The vesicular neurotransmitter transporters: current perspectives and future prospects

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THERE ARE THREE families of vesicular neurotransmitter transporters. The vesicular amine transporters (VATs) are members of the toxin-extruding proton-translocating antiporter family (TEXANs) (1). The vesicular inhibitory amino acid transporter (VIAAT/VGAT) is a member of a second family with limited homology to plant amino acid permeases (2, 3). A vesicular transporter for the excitatory amino acid glutamate (VGlut) has been cloned and functionally characterized (4, 5), and is a member of a separate, third gene family. These three transporter families account for the vesicular transport of catecholamines, serotonin, histamine, acetylcholine, GABA, glycine, and glutamate. The vesicular neurotransmitter transporters (VNTs) are the final arbiters of neurotransmitter entry into the secretory vesicle: they specify the quality and quantity of its content. VNT targeting to specific types of vesicles determines the subcellular locations from which transmitters are mobilized and thus the synaptic dynamics of neurotransmission. Loss of chemical neurotransmission in nematodes deficient in vesicular transmitters and loss of transmitter release in cells cultured from VNT-deficient mice have provided reverse genetic proof of the vesicular hypothesis of neurotransmission (6, 7). Neurodegenerative processes as they occur during human life are being imaged with neurotransmitter transporter ligands (8). The chemical neuroanatomy of the vesicular transporters has revealed novel neuronal phenotypes and demonstrated the existence of amine-handling capability in inflammatory as well as neuroendocrine cells (9). Future prospects include elucidating gene regulatory mechanisms for cell-specific expression of the vesicular transporters; identifying novel proteins required for vesicular transporter targeting; and characterizing discrete classes of neurotransmitter release sites based on vesicular transporter targeting to several types of synaptic vesicle, required for the dynamic range of neurotransmission used by different neuronal subtypes.

Vesicular neurotransmitter transporters are the gatekeepers of the secretory vesicle, responsible for regulated secretion of informational molecules from neurons (i.e., neurotransmitters) and neuroendocrine cells (i.e., hormones, paracrine, or autocrine factors). The history of the vesicular transporter field began with the discovery of neurotransmitter secretory vesicles themselves by Hillarp in 1958 (10), and several reviews have been written on the early development of the field, describing the isolation of catecholamine granules and cholinergic vesicles, development of pharmacological agents for the study of vesicular transport, and the biochemical characterization of the transport process itself (1, 11–18). The cloning of the vesicular amine transporters has revealed much about their involvement in neurotransmission and its regulation. Perhaps most important, vesicular transporters are no longer viewed as static filters for admitting a fixed amount of neurotransmitter into a fixed number of standard-sized vesicles, but as dynamic regulators of where, how, what kind, and how much neurotransmitter is released during synaptic transmission (19). The full extent of how the regulation of vesicular transporter activity fine-tunes neuronal and endocrine informational output is just now being delineated.

Information obtained about the neuroanatomical localization and expression of vesicular transporters is reviewed here by Weihe and Eiden (9). The question of why two monoamine transporters should exist at all in mammals, for example, is answered by the observation that the VMAT1 isoform is restricted to endocrine cells and VMAT2 is the only form expressed in neurons, although VMAT2 is expressed in some neuroendocrine cells, depending on the species examined. A strict neuroendocrine division between VMAT1 and VMAT2 expression exists in the enterochromaffin cells of the gut in all species. Only VMAT1 is expressed in serotonin-accumulating enterochromaffin cells, whereas only VMAT2 is found in enterochromaffin-like histaminocytes of the stomach. The major difference between the two transporters is that VMAT2 efficiently transports histamine and VMAT1 does not. VMAT1 may have evolved during the evolutionary emergence of histamine as a secreted effector molecule, restricting the

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storage of histamine in enterochromaffin cells of the lower gut.

The ability to visualize the vesicular transporters in individual neurons has revealed that several groups of neurons possess some aminergic traits, but lack a complete aminergic phenotype. 'Orphan neurons' include those that possess biosynthetic enzymes for biogenic amines but no vesicular transporters, and the reverse. Will these turn out in the first case to represent neurons with new modes of vesicular secretion and, in the second, neurons that store biogenic amines synthesized by novel biosynthetic pathways?

