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Noradrenergic Innervation of Hypophysiotropic Thyrotropin-Releasing Hormone-Synthesizing Neurons in Rats

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Abstract

Hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons, the central regulators of the hypothalamic-pituitary-thyroid axis, are located in the paraventricular nucleus of the hypothalamus (PVN). These neurons are well-known to be stimulated by cold exposure through activation of ascending brainstem pathways, and are heavily innervated by catecholaminergic axons that contain dopamine-beta-hydroxylase (DBH) and phenylethanolamine-N-methyltransferase (PNMT), enzymes that generate noradrenaline and adrenaline, respectively. However, whether noradrenergic-synthesizing cell groups that lack PNMT contribute to the innervation of TRH neurons is not known. Therefore, triple-labeling immunofluorescence was performed using antibodies against DBH, PNMT and proTRH to determine the relative involvement of adrenaline-synthesizing and noradrenergic neurons in the innervation of TRH neurons in the PVN of rats. Using confocal microscopy, the number of PNMT/DBH (adrenaline-synthesizing) and single-labeled DBH (noradrenergic) boutons juxtaposed to proTRH neurons was quantified. Both noradrenergic and PNMT-containing varicosities were observed in close apposition to virtually all proTRH neurons. An average of 11.8±0.6 PNMT-containing and 7.4±1.0 noradrenergic boutons was present on the surface of proTRH cell bodies and proximal dendrites. Of all catecholaminergic axon-varicosities juxtaposed to proTRH neurons, 63.5±1.2% contained PNMT while the remaining 36.5±1.2% were immunopositive for DBH only. We conclude that both adrenaline-synthesizing and noradrenergic axons innervate hypophysiotropic TRH neurons, although there is a predominance of adrenaline-synthesizing fibers. Since adrenaline-synthesizing and noradrenergic cell groups of the brainstem may respond differently to various physiological stimuli, we hypothesize that the two cell groups are likely to mediate the effects of distinct stimuli toward the hypophysiotropic TRH neurons.

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Keywords

TRH; PNMT; noradrenergic; innervation; PVN

Introduction

Hypophysiotropic thyrotropin-releasing hormone (TRH)-synthesizing neurons located in the medial and periventricular parvocellular subdivisions of the paraventricular nucleus of the hypothalamus (PVN) control the hypothalamic-pituitary-thyroid (HPT) axis. These neurons integrate a variety of humoral and neuronal signals and mediate the effects of these inputs toward the pituitary gland. One of the major regulatory mechanisms over hypophysiotropic TRH neurons is the negative feedback effect of thyroid hormones which maintains euthyroid levels of thyroid hormone in the circulating blood. Under certain circumstances, however, the setpoint for feedback inhibition of hypophysiotropic TRH can be altered by its neuronal inputs (Lechan and Fekete, 2006). For example, cold stress induces central hyperthyroidism, characterized by high levels of circulating thyroid hormones yet increased TRH gene expression in the PVN (Uribe et al., 1993; Zoeller et al., 1990).

The effects of cold exposure on the HPT axis are primarily mediated by catecholaminergic inputs arising from the brainstem (Annunziato et al., 1977; Arancibia et al., 1989; Onaya and Hashizume, 1976; Rondeel et al., 1991; Schettini et al., 1979). This concept is supported by the observation that 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 (Ignar and Kuhn, 1988). Furthermore, β-adrenergic antagonists are capable of blocking cold-induced activation of TRH-synthesis in the PVN (Perello et al., 2007).

Hypophysiotropic TRH neurons are densely innervated by catecholaminergic axons containing dopamin-β-hydroxylase (DBH), an enzyme present in both adrenaline-synthesizing and noradrenergic axons, and phenylethanolamine-N-methyl-transferase (PNMT) (Liposits et al., 1987), an enzyme present only in adrenaline-synthesizing axons. It is not clear, however, whether these axons originate exclusively from the PNMT-containing neurons of the medullary C1-3 regions (Cunningham et al., 1990), or whether noradrenergic neurons of the brainstem are also involved in the innervation of the hypophysiotropic TRH neurons. Therefore, we performed triple-labeling immunofluorescence using specific antisera against proTRH, DBH and PNMT, to determine the relative involvement of adrenaline-synthesizing and noradrenergic neurons in the innervation of the hypophysiotropic TRH neurons.

