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Neurochemical coding compared between varicose axons and cell bodies of myenteric neurons in the guinea-pig ileum

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Abbreviated title: Cholinergic markers in axons and cell bodies of enteric neurons do not always correspond

Key words: acetylcholine, choline acetyltransferase, vesicular acetylcholine transporter, enteric nervous system

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Abbreviations

5-HT, 5-hydroxytryptamine; ChAT, choline acetyltransferase; IR, immunoreactive; NOS, nitric oxide synthase; SOM, somatostatin; SP, substance P; VAChT, vesicular acetylcholine transporter; VIP, vasoactive intestinal polypeptide.

Abstract

The discrete functional classes of enteric neurons in the mammalian gastrointestinal tract have been successfully distinguished on the basis of the unique combination of molecules and enzymes in their cell bodies ("chemical coding"). Whether the same chemical coding exists in varicose axons of different functional classes has not been systematically tested. In this study, we quantified the coexistence of markers that define classes of nerve cell bodies in the myenteric plexus of the guinea-pig ileum, in varicose axons of the same neurons. Profound differences between the combinations of immunohistochemical markers in myenteric nerve cell bodies and in their

varicosities were identified. These discrepancies were particularly notable for classes of neurons that had previously been classified as cholinergic, based on immunoreactivity for choline acetyltransferase (ChAT) in their cell bodies. To detect cholinergic varicose axons of enteric neurons in this study, we used antiserum against the vesicular acetylcholine transporter (VAChT). ChAT-immunoreactivity has been reported to be consistently co-localized with 5-hydroxytryptamine (5-HT) in interneuronal cell bodies, yet only 29±5% (n=4) of 5-HT-immunoreactive varicosities contained vesicular acetylcholine transporter (VAChT). Somatostatin coexists with ChAT-immunoreactivity in a class of descending interneuron but only 21±1% (n=4) of somatostatin-immunoreactive varicosities were VAChT-immunoreactive. Comparable discrepancies were also noted for non-cholinergic markers. The results suggest that chemical coding of cell bodies does not necessarily reflect chemical coding of varicose axon terminals and that the assumption that nerve cell bodies that contain ChAT are functionally cholinergic may be questionable.

Introduction

The enteric nervous system, the “third” division of the autonomic nervous system, is composed of two ganglionated plexus, the myenteric plexus and submucosal plexus, within the wall of the gut. Both plexus contain neural circuits that regulate gastrointestinal functions. In small laboratory animals, the myenteric plexus contains intrinsic sensory neurons, interneurons and motorneurons that regulate the contractility of the circular and longitudinal smooth muscle layers, while the submucosal plexus is largely involved in regulating local blood flow and secretion by enterocytes [7].

The enteric nervous system has been intensively studied in the guinea-pig ileum. Enteric neurons in this preparation have been divided into discrete functional classes based on their electrophysiological properties, axonal projections and the neurochemical coding of their nerve cell bodies [4]. The majority of enteric neurons express choline acetyltransferase (ChAT) in their cell bodies, and have been presumed to be functionally cholinergic. Within the myenteric plexus there are ascending and descending interneurons that underlie polarised reflexes and coordinated patterns of motility [14]. Acetylcholine, acting at nicotinic receptors is a major contributor to fast excitatory post-synaptic potentials, but also causes muscarinic slow excitatory post-synaptic potentials [8,18]. Acetylcholine from enteric motor neurons also contracts the circular and longitudinal smooth muscle layers via muscarinic receptors [15]. Muscarinic and nicotinic antagonists profoundly interfere with gastrointestinal motility [9]. Given the major functional role of cholinergic neurotransmission, identification of cholinergic enteric neurons is a significant undertaking.

Immunohistochemical localisation of the key enzymes or transporters has been used to identify neurotransmitters in nerve cell bodies. One class of descending interneurons is distinguished by immunoreactivity for both 5-hydroxytryptamine (5-HT) and ChAT in cell bodies [4]. It has been widely assumed that markers in cell bodies are also expressed in varicose axons; however, this has never been systematically tested. Whether all varicose axons of these interneurons contain both 5-HT- and ChAT-immunoreactivity has not been reported. To study this, we quantified the co-localization of cholinergic markers in 5-HT-containing varicosities. Vesicular acetylcholine transporter (VAChT) accumulates acetylcholine in synaptic vesicles and is a marker for cholinergic axons [17]. The VAChT coding region is contained within the ChAT gene locus [6]. Transcription of both genes is controlled by the same or neighbouring promoters, which thereby coordinate expression of the two proteins, both of which are required to establish a cholinergic phenotype [5]. In this study, we examined the distribution of VAChT in varicosities in myenteric ganglia and

compared coexistence with previously published reports in which ChAT and other markers were co-localized in cell bodies of myenteric neurons [4].

