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Different subcellular distributions of the vesicular monoamine transporter, VMAT2, in subclasses of sympathetic neurons

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Abstract

A subpopulation of neurons in the rat superior cervical ganglion (SCG) was found to lack immunostaining for VMAT2, an isoform of the vesicular monoamine transporter that loads catecholamines into vesicles for release at the synapse. Double labeling with neuropeptide Y (NPY), a marker for vasomotor neurons, revealed selective cellular colocalization of NPY together with intense perinuclear staining for VMAT2. This implied that VMAT2-negative neurons were likely to have secretomotor and pilomotor phenotypes. We tested this by identifying peripheral noradrenergic axons by their expression of immunoreactivity for tyrosine hydroxylase (TH) and determining whether they also expressed NPY and VMAT2. This analysis revealed the presence of VMAT2-positive, non-vasomotor sympathetic axons in the submandibular gland and at the base of piloerector hairs. Together the results confirm earlier indications that virtually all sympathetic neurons in the rat SCG express VMAT2 and they show for the first time that functional subclasses of cells can be distinguished by different somatic levels of immunoreactivity for VMAT2.

Keywords

superior cervical ganglion; norepinephrine; vasomotor; pilomotor; secretomotor; neuropeptide Y

1. Introduction

Monoaminergic neurons load catecholamines and serotonin into synaptic vesicles through reserpine-sensitive facilitated transport (Henry et al., 1994; Henry et al., 1998). Molecular cloning studies have identified two isoforms of the vesicular monoamine transporter (Erickson et al., 1992; Liu et al., 1992). VMAT1 is preferentially expressed by adrenal chromaffin cells and by small intensely fluorescent cells in sympathetic ganglia (Weihe et al., 1994). By contrast, selective VMAT2 expression has been reported for noradrenergic sympathetic neurons, midbrain dopamine neurons and other monoaminergic neurons in the brain stem (Erickson et al., 1996; Weihe and Eiden, 2000; Weihe et al., 1994). Taken together, this earlier literature conveys the distinct impression that all noradrenergic sympathetic neurons are immunoreactive for VMAT2. It is therefore surprising that one study (Hou and Dahlstrom, 1996), which employed a biochemically well-characterized antibody, reported that only about 70% of neurons in the rat superior cervical ganglion (SCG) appeared immunoreactive for VMAT2. This raises a paradox because approximately 98% of SCG neurons express tyrosine hydroxylase (TH) and are functionally noradrenergic (Gibbins, 1995). Explanation of the

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paradox implies either the existence of a novel vesicular transporter or cellular variation in the expression and trafficking of transporter molecules. To distinguish these possibilities we sought in the present study to determine whether variation in somatic levels of VMAT2 immunoreactivity correlated with functional phenotypes in the rat SCG and then examined VMAT2 staining in terminal fields of these neuronal cell types.

Neuronal phenotypes in the SCG are defined by their selective innervation of end organs and also by peptide expression, size, and rostro-caudal position (Gibbins, 1991; Gibbins, 1995; Headley et al., 2005). Approximately 90% of rat SCG neurons belong to one of three functional groups: secretomotor neurons that project to the submandibular salivary glands, pilomotor neurons that innervate pilli arrector muscles, and vasomotor neurons that control blood flow in the head (Gibbins, 1991; Gibbins, 1995; Voyvodic, 1989). Neuropeptide Y (NPY) is an important cotransmitter in vasoconstrictor sympathetic neurons, which constitute 50–70% of the cells in paravertebral chain ganglia (Gibbins, 1995; Jarvi et al., 1986; Romeo et al., 1994). This suggests cellular variation in somatic staining for VMAT2 might correlate with the NPY-positive neuronal phenotype.

2. Results

The majority of sympathetic neurons scattered throughout the rostrocaudal extent of the SCG exhibited intense VMAT2-immunoreactivity (IR), especially in the perinuclear cytoplasm (Fig. 1A). In addition, many neurons appeared completely negative for VMAT2-IR, while others were very weakly stained, but clearly distinct from the intensely immunoreactive cells. Those neurons located in the vicinity of the ECN that lacked or showed very little VMAT2 staining tended to be larger than intensely stained VMAT2-positive neurons (Fig. 1B). However, near the ICN, VMAT2-negative and VMAT2-positive neurons were similar in size (Fig. 1C). The overall relation between cell size, cell position and VMAT2-IR resembled the ganglionic topography of cellular staining for NPY-IR that has been observed in the rat SCG (Headley et al., 2005). Double labeling for VMAT2 and NPY confirmed the cellular colocalization of these two molecules (Fig. 2). Most NPY-negative neurons in the SCG are either secretomotor cells that control salivary glands or pilomotor cells (Gibbins, 1989; Gibbins, 1991). To determine whether the peripheral axonal processes of these neurons express VMAT2 we examined the end-organs they innervate.

