,E–G). In the confocal images, CART-immunoreactivity organized primarily in a perinuclear distribution reminiscent of the location of the

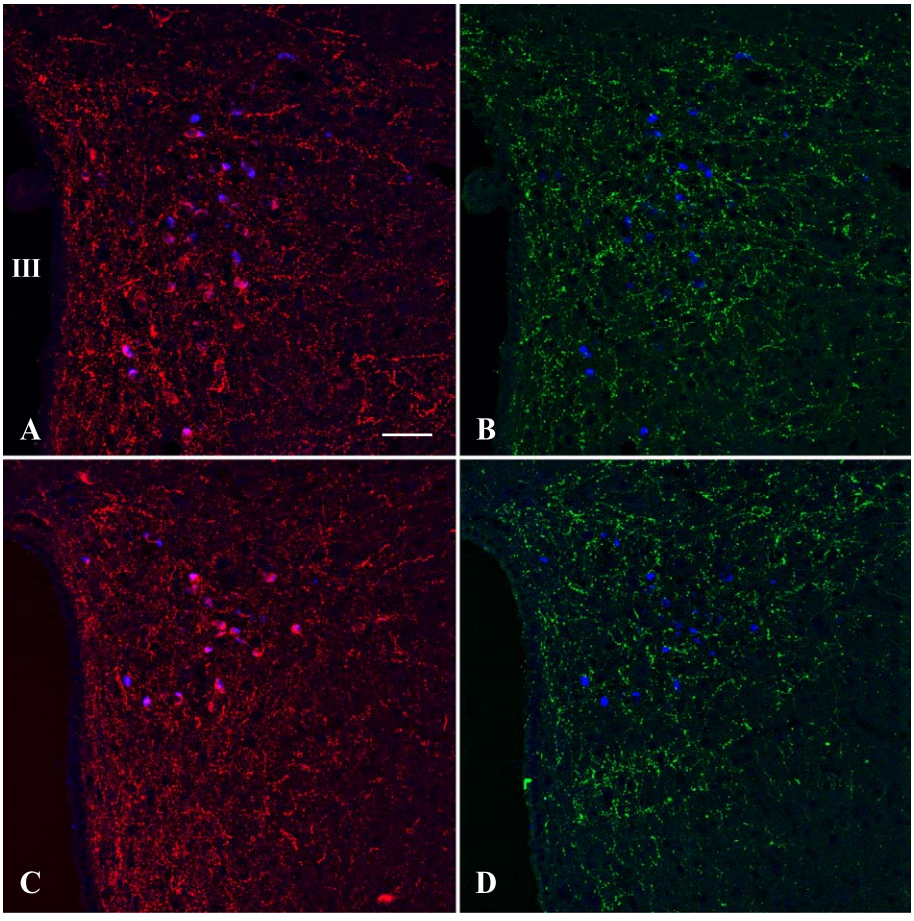
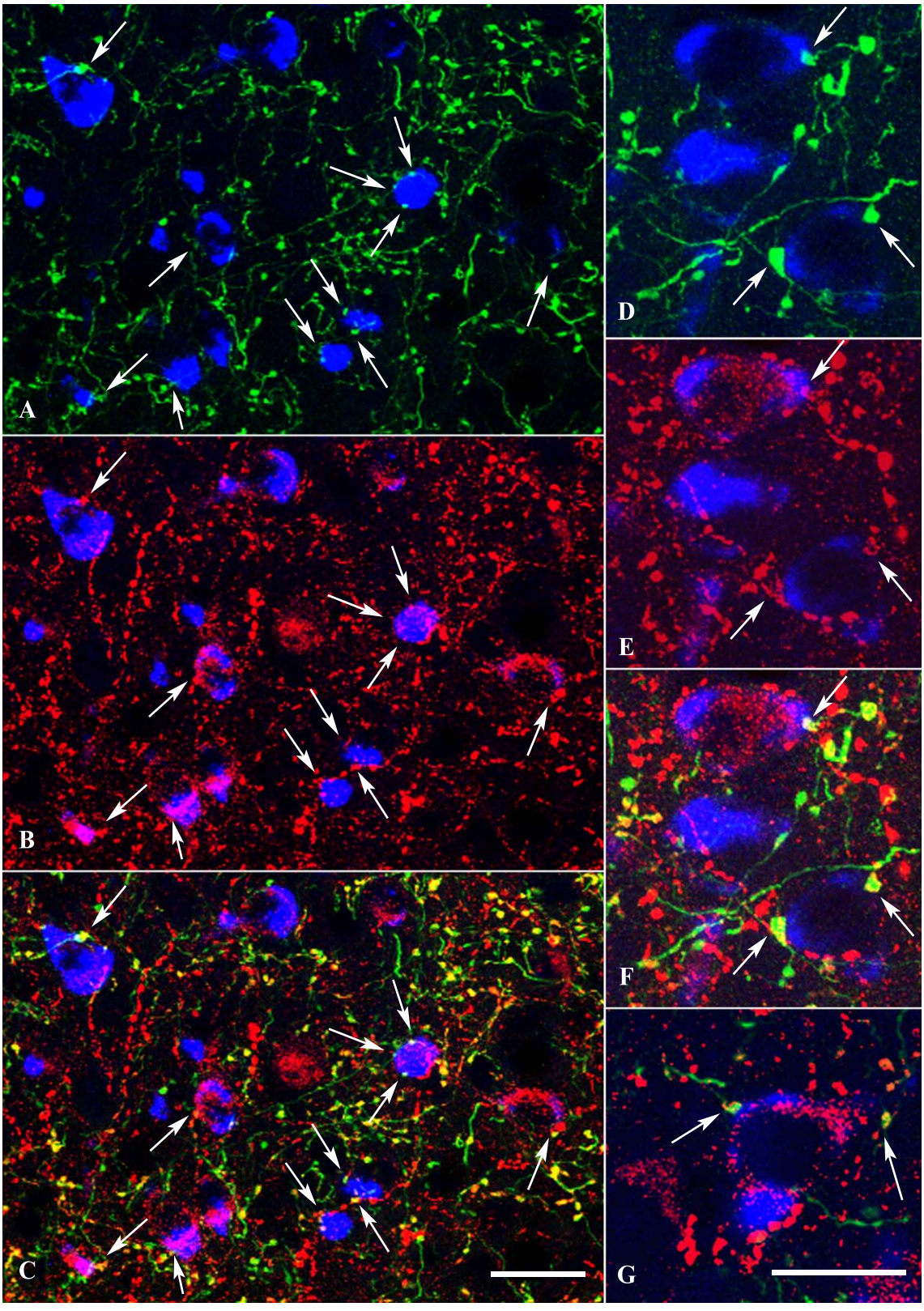


