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Vesicular Neurotransmitter Transporters

Potential Sites for the Regulation of Synaptic Function

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Abstract

Neurotransmission depends on the regulated release of chemical transmitter molecules. This requires the packaging of these substances into the specialized secretory vesicles of neurons and neuroendocrine cells, a process mediated by specific vesicular transporters. The family of genes encoding the vesicular transporters for biogenic amines and acetylcholine have recently been cloned. Direct comparison of their transport characteristics and pharmacology provides information about vesicular transport bioenergetics, substrate feature recognition by each transporter, and the role of vesicular amine storage in the mechanism of action of psychopharmacologic and neurotoxic agents. Regulation of vesicular transport activity may affect levels of neurotransmitter available for neurosecretion and be an important site for the regulation of synaptic function. Gene knockout studies have determined vesicular transport function is critical for survival and have enabled further evaluation of the role of vesicular neurotransmitter transporters in behavior and neurotoxicity. Molecular analysis is beginning to reveal the sites involved in vesicular transporter function and the sites that determine substrate specificity. In addition, the molecular basis for the selective targeting of these transporters to specific vesicle populations and the biogenesis of monoaminergic and cholinergic synaptic vesicles are areas of research that are currently being explored. This information provides new insights into the pharmacology and physiology of biogenic amine and acetylcholine vesicular storage in cardiovascular, endocrine, and central nervous system function and has important implications for neurodegenerative disease.

Index Entries: VMAT1; VMAT2; VAChT; vesicular monoamine transporter; vesicular acetylcholine transporter; cholinergic gene locus; gene knockout; large dense core vesicles; small synaptic vesicles; targeting; neurotoxicity; neurodegeneration; Parkinson's disease; Alzheimer's disease.

Introduction

The neurotransmitters used by neurons and endocrine cells for intercellular communication are contained in secretory vesicles. A unique group of proteins found on secretory vesicles are the transporters required for selective accumulation of transmitters from the

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cytoplasm into these vesicles. At least four different vesicular transporter types have been identified biochemically that are specific for uptake of the classical neurotransmitters: biogenic amines (serotonin [5HT], dopamine, norepinephrine, epinephrine, and histamine), acetylcholine (ACh), glutamic acid, and γ-aminobutyric acid (GABA)/glycine (Njus et al., 1986; Kanner and Schuldiner, 1987; Johnson, 1988; Maycox et al., 1990; Parsons et al., 1993). Together with the enzymes required for the biosynthesis of these neurotransmitters, the vesicular transporters determine the content of neurotransmitter released via exocytosis of the vesicles following cell depolarization (Jahn and Südhof, 1993; Kelly, 1993). Regulation of vesicular transporters and vesicular storage of neurotransmitters therefore play fundamental roles in endocrine and neuronal function. Within the last few years, two types of vesicular neurotransmitter transporters, the vesicular monoamine and ACh transporters (two VMAT isoforms and VAChT) have been cloned and found to comprise a new gene family. Understanding the molecular basis for the cell-specific expression, specific intracellular targeting, bioenergetic properties, and neuropharmacology of the vesicular neurotransmitter transporters will provide insights into the specialized pharmacology, physiology and cell biology of the monoaminergic and cholinergic cell types formed in the mammalian nervous and endocrine systems.

Vesicular Monoamine Transporters (VMAT1 and VMAT2)

In 1992, two groups identified two isoforms of the vesicular monoamine transporter, now designated VMAT1 and VMAT2, by expression cloning. The cDNA screening strategies relied on passive and active uptake processes in intact fibroblastic cells: diffusion of a quaternary amine (1-methyl-4-phenylpyridinium, MPP^+) or neutral amine (^{3}H-5HT) across the plasma membrane followed by sequestration of the charged species into an intracellular compartment (Erickson et al., 1992; Liu et al., 1992a). Despite the lack of synaptic vesicles in these nonneuroendocrine cells, the presence of intracellular organelles, such as endosomes, bearing the ubiquitous vacuolar-type H^+-ATPase (Rudnick, 1986) was sufficient to provide the minimal cellular requirements for reconstitution of VMAT activity and isolation of individual cDNA clones. The direct measurement of ATP-dependent vesicular transport activity was then performed in isolated intracellular organelles (Liu et al., 1992a) or in cultured cells in which the plasma membrane was selectively permeabilized with digitonin in an intracellular-type medium (Erickson et al., 1992). These assays now provide convenient in vitro or in situ systems for expression and structural analysis of vesicle transporter cDNAs. The intracellular expression, ionic requirements, and sensitivity of transport to reserpine distinguishes these vesicular transporters from the high-affinity reuptake transporters present on the plasma membrane (Amara and Kuhar, 1992).

Differential Expression of the VMAT Genes

Two separate genes encode VMAT1 and VMAT2, and their chromosomal localizations in humans are 8p21 (Peter et al., 1993) and 10q26.1 (Erickson and Eiden, 1993; Peter et al., 1993; Surratt et al., 1993), respectively. Previously, it had been assumed that all biogenic amines used the same vesicular transporter with the type of neurotransmitter found in monoaminergic cells governed by expression of the specific biosynthetic enzymes and the appropriate and specific plasma membrane reuptake transporter. The existence of two genes for the VMAT isoforms suggests that they are differentially expressed in various cell types and impart functionally important differences in vesicular amine storage to these cells. VMAT1 mRNA is found in the adrenal gland and is absent from the brain (Liu et al., 1992a), whereas VMAT2 mRNA is expressed in noradrenergic, dopaminergic, and serotonergic cell bodies in the brain stem (Erickson et al., 1992;

Liu et al., 1992a). Immunohistochemical studies with isoform-specific polyclonal antipeptide antibodies have further shown that VMAT1 is expressed in monoaminergic endocrine and paracrine cells, and not in neurons, whereas VMAT2 is expressed predominantly in monoaminergic neurons of the central (CNS), peripheral and enteric nervous systems of rat and human (Weihe et al., 1994; Erickson et al., 1995, 1996a; Peter et al., 1995).

VMAT2 mRNA and immunoreactivity are also abundantly found in the histamine-containing enterochromaffin-like cells of the oxyntic mucosa of the stomach (Erickson et al., 1992, 1995, 1996a; Weihe et al., 1994; Peter et al., 1995; De Giorgio et al., 1996). Histamine is released from the stomach during vagal stimulation and is involved in the regulation of parietal acid secretion and normal gastrosecretory function. The expression of VMAT2 on secretory vesicles of the rat gastric corpus is regulated by factors that modulate histamine biosynthesis (Dimaline and Struthers, 1996). Conditions that produce achlorhydria, such as inhibition of H^+/K^+ ATPase with omeprazole, upregulate the expression of VMAT2 to accomodate the increased histamine biosynthesis and secretion. Further along the digestive tract, VMAT1 immunoreactivity alone is found in the enterochromaffin cells. VMAT1 and VMAT2 are therefore powerful tools for differential diagnosis of gastrointestinal carcinoids (Erickson et al., 1996a; Kölby et al., 1997).

In the rat adrenal medulla, VMAT2 is expressed in a subpopulation (approx 10%) of chromaffin cells, which may be noradrenergic, and in ganglion cells (Mahata et al., 1993; Schäfer et al., 1996). In bovine and primates, however, both VMAT1 and VMAT2 are expressed in all chromaffin cells of the adrenal gland, which may reflect subtle environmental differences in adrenomedullary ontogenesis in various species (Erickson et al., 1996a; Henry et al., 1997). Prolonged K^+ stimulation of cultured bovine chromaffin cells selectively increases the transcription of the VMAT2 gene (Krejci et al., 1994), in addition to inducing tyrosine hydroxylase and dopamine β hydroxylase genes (Thoenen et al., 1969; Patrick and Kirshner, 1971; Stachowiak et al., 1990). This treatment may trigger a shift from an endocrine to a more neuronal phenotype (Unsicker et al., 1983). Thus, the coordinate increased expression of genes controlling the biosynthesis and vesicular packaging of norepinephrine in chromaffin cells of the bovine adrenal medulla is regulated by transsynaptic induction (Thoenen, 1974; Sietzen et al., 1987; Desnos et al., 1992, 1995; Krejci et al., 1994).

