Chemical neuroanatomy of the vesicular amine transporters

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ABSTRACT **Acetylcholine, catecholamines, serotonin, and histamine are classical neurotransmitters. These small molecules also play important roles in the endocrine and immune/inflammatory systems. Serotonin secreted from enterochromaffin cells of the gut epithelium regulates gut motility; histamine secreted from basophils and mast cells is a major regulator of vascular permeability and skin inflammatory responses; epinephrine is a classical hormone released from the adrenal medulla. Each of these molecules is released from neural, endocrine, or immune/inflammatory cells only in response to specific physiological stimuli. Regulated secretion is possible because amines are stored in secretory vesicles and released via a stimulus-dependent exocytotic event. Amine storage—at concentrations orders of magnitude higher than in the cytoplasm—is accomplished in turn by specific secretory vesicle transporters that recognize the amines and move them from the cytosol into the vesicle. Immunohistochemical visualization of specific vesicular amine transporters (VATs) in neuronal, endocrine, and inflammatory cells provides important new information about how amine-handling cell phenotypes arise during development and how vesicular transport is regulated during homeostatic response events. Comparison of the chemical neuroanatomy of VATs and amine biosynthetic enzymes has also revealed cell groups that express vesicular transporters but not enzymes for monoamine synthesis, and *vice versa*: their function and regulation is a new topic of investigation in mammalian neurobiology. The chemical neuroanatomy of the vesicular amine transporters is reviewed here. These and similar data emerging from the study of the localization of the recently characterized vesicular inhibitory and excitatory amino acid transporters will contribute to understanding chemically coded synaptic circuitry in the brain, and amine-handling neuroendocrine and immune/inflammatory cell regulation.—Weihe, E., Eiden, L. E. Chemical neuroanatomy of the vesicular amine transporters. *FASEB J.* 14, 2435–2449 (2000)**

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AMINE-HANDLING CELLS IN mammals are those that synthesize, store, and secrete the quaternary amine neurotransmitter acetylcholine (ACh), or the biogenic amines dopamine, norepinephrine, epinephrine, histamine, or serotonin (5HT). The nervous, neuroendocrine, and immune/inflammatory systems all contain key amine-handling cells. Biogenic amine-containing neurons are grouped into a series of discrete noradrenergic, dopaminergic, serotonergic, and histaminergic brain nuclei. Cholinergic neurons are organized into nine projection nuclei and two intrinsic cell groups in the central nervous system (1). In the peripheral nervous system, amine-containing neurons make up most or all of the neurons of the sympathetic and parasympathetic (autonomic) nervous systems. Amines are largely absent from the sensory nervous system, where the transmitters are neuropeptides and glutamate.

Biogenic amine-containing neuroendocrine cells are derived both from the neural crest (e.g., chromaffin cells) and from local stem cells (e.g., gut enterochromaffin and skin Merkel cells; ref 2). Biogenic amine-handling cells of the immune/inflammatory axis are of presumptive hematopoietic origin (3). These are histamine-containing mast cells and basophils, and serotonin-containing platelets. The proteins expressed by all of these cell populations that make them 'amine handling' include enzymes for synthesizing amines, plasma membrane transporters for scavenging and recycling amines from the extracellular space, and intracellular transporters for amine storage in secretory vesicles. To store amines in secretory organelles, amine-handling cells all express one of three vesicular amine transporters (VATs): the biogenic amine transporters VMAT1 and VMAT2, and the acetylcholine transporter

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TABLE 1. *Demonstrated expression patterns of VATs and amine biosynthetic enzymes, and their chemically coded phenotypes*[a]

TH	AADC	DβH	PNMT	TrH/5-HT	HDC	VMAT 1	VMAT 2	Cell type	Phenotype
+	+	−	−	−	−	+	±	SIF cell	DA
+	+	+	−	−	−	−	+	Neuron of CNS and gut	DA
+	+	+	−	−	−	+	±	Chromaffin cell	NE
+	+	+	−	−	−	−	+	Neuron of CNS and PNS	NE
+	+	+	+	−	−	+	±	Chromaffin cell	Epi
+	+	+	+	−	−	−	+	Neuron of CNS	Epi
−	+	−	−	+	−	+	−	Enterochromaffin cell	5HT
−	+	−	−	+	−	−	+	CNS and enteric neurons	5HT
−	?	−	−	+	−	−	+	Megakaryocyte/platelet	5HT
−	?	−	−	−	+	−	+	Enterochromaffin-like cell	HIS
−	?	−	−	−	+	−	+	Neuron of CNS	HIS
−	?	−	−	−	+	+	−	Merkel cell	??
−	+	−	−	−	+	−	+	Mast cell	HIS
+	+	−	−	−	−	−	−	Olfactory bulb 'nonstored' DA	DA
−	?	−	−	−	−	−	+	Thalamocortical 'orphan neurons'	5HT??
+	±	−	−	−	−	−	−	Hypothalamic 'orphan neurons'	??

[a] Type of neurons or neuroendocrine cells expressing VMAT1 or VMAT2, and various combinations of biosynthetic enzymes for serotonin (TrH, tryptophan hydroxylase), dopamine (TH, tyrosine hydroxylase; AADC, aromatic amino acid decarboxylase), norepinephrine (TH, AADC, and DβH, dopamine β-hydroxylase), epinephrine (TH, AADC, DβH, and PNMT, phenylethanolamine N-methyl transferase), histamine (HDC, histidine decarboxylase) are shown. Expression of various plasma membrane transporters that contribute to efficacious chemical neurotransmission and specific to each transmitter are not listed here. VAChT-positive neurons are not included as there is a single combination (VAChT+ChAT) of biosynthetic and transporter protein required for the cholinergic phenotype, with both proteins expressed from a single gene locus. SIF cell, small intensely fluorescent cell.

VAChT. It is from the secretory granules or vesicles within the cell that amines are released in a highly regulated fashion to act as informationally specific hormones, autocrine or paracrine factors, and neurotransmitters. Thus, the chemical neuroanatomy of amine-handling cells is determined by a precisely regulated coexpression of enzymes imparting synthesis and reuptake capability for a particular amine, together with one of the three VATs.

The expression patterns of biosynthetic enzymes and VATs that give rise to each of the amine-handling phenotypes of the nervous, neuroendocrine, and immune/inflammatory systems are summarized in **Table 1**. In this review, we will describe current information about VAT expression and function that illuminates the role of amine-handling cells in each of these three major homeostatic systems.

AMINERGIC NEURONS

The central cholinergic nervous system

The vesicular acetylcholine transporter (VAChT) gene is transcribed, along with the acetylcholine biosynthetic enzyme choline acetyltransferase (ChAT), from a genomic location called the cholinergic gene locus (4). All cholinergic neurons of the mammalian nervous system, both central and peripheral, therefore express VAChT and ChAT (5–8). Despite their coordinated expression and regulation in mammals (9–12), the relative levels of VAChT and ChAT mRNAs can differ substantially under certain conditions. VAChT and ChAT mRNA levels are differentially up-regulated by NGF in rat cholinergic septal neurons (13). VAChT mRNA is more abundant than ChAT mRNA in whole brain during early development (14). This pattern is due to higher levels of VAChT than ChAT transcripts in nascent cholinergic neurons, and it persists into adulthood in peripheral cholinergic neurons. In the adult central nervous system, ChAT and VAChT mRNA levels are similar (15).