Results

Both DBH- and PNMT-immunoreactive (IR) axons densely innervated the parvocellular division of the PVN, including the medial parvocellular subdivision of the PVN where the hypophysiotropic proTRH neurons are located (Fig. 1A.).

Both single-labeled DBH-IR and DBH/PNMT-IR axon varicosities were found in juxtaposition to the vast majority of proTRH-IR neurons (97.8 ± 0.7% and 100% respectively) (Fig. 1B–D). An average of 7.4 ± 1.0 single-labeled DBH-IR boutons per proTRH cell was observed while 11.8 ± 0.6 PNMT-IR boutons were juxtaposed to proTRH neurons (Table 1). The relative contribution of noradrenergic axons to the catecholaminergic innervation of the hypophysiotropic proTRH neurons was 36.5 ± 1.2%. Adrenaline-synthesizing axons formed the remaining two thirds (63.5 ± 1.2%) of the catecholaminergic innervation of proTRH neurons (Table 1). The results of the quantitative analysis showed that there were significantly

more PNMT-IR varicosities juxtaposed to the proTRH neurons in the lateral part of the medial parvocellular subdivision of the PVN compared to the medial part of this subdivision. Table 2 displays the regional heterogenity.

Discussion

The present data demonstrate that the catecholaminergic innervation of the hypophysiotropic TRH neurons originates from both adrenaline-synthesizing and noradrenergic neurons. Approximately two thirds of the catecholaminergic innervation of the TRH neurons arises from adrenaline-synthesizing neurons, while the remaining one third originates from noradrenergic neurons.

Adrenaline-synthesizing neurons are located only in the C1-3 regions of the medulla. Since all three PNMT-containing cell groups innervate the parvocellular subdivisions of the PVN and have very similar projection fields (Cunningham et al., 1990), it is likely that each of these groups are involved in the innervation of hypophysiotropic TRH neurons. Noradrenergic neurons are organized into 6 cell groups (A1-6) of the brainstem, but only three project to the PVN: the A1, A2 and A6 noradrenergic cell groups (Sawchenko et al., 1985). Since the A1 region mainly innervates the magnocellular part of the PVN and sends only scattered fibers to the periventricular parvocellular subdivision of the PVN (Cunningham and Sawchenko, 1988), this noradrenergic cell group may have only a relatively minor role in the innervation of the hypophysiotropic TRH neurons. The A2 and A6 noradrenergic cell populations, however, innervate the medial and periventricular parvocellular subdivisions of the PVN, indicating that the noradrenergic innervation of the hypophysiotropic TRH neurons may primarily originate from these two medullary noradrenergic neuron populations (Cunningham and Sawchenko, 1988).

DBH is present in the synaptic vesicles and synthesizes noradrenaline in the terminals of both the noradrenergic and adrenaline-synthesizing neurons (Mefford, 1988). In contrast PNMT is present in the cytoplasm of the PNMT-containing terminals, therefore, the adrenaline-synthesis of these terminals is dependent on the noradrenaline concentration in the cytoplasm (Mefford, 1988). The synthesized adrenaline is transported then into the synaptic vesicles (Mefford, 1988). The cytoplasmic noradrenaline pool at least partly originates from reuptake of the released noradrenaline (Mefford, 1988). Therefore, under basal condition, when these neurons may contain primarily noradrenaline, the released transmitter may act primarily on β1 and/or α1 adrenergic receptors (Mefford, 1988). However, after repeated firing when the reuptake of catecholamines is increased, these neurons may contain primarily adrenaline and act on α2 adrenergic receptors (Mefford, 1988). All adrenergic receptors are G protein coupled receptors, but use different classes of G proteins (Lynch and Ryall, 2008). The α1 receptors are coupled to G_q proteins and result in increased intracellular Ca^{2+} levels (Lynch and Ryall, 2008). The α2 receptors are coupled to G_i proteins and therefore, inhibit cAMP synthesis (Lynch and Ryall, 2008). Beta adrenergic receptors are coupled to G_s proteins and stimulate the synthesis of cAMP (Lynch and Ryall, 2008). Therefore, noradrenergic neurons and adrenaline-synthesizing neurons may exert different effects on their postsynaptic targets.