Transport of Monoamines by VMAT1 and VMAT2

The characteristics of vesicular monoamine transport by VMAT1 and VMAT2 from rat, bovine, and human in permeabilized cells or in membrane fractions from transfected fibroblasts have been described in detail (Erickson et al., 1992, 1996a; Liu et al., 1992a; Erickson and Eiden, 1993; Gasnier et al., 1994; Howell et al., 1994; Krejci et al., 1994; Peter et al., 1994; Weihe et al., 1994). Uptake is energy-dependent and is abolished by the proton-translocating ionophore carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) or by the vacuolar H^+-ATPase inhibitors tri(*n*-butyl)tin and bafilomycin. The transport process is saturable, with apparent affinities (K_m) of VMAT1 and VMAT2 for 5HT and the catecholamines in the low micromolar range. Reserpine and ketanserin are potent inhibitors of vesicular monoamine transport, with the apparent affinity (n*M* range) of VMAT2 for both compounds being approx threefold higher than VMAT1. The relative affinities of both VMAT isoforms for the biogenic amines display the following rank order: 5HT>dopamine>epinephrine>norepineprine>histamine. Although the transporters exhibit similar affinity for 5HT (approx twofold difference), VMAT2 shows a slightly higher affinity (approx three- to fourfold) for catecholamine substrates than does VMAT1. In addition, VMAT2 has a higher intrinsic transport rate (40/min) compared to VMAT1 (10/min), perhaps an important feature for neuronal vs endocrine secretion of monoamines.

The cytosolic concentration of amines in monoaminergic neurons and endocrine cells is

estimated to be approx 10–20 μ*M* (Phillips, 1982; Silva and Bunney, 1988), somewhat above the K_m for vesicular transport. Thus, the activities of the VMAT isoforms in chromaffin cells and in monoaminergic neurons may be near saturation in vivo and operating at their maximal rates. In the rat brain, modest increases in both cytoplasmic and vesicular dopamine and norepinephrine concentrations are observed following inhibition of monoamine oxidase A (MAO-A) by clorgyline or pargyline treatments (Buu, 1989). This elevation of cytoplasmic levels is sufficient to prevent depletion of vesicular stores when reserpine is subsequently administered. MAO-A plays a prominent role in the regulation of cytoplasmic monoamine levels, in addition to the biosynthetic enzymes, and has been a therapeutic site to enhance central monoaminergic function in clinical depression (Costa and Sandler, 1972; Mandel and Klerman, 1978). In the adrenal medulla, an increase in VMAT2 content has been observed following prolonged K^+ depolarization and could be an adapative process that occurs to allow a more rapid filling of the granules in continuously secreting cells (Sietzen et al., 1987; Desnos et al., 1992, 1995; Krejci et al., 1994). Thus, vesicular monoamine storage in both neurons and endocrine cells appears to be a potential site for regulation.

The maturation time of monoaminergic vesicles, in terms of the time required to reach steady-state vesicular monoamine accumulation, is highly variable, being approx 2–4 min in the brain, 30–60 min in the sympathetic nervous system, and 30–60 h in the adrenal medulla (Scherman and Boschi, 1988). The reasons for these differences may relate to the presence of different classes of regulated secretory organelles expressing VMAT1 or VMAT2 in endocrine vs neuronal cells. These differences in transport characteristics suggest that recycling and reuse of vesicles after exocytosis play a more important physiological role in central synapses than at the periphery or in endocrine secretion.

Differential Pharmacology of VMAT1 and VMAT2

Inhibition of vesicular monoamine transport by tetrabenazine is a key feature distinguishing VMAT1 from VMAT2 (Erickson and Eiden, 1993, 1996a; Peter et al., 1994). Tetrabenazine inhibits uptake by VMAT2 with a K_i of approx 50 n*M*, whereas concentrations of tetrabenazine as high as 20 μ*M* do not affect transport mediated by human VMAT1. Furthermore, only VMAT2 shows significant binding of dihydrotetrabenazine, a useful imaging marker in human neurologic disorders (Henry and Scherman, 1989; Lehericy et al., 1994; Kuhl et al., 1996; Wilson et al., 1996). Species differences exist, however, in the sensitivity of VMAT1 to tetrabenazine. Whereas human VMAT1 is unaffected by tetrabenazine (Erickson et al., 1996a), a 100-fold difference in sensitivity exists between the rat isoforms (Erickson and Eiden, 1993; Peter et al., 1994; Weihe et al., 1994) and only a 10-fold difference in sensitivity between bovine VMAT1 and VMAT2 is observed (Henry et al., 1997). Tetrabenazine preferentially depletes vesicular monoamine stores in the CNS, compared to the adrenal gland, and has been used successfully to treat various movement disorders in humans (Asher and Aminoff, 1981).

The interaction of histamine with the transporters is a second key feature distinguishing VMAT1 from VMAT2 (Erickson and Eiden, 1993; Peter et al., 1994; Erickson et al., 1996a). In fact, VMAT1 displays a decreased apparent affinity for all unsubstituted aromatic amines tested so far, including histamine, phenylethylamine, amphetamine, and MPP^+, which lack strong electron-donating substituents on the aromatic ring (Fig. 1). Histamine, however, is a relatively poor substrate for VMAT2, exhibiting a K_m for transport approx 200-fold higher than what is observed for 5HT and the catecholamines (Erickson et al., 1995; Merickel and Edwards, 1995). In rat basophilic leukemia cells (RBL-2H3), from which VMAT2 was originally cloned, VMAT2 also has a low affinity ($K_m \approx 200$ μ*M*) for histamine relative to the other monoamines (Kanner and Bendahan, 1985). Thus, VMAT2's affinity for histamine and the expression of VMAT2 in neurons and endocrine cells that store and secrete histamine indicate that VMAT2 is the histamine vesicular transporter in these cells (Erickson et al., 1995; Peter et al., 1995).

Role of VMAT2 in Neurodegeneration

Indirectly acting sympathomimetic amines, such as amphetamine and its substituted derivatives, methylenedioxymethamphetamine (MDMA) and fenfluramine, are known to be potent amine-releasing agents (Weiner, 1985) and particularly neurotoxic to monoaminergic neurons of the CNS in rodents and primates (Ricaurte et al., 1982, 1985, 1988; Kleven et al., 1988). The specificity of the amphetamine compounds relies on the plasma membrane reuptake transporters, which selectively accumulate them into dopaminergic or serotonergic nerve terminals. Once inside the cell, these substrates compete with monoamines for vesicular sequestration and ultimately disrupt vesicular monoamine storage and H^+ electrochemical gradients (Disbrow et al., 1983; Knepper et al., 1988; Sulzer and Rayport, 1990; Rudnick and Wall, 1992). The potency of these compounds (μM range) and in particular, the stereospecificity of the action of amphetamine, support a direct interaction with VMAT2 in vivo (Fig. 1). Although the affinity of VMAT2 for phenylethylamine is similar to that of the amphetamine, phenylethylamine does not produce the same behavioral and neurotoxic effects. This may be explained by the presence of an α-methyl substituent on amphetamine and its substituted derivatives that prevents degradation by monoamine oxidases (Axelrod, 1971). The amphetamines therefore persist in the nerve terminal and are more likely to release monoamines from vesicular storage sites and exhaust metabolic energy in futile maintenance of storage vesicle proton gradients. The resulting increased cytoplasmic concentration of dopamine has two major fates. It can be released from the nerve ending by reversal of the plasma membrane transporter, contributing to the increased locomotor effects and behavioral reward properties of amphetamines (Schuldiner et al., 1993a). In addition, it can be metabolized by monoamine oxidases to generate toxic products, such as hydrogen peroxide, oxygen-derived radicals, semiquinones, and quinones or undergo autoxidation, which

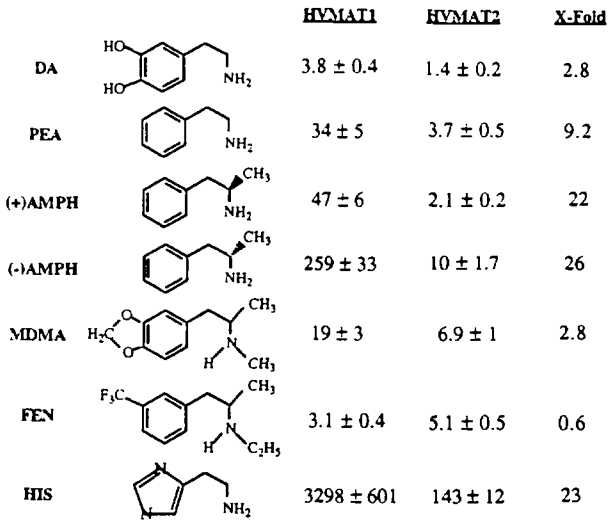

Fig. 1. Pharmacological sensitivity of hVMAT1 and hVMAT2 to substituted and unsubstituted aromatic amines. The VMAT isoforms display a two- to threefold difference in their affinities for dopamine (DA), methylenedioxymethamphetamine (MDMA), and fenfluramine (FEN). VMAT1 displays greatly reduced affinity for all substrates that lack electron-donating substituents on their aromatic ring, compared to VMAT2. The affinity of VMAT2 for histamine (HIS) is considerably reduced compared to amphetamine (AMPH) and phenylethylamine (PEA). K_i values (μM) were determined by nonlinear regression. X-fold values represent the relative higher affinity of the substrates for hVMAT2. Reproduced from Erickson et al. (1996a).

may directly induce intracellular oxidative stress and neurotoxicity (Cubells et al., 1994; Ben-Shachar et al., 1995; Seiden and Sabol, 1996). Thus, the intraneuronal actions of the amphetamines, both psychopharmacologic and toxic, include the specific interaction of these drugs with VMAT2.