Materials and Methods

Adult guinea-pigs of either sex (weight 230–360g) were stunned and killed by exsanguination, in a manner approved by the Animal Welfare Committee of Flinders University, South Australia. Animals were opened along the ventral midline and the ileum was removed, intestinal contents were flushed and the preparation placed in Krebs solution (118mM NaCl, 4.75 mM KCl, 1.0mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.2mM $MgSO_4$, 11.1 mM D-glucose, 2.5mM $CaCl_2$, bubbled with 95%O_2/5%CO_2). Segments of ileum were pinned in a Sylgard-lined petri dish (Dow Corning, Midland, MI) filled with phosphate buffered saline (PBS: 0.15M NaCl, 0.01M NaH_2PO_4, pH 7.2), opened longitudinally along the mesenteric border, and maximally stretched. The wholemount was immersed in modified Zamboni's fixative (2% formaldehyde, 15% saturated picric acid in 0.1M phosphate buffer, pH 7.0) for approximately 24 hours at 4°C. It was then fixed, cleared with three washes of 100% dimethylsulphoxide (DMSO), and stored in PBS at 4°C. The mucosa, submucosa and circular muscle were removed via sharp dissection, yielding strips of circular muscle or preparations with the myenteric plexus and longitudinal muscle intact.

Preparations of myenteric plexus and longitudinal muscle or circular muscle were incubated with antisera to combinations of markers (Table 1) at room temperature for two days. Preparations were rinsed three times in PBS and incubated with secondary antisera (Table 2) for 4 hours at room temperature. After a final rinse with PBS, preparations were equilibrated with 50%, 70%, and 100% carbonate-buffered glycerol, and mounted in 100% carbonate-buffered glycerol (pH 8.6). All antibodies were diluted in 0.1 M PBS (0.3 M NaCl) containing 0.1% sodium azide. Controls for double-labeling were performed by omitting one or more primary antibodies from the procedure,

and by ensuring that all combinations of primary and secondary antisera were free of cross-reactivity.

Specimens were examined on an Olympus IX71 microscope (Japan) equipped with epifluorescence and highly discriminating filters (Chroma Technology Co., Battledore, VT). Images were captured using a Roper scientific (Coolsnap) camera at 1392 x 1080 pixels, using AnalySIS Imager 5.0 (Olympus-SIS, Münster, Germany) and saved as TIFF files. Matched micrographs of immunohistochemically-labeled nerve structures were captured using a 40x objective water-immersion lens and displayed in ImageJ (NIH, Bethesda, MD) as a stack. We used a previously characterized method to identify coexistence of markers in varicosities [13]. We selected varicosities by moving the cursor to a random site in a ganglion and selecting the varicosity closest to the cursor. Varicosities were considered immunoreactive when they were clearly discernible above background labeling. All varicosities that met this criterion were present in micrographs where all pixels less than or equal to three standard deviations above the mean value of background fluorescence had been removed. Varicosities were excluded when: they could not be easily distinguished above background labeling; were out-of-focus; were overlying nerve cell bodies or other immunoreactive structures. Five or ten varicosities were selected at widely separated sites in each ganglion and either 20 or 10 stacks were examined, giving total counts of 100 varicosities, in each of four guinea-pigs. To analyze axons in the muscle layers, a transect line was drawn and the 5 varicosities closest to the line were quantified from each of 20 stacks, resulting in total counts of 100 varicosities in each of the four animals. Group data are expressed as percentage means (mean number of varicosities from a sample of 100) ± standard error of the mean (SEM), with *n* referring to the number of animals.

Figures were generated from grayscale images adjusted for contrast and brightness in Adobe Photoshop CS5 and were cropped and resized to improve display of varicosities of interest.