The action of catecholamines on the TRH neurons may also be modified by co-released transmitters. Catecholaminergic cell groups of the brainstem projecting to the PVN co-synthesize several neuropeptides. One of these neuropeptide is neuropeptide Y (NPY), which is expressed in approximately 75–90% of the adrenaline-synthesizing C1-3 regions of the brainstem, approximately 50–60% of the noradrenergic neurons of the A1 cell group, and 10–15% of the neurons of the A2 and A6 cell groups (Sawchenko et al., 1985). NPY has a potent inhibitory effect on TRH neurons (Fekete et al., 2001; Sarkar and Lechan, 2003), but it is known that NPY can also affect catecholaminergic neurons by increasing the number of the

presynaptic α_2 catecholaminergic receptors (Agnati et al., 1983). An inhibitory presynaptic action of NPY has also been reported in which NPY can decrease the synaptic activity of A1 catecholaminergic neurons by acting on Y2 presynaptic receptors (Khanna et al., 1993).

Other co-transmitters of the catecholaminergic neurons, such as cocaine and amphetamine regulated transcript (CART) and pituitary adenylate cyclase activating polypeptide (PACAP), also regulate the TRH neurons in the PVN, possibly by potentiating the effects of catecholamines on these neurons (Agarwal et al., 2005; Fekete et al., 2000b; Lee et al., 1988). CART is mainly expressed in adrenaline-synthesizing C1-3 regions (Dun et al., 2002; Fekete et al., 2004). Approximately half of the CART-IR innervation of the TRH neurons derives from the brainstem, suggesting that the C1-3 regions are of considerable importance in the regulation of the TRH neurons (Wittmann et al., 2004). Although CART increases the TRH mRNA level in the PVN (Fekete et al., 2000b), both the receptor and the signaling mechanism are unknown. PACAP is expressed in the C1 region and probably stimulates the transcription of the TRH gene (Agarwal et al., 2005; Lee et al., 1988), but experimental confirmation is needed. Finally, a subgroup of noradrenergic neurons of the A6 co-expresses galanin (Levin et al., 1987).

The only known physiological condition in which catecholaminergic neurons regulate the TRH neurons is cold stress (Krulich, 1982; Perello et al., 2007; Sanchez et al., 2001; Uribe et al., 1993; Zoeller et al., 1990). The hypothesis that cold-induced stimulation of TRH neurons is mediated by catecholamines is supported by evidence that α-adrenoreceptor antagonists lower the basal level of TSH and prevent cold-induced stimulation of TSH secretion, similar to pharmacological depletion of the central catecholaminergic pathway (Krulich, 1982). In addition, Perello et al. (Perello et al., 2007) have recently demonstrated that cold-induced proTRH biosynthesis and TRH-release can be blocked by centrally administered β-adrenoreceptor and α-adrenoreceptor antagonists, respectively. It is likely that adrenergic receptors are present on TRH neurons, since β-adrenoreceptor antagonists can block CREB phosphorylation (Perello et al., 2007), a factor which strongly enhances proTRH synthesis (Harris et al., 2001; Lee et al., 1988). Cold-induced TRH release might also be mediated by α1-adrenoreceptors located on TRH axon terminals in the median eminence, because α1-adrenoreceptor agonist infusion into the median eminence can increase TRH secretion (Tapia-Arancibia et al., 1985). A large amount of pharmacological data supports the effects of catecholamines on TRH neurons (Cote-Velez et al., 2005; Krulich, 1982; Perello et al., 2007; Tapia-Arancibia et al., 1985), but there is still no morphological evidence for the existence of adrenergic receptors on the surface of TRH neurons. Interestingly, however, c-fos expression has not been described in catecholaminergic neurons after cold exposure (Baffi and Palkovits, 2000). Therefore, application of other activation markers will be necessary to determine whether adrenaline-synthesizing or noradrenergic cell groups or both mediate the effects of cold exposure on the hypophysiotropic TRH neurons.