VAChT staining is a valuable adjunct to ChAT in investigating the chemical neuroanatomy of cholinergic systems. Staining for VAChT has been largely confirmatory of the existence of cholinergic neuronal cell groups in both the central nervous system (CNS) and peripheral nervous system (PNS), inferred from staining for acetylcholine esterase and ChAT. Beyond this, VAChT has the special advantage of staining cholinergic nerve terminals in proportion to their density of synaptic vesicle clusters (**Fig. 1**). For this reason, VAChT immunohistochemistry has allowed identification of previously unsuspected or uncharacterized cholinergic projection fields in several areas of brain and in the periphery. A dense plexus of cholinergic nerve endings is now known to exist, for example, in the hypothalamic median eminence in mouse, rat, and primate (6, 8, 16, 17) as previously only inferred by measurement of ChAT enzymatic activity (18). The primary sen-

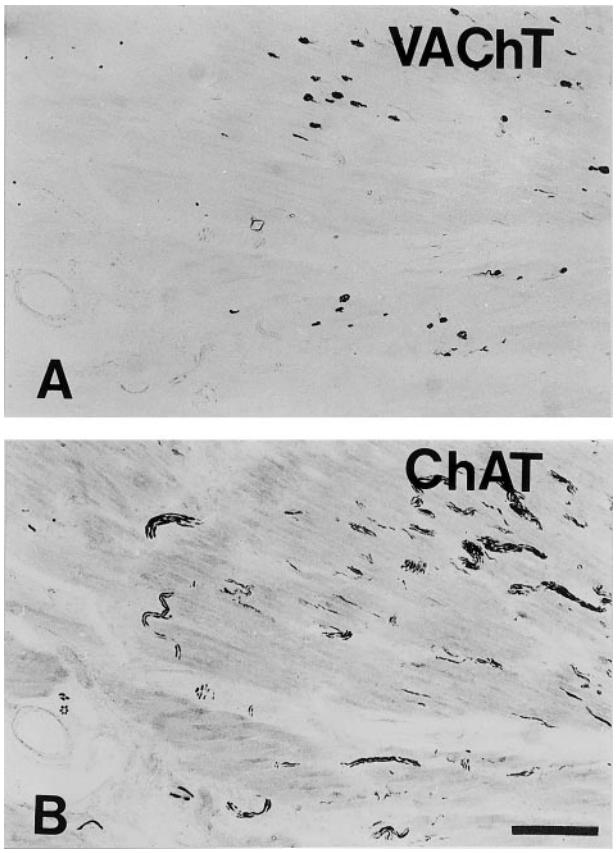

Figure 1. Complementary immunostaining of cholinergic motor neurons for ChAT and VAChT. Complementary patterns of *A)* terminal staining with VAChT and *B)* fiber staining with ChAT in rat limb skeletal muscle. Bar indicates 50 μm. From Weihe et al., 1996; ref 16.

sory mesencephalic nucleus has been shown to express the cholinergic phenotype based on VAChT− and ChAT-positive staining. Cholinergic hypothalamic nuclei, including an arcuate cell group that may be the source of cholinergic projections to the median eminence, have been visualized with VAChT staining and *in situ* hybridization histochemistry in both mouse and rat (5, 6, 8, 16, 17, 19).

VAChT immunoreactivity in cell bodies and nerve terminals allows greater ability to detect pathophysiological alterations in cholinergic neurons of the CNS by providing an estimate of dynamic changes in cholinergic terminal field density and allowing colocalization of VAChT with other neurotransmission-relevant molecules. For example, down-regulation of cyclooxygenase immunoreactivity in basal forebrain cholinergic neurons and decreased cholinergic projection density in cortex have both been observed in SIV-infected primates with qualitative and semiquantitative VAChT immunohistochemistry (20). Misawa et al. have noted the loss of VAChT-positive homotypic synapses on ventral horn spinal motor neurons in amyotrophic lateral sclerosis spinal cord, suggesting a role for these synapses in the initiation or progression of cholinergic motor neuron dysfunction in this disease (21). Debeir and co-workers have noted a specific decrease in VAChT-immunoreactive synapses compared to synaptophysin (total) immunoreactive synapses in rat cortex after local administration of anti-NGF antibody or TrkA peptide antagonist (22)

Biogenic amine-containing neurons of the CNS

VMAT1 and VMAT2 represent isoforms of the vesicular monoamine transporter that are in general segregated into endocrine and neuronal monoamine-containing cells, respectively (23, 24). VMAT2 is the exclusive VMAT isoform found in serotoninergic, noradrenergic, dopaminergic, histaminergic, and adrenergic neurons of the CNS (see Table 1). It has been suggested that VMAT1-positive neurons exist in the developing rat CNS based on *in situ* hybridization histochemical data (25). However, others have obtained no evidence for neurons positive for VMAT1 mRNA or protein in either developing or adult rat CNS (23, 24, 26). The lack of catecholamine storage in the CNS of VMAT2-deficient mice during development is also consistent with exclusive VMAT2 expression in CNS monoaminergic neurons in the rodent (27).

Expression of VMAT2 in developing and adult CNS neurons defines cell groups with an incomplete ('orphan') biogenic amine phenotype

As expected, VMAT2 is a general histochemical marker for biogenic amine-handling neurons of the CNS. However, neurons have now been identified that, despite being strongly positive for VMAT2, apparently lack biosynthetic enzymes for any of the known biogenic amines. Other neuronal cell groups contain a full complement of biosynthetic enzymes but lack VMAT2 expression. For example, distinct thalamocortical and intrathalamic neuronal cell groups have been identified that are transiently VMAT2 positive during late gestation and early postembryonic life in mouse and rat, but lack tyrosine hydroxylase (TH) necessary to produce catecholamines or tryptophan hydroxylase for the synthesis of serotonin (26, 28, 29). These neurons express in addition to VMAT2 the serotonin plasma membrane transporter and under certain conditions may function as transient serotoninergic neurons by releasing exogenous serotonin scavenged from the extracellular space (28, 29).

These observations highlight the participation of VMAT2 in multiple overlapping neurotransmitter 'regulons' in the brain and raise the possibility that neurotransmitter circuits utilizing nonclassical neurotransmitter substrates for VMAT2 remain to be discovered. VMAT2 recognizes as transport substrates the

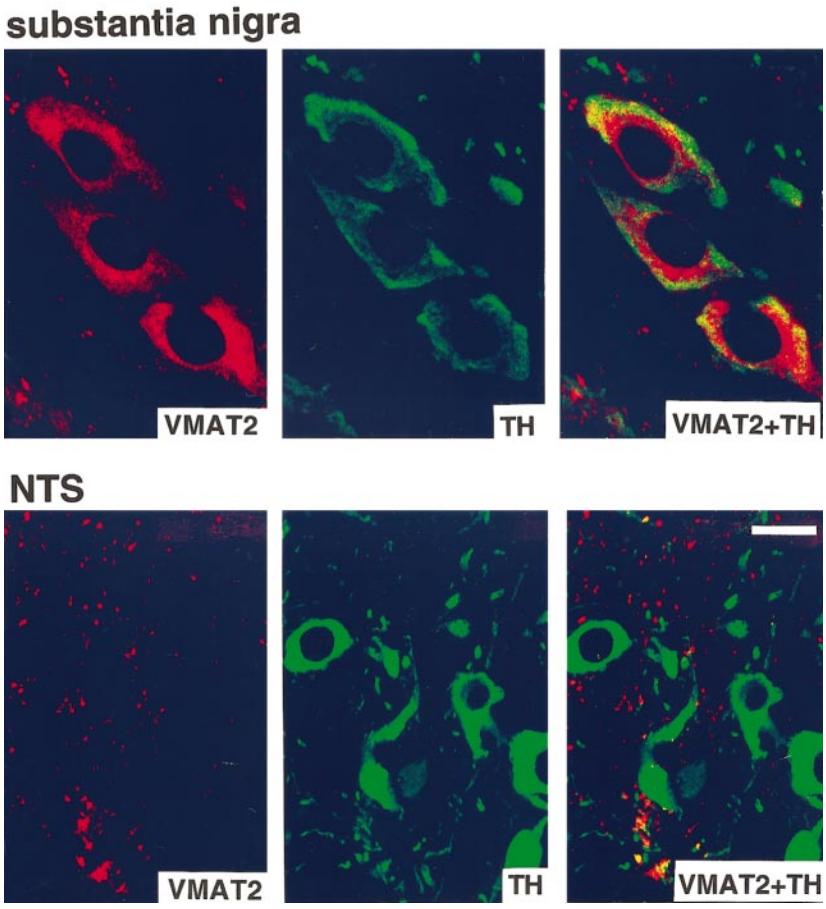

Figure 2. Dissociated expression of monoaminergic traits in CNS. Dopaminergic (VMAT2+/TH+) neurons of substantia nigra and TH+/VMAT2− neurons of the nucleus tractus solitarius (NTS) of the rhesus monkey. TH-positive neurons of the NTS lack transporter expression, although both catecholaminergic (VMAT2+/TH+) and presumptive serotonergic (VMAT2+/TH−) nerve fibers can be seen projecting into the NTS. Bar: 10 μm.

biogenic amines phenylethylamine, tyramine, and octopamine (30, 31), which are present in trace amounts in adult and developing rodent brain and sympathetic nervous system (32, 33). Neurons that contain aromatic amino acid decarboxylase and VMAT2 would be candidate tyraminergic cells; those also containing dopamine β-hydroxylase would be candidate octopaminergic mammalian neurons. Flies with a mutation in tyrosine decarboxylase, an aromatic amino acid decarboxylase (AADC) that converts tyrosine to tyramine, have reduced levels of tyramine and are resistant to the sensitizing effects of repeated administration of cocaine (34). It is not yet known whether these neurons use VMAT2 as their vesicular transporter.