Sympathetic axons in the submandibular gland were identified by their immunoreactivity for TH-IR and then examined for co-expression of VMAT2. All axons containing TH-IR were VMAT2-positive (Fig. 3A,B). In sections double labeled for NPY and VMAT2, some axons were positive for both markers and others only expressed VMAT2-IR (Fig. 3C, D). As expected the NPY-positive fibers tended to be densely packed and prominent near blood vessels. This contrasted with VMAT2-positive, NPY-negative axons, which were sparsely distributed amongst the secretory cells of the gland. In addition our methodology produced some non-specific staining of glandular ducts (Fig 3A) that persisted when the VMAT2 primary antibody was omitted from the staining protocol (not shown).

All of the TH-positive axons localized near pilli arrector muscles at the base of hair follicles lacked NPY-IR (Fig. 4A,B) and were therefore identified as pilomotor fibers (Gibbins, 1989; Gibbins, 1991). Double labeling of pilomotor axons revealed they were positive for both TH-IR and VMAT2-IR (Fig. 4C,D).

3. Discussion

In the present study, we replicated an earlier observation (Hou and Dahlstrom, 1996) that a substantial fraction of neuronal cell bodies in the rat SCG do not seem to express

immunoreactivity for VMAT2 (Fig. 1). Double labeling then revealed that neurons expressing intense VMAT2-IR matched with the cells that express immunoreactivity for NPY (Fig. 2). This colocalization implies that cellular variations in VMAT2 expression are tightly correlated with the functional identities of sympathetic neurons - cell bodies of vasomotor neurons are VMAT2-positive, while secretomotor and pilomotor neurons appear VMAT2-negative. The identification of NPY-negative cells as mainly secretomotor or pilomotor was based on previous tracing studies and measurements of cell size and position (Bowers and Zigmond, 1979; Gibbins, 1991; Headley et al., 2005; Voyvodic, 1989). Direct examination of the submandibular salivary glands (Fig. 3) and skin from the forehead (Fig. 4) revealed that non-vasomotor sympathetic axons, as identified by TH expression and the absence of NPY-IR, did contain VMAT2-IR. This implies that virtually all neurons in the rat SCG express VMAT2, but that the subcellular processing of this transporter is different in functional subclasses of sympathetic neurons.

Given that secretomotor and pilomotor axons were VMAT2-positive, it seems unlikely that the somata of these cells were truly negative for VMAT2. A more plausible explanation is that levels of the transporter in these cell bodies were simply below the detection threshold of immunocytochemistry. This is also consistent with the observation that some NPY-negative cell bodies appeared weakly positive for VMAT2. Such a situation could arise if VMAT2 was synthesized at relatively low rates by secretomotor and pilomotor neurons or if it was more rapidly trafficked away from the Golgi apparatus into axons and synaptic terminals. These possibilities raise the interesting notion that levels of VMAT2 may vary at different types of neuroeffector synapses. Our findings may also prove relevant to the central nervous system, where it has recently been found that some neuronal cell bodies are immunoreactive for TH, but not VMAT2 (Weihe et al., 2006).

4. Experimental Procedures

Animal and Tissue Preparation

All procedures pertaining to the care and use of live animals were conducted in accord with a protocol approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Six adult male Sprague-Dawley rats (150 – 250 g, Charles River Laboratories) were euthanized with an overdose of isoflurane (Abbott). They were then perfused through the heart with 50 ml of phosphate-buffered saline, pH 7.3 (PBS) followed by 400 ml of fixative containing 2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.3 (PB). Both SCGs were dissected from each animal and post-fixed for 1 hour while the connective tissue capsules were removed. Samples of skin from the forehead were cut into 2–3 mm pieces, stretch mounted in Sylgard coated dishes and post-fixed overnight at 4°C. Submandibular salivary glands were processed in a similar way, but not stretched.