Results

Reliability of VAChT and ChAT antisera to label varicosities

We first quantified the coexistence of VAChT and ChAT in varicosities (Fig. 1). The two markers coexisted in varicosities in the deep muscular plexus (ChAT+/VAChT+: 85 ± 1.3%; VAChT+/ChAT+: 86 ± 2%, n=4) and tertiary plexus (ChAT+/VAChT+: 66 ± 4.3%; VAChT+/ChAT+: 70 ± 3.8%, n=4). In myenteric ganglia, 75 ± 0.6% of ChAT-IR varicosities were VAChT-IR. The high density of ChAT labeling in myenteric ganglia precluded quantification with VAChT the other way around. These observations suggest that both ChAT and VAChT labeling might underestimate the total population of cholinergic varicosities.

Coexistence of neuronal markers in myenteric ganglia

We tested whether markers present in the cell bodies of different classes of enteric neurons were also present in their varicosities. The first classes studied were the ascending and descending interneurons and intrinsic sensory neurons. In myenteric ganglia, varicosities belong mostly to these populations [3]. All cell bodies of ascending interneurons are immunoreactive for calretinin and ChAT [4], but just 29 ± 4.8% (n=4) of calretinin-IR varicosities in myenteric ganglia were immunoreactive for VAChT (Fig. 2). One class of descending interneuron contain both somatostatin (SOM) and ChAT in their cell bodies [4]; only 21 ± 0.8% (n=4) of SOM-IR varicosities were VAChT-IR in myenteric ganglia (Fig. 2). Another class of descending interneurons has been defined by 5-HT and ChAT in their cell bodies [4], yet just 28 ± 2.9% (n=4) of 5-HT-IR varicosities were immunoreactive for VAChT in myenteric ganglia (Fig. 2). These results indicate that there may be differences in the expression of cholinergic markers between cell

bodies and axon terminals of some interneurons, which are not reflected by other neurochemical markers such as calretinin, SOM and 5-HT.

All nitric oxide synthase (NOS)-IR cell bodies in myenteric ganglia are immunoreactive for vasoactive intestinal polypeptide (VIP) [4]. To test whether all varicose axons of descending interneurons were immunoreactive for NOS and VIP, we quantified their coexistence in myenteric ganglia. Just 43 ± 2.9% (n=4) of NOS-IR varicosities were VIP-IR. Similarly, the cell bodies of all calretinin-IR interneurons are reported to be SP-IR [4], but when we studied the axon terminals of these neurons in myenteric ganglia, only 6 ± 1.5% (n=4) of calretinin-IR varicosities were SP-IR. These findings indicate that the neurochemical coding of cell bodies of descending and ascending interneurons may differ from that of their varicose axons, in non-cholinergic markers too.

We also quantified the coexistence of calbindin, substance P (SP) and VIP with VAChT in varicosities in myenteric ganglia. Calbindin is present in most cell bodies of intrinsic primary afferent neurons, some of which contain ChAT in their cell bodies [4]. VIP and ChAT coexist in all cell bodies of a class of descending interneurons [4]; VIP also coexists with NOS in the cell bodies of another class of descending interneurons [4]. SP coexists with ChAT in the same class of ascending interneurons that contain calretinin, but is also present in some cell bodies of intrinsic primary afferent neurons [4]. Therefore, interpretation of VAChT with these other neuronal markers in varicosities is difficult because not all nerve cell bodies that contain calbindin, SP and VIP contain ChAT. Of calbindin-IR varicosities, 43 ± 6.1% (n=4) were VAChT-IR. Just 8 ± 0.9% (n=4) of VIP-IR varicosities were VAChT-IR and 18 ± 0.9% (n=4) of SP-IR varicosities were VAChT-IR. We tested whether NOS coexisted with the cholinergic marker, VAChT, in varicosities in myenteric ganglia. On average, 20 ± 1% (n=4) of NOS-IR varicosities in ganglia were VAChT-IR, suggesting the presence of a class of enteric neurons that utilize both nitric oxide and acetylcholine.