Another class of neurons in the rodent CNS has been identified that express biogenic amine biosynthetic enzymes, but not a vesicular monoamine transporter. In the principal olfactory bulb, periglomerular neurons clearly express both TH and AADC and presumably synthesize dopamine (35), but lack VMAT2 (24). The dynamic regulation of TH in these neurons suggests a role for the synthesized catecholamine (36), but the functional status of these 'dopaminergic' cells is presently unclear. Other neurons that express tyrosine hydroxylase but lack VMAT2 include extensive plexus of TH+/VMAT2− neurons in the supraoptic nucleus, area postrema, and nucleus tractus solitarius (**Fig. 2**). The absence of VMAT2 in TH-positive neuronal cell groups previously classified as 'catecholaminergic' invites consideration of the functional significance of amine synthesis and storage in neurons that lack the capability for vesicular accumulation of transmitter. The possibilities of nonvesicular catecholamine release or the presence of vesicular transporters other than VMAT2 have yet to be explored.

VMAT2 and VAChT expression in the autonomic nervous system

Autonomic regulation of visceral functions depends on a dual innervation from the sympathetic and parasympathetic nervous systems. Centrally located preganglionic cholinergic cell groups are connected to sympathetic ganglia whose cell bodies are arranged within the paravertebral and prevertebral sympathetic chains and to parasympathetic ganglia contained both intrinsically in target organs such as heart, gut, iris, genitourinary tract, and lung, as well as in cranial and sacral extrinsic ganglia. Sympathetic and parasympathetic ganglia in turn supply dual innervation to the visceral organs, cardiovascular system, and the skin. Sympathetic neurons are in general noradrenergic, and parasympathetic neurons are, like the preganglionic neurons, cholinergic. An exception to the simple rule of chemical

coding for noradrenergic neurotransmission in sympathetics and cholinergic neurotransmission in parasympathetics is found in the paravertebral sympathetic chain. Here, ~5% of the ganglionic neurons are cholinergic, and project to the eccrine sweat glands, the skeletomuscular vasculature, and the periosteum (7, 37, 38). These targets are also unique in receiving no parasympathetic innervation (see ref 38 and references therein).

Expression of the vesicular amine transporters in the developing as well as mature sympathetic and parasympathetic nervous systems has provided insight into the mechanisms of lineage determination leading to chemical coding of neurotransmission (26, 39, 40).

Sympathetic neurons

The paravertebral sympathetic chain contains mainly TH+ neurons that coexpress VMAT2. A second population of cells exists that is TH-/VAChT+/VMAT2−. Depending on the rostral-caudal level, this population varies from 1 to 5% of the total number of principal neurons in the ganglion. The prevertebral chain contains exclusively TH+/VMAT2+ noradrenergic neurons in the rat, but in some species may occasionally contain rare prevertebral cholinergic (VAChT-positive) neurons as in the paravertebral chain. Developmentally, the noradrenergic neurons of the sympathetic chains appear to express vesicular transporter as early as they express catecholaminergic biosynthetic enzymes, so that the ability to store and release catecholamines is an early emerging feature of the sympathetic nervous system. As with VAChT, VMAT2 immunohistochemistry allows facile visualization of terminal fields for all autonomic target organs (**Fig. 3**).

Cholinergic differentiation in the sympathetic nervous system is a more complex matter. VAChT expression in the developing sympathetic chain appears to be coincident with both VMAT2 appearance and preganglionic innervation of the chain anlage. We recently quantified the number of VAChT-positive neurons in the developing sympathetic chain. They represent approximately the adult proportion (5% in stellate) as early as embryonic day 16 (Gördes and E. Weihe, unpublished observations). Since VAChT-positive fibers are also seen innervating sweat glands as early as postembryonic day 2, when forepaw sweat glands are still developing, the initial cholinergic sympathetic differentiation appears to be independent of the establishment of synaptic relationships between these neurons and their final peripheral targets (40). It has been suggested that the cholinergic sympathetic neurons that innervate the sweat glands, which form only after birth, achieve the

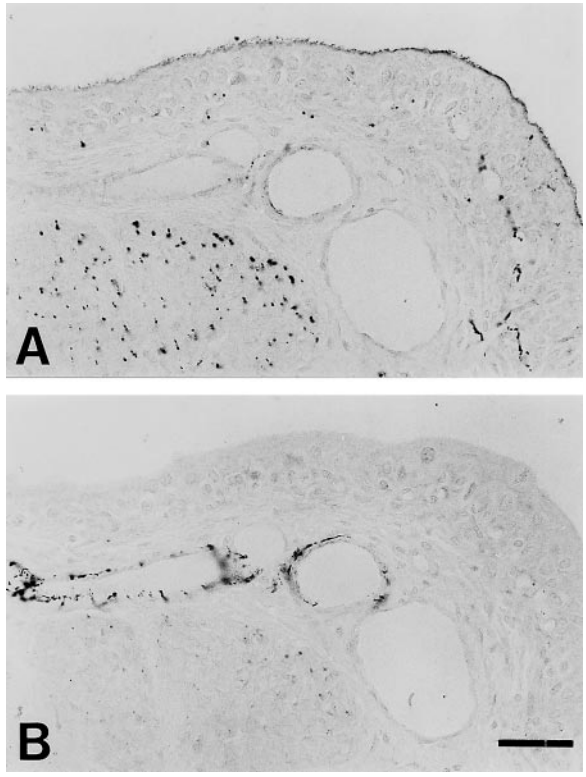

Figure 3. Visualization of VMAT2-positive (noradrenergic sympathetic) and VAChT-positive (cholinergic parasympathetic) innervation of peripheral organs. *A)* VAChT+ parasympathetic terminals innervate smooth muscle and *B)* VMAT2+ sympathetic terminals innervate blood vessels in the urinary bladder of the rat. Bar: 50 μm. From Schäfer et al., 1998.

cholinergic phenotype by switching from a noradrenergic to a cholinergic expression program under the influence of factors secreted from the sweat glands around postembryonic day 2 until about postembryonic day 10 (37, 38, 41, 42). Several studies suggest that the cholinergic phenotype can be elicited *de novo* from nearby neurons that are purely noradrenergic when induced to innervate a transplant consisting in normally cholinergically innervated target tissue (e.g., sweat gland, periosteum) (38, 41). On the other hand, VAChT+ neurons exist, at least in the stellate ganglion, as early as embryonic day 17 and comprise a fairly constant 5–7% of the total cell number of the ganglion through postembryonic day 10 (15, 40). Costaining with VAChT and VMAT2, along with TH, indicates that at least three populations of neurons exist in the stellate ganglion innervating the sweat glands and other peripheral targets. These include the majority of principal ganglion cells, which are fully and exclusively noradrenergic (TH+/VMAT2+/VAChT−), and two populations of VAChT+/VMAT2− cells—one positive for TH. The VAChT+/TH+/VMAT2− neurons innervate the sweat glands (**Fig. 4**). The VAChT+/TH−/VMAT2− neurons likely are non-