We conclude that the adrenaline-synthesizing and noradrenergic neurons of the brainstem contribute unequally to the catecholaminergic innervation of hypophysiotropic TRH neurons. We propose that adrenaline-synthesizing and noradrenergic neurons may each mediate the effects of different physiological conditions on the hypothalamic-pituitary-thyroid axis through direct effects on hypophysiotropic TRH neurons.

Materials and methods

Animals

The experiments were carried out on adult, male rats (Wistar and Sprague-Dawley), weighing 280–320 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.

Tissue preparation for immunocytochemistry

Because colchicine treatment is necessary to visualize the perikarya and dendrites of hypophysiotropic proTRH neurons, and our preliminary studies indicated that a low dose of colchicine (40 μg/animal) does not alter the staining pattern of DBH or PNMT 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 injected intracerebroventricularly with 40 μg colchicine in 2 μl 0.9% saline under stereotaxic control. After 20 h, the animals were perfused transcardially with 20 ml 0.01 M phosphate buffered saline, pH 7.4 (PBS), followed by 150 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were rapidly removed, and blocks containing the hypothalamus cryoprotected in 30% sucrose in 0.01 M PBS overnight at 4 °C and snap frozen on dry ice. Serial 30 μm thick coronal sections through the PVN were cut on 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.

Triple-labeling immunofluorescence for DBH, PNMT, and PROTRH

To elucidate the relative contribution of noradrenergic and adrenaline-synthesizing cell groups of the brainstem to the catecholaminergic innervation of hypophysiotropic TRH neurons, triple-labeling fluorescent immunocytochemistry was performed on every fourth section of the hypothalamus. The sections from each brain were treated with 0.5% Triton X-100/0.5% H_2O_2 in PBS for 20 min. An additional, 10 min incubation in 0.5% Triton X-100 was applied to further improve antibody penetration. To reduce non-specific antibody binding, the sections were treated with 2% normal horse serum in PBS for 20 min. The sections were then incubated first in the following mixture of primary antibodies for 3 days at 4 °C: murine monoclonal antibody to DBH (Cat# MAB308, Millipore, Billerica, MA) at 1:1000 and rabbit anti-PNMT serum (gift from Martha C. Bohn, Northwestern University Medical School, Chicago, IL) at 1:1000. After rinses in PBS, the sections were incubated in biotinylated donkey anti-rabbit IgG, 1:250 (Jackson Immunoresearch, West Grove, PA) overnight. After further washing in PBS the sections were incubated in rabbit-anti-proTRH serum (178–199) (gift from Éva Rédei, Northwestern University, Chicago, IL) at 1:2500 for 2 days. After rinses in PBS, the sections were incubated in a mixture of fluorochrome-conjugated reagents for one day at 4 °C. The fluorescent markers were as follows: fluorescein isothiocyanate (FITC)-conjugated Streptavidin (Jackson Immunoresearch) at 1:300 dilution, CY^3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) at 1:200 dilution and CY^5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) at 1:100 dilution. Primary and secondary antibodies and FITC-Streptavidin 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.

Although rabbit antiserum was used for the detection of both the PNMT- and proTRH-IR structures, because PNMT antiserum and biotinylated donkey anti-rabbit IgG was applied before the use of rabbit anti-proTRH serum, the avidin-conjugated FITC labeled only the PNMT-IR structures. CY^5-conjugated anti-rabbit IgG labeled both PNMT- and proTRH-IR elements, but PNMT-containing neurons are not present in the PVN, therefore, all CY5-labeled perikarya in the PVN are TRH-IR perikarya. Similar approach was successfully used in our earlier studies (Fekete et al., 2000a; Wittmann et al., 2002). To further improve the specificity of the immunostaining, the triple-labeling was also performed with a recently generated sheep TRH antiserum (Wittmann et al., 2009). The immunolabeling was performed as described above just the rabbit TRH serum was replaced with the sheep TRH antiserum in 1:8000 dilution.