It has long been known that reserpine-sensitive transporter activity exists in amine-handling cells of the immune/inflammatory system, including histamine-secreting basophils and mast cells and serotonin-secreting platelets. All three cell types express VMAT2, as predicted from the substrate preference for histamine of VMAT2. The presence of VMAT2 in platelets validates the study of amine storage and amine storage defects in these accessible cells as models for amine-handling in central nervous system (CNS) neurons (20). An intriguing aspect of VMAT2 expression in histamine-storing mast cells is that little or no VMAT2 is expressed in mast cells of the lamina propria of the stomach, whereas VMAT2 is easily visualized in mast cells of other tissues. Perhaps VMAT2 is down-regulated in mast cells entering specific tissue milieux, to allow acute histamine release but prevent chronic histamine secretion.

Transport assays in cells into which wild-type and mutant transporter cDNAs are admitted and expressed have accelerated the study of transporter function at the molecular level. Insights have been gained into the evolution of the vesicular transporters as a diverse set of proteins that all exploit the presence of vacuolar ATPase, and the relative proton impermeability of the storage vesicle, to drive transport of positively charged, zwitterionic, and negatively charged species into a positively charged environment. The driving force for transport is generated by the proton gradient in all cases, but dominated by proton loss *per se* (chemical gradient) for positively charges species, by both proton loss and charge dissipation (electrochemical) for zwitterions, and by dissipation of the electrical and loss of the chemical gradient for negatively charged species. Here, Stan Parsons analyzes the bioenergetics and structure–activity relationships for the vesicular amine transporters, focusing mainly on VAChT and the common mechanisms of transport shared by VAChT and VMATs (21). Experimental verification of the exchange of two protons for one amine for both VAChT and VMAT transport has emphasized the experimental validity of using information from the study of both VAChT and the VMATs to develop a common mechanistic explanation of VAT transport of amines. Understanding the molecular pathway that protons must take to exit the vesicle through the vesicular transporter should reveal the essential commonalities among all proton-coupled transporters, along with the unique properties of VATs, VIAATs, and VGlut that are potentially exploitable pharmacologically.

The current status of how the transport machinery is mobilized to subpopulations of secretory vesicles is reviewed here by Erickson and colleagues (22). Key issues in this area include the trafficking of vesicular transporters to their final destinations on different classes of secretory vesicles. The original observation made in PC12 cells that VAChT may be transported to small synaptic vesicles via large dense-core vesicles (LDCVs) (23) has received additional support from studies that indicate VAChT phosphorylation may reversibly direct it either to LDCVs or SSVs (24). What remains to be seen is whether the LDCV is always a trafficking pathway for VAChT and if, in some neurons, it is VAChT's final destination. Resolution of this issue not only in PC12 cells, but also in actual neurons, will have major implications for understanding the cell biology of chemically coded neurotransmission.

Rand and colleagues review in this issue the neurogenetics of transporter function in *Caenorhabditis elegans* (25). Historically, worm genetics has played a prominent role in the study of vesicular neurotransmitter transporters. The strong homology of unc-17 to mammalian VMAT, along with other evidence, allowed Alfonso et al. to posit that the protein encoded by unc-17 was a vesicular acetycholine transporter even without direct evidence of transport activity (6). The unc-17 cDNA in turn provided a probe for mammalian VAChTs that could be directly assayed for acetylcholine transporter activity (26). The discovery of unc-17 as part of a primary transcript also generating choline acetyltransferase by alternative splicing in the worm (27), in turn led to the discovery of the cholinergic gene locus in mammals (28).

C. elegans is the first complex metazoan organism with a fully defined genome (25). In *C. elegans*, 'saturation' genomics predicts the total number of transporters and transporter-related gene-encoded proteins in the genome and allows functional evaluation of each. Thus, the presence of a single VMAT in the *C. elegans* genome indicates that VMAT1 and VMAT2 likely emerged as vesicular monoamine transporter variants after duplication of a single ancestral gene. Chalfie and Jorgensen have pointed out that the worm nervous system is ideally suited to both forward and reverse genetic analysis of neuronal function, because even severe neuronal dysfunction does not cause developmental lethality in this