Limited homology of the vesicular amine transporter genes with the toxin extruding antiporters of bacteria (TEXANS) have suggested a role for these proteins in the human susceptibility to neurodegenerative diseases, such as idiopathic Parkinson's disease (Schuldiner et al., 1995). These transporters are all reserpine-sensitive pumps that act to reduce the cytoplasmic concentrations of potentially toxic amines (Yelin and Schuldiner, 1995). Parkinson's disease is associated with an increase in oxidative stress

within dopaminergic synapses, which may be caused, in part, by the actions of dopamine itself (Fahn and Cohen, 1992; Jenner and Olanow, 1996).

The selective neurotoxicity associated with MPP^+ has become a useful model for Parkinson's disease (Davis et al., 1979; Langston et al., 1983). It has been proposed that secretory organelles in neurons and neuroendocrine cells provide protection following exposure to this neurotoxin by sequestering cytoplasmic MPP^+, thus preventing mitochondrial damage and cell death (Ramsay and Singer, 1986; Reinhard et al., 1988). The ability of VMAT1 to provide a reserpine-sensitive mechanism of resistance to MPP^+ poisoning in PC12 cells has now clearly been established (Liu et al., 1992b). As observed for the unsubstituted aromatic primary amines, VMAT2 shows a higher apparent affinity for MPP^+ than VMAT1 (Peter et al., 1994; Erickson et al., 1996a). Yet, paradoxically, the dopaminergic projection neurons of the substantia nigra are particularly vulnerable to the neurotoxic effects of MPP^+ despite the high level of expression of VMAT2 (Davis et al., 1979; Langston et al., 1983; Erickson et al., 1996a). This may be because high-affinity plasma membrane catecholamine transporters, required for the neuronal uptake of MPP^+, are more abundant in the brain than in chromaffin cells of the adrenal medulla or in PC12 cells, resulting in higher levels of cytoplasmic MPP^+ in central dopamine- and norepinephrine-containing neurons (Javitch et al., 1985). Furthermore, the limited vesicular storage capacity of dopaminergic synaptic vesicles, compared with chromaffin granules and noradrenergic dense cored granules, (McMillen et al., 1980; Takimoto et al., 1983) may be insufficient to clear the cytoplasm of MPP^+ in human dopaminergic neurons and therefore, render them more susceptible to neurotoxin damage.

The cellular expression of both the plasma membrane dopamine transporter and VMAT2 mRNA is significantly reduced in sections of the human postmortem substantia nigra in Parkinson's disease tissue compared to controls (Harrington et al., 1996). Much of the reduced expression is caused by a marked loss of dopamine-containing cells, but a reduction in the expression of both gene transcripts per cell in the surviving pigmented neurons is observed as well. The reduction in mRNA may however, reflect cell damage rather than specific compensatory downregulation resulting from neuronal dysfunction.

VMAT2 in Psychiatric Disorders

Presynaptic hypofunction at central monoaminergic synapses have been postulated for human depressive illness, based on pharmacological studies using reserpine (Frize, 1954; Praag, 1978). Hyperdopaminergic function is associated with schizophrenia (Meltzer and Stahl, 1976). Sequence analysis from patients with affective disorder and schizophrenia, however, have not indicated that a defect in the neuronal VMAT2 isoform exists (Lesch et al., 1994; Percisco et al., 1996). Possible differences in the level of VMAT2 expression and vesicular monoamine storage in affected central monoaminergic neurons has not been examined.

VMAT2 Gene Knockouts

Gene knockout experiments, performed in worms and mice, have enabled further evaluation of the role of VMAT2 in monoamine-specific behavior and neurotoxicity. Two decades ago, Brenner and colleagues characterized the phenotype of *cat*-1 mutants in *Caenorhabditis elegans*, which have defects in the formaldehyde-induced fluorescence and lack dopamine in the nerve endings (Sulston et al., 1975). Worms that lack the CAT-1 protein do not die but display defects in thermoregulation, fertility, locomotion, and in their ability to seek food. Although the behavioral phenotype is consistent with a reduced monoamine secretion, the secretory vesicles in the *cat*-1 mutant are normal in appearance and distribution. Rand and colleagues have cloned the cDNA corresponding to the *cat*-1 mutant and found it to be a VMAT, based on its homology to verte-

brate VMATs, association with synaptic vesicles of known biogenic amine-containing neurons, demonstration of reserpine- and tetrabenazine-sensitive transport of 5HT in a reconstituted *in situ* system, and phenotypic rescue by transgenic expression of mammalian VMAT1 or VMAT2 (Duerr et al., manuscript in preparation).

Uhl and colleagues recently generated VMAT2 gene knockout mice. Whereas homozygotes are very short lived because they do not feed, heterozygotes are viable into adulthood and can be studied. They express VMAT2 levels that are approximately half of wild-type, and their weight gain, fertility, habituation, passive avoidance, and locomotor activities are similar to wild-type littermates. Heart rate and blood pressure are somewhat reduced compared to wild-type. Interestingly, in these heterozygotes, amphetamine produces the typical enhanced locomotor response as observed with wild-type, but diminished behavioral reward. In addition, administration of the MPP^+ precursor MPTP to heterozygotes produces more than twice the dopamine cell losses found in wild-type mice. These results confirm a major role for VMAT2 in the actions of monoaminergic psychostimulants and neurotoxins. Furthermore, these studies suggest that monoaminergic synaptic vesicles contribute more to amphetamine-conditioned reward than to amphetamine-induced locomotion and therefore play a central role in drug abuse (Takahashi et al., 1997).

Vesicular Acetylcholine Transporter (VAChT)

In 1993, Rand and colleagues cloned the *unc-17* gene from the nematode *C. elegans*, and discovered that it codes for a protein having approx 40% amino acid sequence identity with the VMATs and a similar predicted structure (Alfonso et al., 1993). Based on this striking homology, its expression on synaptic vesicles in cholinergic nerve endings, and the observation of behavioral defects that were consistent with impaired cholinergic neurotransmission in *unc*-mutants, they proposed that *unc-17* encodes a vesicular acetylcholine transporter (VAChT). The model system for the study of VAChT is the cholinergic synaptic vesicle purified from the electric organ of the marine ray *Torpedo* (reviewed in Parsons et al., 1993). The electric lobe, containing the cell bodies of neurons that innervate the electic organ, was used to prepare cDNA libraries from which the *unc*-17 homolog from *Torpedo* was obtained by low stringency screening (Varoqui et al., 1994). The *Torpedo* protein, expressed in CV-1 fibroblast cells, possessed a high-affinity binding site for vesamicol, a drug that blocks in vitro and in vivo ACh accumulation in cholinergic vesicles. Using a probe derived from the *Torpedo* vesamicol-binding protein cDNA, mammalian homologs from rat neuroendocrine PC12 and human neuroblastoma SK-N-SH cDNA libraries were isolated (Erickson et al., 1994; Roghani et al., 1994). Expression of the rat VAChT in fibroblasts enabled intact cells to sequester ACh in a vacuolar ATPase-containing compartment by a process that was inhibited by L-vesamicol (Erickson et al., 1994). Recently, active transport of ACh has been measured in secretory organelles prepared from PC12 cells stably expressing the human VAChT cDNA, providing the first kinetic analysis of mammalian VAChT (Varoqui and Erickson, 1996a).

High levels of expression of VAChT mRNA have been observed in all major cholinergic cell groups examined, including peripheral postganglionic parasympathetic cells, preganglionic sympathetic and parasympathetic cells, ventral spinal cord and brainstem motoneurons, and scattered cell groups in the basal forebrain, the habenula, and striatum (Erickson et al., 1994; Roghani et al., 1994; Schäfer et al., 1994). This distribution is identical to that previously reported for the mRNA encoding the biosynthetic enzyme for ACh, choline acetyltransferase (ChAT) (Paxinos and Butcher, 1985). Thus, VAChT and ChAT gene expression appears to be tightly linked in mammalian neurons, with both transcripts being produced

from a common 'cholinergic' gene locus (Béjanin et al., 1994; Erickson et al., 1994).