Triple-labeled sections were examined using a Radiance 2100 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK). From each brain, every fourth section was analyzed from different rostro-caudal levels of the medial parvocellular subdivision of the PVN in which hypophysiotropic TRH neurons reside. The atlas by Paxinos and Watson (Paxinos and Watson, 1998) was used to identify the subdivisions of the PVN.

With 60× oil lens, serial optical sections of 180 × 180 μm areas covering the entire medial parvocellular subdivision of the PVN were recorded in each section. The sections subjected to triple labeling immunofluorescence were scanned in one step for FITC, CY^3 and CY^5 (laser excitation lines were 488 nm for FITC, 543 nm for CY^3, and 637 nm for CY^5; dichroic/emission filters, 560/500–530 nm for FITC, 650/565–625 nm for CY^3, and a 660-nm-long pass filter for CY^5). Pinhole sizes were set to obtain optical slices less than 0.8 μm thick, and the series of optical slices were recorded with a 0.6 μm Z step. The series of optical sections were merged and displayed with LaserVox (BioRad Laboratories) software and an IBM compatible personal computer. Perikarya and proximal dendrites of the proTRH neurons were traced through the optical slices, and the different types of boutons juxtaposed to proTRH neurons were counted. Data are presented as mean ± SEM.

To illustrate triple labeling, proTRH, DBH and PNMT immunoreactivities were displayed in one image, using the three basic colors (red, green, and blue). DBH and PNMT immunoreactivities are displayed in *red* and *green*, respectively, while *blue* represents proTRH immunoreactivity. Therefore, DBH/PNMT double-labeled axons appear *yellow* due to red and green color mixing. All images represent single optical slices. Images captured through a 20× objective are less than 2.1 μm thick, whereas images captured through 60× oil lens are less than 0.8 μm thick.

Boutons that contained only DBH-immunoreactivity without PNMT-immunoreactivity were considered noradrenergic, whereas fibers containing both DBH-and PNMT-immunoreactivity were considered adrenaline-synthesizing axons. To examine the regional differences in the catecholaminergic innervation of the TRH neurons, the region where the hypophysiotropic TRH neurons reside were divided to two parts: medial and lateral (Figure 2). The regional heterogeneity of catecholaminergic boutons were analyzed with Student T-test. $p < 0.05$ was considered significant.