Coexistence of neuronal markers in the deep muscular plexus

The circular muscle layer in the gut wall is innervated by excitatory and inhibitory motorneurons that have their cell bodies in the myenteric plexus [4]. The axons of these neurons are contained mostly in the deep muscular plexus, with some in the circular muscle plexus [4]. All cell bodies of excitatory motorneurons to the circular muscle are ChAT-IR and SP-IR [3]; all inhibitory motorneurons to the circular muscle express immunoreactivity for NOS and VIP in their cell bodies [3]. In the deep muscular plexus, there was almost complete overlap of NOS with VIP and VAChT with SP in varicosities (Fig. 3). Nearly all SP-IR varicosities were VAChT-IR (96 ± 0.8%, n=4), and most VAChT-IR varicosities were SP-IR (95 ± 1.5%, n=4). Of VIP varicosities, 93 ± 2.2% (n=4) were NOS-IR, and 86 ± 1.5% (n=4) of NOS varicosities were VIP-IR. There was almost no overlap between these two distinct populations, as only 0.5 ± 0.3% (n=4) of ChAT-IR varicosities were NOS-IR, and 0.5 ± 0.3% (n=4) of NOS-IR varicosities were ChAT-IR. These results confirm previous findings that there are just two neurochemically-distinct populations of motorneurons that supply the circular muscle [10].

Coexistence of neuronal markers in the tertiary plexus

The longitudinal muscle receives innervation from excitatory and inhibitory motorneurons in the myenteric plexus. The axons of these neurons lie in the tertiary plexus [4]. The cell bodies of inhibitory motorneurons are immunoreactive for NOS and VIP [4]. Cell bodies of excitatory motorneurons are immunoreactive for ChAT or SP, or both [4]. Coexistence of NOS and VIP in varicosities in the tertiary plexus was limited (Fig. 3). Just 22 ± 2.8% (n=4) of VIP-IR varicosities were NOS-IR, and 33 ± 2.1% (n=4) of NOS-IR varicosities were VIP-IR. Of VAChT-IR varicosities, 29 ± 1.9% (n=4) were SP-IR, and 31 ± 2.3% (n=4) of SP-IR varicosities were VAChT-

IR. There was no overlap between VIP and ChAT in varicosities (ChAT+/VIP+: 0 ± 0%; VIP+/ChAT+: 0 ± 0%, n=4).

Discussion

In this study, we determined if the neurochemical coding of nerve cell bodies predicts the coding of varicose axons, particularly for cholinergic markers, in the myenteric plexus of the guinea-pig ileum. Our results suggest that coexistence of markers in nerve cell bodies is often not a good predictor for coexistence in varicose axons in the guinea-pig ileum. For example, all nerve cell bodies containing 5-HT-, calretinin- and SOM-immunoreactivity in the myenteric plexus of the guinea-pig ileum are immunoreactive for the rate-limiting enzyme for acetylcholine synthesis; choline acetyltransferase (ChAT) [4]. However, fewer than 30% of varicosities immunoreactive for 5-HT, calretinin or SOM were VAChT-IR in myenteric ganglia. Likewise, all NOS-IR cell bodies in the guinea-pig ileum myenteric plexus are VIP-IR [4], but fewer than 50% of NOS-IR varicosities in myenteric ganglia were VIP-IR, and fewer than 30% of NOS-IR varicosities in the tertiary plexus were VIP-IR. In contrast, all SP-IR nerve cell bodies are also ChAT-IR, and in the deep muscular plexus, VAChT- and SP-immunoreactivity almost always coexisted in varicose axons of motorneurons, as did NOS- and VIP-immunoreactivity. In combination, these results suggest that there are large differences in the extent to which levels of neurochemical markers in varicose axons resemble those in nerve cell bodies. To our knowledge, this is the first report of such a phenomenon in the enteric nervous system.

The discrepancies in labeling of cell bodies and varicose axons were most profound for the classes of enteric neurons that express immunoreactivity for ChAT in their cell bodies. This could be explained by failure of the VAChT and ChAT antiserum to adequately reveal these proteins in many varicosities. It is possible that VAChT and ChAT would have been detected in previously

unlabeled varicosities with higher concentrations of antisera, potentially even in varicosities where it would not be expected to be expressed. However, the dilution of antisera used provided the highest signal-to-noise and increasing the concentration would have compromised our ability to quantify coexistence. Therefore, this possibility was not investigated. These observations illustrate the limits of the immunohistochemical detection technique, wherein the apparent absence of immunoreactivity for a marker is often taken for granted when there is a clearly defined pattern of cell-selective expression.