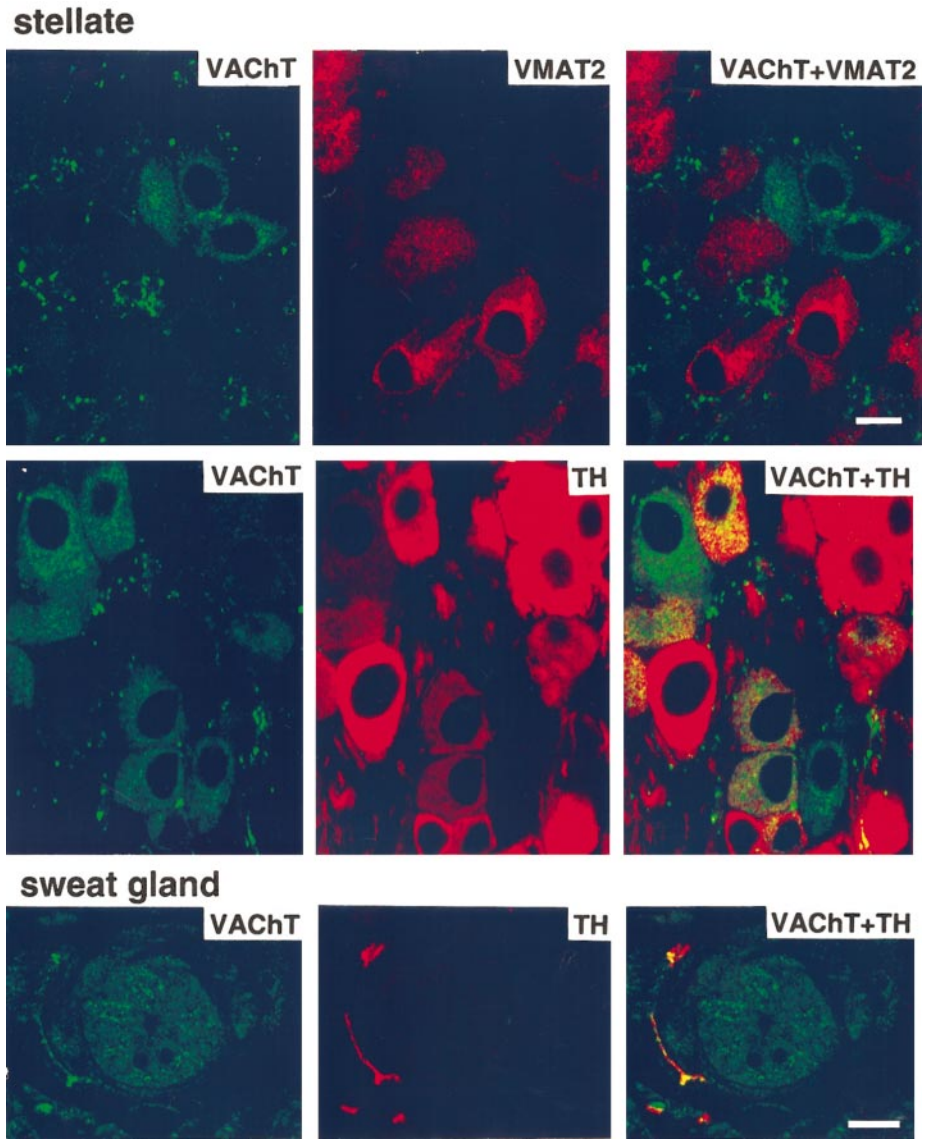

Figure 4. Development of neurons possessing both cholinergic and noradrenergic phenotypes in rat sympathetic ganglia. Upper panel: VAChT+/VMAT2− cholinergic and VAChT−/VMAT2+ noradrenergic principal ganglion cells in rat stellate ganglion, postembryonic day 6. Middle panel: One population of noradrenergic (TH+/VAChT−) and two populations of cholinergic (VAChT+/TH−; VAChT+/TH+) neurons in rat stellate ganglion, postembryonic day 6. VAChT−/TH+ neurons in this panel correspond to VMAT2+ neurons in upper panel. Lower panel: VAChT+/TH+ cholinergic fibers in rat sweat gland, postembryonic day 6. These fibers are also VMAT 2− and are presumably the projections of VAChT+/TH+ principal ganglion cells shown in the middle panel. VMAT2 antibody 80182 was raised in rabbit; TH antibody (Chemicon) was raised in sheep. Two VAChT antibodies were used: one raised in rabbit (80259, middle and bottom panels) and one in goat (VAChTcom, top panel). Bar: 10 μm.

sudomotor and may innervate the skeletal muscle vasculature.

Thus, while copious evidence exists for increased expression of cholinergic markers at sweat gland nerve terminal during postnatal development (see above), there is also increasing evidence that cholinergic neurons, both those coexpressing noradrenergic traits and those not, already exist in the stellate ganglion, innervating the forepaw sweat glands, and that VAChT-positive terminals exist at sweat glands as early as the glands themselves exist. One possible resolution of this seeming paradox is that the dramatic increase in the density of VAChT-positive terminals in the sweat glands and periosteum that occurs postnatally is due not to up-regulation of the cholinergic gene locus at the transcriptional level, but to reorganization of vesicle trafficking and increased numbers of small synaptic vesicles at these nerve terminals as the sympathetic cholinergic innervation of these target tissues matures.

Parasympathetic neurons

Parasympathetic postganglionic neurons are organized into ganglia extrinsic to the innervated organs in the cranial and sacral regions and in intrinsic ganglia throughout the viscera of the head and neck, and the thoracic, abdominal and pelvic cavities. These neurons are purely cholinergic (VAChT+/VMAT2−) in the adult, but initially differentiate as VAChT/VMAT2 copositive cells in the otic, sphenopalatine, and submandibular cranial parasympathetic ganglia (26). Since these neurons are TH− during the period of expression of VMAT2, the functional significance of transient VMAT2 expression, as in the CNS, is unclear. The ciliary parasympathetic ganglion also expresses VAChT prenatally. However, the ciliary ganglion expresses no VMAT2 but, rather, a TH phenotype that persists into adulthood (43 and references therein). Thus, the ability to visualize multiple components of the noradrener-

gic and cholinergic phenotypes, including the VATs, reveals a complex coexpression of noradrenergic traits in both sympathetic and parasympathetic neurons. This coexpression indicates that some neuronal populations pass through a developmental stage in which neurotransmitter-associated traits are not as stringently regulated as in the fully differentiated neuron. It may impart properties of neurotransmission that are unique to developing neurons.

Since VAChT and ChAT are invariably coexpressed in cholinergic neurons, VAChT has become the marker of choice for detailed examination of cholinergic terminal fields, particularly in the PNS (7). Visualization of target organs and tissues of the parasympathetic nervous system with VAChT antibodies has revealed a far more extensive innervation in 'classical' targets of the cholinergic parasympathetic nervous system than previously appreciated. This is true for all three major categories of parasympathetic innervation: the secretory cells and contractile myoepithelial investment of exocrine secretory glands (e.g., salivary glands, lacrimal glands, exocrine pancreas); visceral organ vasculature; and visceral organ smooth muscle walls (e.g., gut, urinary bladder, ureter, urethra, and vas deferens). Especially striking is the density of cholinergic innervation of ventricular striated muscle in the heart, which is much more prominent in both rodent and primate than reported using ChAT as a marker. Coronary microvessels within the heart itself are also heavily innervated by VAChT-positive terminals (7).

Intrinsic neurons of the gut

Biogenic amine intrinsic innervation of the gut revealed by VMAT2 costaining with biogenic amine markers

The nature of chemical coding of biogenic amine neurons in the gastrointestinal tract, i.e., the enteric nervous system, has been intensively studied during development and in the adult, yet several major unanswered questions remain about amine-handling cell populations of the enteric nervous system remain. This is due in large part to the historical assumptions that if a neuron exhibits a particular marker for a chemically coded phenotype, it possesses the other attributes of that functional phenotype. Thus, TH-positive neurons without DβH are often assumed to be dopaminergic, neurons expressing the serotonin plasma membrane transporter (SERT) are assumed to be fully serotonergic, and so forth. As indicated in Table 1 and **Table 2**, histochemical evidence for a full functional neurotransmitter phenotype in the gut requires the visualization of all components contributing to the phenotype. Even the use of labeled serotonin uptake as an indicator of functional phenotype is suspect, since exogenous serotonin can be accumulated by VMAT2/SERT-positive neurons that lack tryptophan hydroxylase (26, 28).