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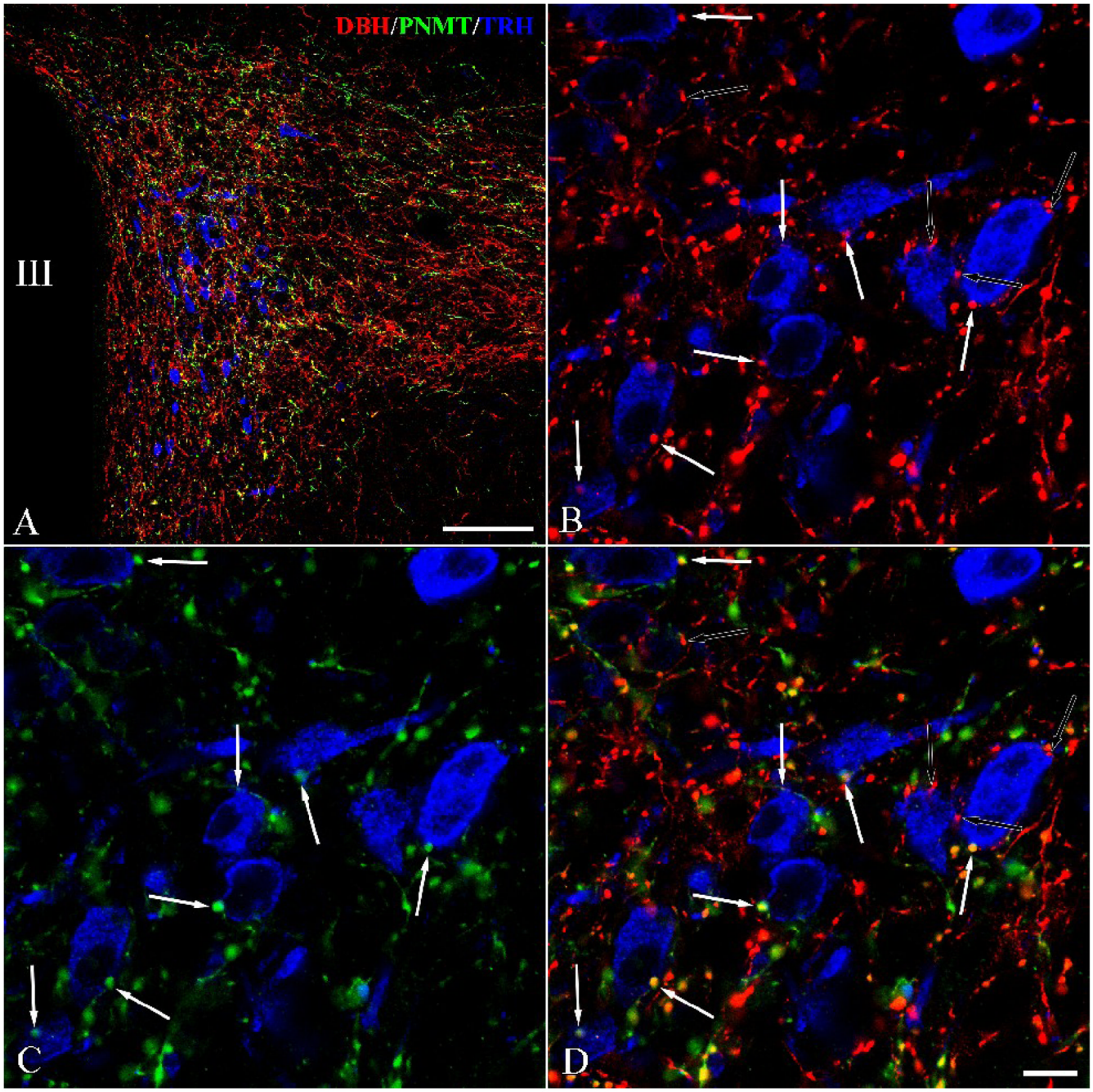

Figure. 1.

Relationship between DBH- (red) and PNMT-IR (green) axons and TRH-containing neurons (blue) in the PVN. Low power magnification image (A) of the PVN illustrates that TRH-synthesizing neurons are embedded in a dense network of DBH- and double-labeled DBH/PNMT-IR (yellow) axons. High magnification confocal images of the same field (B–D) demonstrate DBH- (B) and and PNMT-IR (C) varicosities on the surface of TRH-containing neurons (blue) in the medial parvocellular subdivision of the PVN. The composite image (D) demonstrate that both noradrenergic (DBH-IR only, red) and DBH/PNMT-IR, adrenaline-synthesizing axons (yellow) establish contacts with the TRH neurons. III, Third ventricle. Scale bars, 100 μm for A 10 μm for B, C, D