Fig. 1. Relationship between CART- (red) and PNMT-IR (green) axons and proTRH mRNA-containing neurons (blue) in the PVN. Low power magnification images of the same field from the mid (A,B) and caudal (C,D) levels of the PVN illustrates that proTRH-synthesizing neurons are embedded in a dense network of CART- (A,C) and PNMT-IR (B,D) axons. III: Third ventricle. Scale bare = 50 μm.

Golgi apparatus, and appeared to be separately compartmentalized from that of the proTRH hybridization signal, (Fig. 2E–F). In other neurons, however, CART-immunoreactivity was more generally dispersed in the perikarya, intermingling with the proTRH hybridization signal (Fig. 2B,C).

CART-IR and PNMT-IR nerve fibers showed a similar distribution in all parvocellular subdivisions of the PVN,



although the density of CART-IR fibers was greater. In the periventricular and medial parvocellular subdivisions, where hypophysiotropic TRH neurons are located, both CART- and PNMT-IR axon varicosities were found in close juxtaposition to nearly all proTRH-containing neurons (Fig. 1). By semiquantitative analyses, the number of CART- and PNMT-IR axon varicosities on the surface of proTRH neurons were approximately equal in number in the medial parvocellular subdivision, while in the periventricular subdivision, PNMT-IR varicosities exceeded CART-IR varicosities by 1.4-fold. Superimposed images of the three fluorochromes revealed that PNMT-immunoreactivity was present in 44.0 ± 3.6% of CART-IR axon varicosities in close apposition to proTRH neurons in the PVN. Conversely, approximately half of the PNMT-IR varicosities contact with proTRH mRNA-containing neurons also contained CART-IR in both the periventricular (51.3 ± 5.6%) and medial (53.2 ± 1.1%) parvocellular subdivisions.

4. Discussion

While previous studies from our laboratories demonstrated that CART-containing axon terminals in contact with TRH neurons in the PVN arise from the hypothalamic arcuate nucleus [12], these nerve fibers terminate preferentially on TRH neurons in the periventricular parvocellular subdivision and contact only approximately one-third of TRH neurons in the medial parvocellular subdivision. Since the medial parvocellular subdivision of the PVN contains the majority of hypophysiotropic neurons and are equally as well inundated by CART-containing axon terminals as the periventricular parvocellular subdivision TRH neurons, the origin of the CART projections to TRH neurons in the medial parvocellular subdivision were largely unknown. Given the rich innervation of TRH neurons in the medial parvocellular subdivision of the PVN by catecholaminergic fibers originating in the brainstem [23], we were intrigued by our recent observations using retrogradely transported markers, that other candidate cell populations giving rise the CART axonal innervation to the PVN are located in the medulla including the adrenergic C1–3 neurons and the nucleus tractus solitarius [14].

In the present study, we demonstrate that nearly 50% of CART-containing axons in contact with proTRH mRNA-expressing perikarya in the medial and periventricular parvocellular subdivisions of the PVN are immunoreactive for PNMT, a specific marker for adrenaline-producing neurons. Since PNMT and CART coexist only in the C1–3 regions of the medulla [14], these data would suggest that C1–3 medullary neurons contribute substantially to the CART innervation of hypophysiotropic proTRH neurons.

Since it was necessary to use in situ hybridization histochemistry to identify proTRH neurons because CART-immunoreactivity in fibers innervating the PVN was markedly reduced by colchicine administration necessary for adequate visualization of TRH-IR perikarya, we were only able to assess the ratio of double labeled varicosities in contact with perikarya of these proTRH neurons. While proTRH mRNA extended in the proximal dendrites of some neurons, the dendritic extensions of most neurons were largely unseen. Since it is likely that axons containing CART and/or PNMT also establish contacts with dendrites of TRH neurons [23], it is possible that the percentage of double-labeled axons contacting dendrites of proTRH cells is even greater than determined in this study.

The colocalization of CART and adrenaline in axon varicosities innervating the perikarya of TRH neurons implies a potential role for CART in modulating the effects of catecholamines in the regulation of TRH in the PVN. Indeed, both substances have been shown to have activating effects on hypophysiotropic TRH neurons in vitro and in vivo [12], both by increasing TRH gene expression [12] and TRH secretion [3,12]. In addition, Vaarmann and Kask [30] have shown that CART peptides are capable of increasing monoamines in several regions of the rat brain, and in rabbits, Matsumura et al. [24] have demonstrated that the intracerebroventricular injection but not intravenous injection of CART elicits a dose-related increase in sympathetic nerve activity and circulating levels of adrenaline and noradrenaline.