In contrast to VMATs, a single protein species can account for ACh vesicular transport throughout the cholinergic nervous system. Polyclonal antipeptide antibodies raised against the C-terminus of VAChT have shown that it is a more superior antigen for mapping cholinergic nerve terminals than ChAT, consistent with a cytosolic localization of ChAT and the concentration of VAChT where secretory vesicles are clustered (Schäfer et al., 1995; Erickson et al., 1996b; Gilmore et al., 1996; Weihe et al., 1996; Arvidsson et al., 1997). This enhanced visualization of cholinergic terminal fields with VAChT has enabled several areas of cholinergic neurobiology, currently controversial and unclear, to be more precisely defined. For instance, it is now clear that determination of the cholinergic phenotype in the stellate ganglion precedes sweat gland innervation, and that cholinergic innervation of the sweat gland epithelium proceeds without a prior switch from a noradrenergic phenotype at the level of the synaptic vesicle of the nerve terminal (Schäfer et al., 1997). An autocrine/paracrine role for ACh in regulating neuroendocrine function is supported by the observation of dense innervation of the median eminence of the hypothalamus with VAChT-containing terminals, arising most likely from cholinergic cell bodies in the arcuate nucleus (Schäfer et al., 1994; Weihe et al., 1996; Arvidsson et al., 1997). The origin, physiology, and regulation of the unique cholinergic C-bouton, closely apposed to cranial and spinal motoneuron perikarya, can now be better understood using VAChT as a marker (Gilmore et al., 1996; Arvidsson et al., 1997). In addition, visualization of an intrinsic cholinergic system in the cerebral cortex, in addition to the extrinsic cortical cholinergic innervation arising from the basal forebrain, may be of significance in understanding the role of cholinergic neurotransmission in cognitive brain functions and of potential clinical significance in degenerative brain diseases, such as senile dementia of the Alzheimer's type (Davies and Maloney, 1976; Perry et al., 1977; Geula and Mesulam, 1989; Schäfer et al., 1994, 1995; Arvidsson et al., 1997).

The "Cholinergic" Gene Locus

The entire VAChT gene is contained within the first intron of the ChAT gene, an organization that prompted the term "cholinergic" gene locus (reviewed in Usdin et al., 1995). This nested gene organization is conserved from worm to human and is likely to be important for the coordinated expression of both genes establishing and maintaining the cholinergic phenotype (Alfonso et al., 1994; Béjanin et al., 1994; Erickson et al., 1994). ChAT and VAChT mRNA and protein are coexpressed throughout the mammalian central and peripheral cholinergic nervous system. In addition, these genes are coregulated by various extracellular factors, including nerve growth factor, neurotrophins, retinoids, and other signalling pathways (Berrard et al., 1995; Berse and Blusztajn, 1995; Misawa et al., 1995; Tian et al., 1996). Multiple promoters for both VAChT and ChAT may produce the collection of mRNAs, differing only in their respective 5′ noncoding regions that have been observed (Kengaku et al., 1993; Cervini et al., 1994, 1995). Under normal physiological conditions the expression of ChAT and VAChT from this cholinergic "regulon" is tightly controlled. Uncoupling of ChAT and VAChT gene expression may lead to a reduction in vesicular ACh pools, resulting in presynaptic cholinergic hypofunction.

Age-associated memory loss and cognitive decline in Alzheimer's disease correlate with the failure of cholinergic transmission in neurons of the basal forebrain (Perry et al., 1978; Whitehouse et al., 1981; Rossor et al., 1982; Pepeu et al., 1993). It is possible that this results from a decrease in synthesis and vesicular storage of ACh. ACh synthesis depends on high-affinity choline uptake, acetyl CoA supply, and ChAT activity. Wurtman and colleagues have proposed that cholinergic projection neurons of the basal

forebrain "autocannibalize" in Alzheimer's disease because of a primary defect in choline metabolism. They suggest that these neurons ultimately consume their own membrane phosphatidylcholine to acquire additional choline to synthesize ACh (Wurtman, 1992) and in the process, potentially expose intramembraneous proteins, such as amyloid precursor protein, to proteases (Dyrks et al., 1988). Normally, if choline is in short supply and cholinergic neurons are physiologically active, all available choline is used primarily for ACh synthesis, sustaining neurotransmission at the expense of membrane building (Maire and Wurtman, 1985; Ando et al., 1987; Ulus et al., 1989). An important question is whether one or all of the components of the ACh synthetic and sequestration machinery become impaired during aging and senile dementias. In cortical neurons of Alzheimer's disease brain, the abundance of the plasma membrane high-affinity choline transporter is actually increased, despite a loss of cholinergic projections (Slotkin et al., 1994). Vesamicol binding to VAChT in diseased cortical tissue is reported to be unchanged (Kish et al., 1990; Ruberg et al., 1990). A marked reduction in ChAT mRNA and protein in surviving neurons of Alzheimer's disease brain is, however, a hallmark feature of Alzheimer's disease (Ruberg et al., 1990; Strada et al., 1992; Boissière et al., 1997). Thus, an analysis of the abundance of VAChT mRNA and protein relative to the expression of ChAT in cholinergic neurons in normal and aging brain and in Alzheimer's disease may provide valuable information regarding the potential uncoupling of the expression of these genes and the importance of this locus in the maintenance of patent cholinergic neurotransmission.

Active Transport of ACh by VAChT

An active ACh transport assay following transfection of VAChT cDNA has recently been developed (Varoqui and Erickson, 1996a). Stable lines of PC12 cells expressing the human VAChT cDNA were selected using species-specific anti-VAChT antisera. Active ACh transport increases 20-fold in homogenates containing secretory vesicles in stable PC12 cells expressing human VAChT cDNA compared to control PC12 cells expressing only low levels of the endogenous rat VAChT protein. The transport of ACh by VAChT is dependent on exogenous ATP at 37°C and abolished by low temperature (4°C), the protonophore FCCP, and the vesicular H^+-ATPase inhibitor bafilomycin. Uptake is stereospecifically inhibited by L-vesamicol (n*M* range) and is not affected by reserpine or tetrabenazine. The initial rate of ACh uptake by VAChT is saturable, with an apparent K_m of approx 1 m*M* and a turnover (V_{max}/B_{max}) of approx 65/min (Fig. 2A).

The vesicular storage compartment in human VAChT-expressing PC12 cells in vitro appears to be dynamic, reflecting the concentration of ACh present in the medium (Fig. 2B). A comparison of the time-course of active transport by VAChT at various subsaturating concentrations of ACh in the medium reveals that different levels of maximal ACh accumulation are attained at steady-state. Using a wide range of ACh concentrations, the maximal vesicular accumulation of ACh increases linearly up to 1 m*M* exogenous ACh and saturates at approx 4 m*M*. Since the concentration of ACh in the cytoplasm of nerve terminals of mammalian brain has been estimated to be at 0.2–1 m*M* (Parsons et al., 1993), and given the K_m of ACh for VAChT (approx 1 m*M*), active transport of ACh by synaptic vesicles may not be saturated in vivo.

The low apparent affinity of VAChT for ACh and the concentration-dependent steady-state levels of vesicular ACh accumulation attained in vitro support the notion that pertubation in the level of ACh synthesis would have corresponding effects on the storage and release of ACh in vivo. Treatments that increase brain choline levels result in increased synthesis and release of ACh (Cohen and Wurtman, 1975; Haubrich et al., 1975; Blusztajn and Wurtman, 1983; Koshimura et al., 1990). In addition, transfected rat PC12 cells that overexpress the

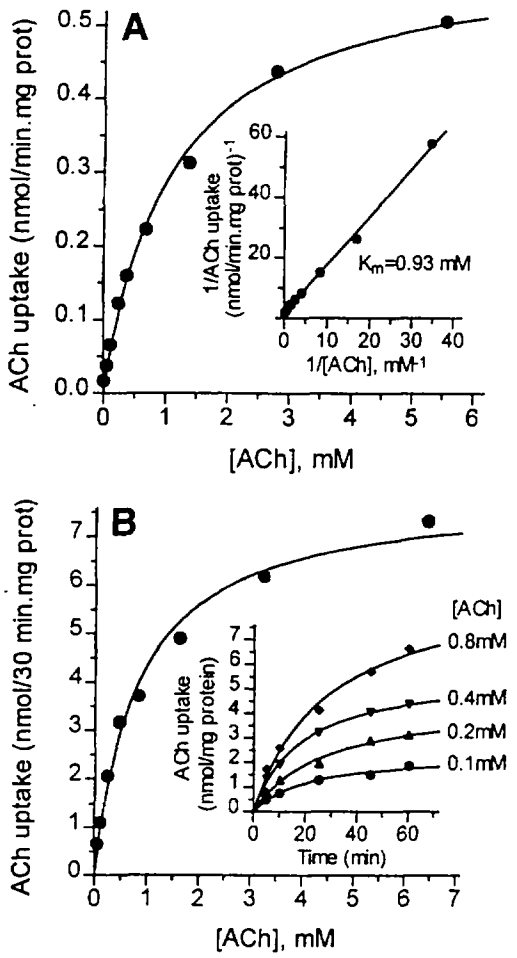

Fig. 2. Active transport of acetylcholine (ACh) by the human vesicular acetylcholine transporter (hVAChT). Uptake of ^{3}H-ACh is performed with crude vesicles prepared from human VAChT-expressing stable PC12 cells. Uptake mediated by the endogenous rat VAChT protein was less than twofold greater than that observed in the presence of 2 μM L-vesamicol. Thus, increasing the amount of VAChT expressed leads to an increase in the amount of ACh accumulated. **(A)** Kinetic analysis of uptake by hVAChT. The initial velocity of vesamicol-sensitive ACh transport by VAChT is saturable and VAChT displays a relatively low affinity for ACh. **(B)** Maximal levels of ^{3}H-ACh accumulation by hVAChT. The steady-state accumulation of ACh by vesicles is also saturable with maximal vesicular levels attained at ACh concentrations in the medium approximately four times higher than the K_m for ACh transport. The time courses of uptake are similar, using several concentrations of ACh in the medium that are below the K_m for ACh transport. These results indicate that the storage compartment in PC12 cells does not simply accumulate ACh until it is full, but rather is dynamic, reflecting the amount of free ACh available in the medium. Reproduced from Varoqui and Erickson (1996a).