ChAT and VAChT are synthetic enzymes required for the synthesis and uptake of acetylcholine into synaptic vesicles, respectively [5]. The ChAT gene locus contains the entire sequence of VAChT cDNA, enabling transcription of VAChT and ChAT mRNA from the same or neighboring promoters [6]. Our observations suggest that there is a mechanism that operates to regulate the level of ChAT protein expression in nerve cell bodies independently of their axons. Such a mechanism may be regulated by a number of factors [1]. If ChAT- and VAChT-immunoreactivity are markers of functionally cholinergic presynaptic terminals, our results raise the intriguing possibility that a single neuron is not either cholinergic or non-cholinergic, but that it may show different levels of cholinergic transmission at different times. We speculate that axons might cyclically change in their functionality, playing a major role in cholinergic transmission at some times and less of a role at others. Alternatively, or perhaps in addition, there may be a reserve pool of axons in some classes of cholinergic enteric neurons, which are not normally involved in cholinergic transmission but may switch to a cholinergic phenotype under some conditions. They might assist in maintaining gastrointestinal function in the face of age-related loss of cholinergic neurons in the myenteric plexus [12] or be recruited to a functionally cholinergic state under pathological situations, such as inflammation [11]. The notion of a reserve pool of axons capable of changing their functionality is

supported by functional studies showing the apparent absence of neurotransmitter release from enteric and autonomic nerve terminals invaded by an action potential [2, 16].

In conclusion, we have shown that coexistence of markers in nerve cell bodies is often not a good predictor for coexistence in varicose axons in the guinea-pig ileum. Whether this is common to other regions of the nervous system remains to be investigated.

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Figure 1. Paired micrographs of myenteric ganglia (**A**&**B**), tertiary plexus (**C**&**D**), and deep muscular plexus (**E**&**F**) from guinea-pig ileum immunohistochemically-labeled with antisera against ChAT and VAChT. At all three sites, there were varicosities that contained both VAChT- and ChAT-immunoreactivity, which are indicated by arrows. Scale bars = 20μm. **Abbreviations:** ChAT, choline acetyltransferase; VAChT, vesicular acetylcholine transporter.

Figure 2. Paired micrographs of myenteric ganglia from guinea-pig ileum immunohistochemically-labeled with antisera against two different markers. Arrows indicate varicosities immunoreactive for both markers and arrowheads indicate varicosities immunoreactive for one of the two markers. **A**&**B**: Immunoreactivity for VAChT and 5-HT coexisted in a proportion of varicosities in myenteric ganglia. **C**&**D**: Immunoreactivity for VAChT and calretinin coexisted in subsets of varicosities in myenteric ganglia. **E**&**F**: VIP- and NOS-immunoreactivity did not coexist in all labeled varicosities in myenteric ganglia. **G**&**H**: Just a subset of SOM-IR varicosities were VAChT-IR in myenteric ganglia. Scale bars = 20μm. **Abbreviations:** 5-HT, 5-hydroxytryptamine; IR, immunoreactive; NOS, nitric oxide synthase; SOM, somatostatin; VAChT, vesicular acetylcholine transporter; VIP, vasoactive intestinal polypeptide.

Figure 3. Paired micrographs of deep muscular plexus (**A**-**D**) and tertiary plexus (**E**-**H**) from guinea-pig ileum immunohistochemically-labeled with antisera against either VAChT and SP or NOS and VIP. Arrows indicate varicosities immunoreactive for both markers and arrowheads indicate varicosities immunoreactive for one of the two markers. Almost always, VIP- and NOS-

immunoreactivity, and VAChT- and SP-immunoreactivity, coexisted in varicosities in the deep muscular plexus. However, in the tertiary plexus, only a subset of varicosities contained both NOS and VIP, and VAChT and SP. Scale bars = 20μm. **Abbreviations:** IR, immunoreactive; NOS, nitric oxide synthase; SP, substance P; VAChT, vesicular acetylcholine transporter; VIP, vasoactive intestinal polypeptide.