organism (29). Consequently, testing of the nonvesicular hypothesis of neurotransmission can be carried out in whole organisms completely lacking a given vesicular neurotransmitter transporter. Worm mutants in which either VAChT or VIAAT/VGAT are nonfunctional or absent exhibit a complete loss of cholinergic or GABAergic function, respectively, even though cytoplasmic levels of ACh or GABA are in fact elevated rather than reduced (6, 30). The same is true for dopaminergic function in VMAT-deficient (cat-1) worms (31). Thus, the nonvesicular secretion of acetylcholine through the mediatophore (a subunit of the vacuolar ATPase that powers vesicular uptake of all known neurotransmitters), once proposed as a potential alternative to secretion by exocytosis of acetylcholine from synaptic vesicles, appears to be without physiological relevance (32). However, nonvesicular secretion of other neurotransmitters, as proposed for GABA via reversal of the plasma membrane GABA transporter, may well occur (33). Undoubtedly, knockout or conditional knockout models for the vesicular GABA transporter will be useful in further testing of nonexocyotic models for neurotransmission. The issue of 'orphan' neurons, in this case expressing VMAT but not dopamine or serotonin, is again raised in *C. elegans* as in mammals. These neurons may be histaminergic, octopaminergic, or they may store and secrete a novel neurotransmitter, perhaps synthesized by one of the four amino acid decarboxylases of *C. elegans* with an as yet unassigned biosynthetic function.

In contrast to the cat-1 mutation in *C. elegans*, the VMAT2 null mutation is lethal in mice soon after birth, and thus adult nulls cannot be studied (7, 34, 35). The absence of biogenic amine storage in the CNS of neonatal VMAT2-deficient mice, however, confirms that VMAT2 is the only functional biogenic amine vesicular transporter in the mammalian CNS (36) and that vesicular transporter activity is absolutely required for storage of significant amounts of biogenic amines, even when their synthesis is completely unimpaired. VMAT2 heterozygous mice do exhibit haploinsufficiency at the VMAT2 gene. Remarkably, mice with a single VMAT2 gene express about half the normal amount of VMAT2 protein, and nervous tissue contains about half the normal amount of serotonin, dopamine, and norepinephrine (7, 34, 35). Uhl and colleagues review here the VMAT2 heterozygous knockout haploinsufficiency syndrome in mice, and its implications for VMAT2 as a potential target in drug-seeking behavior and neuropsychiatric disease in humans (37). Monoamine oxidase inhibition increases serotonin and norepinephrine, but not dopamine levels, while amphetamine causes significant dopamine release in neurons cultured from VMAT2 null mice and rescues VMAT2-deficient mice for several days after birth (7). These observations have direct relevance to the neuropharmacology of these agents and the potential etiologies of the human diseases that they effectively treat (7).

Amine transport by the VATs can be blocked by particularly potent heterocyclic organic inhibitors: vesamicol for VAChT and tetrabenazine and reserpine for VMATs. These ligands also bind the VATs with high affinity when administered *in vivo*, and thus provide a way to image the nerve terminal and study synaptic patency in human brain in neurodegenerative and other diseases. Simon Efange reviews the development of vesamicol- and tetrabenazine-based radioligands for the visualization of VAChT and VMAT2, respectively, in primate and human brain (38). The use of these agents in the *in vivo* imaging of human brain during neurodegenerative and psychiatric illness, and after traumatic brain injury, is potentially enormous. Frey and colleagues, for example, have demonstrated in mild Parkinson's disease (PD) that tetrabenazine binding is greatly decreased in posterior but not yet in anterior putamen as in later stages of PD, suggesting an anterograde progression of dopaminergic neuronal destruction in this disease (8). Efange reviews the critical aspects of ligand development for *in vivo* studies that allow mechanistic conclusions to be drawn from these somewhat empirical imaging studies. Fully interpretable imaging studies, in turn, allow a host of interesting and important questions about human neurodegenerative disease to be raised. These include asking what neurodegeneration really consists in (e.g., loss of neurons, loss of neurotransmitter synthetic capacity, loss of storage capacity, dysregulation of targeting or synapse formation with neuronal death secondary to synapse loss, etc.). Imaging allows these questions to be addressed in early stages of the illness, and moves neuropathological assessment from attempts to deduce disease mechanism from autopsy findings alone, to clinical diagnosis and monitoring of disease in a dynamic way.