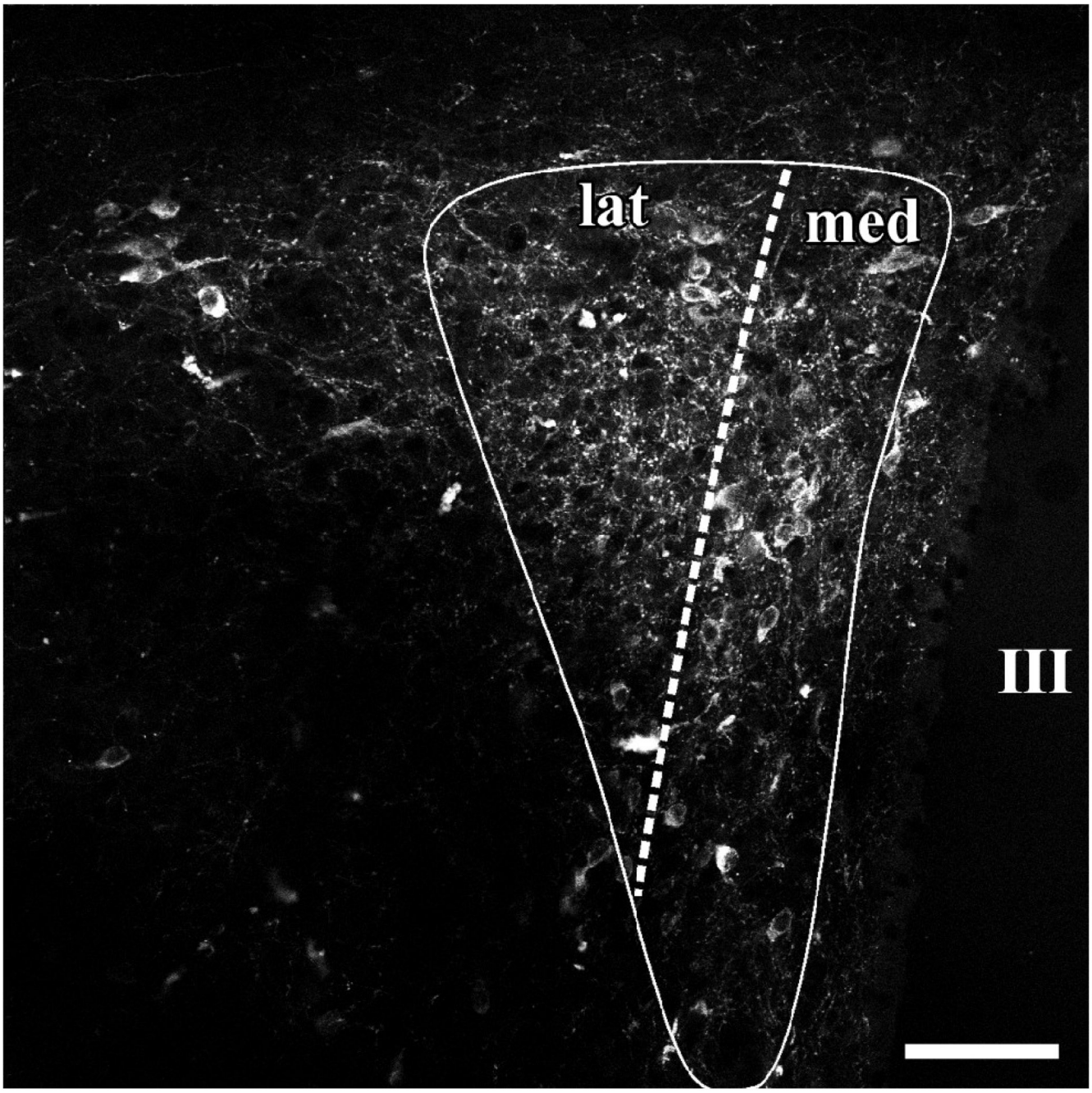

Figure 2.
Photomicrograph of TRH-immunoreactivity in the PVN illustrates the medial and lateral division of the medial parvocellular subdivision of the PVN used for quantification of the DBH- and PNMT-containing innervation of the TRH neurons. med: medial part of the medial parvocellular subdivision, lat: lateral parvocellular part of the medial parvocellular subdivision, III: third ventricle. Scale bar = 100 μm

Table 1

Type of catecholaminergic neurons	Percentage of contacted proTRH neurons	Average number of catecholaminergic varicosities per innervated proTRH neuron	Percentage of all catecholaminergic boutons in contact with proTRH neurons
single DBH	97.8 ± 0.7	7.4 ± 1.0	36.5 ± 1.2
DBH/PNMT	100	11.8 ± 0.6	63.5 ± 1.2
all catecholaminergic	100	19.8 ± 2.2	100

Table 2

Type of catecholaminergic neurons	Average number of DBH varicosities per proTRH neuron	DBH boutons (%)
Medial part of medial parvocellular subdivision		
single DBH	7.0 ± 0.7	
DBH/PNMT	11.3 ± 1.0*	
all catecholaminergic	18.4 ± 1.7	38.2 ± 0.9*
Lateral part of medial parvocellular subdivision		
single DBH	6.3 ± 0.9	
DBH/PNMT	14.7 ± 0.6*	
all catecholaminergic	21.0 ± 1.5	29.5 ± 2.2*

* $p<0.05$