After post-fixation, SCGs and pieces of submandibular gland were washed several times in PBS and dehydrated through graded ethanols. Tissues were infiltrated with polyethylene glycol (PEG, MW 1000, Sigma P-3515) for 30 min at 47°C under vacuum and then embedded in PEG (MW 1450, Sigma P-5402). Microtome sections (10 µm, Leica RM2165) were placed into PBS and processed while free-floating.

Skin samples were infiltrated with 30% sucrose in 0.1M PB (overnight, 4°C) and then frozen on dry ice in a 1:2 mixture of 30% buffered sucrose and Tissue-Tek O.C.T. compound (Sakura). Cryostat sections (10 µm, Leica CM3050S) were mounted directly onto Superfrost/Plus glass slides (Fisher Scientific), and dried overnight before processing for immunofluorescence.

Immunohistochemistry

Immunostaining and double labeling utilized three primary antibodies made in different species: rabbit anti-NPY (1:1000, Sigma), mouse monoclonal anti-TH (1:2000, Chemicon) and goat anti-VMAT2 (1:500, Santa Cruz #sc-7721). All antibodies were diluted in PBS containing 1.5% normal donkey serum (Jackson ImmunoResearch) and 0.3% Triton X-100 (Sigma) (PBSDT).

Secondary antibodies were donkey anti-rabbit CY2, donkey anti-rabbit CY3, donkey anti-mouse CY3 and donkey anti-goat CY2. All secondaries were obtained from Jackson ImmunoResearch and diluted 1:200 in PBSDT.

To minimize non-specific background, skin sections were pretreated in 0.5% sodium borohydride (Sigma) for 10 min. All tissue sections were then preincubated in PBSDT for 90 min at room temperature and then incubated in primary antibodies overnight at 4°C. After six rinses in PBS, sections were incubated for 90 minutes at room temperature in secondary antibodies diluted in PBSDT, rinsed six times and mounted.

After processing for immunocytochemistry, sections of the SCG and submandibular gland were rinsed in distilled water, mounted on Superfrost/Plus slides and air dried overnight. The mounted sections were then dehydrated through graded ethanols, cleared in xylenes, and coverslipped with Krystalon mounting medium (Harleco). Frozen skin sections on glass slides were dehydrated immediately after immunostaining, cleared, and coverslipped with Krystalon.

Omission of primary antibodies from the staining protocols eliminated all neuronal staining for VMAT2, NPY and TH. Cross-reactivity controls showed that all secondary antibodies reacted specifically with the appropriate primary antibodies in double labeling protocols.

Immunofluorescent sections were viewed using a Zeiss Axioskop 2 microscope and photographed with a Zeiss Axiocam camera and AxioVision 4.4 software. Figures were prepared using Adobe Illustrator.

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ABBREVIATIONS

VMAT	vesicular monoamine transporter
SCG	superior cervical ganglion
NPY	neuropeptide Y
TH	tyrosine hydroxylase
IR	immunoreactivity
ECN	external carotid nerve
ICN	internal carotid nerve

Figure 1.

VMAT2 immunoreactivity in the SCG is restricted to a subset of sympathetic neurons. (A) Low power micrograph (10x) showing VMAT2-positive cell bodies present along the rostro-caudal axis of the ganglion. The external carotid nerve (ECN) exits near middle of the ganglion and internal carotid nerve (ICN) marks the rostral pole of the ganglion. (B, C) Higher power images (40x) show that some neurons appear negative or weakly stained for VMAT2-IR. White boxes in panel A show the positions of the micrographs in B and C and white dashed lines outline examples of non-immunoreactive neurons. Note that near the ECN (B), the negative cells are relatively large and near the ICN (C), the negative and positive neurons are similar in size.

Figure 2.
Cellular colocalization of immunoreactivity for (A) NPY and (B) VMAT2 in the SCG.

Figure 3.

Double labeling of the submandibular gland reveals VMAT2-IR in secretomotor and vasomotor sympathetic axons. Colocalization of VMAT2-IR (A) and TH-IR (B) in axons running between gland cells (white arrowheads) and in small blood vessels (grey arrowheads). Staining of the ducts in panel A was non-specific. Colocalization of VMAT2-IR (C) and NPY-IR (D) in the submandibular gland was restricted mainly to blood vessels.

Figure 4. Immunoreactivity of pilomotor sympathetic axons (arrowheads) at the base of cutaneous hairs. Double labeling reveals that TH-positive axons (A, C) near the pilli arrector muscles are negative for NPY (B) and positive for VMAT2 (D).