CONCLUDING REMARKS

It is appropriate to conclude this overview of the reviews of the vesicular neurotransmitter transporters gathered together here in this special issue of *The FASEB Journal* with a summary of important unresolved issues and future prospects in this rapidly progressing field.

First, the vesicular neurotransmitter transporters are now recognized as providing a critical avenue of experimental access to the study of the cell biology of vesicular function that focuses on the environmental content of secretory vesicles. The analysis of SNARE

proteins and their function in guiding synaptic and secretory vesicles to the nerve terminal and positioning them for exocytotic release is recognized as a mature area of research rapidly nearing completion (39). Studying the molecular mechanisms for vesicular neurotransmitter transporter trafficking now promises to provide the tools required for identification of the additional set of proteins that sorts the VNTs to their final destinations, so that they can determine the informational content of individual types of synaptic vesicles, much as the SNARE proteins and their interactors determine where the vesicles themselves end up in each type of chemically coded neuron or neuroendocrine cell.

Second, the regulation of VNT abundance, location, and activity by transcriptional and posttranslational mechanisms and by interaction with other vesicle-associated proteins is now appreciated to play a major role in determining vesicular quantal size, the 'currency' of synaptic neurotransmission.

Third, the generality of proton antiport as the engine that drives vesicular uptake of neurotransmitters is now fully appreciated through mechanistic studies of the type pioneered by Parsons and co-workers. Although proton exchange powers amine uptake, however, it may be merely permissive for the uptake of glutamate and aspartate by providing charge neutralization for each substrate molecule transported. Thus, although the universal driver for neurotransmitter uptake into vesicles is the proton gradient generated by the vesicle's vacuolar ATPase, proton antiport through the transporter itself may be unique to the amine transporters compared to other subfamilies of vesicular transporters. Several other vesicular transporters remain to be characterized besides the excitatory amino acid carriers. These include the carrier for vesicular ATP and the carrier for intravesicular calcium. SV2, which is strikingly homologous to the VATs, is a potential vesicular calcium transporter (40–42). If so, it would be predicted to be itself a proton antiporter since it carries a net positively charged cation into the vesicle. For each of these transporters, drugs that modulate VNT activity, and thus increase or decrease quantal size and the efficacy of neurotransmission, can be envisioned.

Fourth, the study of VNT expression during development and in the mature nervous system have provided important insight into the regulation of genomic neuronal regulatory units and how they are mobilized to provide functional phenotypes for the chemical coding of neurotransmission underlying the complex ionotropic and metabotropic intercellular communication in which nervous system function wholly consists. The most obvious example is the cholinergic gene locus, the first known neuronal operon. Here, cholinergic chemical coding is ensured by coregulation of the transcripts for VAChT and ChAT from the same gene locus (43). The 'regulons' that are transcriptionally activated to produce serotonergic, GABAergic, glycinergic, dopaminergic, and other chemically coded neurons must also involve differential regulation of VNT genes in various neuronal populations. The recent discovery that the transcriptional activator unc-30 regulates the genes encoding glutamic acid decarboxylase and the vesicular GABA transporter in *C. elegans* is an important example, and the regulation of dopamine-β-hydroxylase and other components of the noradrenergic phenotype by Phox2a is another (44, 45). However, transcriptional regulation of the VNTs that are shared by multiple chemically coded phenotypes such as VMAT2 will be particularly interesting to examine. Their regulation in serotonergic vs. noradrenergic neurons, for example, will reveal important facets of the transcriptional regulator cascades operating during the establishment of chemical coding in the nervous system. It is noteworthy that at present, the VGlut is the only known protein marker associated with the excitatory amino acid neuronal phenotype. Understanding the regulation of the VGlut gene will provide an important avenue into the identification of transcriptional activators that determine the chemical phenotype of glutamatergic neurons, one of the most ubiquitous chemically coded phenotypes in the nervous system.

Finally, the ability to image vesicular transporters, if extended to those for inhibitory and excitatory neurons, will make the synaptic organization of the brain, and its derangement in nervous system dysfunction transparent to clinicians at stages of disease amenable to actual prevention of neurodegeneration, rather than repair or limiting of overt damage. FJ

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