VMAT2 is a pan-biogenic amine neuronal marker in the gut, providing a histochemical 'denominator' by which fractional expression of markers for dopamine, norepinephrine, and serotonin can be examined in the enteric nervous system. In both rodent and human, the greatest abundance of VMAT2-positive nerve fibers are found at blood vessels and around enteric ganglia. These neurons are also positive for TH and DβH, and represent the sympathetic postganglionic innervation of the gut. Rare VMAT2-positive fibers in muscle and mucosal layers of gut appear to lack TH expression and may correspond to projections of intrinsic serotonergic neurons, i.e., the also rare submucous and myenteric plexus cell body staining for VMAT2, which in adjacent sections appear to lack TH and DβH expression. These neurons have not, however, been demonstrated to express tryptophan hydroxylase or aromatic amino acid decarboxylase. Those that express SERT may obtain serotonin either by direct biosynthesis or via scavenging of serotonin released from enterochromaffin cells within the lamina propria/intestinal epithelial villi. The extensive distribution of serotonin receptors in the intestine and the

TABLE 2. *Phenotypic markers and chemical coding of monoaminergic neurotransmission in the rat and human enteric nervous system*

Neuronal element	Rat	Human
Fibers: TH+/DβH+/VMAT2+ (noradrenergic)	At blood vessels and around sympathetic postganglionic ganglia	At blood vessels and around sympathetic postganglionic ganglia
Fibers: TH+/DβH−/VMAT2+ (dopaminergic)	None visible	Submucous smooth muscle; circular and longitudinal muscle layers
Fibers: TH−/DβH−/VMAT2+ (serotonergic)	Muscle layer (rare) mucosa (lamina propria; rare)	In muscle layer (very rare) mucosa (lamina propria; very rare)
Soma: TH+/DβH+/VMAT2+ (noradrenergic)	None	None
Soma: TH+/DβH−/VMAT2+ (dopaminergic)	None	Myenteric and submucous plexus (5% total)
Soma: TH−/DβH−/VMAT2+ (serotonergic)	Submucous>myenteric plexus (very rare)	Submucous>myenteric plexus (very rare)

many effects of endogenous serotonin application on enteric nervous system function make this an important area for further study. An interesting population of biogenic aminergic neurons of the human, but not the rodent gut, are the VMAT2-positive cells of the myenteric and submucous plexus that are TH+/DβH− and therefore presumably dopaminergic. The function of these neurons and whether they represent in human a stable continuation of the so-called transiently catecholaminergic neurons of the rodent developing gut (44) are open questions.

Cholinergic innervation of the gut

The use of VAChT for staining of nerve terminals with efficiency equivalent to that for vasoactive intestinal polypeptide (VIP) and other neuropeptides has allowed a better neuroanatomical accounting of the NANC (nonadrenergic, noncholinergic) neuronal system in the gut (45). It is now clear that most intrinsic neurons of the gut found in myenteric or submucous ganglia are in fact cholinergic as well as VIPergic, with only a small proportion lacking VAChT (**Fig. 5**). In the colon, the percentage of VIP+/VAChT− neurons is increased, and these may represent NANC intrinsic sensory neurons of the gut. Nonadrenergic, noncholinergic (e.g., VIPergic) neurons may be those that both contain and release ACh and VIP, but at different frequencies of stimulation. By our estimate (M. Anlauf, L. E. E., and W. E., unpublished results), non-VIPergic NANC may comprise up to 20% of the total neuronal numbers of the human gastrointestinal tract, which are neither VAChT nor VMAT2 expressing. Recent studies of the ChAT/VAChT ratio of the peripheral vs. central nervous system also demonstrate this ratio is rather low in the PNS compared to the CNS (15). This, together with the recent finding of alternatively spliced 'peripheral' forms of ChAT (46) of potentially lesser immunoreactivity for conventional ChAT antibodies, may account for the inability to stain for ChAT even in large proportions of apparently cholinergically competent neurons and explain the previously rather high estimate of the proportion of intrinsic neurons in gut and urogenital tract that are noncholinergic.

The concordance of VIP and VAChT in most if not all 'parasympathetic' or 'autonomic' intrinsic neurons allows further distinctions to be made between VIP+/VAChT− putative sensory neurons and VIP+/VAChT+ enteric autonomic neurons. For example, the percentage of VIP+/VAChT− intrinsic neurons in the submucous plexus of the colon (see above) approaches 30%, whereas the numbers of these neurons in the submucous plexus of the proximal gut and in the myenteric plexus at all levels is much lower (3–10%; M. Anlauf et al., unpublished observations).

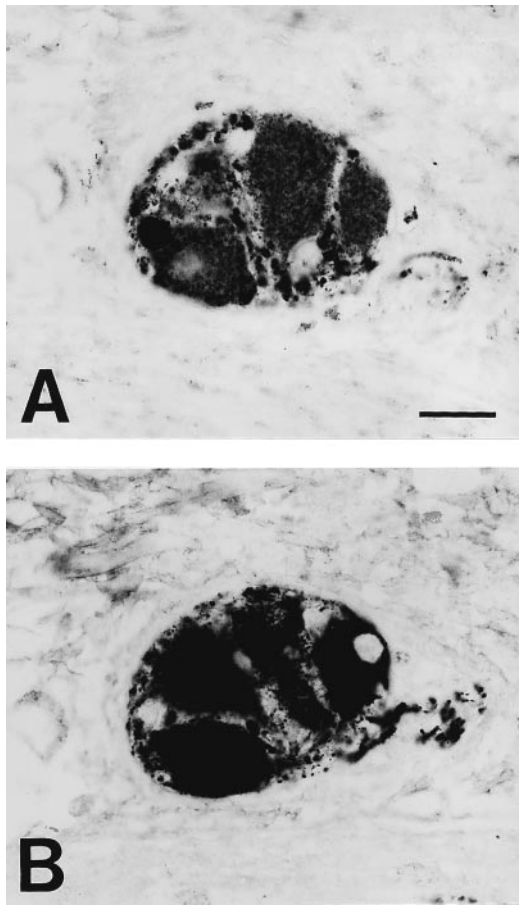

Figure 5. VAChT and VIP coexistence in the human gastrointestinal (enteric) nervous system. Coexistence of *A)* VAChT and *B)* VIP immunoreactivity in adjacent sections of the human myenteric plexus. Bar: 15 μm.

BIOGENIC AMINE-CONTAINING ENDOCRINE CELLS AND VMAT EXPRESSION

VMAT1 is the 'endocrine' and VMAT2 the 'neuronal' vesicular amine transporter for neural crest-derived autonomic and neuroendocrine cell types

The differential expression of VMAT1 and VMAT2 in the central nervous system and the periphery suggests that lineage determination mechanisms for central and peripheral aminergic cells are fundamentally different (26). As discussed above, neuronal elements of the autonomic nervous system that develop into noradrenergic neurons express VMAT2 and not the VMAT1 isoform of the vesicular monoamine transporter (see previous text). In neuroendocrine cell groups and tissues derived from the neural crest, the dominant transporter is VMAT1, although depending on the cell type and species

examined, VMAT2 may also be expressed in some neural crest-derived neuroendocrine cells. The small intensely fluorescent (SIF) cells of the sympathetic ganglia, for example, comprise less than 1% of the total monoaminergic cells of a typical sympathetic ganglion and store dopamine in large dense core vesicles that contain VMAT1 as well as VMAT2 in the rat and mouse (26). VMAT1 and VMAT2 coexpression is also seen in SIF cells in parasympathetic and placodal (vagal and glossopharyngeal) ganglia in the rat (E. Weihe, unpublished results).

The adrenal medulla contains norepinephrine and epinephrine-storing chromaffin cells. In rat and mouse, VMAT1 is the dominant transporter for both types of cells, whereas in human and rhesus monkey, VMAT1 and VMAT2 are both expressed in apparently all of the cells of adult adrenal medulla (23, 24, 30). Other 'chromaffin-like' cells of autonomic origin include the glomus cells of the carotid and aortic bodies. These cells are dopaminergic, have short processes, and function as chemoreceptive units signaling changes in blood gas levels to the cardiorespiratory centers of the brain stem. Glomus cells have as their vesicular transporter predominantly VMAT1 in the adult, but express quite high levels of VMAT2 early in development, with a decline into adulthood.