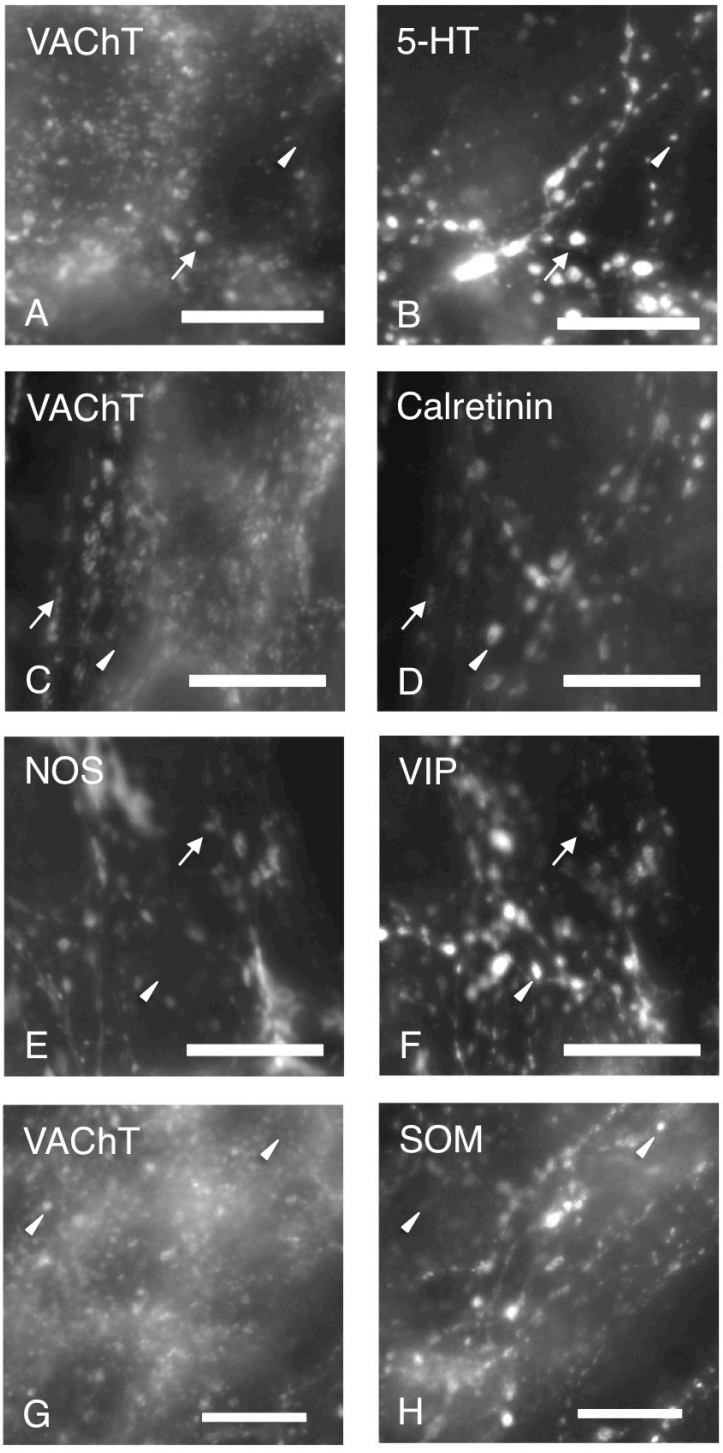

Figure 1. Paired micrographs of myenteric ganglia from guinea-pig ileum immunohistochemically-labeled with antisera against two different markers. Arrows indicate varicosities immunoreactive for both markers and arrowheads indicate varicosities immunoreactive for one of the two markers. **A&B**: On preparations labeled with antisera against VAChT and 5-HT, immunoreactivity for VAChT and 5-HT coexisted in a proportion of varicosities. **C&D**: Immunoreactivity for VAChT and calretinin coexisted in subsets of varicosities in myenteric ganglia. **E&F**: Surprisingly, VIP- and NOS-immunoreactivity did not coexist in all labeled varicosities in myenteric ganglia. **G&H**: Again, only a subset of SOM-IR varicosities were VAChT-IR in myenteric ganglia. Scale bars = 20μm