ACh biosynthetic enzyme ChAT show increased vesicular ACh levels and increased stimulated release of ACh following choline loading (Misawa et al., 1994). Pertubation in the level of VAChT in vitro likewise affects the amount of ACh that can be accumulated by secretory vesicles (Varoqui and Erickson, 1996a) and may therefore also have corresponding effects on the storage and release of ACh in vivo. Recently, Poo and colleagues overexpressed the rat VAChT in developing *Xenopus* spinal neurons and found a marked increase in the amplitude and frequency of miniature excitatory postsynaptic currents at neuromuscular synapses (Song et al., 1997). An over 10-fold increase in the vesicular packaging of ACh appeared in developing neurons even before synaptogenesis; this effect was blocked by L-vesamicol. These results demonstrate that the ACh quanta released from cholinergic nerve terminals can be regulated by changes in the activity of ChAT or VAChT.

Molecular Analysis of VMATs and VAChT

The predicted amino acid sequences of human VMAT2 and VAChT, and their proposed topology in the vesicular membrane, are shown in Fig. 3. VMAT1 and VMAT2 exhibit approx 60% sequence identity and share approx 40% identity with VAChT. Considerably higher homology among these proteins exists within their putative 12 transmembrane domains (TMD). Potential glycosylation sites are observed in the poorly conserved intravesicular loop located between putative TMD I and II. The hydrophilic cytoplasmic amino and carboxy termini are also poorly conserved among VMAT1, VMAT2, and VAChT. A generally similar 12-TMD architecture has been proposed for many secondary transport proteins

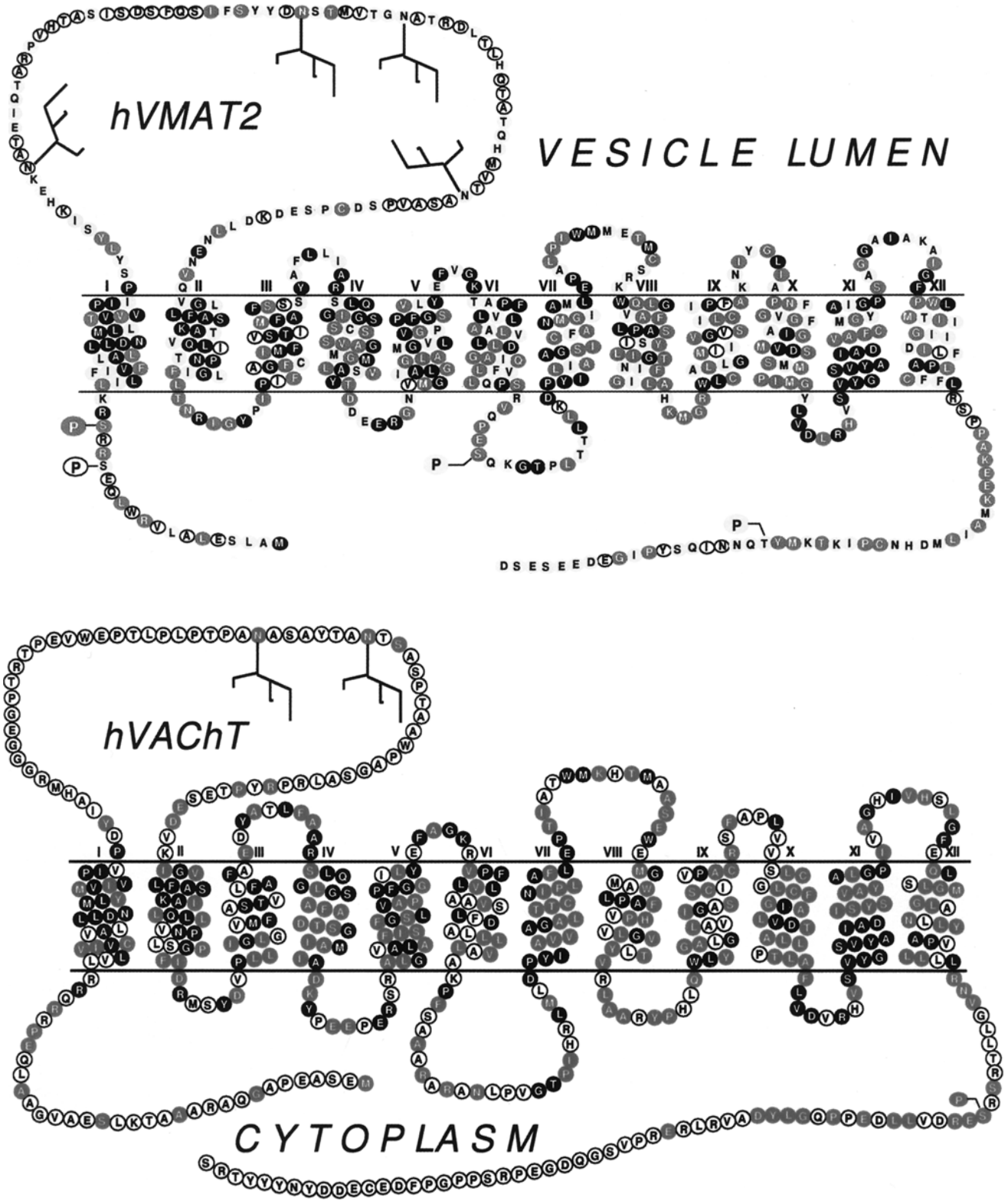

Fig. 3. Primary amino acid sequence and predicted secondary structure of hVMAT2 and hVAChT. Twelve putative transmembrane domains (TMD I–XII), potential sites for N-linked glycosylation, and potential sites for phosphorylation by protein kinase C are indicated. The VMAT isoforms and VAChT are glycolsylated. As yet, no evidence for regulated phosphorylation of these proteins exists. Black indicates amino acids conserved among VMAT1, VMAT2, and VAChT proteins (VMATs from rat, bovine, and human and VAChTs from rat, human, and *Torpedo*). Blue indicates amino acids unique to all VMATs. Yellow indicates amino acids conserved among VMAT2 isoforms. Red indicates amino acids unique to all VAChTs.

that utilize transmembrane electrochemical gradients established by various ATPases. Sequence alignments demonstrate a limited homology with several bacterial drug-resistance transporters, but none with the biogenic amine plasma membrane transporters (Amara and Kuhar, 1992).

The monoamine and ACh vesicular transporters are thought to employ a common bioenergetic mechanism for substrate transport. These transporters exchange two intravesicular protons, accumulated in the vesicles by the vesicular H^+-ATPase, for one cationic amine substrate (Knoth et al., 1981; Nguyen and Parsons, 1996). Sequence conservation of charged and polar amino acids within the 12 putative TMDs suggests their involvement in maintaining native conformations of the transporter, in particular for substrate binding and H^+/antiport. Aspartic acid residues within putative TMDs I, VI, X, and XI are conserved in VMATs and VAChT in all species obtained so far. An additional aspartic acid in TMD IV is unique to all VAChTs. The aspartic acid residues as well as other polar amino acids (e.g., serine) within the TMDs of the VMATs and VAChT are likely to be part of a "pore" within the vesicle membrane and be important for the binding and exchange of H^+ and substrates. Indeed, earlier biochemical studies using bovine chromaffin granules and *Torpedo* synaptic vesicles have indicated that a membrane embedded aspartic acid is required not only for substrate transport function but for the binding of tetrabenazine and vesamicol (Gasnier et al., 1985; Diebler, 1992).