VMAT2 is absent from the well-studied rat pheochromocytoma-derived cell line PC12, although VMAT2 is expressed in the rat adrenal medulla (*vide supra*). This has enabled an interesting set of experiments in which VMAT2 expression has been forced in PC12 cells and its secretory vesicle destination followed by immune electron microscopy and subcellular fractionation (16, 47). Contrary to expectation, VMAT2 does not target to small synaptic vesicles that contain VAChT in these cells, but exclusively to the large dense core VMAT1-positive secretory vesicles, implying that VMAT2 expression in small synaptic vesicles in noradrenergic neurons requires an as yet unidentified additional competence/chaperone factor not expressed in PC12 cells. These findings suggest an additional molecular layer of regulation in amine handling within secretory vesicles of the nervous and endocrine systems.

The gastroenteropancreatic neuroendocrine system

Besides adrenomedullary chromaffin cells, glomus cells and SIF cells derived from the neural crest, vesicular monoamine transporters are expressed on a wide variety of endocrine cells of the stomach, intestine, and pancreas. The numerous enterochromaffin cells of the intestine are found in the crypts and villi of the intestinal epithelium and generated along with other gut epithelial cell types (Paneth, enterocytic, and goblet cells) from the crypt stem cell (2). Enterochromaffin cells are sensory transducers of the gut epithelium that store and release serotonin to activate local sensory afferent neurons that modulate gut motility (48). These cells express exclusively VMAT1 and, unlike the endocrine cells of neural crest origin, do not go through a VMAT2-expressing stage (23, 26). In the oxyntic mucosa of the stomach, a second population of enterochromaffin-like (ECL) cells stores and releases histamine in response to gastrin secretion, leading to paracrine activation of acid secretion from neighboring parietal cells. These cells express exclusively VMAT2 (23, 49, 50) and, again unlike neural crest-derived endocrine cells, do not pass through a VMAT2/VMAT1-coexpressing developmental stage. A critical difference in VMAT1 and VMAT2 substrate recognition may provide a clue to the purpose of this restriction: VMAT1, unlike VMAT2, does not efficiently transport histamine (51, 52). In contrast, the primordial VMAT of *Caenorhabditis elegans* does transport histamine (31), suggesting that the VMAT1 isoform may function to restrict paracrine/autocrine cells from scavenging and storing the ubiquitous autacoid histamine. The Merkel cell of the skin expresses histidine decarboxylase (E. Weihe, unpublished observations) yet expresses VMAT1 rather than VMAT2. VMAT1 may be a 'transporter of opportunity' in these cells, similar to the proposed role of VMAT2 in transient thalamocortical serotonergic neurons of the CNS—in this case, functioning to take up and store histamine only when concentrations of the latter are very high, and serotonin or another biogenic amine when they are not.

Recently, two laboratories have reported on the presence of VMAT1 in a population of G cells (gastrin-containing cells) of the stomach and duodenum of the rat (53, 54). Two roles for VMAT1 in these cells have been postulated. The first is that G cells, in addition to the well-characterized ECL cells (see above), store and secrete histamine in the gut (54). The second is that VMAT1 allows these cells to accumulate dietary biogenic amines whose accumulation in secretory vesicles retards the processing of progastrin to the active gastrin peptide (53). Other studies have failed to document specific VMAT1 staining in G cells however (55), so that the presence and role(s) of VMAT1 in G cells remain interesting but open questions.

Significantly, many endocrine tumors of the stomach are derived from either VMAT1-positive EC or VMAT2-positive ECL cells (55; **Fig. 6**). VMAT2 has been proposed as a specific marker for gastric enterochromaffin-like cell tumors, since it is absent from all other endocrine tumors that arise in the stomach and small intestine, including gastrin (G cell), somatostatin, EC, and poorly differentiated endocrine carcinomas (55, 56).

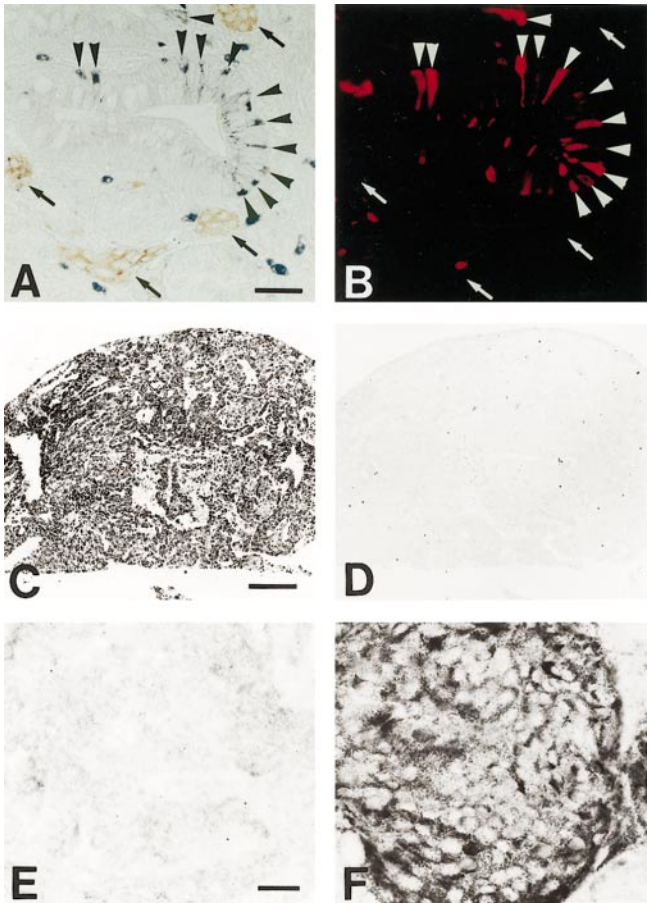

Figure 6. Gastroenteropancreatic expression of VMAT1 and VMAT2. *A)* VMAT1 (black, arrowheads) and VMAT2 (brown, arrows) staining of enterochromaffin (EC) and enterochromaffin-like (ECL) cells, respectively, in duodenum. *B)* 5HT costaining of only the VMAT1-positive (EC, arrowheads) population. *C)* VMAT2-positive and *D)* VMAT1-negative ECLoma; *E)* VMAT2-negative and *F)* VMAT1-positive EComa. From Eissele et al., 1999 (55). Bars: *A, E)* 20 μm; *C)* 200 μm.

VMAT1, VMAT2, and VAChT may all be expressed in human pheochromocytoma, based on the cloning of their cDNAs from pheochromocytoma tissue (30). A systematic examination of pheochromocytoma subtyping based on the relative expression of the three VATs has not yet been assayed. This will be of particular interest in view of the presence of VIP, which is coexpressed with VAChT in most cholinergic peripheral neurons in a subset of human chromaffin cells and pheochromocytomas (57).

VMAT2 is expressed in β cells of the pancreas, as well as in pancreatic tumors (E. Weihe, unpublished observations; **Fig. 7**). Whether VMAT2 is expressed in large dense core vesicles or small vesicles will be of interest to determine, particularly in light of the observations that other markers generally associated with the synaptic vesicle in neurons are present in pancreatic islet neuroendocrine cells (58).

THE INFLAMMATORY SYSTEM

Identification of VMAT isoforms present in basophils, platelet-forming megakaryocytes, and mast cells of the hematopoietic lineage, and antigen-presenting Langerhans cells of human skin, contribute to a better understanding of the pharmacology of amine storage and handling in these cells, which are critical to their function in host defense and inflammatory processes.

Megakaryocytes/platelets

The major role of platelet secretory granule activation in most species appears to be the local release of serotonin. That histamine is a major component of the platelet granule in some species may be due to the fact that VMAT2 is the platelet vesicular transporter (E. Weihe, unpublished results; **Fig. 8**) and has an affinity for histamine that approaches that for serotonin in some species. The species-specific presence of HDC in platelets has not yet been examined.