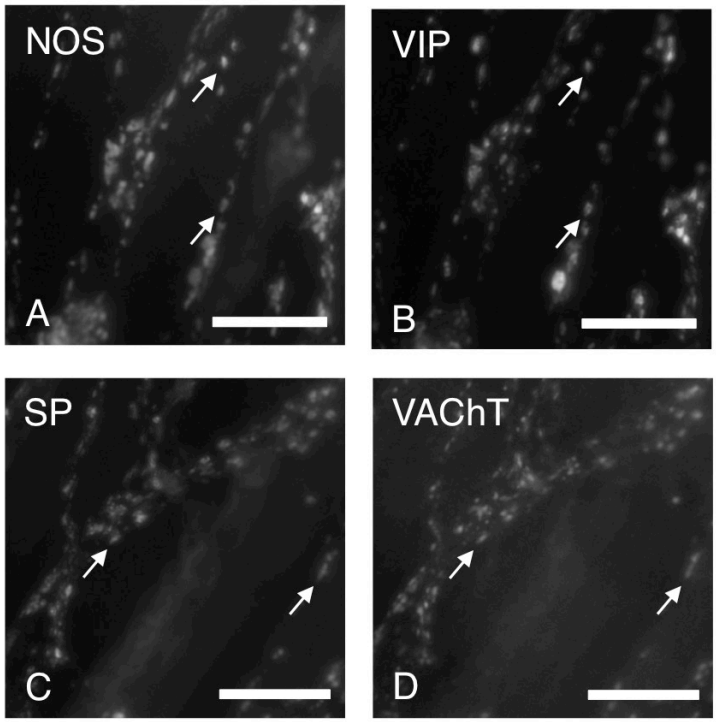

Figure 2. Paired micrographs of deep muscular plexus from guinea-pig ileum immunohistochemically-labeled with antisera against either VAChT and SP or NOS and VIP. Arrows indicate varicosities immunoreactive for both markers and arrowheads indicate varicosities immunoreactive for one of the two markers. **A&B**: Almost always, VIP- and NOS-immunroeactivity coexisted together in varicosities. **C&D**: SP- and VAChT-immunoreactivity almost always coexisted together in varicosities. Scale bar = 20μm

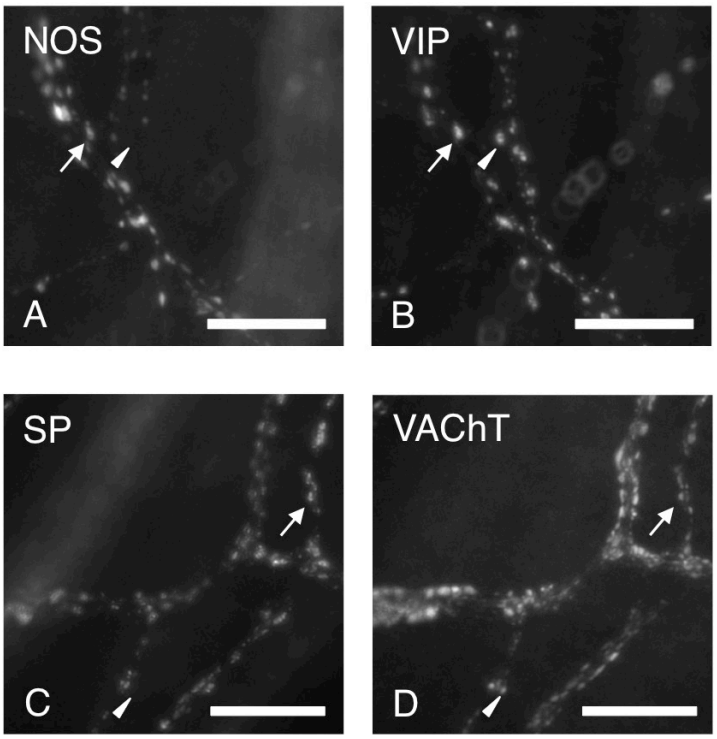

Figure 3. Paired micrographs of tertiary plexus from guinea-pig ileum immunohistochemically-labeled with antisera against either VAChT and SP or NOS and VIP. Arrows indicate varicosities immunoreactive for both markers and arrowheads indicate varicosities immunoreactive for one of the two markers. **A&B**: NOS- and VIP-immunoreactivity did not coexist together in all varicosities. **C&D**: Only a subset of SP-IR and VAChT-IR varicosities were immunoreactive for the other marker. Scale bar = 20μm