VAChT ($K_m \approx$ m*M* range) has been shown in *Torpedo* synaptic vesicles to be the least selective among known ACh-binding proteins (Marshall and Parsons, 1987). The substrate quaternary and carbonyl groups are essential, and increased hydrophobic/aromatic bulk increases VAChT's affinity for various ACh analogs approx 10–100-fold (Clarkson et al., 1992). The increase in affinity for the larger ACh analogs is probably caused by increased contact points with specific amino acids in TMDs. The VMATs also transport a variety of compounds, including the catecholamines, indolamines, such as 5HT, the adrenal imaging agent m-iodobenzylguanidine, the neurotoxin MPP^+, and a number of neuroactive unsubstituted and substituted aromatic amines, but with considerably higher affinity (μM range) than VAChT displays for ACh (Njus et al., 1986; Kanner and Schuldiner, 1987; Johnson, 1988). Despite the broad range of substrate specificity, VMATs and VAChT do not transport each other's substrates.

Model for Vesicular Amine Transport

The binding site for reserpine is thought to overlap with the high-affinity monoamine uptake site in both VMAT isoforms based upon the facts that it competitively inhibits monoamine transport with an inhibition constant (K_i) in the nanomolar range, it binds to the transporters with a dissociation constant (K_d) similar to its K_i, and the binding of reserpine is competitively inhibited by concentrations of monoamines equivalent to their K_m for transport (Schuldiner et al., 1995). It has been proposed that tetrabenazine and ketanserin bind to a site in VMAT2 that faces the vesicle lumen and represents a low-affinity discharge site for the substrates (Henry and Scherman, 1989). These inhibitors block vesicular monoamine transport noncompetitively, but in lysed preparations of vesicles, their binding is competitively displaced by the monoamine substrates at concentrations approx 100 times greater than their K_m for uptake. Tetrabenazine also inhibits reserpine binding, suggesting that the high- and low-affinity monoamine recognition sites interact (Darchen et al., 1989). This model is based largely on studies performed in bovine chromaffin granules (Scherman and Henry, 1984; Henry et al., 1987; Darchen et al., 1989) and guides current work with the cloned genes.

There is no reserpine-like compound that inhibits VAChT at the high-affinity ACh recognition site. Instead, the action of vesamicol resembles that of tetrabenazine. Vesamicol inhibits ACh transport with an inhibition constant (K_i) similar to its dissociation constant

(K_d). However, inhibition is of the mixed noncompetitive type (Bahr and Parsons, 1986; Diebler and Morot Gaudry-Talarmain, 1989), indicating that vesamicol does not bind to the ACh uptake site. In addition, the binding of vesamicol is competitively displaced in lysed vesicles by ACh at concentrations approx 10 times greater than the K_m for ACh uptake (Varoqui and Erikson, unpublished data). It has, however, been postulated that the vesamicol "receptor" allosterically modulates VAChT at a site that faces the cytoplasm (Kornreich and Parsons, 1988).

A model for the transport of substrates by the vesicular amine transporters is shown in Fig. 4. The binding of reserpine to VMATs is accelerated following imposition of either the electrical or chemical component (ΔpH or $\Delta\psi$) of the transmembrane H^+ gradient (Erickson and Eiden, 1993; Schuldiner et al., 1993b) and is thought to result from a H^+-induced conformational change in the transporter (Weaver and Dupree, 1982; Rudnick et al., 1990). It is believed that the high-affinity uptake recognition site is exposed to the cytoplasm following translocation of one H^+ from the interior of the vesicle. Recent site-directed mutagenesis work has shown that protonation of a histidine residue (His^{419} in rat VMAT1 at the putative junction of the cytoplasmic loop and TMD XI) is involved in this effect (Shirvan et al., 1994). The energy available in the protonated transporter may be released by ligand binding and converted into vectorial movement of substrate through the pore of the transporter and across the membrane (Schuldiner et al., 1995). A second conformational change results in the substrate-binding site being exposed to the vesicle interior, where the substrate can dissociate. The second H^+ in the cycle may be required to facilitate this second conformational change. The aspartic acid in TMD X may play a specific role in proton movement through the transporter (Steiner-Mordoch et al., 1996; Song et al., 1997). Interestingly, the apparent K_i of substrates competing for tetrabenazine binding increases as the pH decreases (Darchen et al., 1988). This could reflect a mechanism for

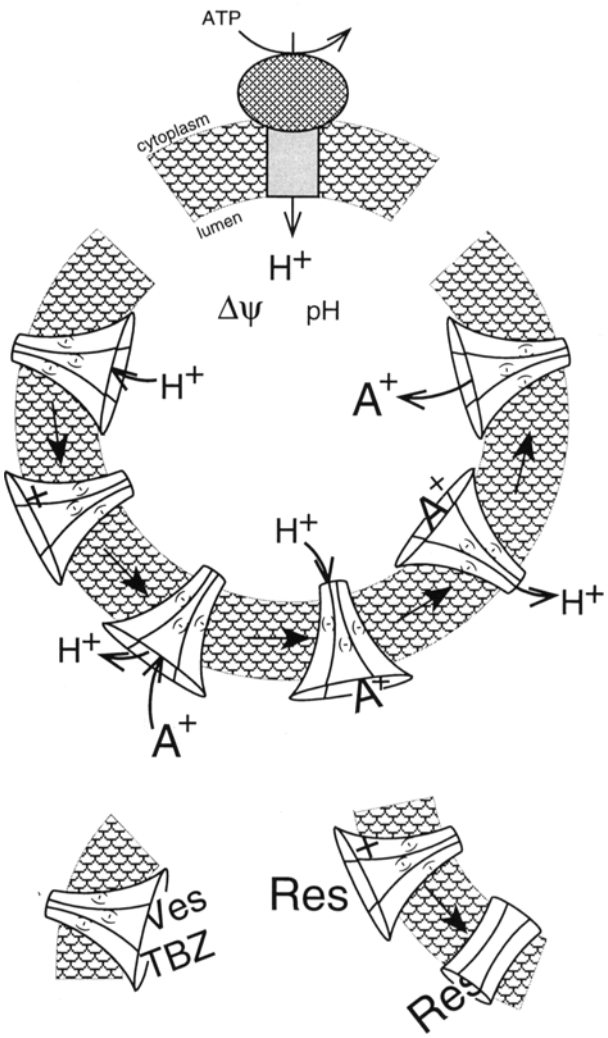

Fig. 4. Model for vesicular transport of monoamines and acetylcholine. Active vesicular transport of amines utilizes the energy of a transmembrane proton gradient generated by a vacuolar-type H^+-ATPase. Two protons are thought to be involved in the translocation of one protonated amine (A^+). (Counterclockwise) The first proton induces a conformational shift exposing a high-affinity substrate binding site to the cytoplasm. Following binding of the amine, another proton is exchanged during a second conformational change that exposes the substrate to the vesicle lumen, where it can dissociate. The inhibitors reserpine (RES), tetrabenazine (TBZ), and vesamicol (VES) interrupt the transport cycle (see text for details).

releasing the substrate in the acidic milieu of the vesicle lumen. Subsequent H^+ translocation regenerates the high-affinity substrate recogni-

tion state. When reserpine is present, it restricts the second conformational change so that instead of being released on the vesicle interior it becomes trapped within the protein.

Amino Acids Involved in Amine Recognition

Studies on G protein-coupled receptors for monoamines have shown that a membrane-embedded aspartic acid residue interacts with the amino group and a serine residue interacts with the hydroxyl groups of the substrate (Strader et al., 1988, 1989). Similarly, the aspartic acid in TMD I and serine residues in TMD III of plasma membrane monoamine transporters may be involved in substrate recognition (Kitayama et al., 1992). Furthermore, aspartic acid in TMD I and serine residues in the evolutionarily related bacterial H^+/antiporters are also required for transporter function (Yamaguchi et al., 1992a,b). The conserved aspartic acid residue in TMD I of VMATs have recently been implicated in high-affinity monoamine recognition. Replacement of this aspartic acid residue with glutamate significantly reduces transport activity, but this substitution has no effect on substrate affinity. However, elimination of the negative charge, i.e., replacement of the aspartate residue by asparagine or valine, abolishes transport (Merickel et al., 1995; J. D. Erickson, unpublished data). Interestingly, the asparagine mutant expresses normal levels of protein and retains the ability to bind reserpine, thus indicating that the first H^+-induced conformational change of the transporter is still occuring but monoamines now have lost the ability to displace the bound reserpine.