Basophils

The original cloning of VMAT2 from a rat basophilic leukemia cell expression library focused interest on

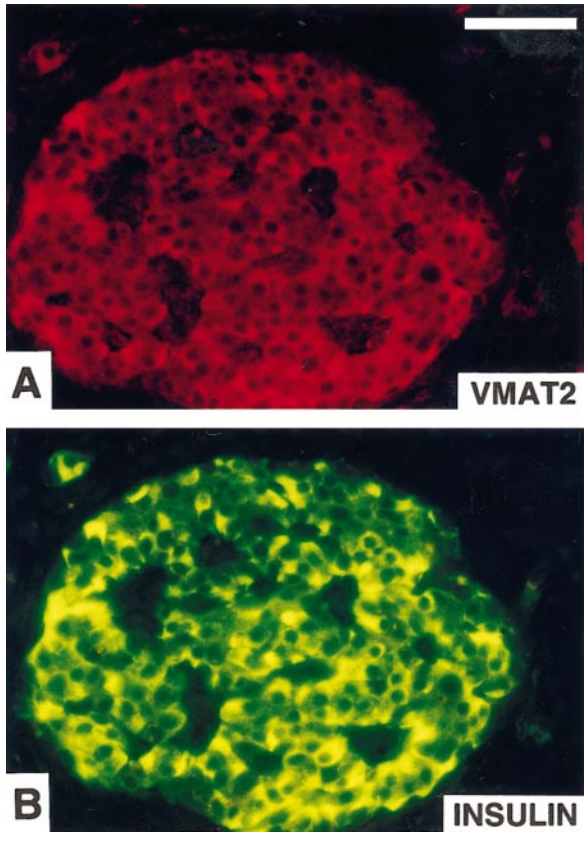

Figure 7. VMAT2 expression in pancreatic β cells. Coexpression of VMAT2 and insulin in human pancreatic islets. Bar: 40 μm.

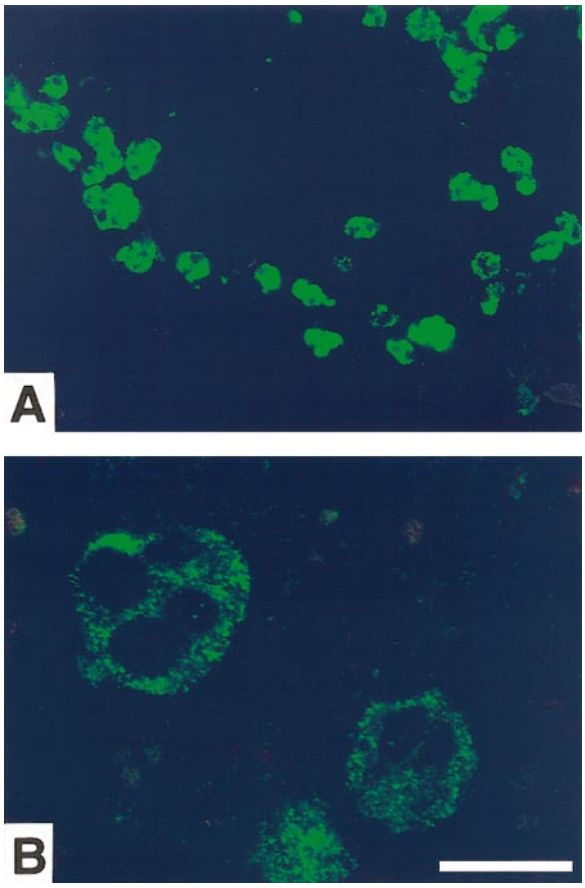

Figure 8. VMAT2 in bone marrow and splenic megakaryocytes. *A)* VMAT2-positive hematopoietic cell colonies in bone marrow of the rat; *B)* VMAT2-positive megakaryocytes in mouse spleen visualized with a rabbit antiserum against the mouse VMAT2 epitope TQNNVQPYPVGDDEESESD. Bar: 20 μm.

VMAT2 expression and function in the basophil lineage (59). Plasma basophils (E. W. and L. E. E., unpublished observations) and tissue mast cells (see below) both express VMAT2, consistent with the apparent generation of both basophils and mast cells from CD34+ pluripotent bone marrow progenitor cells and the storage of histamine, a preferred substrate for VMAT2 compared to VMAT1, in granules of both cell types (60, 61).

Tissue mast cells

VMAT2 is found in histamine-storing and secreting tissue mast cells of brain, tonsils, and skin (**Fig. 9**). Visualization of VMAT2 in mucosal mast cells of the stomach has been difficult compared to other tissues (23). For example, gut tissue mast cells in adventitia and muscle layers are clearly VMAT2 positive as are tonsillar mast cells (Fig. 9). We have previously speculated that VMAT2 down-regulation in stomach mast cells may function to limit mast cell action to histamine release there and to prevent chronic reaccumulation and release of histamine by these cells.

Dendritic cells

As in the basophil, the dendritic cell function of VMAT2 remains unclear, as do the domains of the VMAT2 gene that determine its expression in the hematopoietic cell lineage. It is of interest that pre-B cells express the VMAT2 gene, which is up-regulated along with the histidine decarboxylase gene by increased intracellular calcium (62).

VISUALIZATION OF OTHER VESICULAR NEUROTRANSMITTER TRANSPORTERS IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

Characterization by forward genetics of genes involved in GABAergic function in the worm *C.*

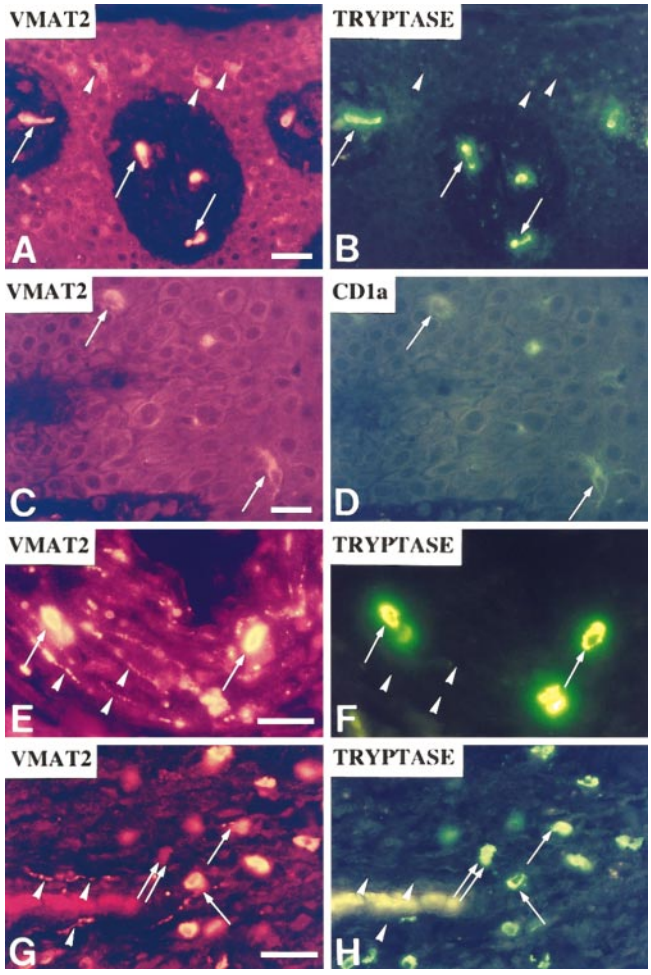

Figure 9. VMAT2 expression in human mast cells and dendritic (Langerhans) cells. Costaining for VMAT2 (*A, C, E, G*), mast cell (tryptase; *B, F, H*), and dendritic cell (CD1A; *D*) markers in skin (*A–D*), duodenum (*E, F*), and tonsil (*G, H*). Arrows mark VMAT2+/tryptase+ mast and dendritic cells; arrowheads mark VMAT2+/tryptase− neuroendocrine cell and neuronal fiber staining. Bars: *A—D, G, H*) 10 μm; *E, F*) 5 μm.