Perhaps one of the best recognized functions of brainstem catecholamine-producing neurons on hypophysiotropic TRH is to increase thyroid hormone levels in response to cold exposure [4]. Thyroid hormone levels and TSH are acutely elevated by cold exposure in several animal species due to a rapid increase in TRH mRNA in the PVN, peaking within 30 to 60 min of the stimulus [27,32], and is associated with an increase in the hypothalamic concentration of adrenaline and noradrenaline by

Fig. 2. Colocalization of CART- and PNMT-immunoreactivity in axon terminals in contact with proTRH mRNA containing neurons in the PVN. Medium magnification confocal images of the same field (A–C) demonstrate (A) the PNMT-IR (green) and (B) CART-IR (red) innervation of proTRH mRNA containing neurons (blue) in the medial parvocellular subdivision at the mid level of the PVN. Axons containing both CART and PNMT appear yellow in the composite image (C). Arrows denote axon varicosities containing both CART and PNMT juxtaposed to proTRH neurons. High power confocal photomicrographs (D–F) illustrates (D) the PNMT-IR (green) and (E) CART-IR (red) innervation of proTRH mRNA containing neurons (blue) in the medial parvocellular subdivision at the caudal level of the PVN. (F) Composite image showing colocalization of PNMT and CART in axons (arrows) juxtaposed to proTRH neurons. (G) A high power confocal photomicrograph from the periventricular parvocellular subdivision of the PVN showing two axon varicosities (arrows) containing PNMT and CART in juxtaposition to a proTRH neuron. Note presence of CART-immunoreactivity in the majority of proTRH neurons (B,C,E–G). The tendency for CART to be primarily located in a perinuclear region of the cytoplasm is particularly well seen in (E–G). Scale bar = 25 μm in (C) corresponds to (A–C); Scale bar in (G) = 15 μm corresponds to (D–G).

push–pull perfusion [26]. Furthermore, the rise in circulating thyroid hormone levels with cold exposure does not occur within the first 10 days after birth in the rat when the hypothalamic catecholamine innervation is still immature [15]. Since an increase in thyroid hormone would ordinarily inhibit TRH gene transcription at the level of the PVN through negative feedback effects [22], catecholamines are believed to increase the setpoint for inhibition of TRH gene expression by T3, thereby permitting high circulating levels of thyroid hormone to contribute to increased thermogenesis. The potential role of CART in this response has not been explored, and thereby, its physiological role in activation of the hypothalamic–pituitary–thyroid axis during cold exposure either through direct effects on hypophysiotropic TRH neurons or via potentiation of catecholamine secretion should now be considered. It is conceivable therefore, that under these conditions, the percentage of the PNMT axons that innervate the TRH neurons and co-contain CART may be increased as a result of increased production and transport of CART from medullary neurons.

The observation that hypophysiotropic TRH neurons are contacted by axon varicosities containing only adrenaline and/or axon varicosities that co-contain adrenaline and CART indicates that the brainstem catecholaminergic projection fields to TRH neurons in the PVN are diverse. In fact, within the C1–3 areas in the brainstem, PNMT neurons that co-express CART are localized in the cranial portion of C1 and in the C2,3 regions, whereas neurons that express PNMT, alone, reside in the caudal part of the C1 area. As a further matter, neuropeptide Y (NPY), a 36 amino acid peptide with inhibitory effects on TRH gene expression [13], is co-expressed in the majority of adrenergic neurons in the C1–3 areas [28]. Recent studies from our laboratories have shown that 75% of all adrenergic axons innervating TRH neurons in the PVN co-contain NPY [31]. By inference, therefore, as many as 50% of PNMT-containing axon varicosities in contact with TRH neurons in the PVN could contain all three substances. Thus, the catecholamine projection fields to TRH neurons in the PVN may subserve a number of different functions that involve both the stimulation and inhibition of hypophysiotropic TRH neurons.

We conclude that a major proportion of CART-containing axons that innervate TRH neurons in the medial and periventricular parvocellular subdivisions of the PVN originate from adrenergic CART neurons of the medulla. Therefore, CART may play an important role in the modulation of adrenergic influences on the hypothalamic–pituitary–thyroid axis.

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