Edwards and colleagues have also performed functional studies following simultaneous replacement of three serine residues (serine$^{180-182}$) with alanine (near the putative junction between TMD III and the intralumenal loop of rVMAT2) and suggest that they are important for the interaction of the hydroxy moiety of the monoamines with the transporter (Merickel et al., 1995). Monoamine transport activity is abolished in the triple serine mutant of rVMAT2, yet normal levels of protein are synthesized and the coupling of reserpine binding to the H^+-gradient remains intact. In addition, 5HT fails to inhibit reserpine binding to this mutant, suggesting that these amino acids are involved in the recognition site for 5HT. Interestingly, the triple serine mutant of rVMAT2 also fails to transport histamine (Merickel and Edwards, 1995). Thus, although serine$^{180-182}$ in rVMAT2 may be an important part of the monoamine translocation channel across the membrane, they may neither be specifically interacting with the hydroxy moiety of the monoamines nor be involved in the differential histamine sensitivity between VMAT1 and VMAT2. Only two of these three serine residues (the first and third) are conserved in rat, bovine, and human VMAT2. The corresponding amino acids in VMAT1 from these species are serine, glycine, and threonine. It is possible that these sequence differences between VMAT isoforms account for the approximately threefold differences observed for catecholamine affinity or in the larger differences observed in sensitivity to other unsubstituted aromatic amines, such as phenylethylamine, amphetamine, or MPP^+.

Chimeric Analysis of VMATs

Functional chimeras have been constructed between VMAT1 and VMAT2 to identify domains responsible for the observed pharmacologic differences between these proteins. Tetrabenazine and two unsubstituted aromatic amines, amphetamine and histamine, have been evaluated because of the marked differences in their interaction with VMAT1 and VMAT2 (*see above*). Chimeras between rat VMAT1 and VMAT2 have indicated that the same domains that affect histamine recognition similarly influence sensitivity to tetrabenazine (Peter et al., 1996). Two putative domains of rat VMAT2, TMDs V–VIII, and TMDs IX–XII, are each required for increased substrate affinity and sensitivity to tetrabenazine, but neither alone suffice to confer VMAT2-like interaction.

Functional chimeras between human VMAT1 and VMAT2 are able to dissociate the interaction of tetrabenazine and amphetamine from that of histamine (Erickson, 1997). For instance, a chimera in which the region between Ile^{265} and Tyr^{418} of human VMAT2 (roughly TMDs VII–X) is replaced with the corresponding VMAT1 sequence displays VMAT2-like sensitivity to tetrabenazine and amphetamine, whereas it exhibits VMAT1-like sensitivity to histamine. Further analysis indicated that specific amino acid residues between Ile^{265} and Trp^{328} of human VMAT2 are important for the increased potency of histamine to compete for uptake with 5HT compared with VMAT1.* This region of VMAT2 alone, however, is not sufficient to confer histamine sensitivity to VMAT1, indicating that a second important domain probably exists in VMAT2 between TMDs XI and XII.

Multiple domains within VMAT2 are important for the translocation of histamine and unsubstituted amphetamine-like compounds across the vesicle membrane. Some of these domains involved in the interaction with amphetamine and histamine might be shared. Indeed, both compounds are direct competitors of 5HT for uptake by VMAT2* and the transport of histamine by VMAT2 is sensitive to inhibition by reserpine (Erickson et al., 1995; Merickel and Edwards, 1995). Therefore, the approx 100-fold difference in the affinities of VMAT2 for amphetamine and histamine could be caused by differences in the interaction of their respective aromatic rings within a same domain of VMAT2. The preferential interaction of both of these compounds with VMAT2, compared to VMAT1, could also be caused by differences at that site between the isoforms. Other domains might also be involved in the differential interaction of these compounds with VMAT1 and VMAT2. The dissociation of the differential sensitivity of the VMAT isoforms to amphetamine and histamine suggests that different sites within the protein are important in the translocation of these amines to the vesicle interior.

* Varoqui and Erikson, manuscript in preparation.

The high-affinity interaction of tetrabenazine with VMAT2 also involves multiple domains of the protein. Photolabeling of VMAT2 with 7-azido-8-[^{125}I]iodoketanserin ([^{125}I]AZIK) is inhibited by tetrabenazine and has been a useful probe to identify at least one amino acid of VMAT2 important for the binding of this drug. Early studies suggested that the [^{125}I]AZIK photolabeled domain of VMAT2 was located within the first putative 8 TMDs based on the fact that a tetrabenazine-sensitive photolabeled VMAT2 proteolytic cleavage product is glycosylated (Erickson et al., 1992). Recently, peptide purification and radiomicrosequencing of [^{125}I]AZIK-labeled VMAT2 indicated derivatization of Lys^{20} at the putative junction between the N-terminus and TMD I (Sievert and Ruoho, 1996; Sagné et al., 1997). Although the Lys^{20} is conserved in rat, bovine and human VMAT2, it is also present in rat and bovine VMAT1. Accordingly, a chimera in which the N-terminus region of bovine VMAT1 is present in VMAT2 retains sensitivity to tetrabenazine (Henry et al., 1997). Interestingly, human VMAT1 has a Gln at this position (Gln^{20}) which may contribute to the fact that tetrabenazine does not interact with human VMAT1 at all compared to only 10-fold difference in potency between the bovine isoforms. Hence, a chimera in which the N-terminus region of hVMAT1 is present in hVMAT2 showed greatly reduced sensitivity (IC_{50} = 5 μM) to tetrabenazine (Erickson, 1997). A chimera in which TMDs VIII–X of human VMAT2 are replaced with VMAT1 sequences (Trp^{328}–Tyr^{418}) displays high affinity for tetrabenazine.* Thus, in addition to TMD I, domains of VMAT2 thought to be important for the binding of tetrabenazine include TMDs V–VII and TMDs XI–XII (Peter et al., 1996; Erickson, 1997).

ACh Transport Function of VAChT

Several *unc-17* point mutants in the nematode *C. elegans* display phenotypes consistent with a reduction of vesicular transport activity and ACh release, indicating that unique amino

acid residues can affect the function of VAChT (Brenner, 1974; Alfonso et al., 1993). Worms completely devoid of the UNC-17 protein die within a few days after they hatch, indicating that the transporter is essential for neural function and for survival. The point mutations that are not lethal are particularly interesting because the VAChT protein is expressed and some degree of activity is retained. Although the mutant proteins have not yet been directly examined in in vitro transport assays, the behavioral data suggest that alterations in the ACh transport site of VAChT itself affect the storage and release of ACh in vivo.

An active transport assay for ACh following expression of VAChT cDNA has only recently been developed and should prove useful for the structure/function analysis of VAChT (Varoqui and Erickson, 1996a). Functional chimeras and site-directed mutagenesis of VAChT have begun to address whether the TMDs that contribute to the ACh transport recognition site differ from the corresponding TMDs in the VMATs involved in the transport of monoamines. A chimera in which the N-terminal portion (up to TMD II and including the lumenal glycosylated loop) of VAChT is replaced with VMAT2 sequences (2/V chimera) binds vesamicol with an affinity similiar to wild-type. However, examination of the transport properties of this 2/V chimera indicates that its apparent affinity for ACh is reduced approx 10-fold compared to wild-type (Varoqui and Erickson, 1997). These data suggest that other amino acids within TMD I, in addition to the conserved aspartic acid residue, are important for ACh transport function. Recently, Poo and colleagues have shown that the unique aspartic acid residue in TMD IV of VAChT is important for ACh transport function. This VAChT mutant can no longer generate an enhanced ACh packaging in *Xenopus* oocytes as observed following injection of wild-type rat VAChT (Song et al., 1997). Furthermore, these investigators indicate that the aspartic acid in TMD X plays a specific role in proton translocation as has been previously suggested for the VMATs. The mutation in TMD X displays a dominant negative effect, reducing even the secretion of ACh by the endogenous VAChT protein (Song et al., 1997). Thus, the analysis of the sites involved in the interaction of ACh with VAChT provides insight not only into the mechanism of vesicular transport but whether particular mutants display altered phenotypes such that vesicular accumulation of ACh, and subsequent regulated ACh release, are affected.

Subcellular Localization of VMATs and VAChT

Large and small dense cored vesicles (LDCV and SDCV) and small synaptic vesicles (SSV) are the organelles used by neurons and endocrine cells for regulated monoamine and ACh secretion, respectively. DCVs are heterogenous is size, varying from 70 to more than 200 nm in diameter, whereas SSVs are more homogeneous and approx 50 nm in diameter. Soluble proteins and neuropeptides are specific to LDCVs, whereas synaptophysin (p38) is thought to be specific to SSVs (Jahn et al., 1985; Navone et al., 1986). SDCVs (70–90 nm) are the synaptic vesicles of monaminergic neurons and may be a hybrid between LDCVs and SSVs (Bauerfeind et al., 1995). The exocytosis of SSVs and LDCVs is differentially regulated and takes place at distinct cellular sites (Thureson-Klein et al., 1988; Lundberg et al., 1994). SSVs are specialized for fast neurotransmission at the nerve ending compared to the neuropeptide- and monoamine-containing LDCVs located throughout monoaminergic endocrine cells and neurons.