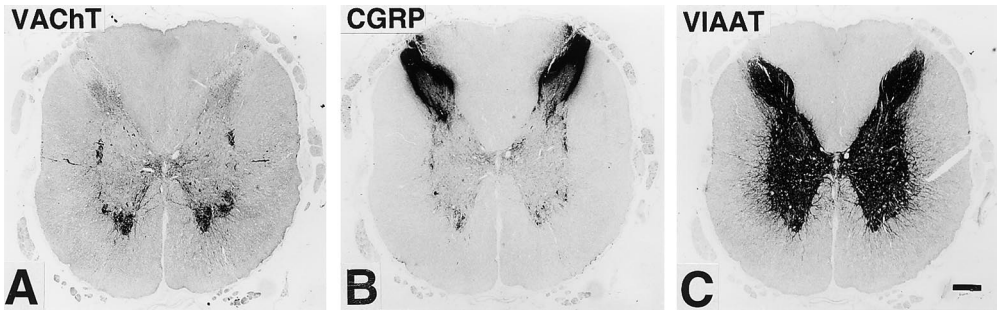

Figure 10. Staining for VIAAT, VAChT, and CGRP in the monkey spinal cord. Monkey thoracic spinal cord stained for VAChT, CGRP, and VIAAT. VIAAT antibody was raised against a carboxyl-terminal peptide from the published rat VIAAT/VGAT sequence and recognizes VIAAT immunoreactivity in all mammalian species examined (E. Weihe and L. E. Eiden, unpublished results). Note abundance of VIAAT (GABA- and glycinergic) compared to peptidergic and cholinergic nerve terminals in spinal cord. Bar: 640 μm.

elegans has resulted in the identification of a putative vesicular gamma-aminobutyric acid (GABA) transporter (VGAT). *C. elegans* VGAT is encoded within the unc-47 gene locus, the location of mutants with impaired GABAergic function at a presynaptic locus accompanied, paradoxically, by increased cellular levels of GABA (63). The functional identity of this protein in the worm was based on three important pieces of circumstantial information: *1)* its occurrence in identified GABAergic neurons, *2)* its mislocalization to cell bodies in mutants deficient in axonal transport of synaptic vesicles to nerve terminals, and *3)* its homology to a family of amino acid permeases in bacteria (64). In the same report, the functional characterization of a mammalian homologue to unc-47 as a GABA transporter was described. Independently, Gasnier and co-workers identified a mammalian homologue of the likely unc-47 cDNA in silico and subsequently characterized the corresponding protein as a mammalian GABA and glycine transporter (65). This protein, perhaps more appropriately called VIAAT (for vesicular inhibitory amino acid transporter), is present in both glycinergic and GABAergic nerve terminals in mammals (66, 67); it is speculated that the higher affinity of VIAAT for GABA compared to glycine explains the preferential accumulation of GABA in neurons that express glutamic acid decarboxylase and therefore synthesize GABA (66). Evidence exists for GABA/glycine cotransmission in the mammalian spinal cord, consistent with VIAAT's dual transporter specificity (67, 68).

The availability of antibodies against each of the major known vesicular neurotransmitter transporters provides the unique opportunity to compare directly the density of each type of chemically defined synapse in a given brain region as well as the synaptic patency for a given chemically coded neuronal projection system in health or disease. **Figure 10** demonstrates cholinergic cell bodies and homotypic synapses in ventral horn motoneurons, preganglionic sympathetic cell bodies in the intermediolateral column, and cholinergic terminal and fibers in the dorsal horn; calcitonin gene-related peptide (CGRP) -positive motoneurons in ventral horn and sensory input from dorsal root ganglion in ventral horn; and GABA/glycinergic synapses in the same area visualized with an antibody raised against a carboxyl-terminal peptide containing sequences common to rat and mouse VGAT (64, 65). The differences in density of cholinergic, peptidergic, and inhibitory (GABA+glycinergic) innervation in the spinal cord is striking: the amount of inhibitory innervation far exceeds that contributed by either the sensory or motor systems in the spinal cord. Thus, comparative staining density and intensity for vesicular transporters is likely in general to be highly indicative of the relative contributions of discrete types of chemically coded neurotransmission at specific neuroanatomical locations. Likewise, changes in the intensity and density of staining for a given vesicular neurotransmitter transporter indicate directly the loss of synaptic patency that can occur early in the course of some neurodegenerative diseases (20, 21, 69–71). The application of antibodies directed against the vesicular GABA/glycine and glutamate transporters (72, 73) is likely to contribute to the understanding of excitatory and inhibitory neurotransmission in the failing brain, with the high degree of anatomical specificity required to unravel mechanisms of neurodegeneration operative in stroke and other human diseases.

CHEMICAL CODING OF AMINE-HANDLING CELLS: FUNCTIONAL AND PHARMACOLOGICAL IMPLICATIONS AND FUTURE PROSPECTS

The ability to visualize the VATs in nervous, endocrine, and inflammatory compartments has contrib-

uted to an expanded view of the development of chemical coding of amine-handling cells in these systems. This expanded horizon includes previously unrecognized patterns of coexpression with other transmitters, with implications for understanding, diagnosing, and treating neuronal and immune disease.

Developmentally, it is clear that the VATs are regulated in many cases independently of the biosynthetic enzymes for any of the major classical neurotransmitters. Insofar as neurons expressing these biosynthetic enzymes do not express the appropriate VAT, the phenotype of neurons thought to be catecholaminergic due to the expression of TH cannot be considered fully functional unless VMAT2 is concomitantly expressed. Neurons expressing VATs and containing no known classical neurotransmitter have orphan status. Potential neurotransmitter candidates for orphan neuronal systems, such as the transient thalamocortical VMAT2+ neurons of the developing brain, include serotonin as a transmitter of opportunity and possibly so-called trace biogenic amines. For example, tyramine can be generated from tyrosine and stored in neurons that contain only aromatic amino acid decarboxylase and VMAT2; octopamine can be generated in such neurons if tyramine β-hydroxylase is expressed. The latter enzyme has been identified in *Drosophila* but not in mammals (74). A classical neurotransmitter may even exist in some neuronal cell groups but remain uncharacterized: for example, a potential histaminergic phenotype for VMAT2+/TH− neurons of the developing parasympathetic nervous system would be conferred by the presence of histidine decarboxylase, a possibility not yet examined in these neurons. Special emphasis should be placed on the prominent 'nonclassical' amine-synthesizing neuronal cell groups now known to exist in the CNS. These include the nucleus tractus solitarius TH+/VMAT2− neuronal cell group, for which the mode of secretion of dopamine (assuming these neurons contain AADC) remains unknown. These neurons are concentrated in a small region of the brain known to be critical for the central regulation of blood pressure, making the identification of their transmitter status of particular importance.

The presence of VMAT2 in unique and previously unsuspected locations, including tonsils and developing brain, provides a potential for imaging with reagents that bind VMAT2 in the clinical contexts of neuroneonatology and inflammatory/immune disease, such as for diagnosis of neurodegeneration of central cholinergic neuronal systems with vesamicol-like reagents (see ref 75).

Finally, the superior visualization of aminergic systems with antibodies directed toward the VATs will enable cotransmitters to be identified, particularly subtypes of amine-handling cells in the brain, endocrine system, and inflammatory axis. Examples already cited include coexpression of the prostaglandin biosynthetic enzyme cyclooxygenase along with VAChT in primate basal forebrain, but not spinal motoneuron projection systems (20). This finding implies cotransmitter specificity between subdivisions of the cholinergic nervous system with functional implications for the treatment of neurodegenerative diseases, such as Alzheimer's disease, that involve cholinergic function. Measurements of synaptic patency in the CNS have also been assayed for the cholinergic system using VAChT staining corrected for synaptophysin as a general synaptic marker (22). A transient population of VMAT2-positive thalamocortical projection neurons has been identified in mammals (26, 28, 29). Cholinergic and monoaminergic innervation of immunocytes in the gut has been documented with VAT immunohistochemistry. These studies mark a new beginning in the *in situ* exploration of plasticity, regulation, and degeneration of specific sets of amine-handling neurons in the brain and periphery, and the function of amine-handling inflammatory and immune cells. FJ

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