The examination of the subcellular localization of the vesicular transporters indicates that these proteins are targeted to distinct vesicle populations in neurons and neuroendocrine cells. The endocrine-specific VMAT1 isoform is expressed on LDCVs of the rat adrenal medulla (Liu et al., 1994). Tetrabenazine-sensitive transport of dopamine in the CNS occurs in vesicle structures that resemble SSVs (Phillipu and Beyer, 1973; Erickson et al., 1990; Floor et al., 1995), yet in rat central monoaminergic neurons, higher levels of

VMAT2 have been observed by immunoelectron microscopy on LDCVs and tubulovesicular structures than on SSVs (Nirenberg et al., 1995, 1996). VAChT, on the other hand, is abundantly expressed on SSVs in cholinergic nerve terminals in *Torpedo* electric organ and in the rat CNS (Gilmore et al., 1996; Varoqui et al., 1996).

Targeting of VMATs and VAChT to Secretory Organelles

The trafficking of VMATs and VAChT can be studied using the pheochromocytoma (PC12) cell line (Fig. 5). PC12 cells are mixed monoaminergic/cholinergic cells that synthesize, store, and secrete ACh as well as dopamine (Green and Rein, 1977a,b; Schubert and Klier, 1977). Earlier subcellular fractionation of PC12 cells has revealed that the LDCVs possess a reserpine-sensitive transport system for monoamines, whereas membrane fractions containing p38 display low levels of specific ACh transport and vesamicol binding (Blumberg and Schweitzer, 1992; Bauerfeind et al., 1993). VMAT1 is expressed almost exclusively on LDCVs in untreated PC12 cells (Liu et al., 1994). These observations have been confirmed using pre-embedding immunogold localization of VMAT1 in NGF-differentiated PC12 cells. In addition, endogenous rat VAChT or stably expressed of human VAChT is shown to be preferentially localized to SSVs in these cells by immunoelectron microscopy (Erickson et al., 1996b; Varoqui and Erickson, 1996b; Weihe et al., 1996). Interestingly, stably expressed rat or human VMAT2 localizes to LDCVs and is not found on SSV clusters in NGF-treated PC12 cells (Varoqui and Erickson, 1996b; Weihe et al., 1996). Whether the neuronal cell background is important and PC12 cells lack some component to allow VMAT2 to be expressed on SSVs, or dopaminergic and cholinergic SSVs are produced by unique biosynthetic pathways remains an open question.

Current work focuses on the identification of the signals responsible for the differential targeting of VMATs and VAChT to LDCVs and SSVs, respectively. Recently, stable PC12 cell lines expressing chimeras between human VAChT and VMAT2 have indicated that structural information resides within the cytoplasmic tail of VAChT, which specifically targets it to SSVs (Varoqui and Erickson, 1997). Thus, several SSV-destined proteins, including synaptophysin (Linstedt and Kelly, 1991a), synaptobrevin (Grote et al., 1995) and SV2 (West et al., 1997), and VAChT contain within cytoplasmic domains the structural information required to direct them to SSVs.

Biogenesis of Monoaminergic and Cholinergic Secretory Organelles

The examination of axonal transport of VMATs and VAChT from the cell body to each type of regulated secretory vesicle would shed light on the biogenesis of monoaminergic and cholinergic secretory organelles in central and peripheral nerves and in neuroendocrine cells. These studies could determine whether divergence of the sorting of these proteins occurs at the level of the *trans* Golgi network or at the early endosome. Synaptophysin, unique to SSVs and SDCVs, reaches this compartment following sorting in the early endosome via a constitutive vesicle (Clift-O'Grady et al., 1990; Cameron et al., 1991; Linstedt and Kelly, 1991b; Régnier-Vigouroux et al., 1991). SV2, found on both DCVs and SSVs, sorts differently from synaptophysin and may reach the nerve terminal by a pathway other than constitutive vesicles, perhaps the LDCVs (Feany et al., 1993; Tanner et al., 1996). It has been suggested that cholinergic vesicles are generated from early endosomes after delivery of their components to the nerve terminal via constitutive vesicles (Kelly and Grote, 1993; Régnier-Vigouroux and Huttner, 1993; Bennett and Scheller, 1994; Mundigl and De Camilli, 1994). However, VAChT-associated gold particles are observed on LDCVs along the fibers of control and human VAChT-expressing PC12 cells (Fig. 5). Whether this observation reflects the biosynthetic route of VAChT from the nucleus to the nerve terminal remains to be determined. LDCVs are the majority of vesicles observed in NGF-differentiated PC12 cells, in contrast to the situation in cholinergic nerves in vivo, where SSVs predominate. Furthermore, in cholinergic nerves,

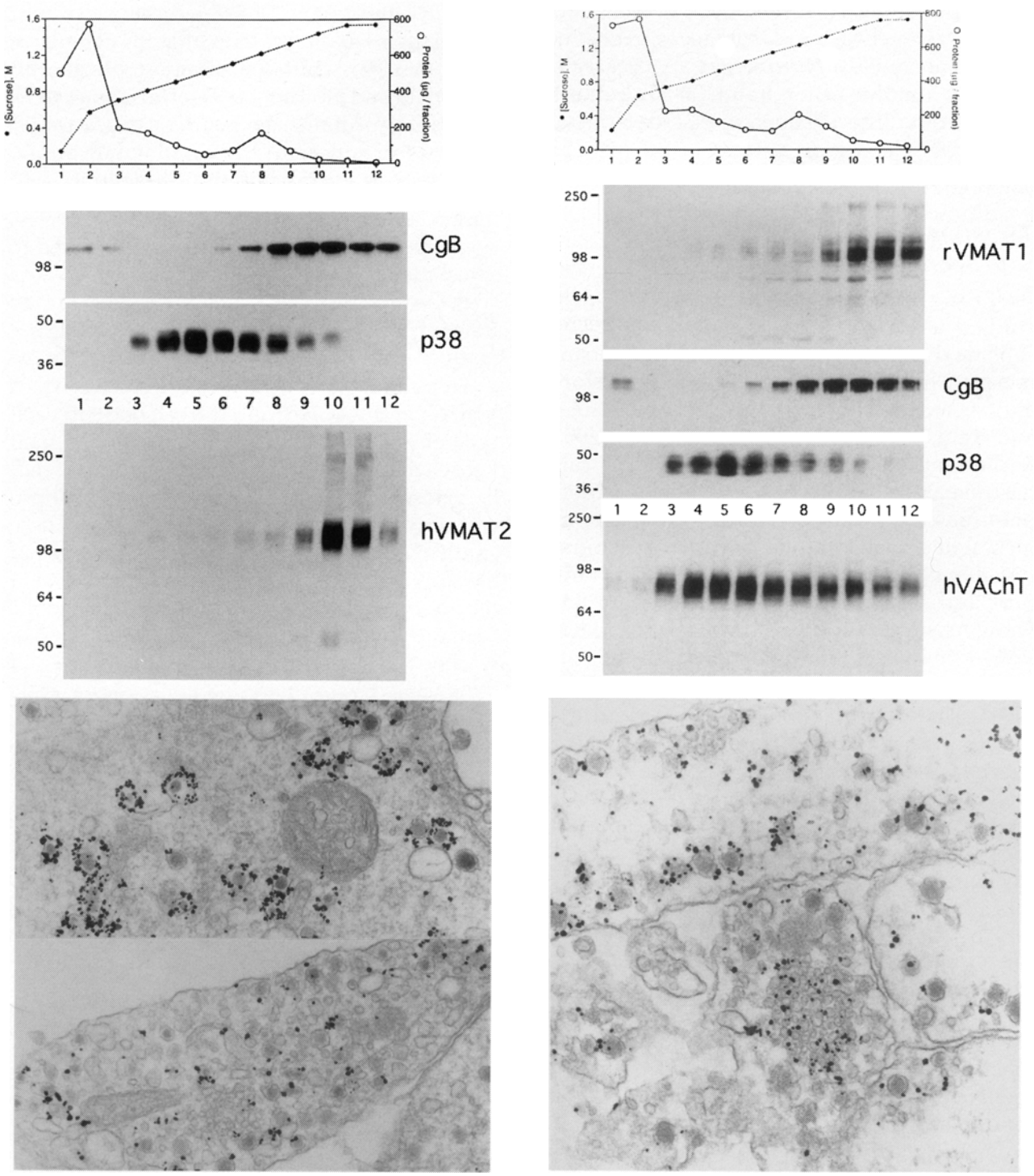

Fig. 5. VMAT1 and VMAT2 are targeted to LDCVs and VAChT is preferentially expressed on SSVs in PC12 cells. Equilibrium density fractionation of PC12 cells expressing hVMAT2 **(left)** and hVAChT **(right)**. Postnuclear homogenates of PC12 cells expressing hVMAT2 or hVAChT were fractionated on a continuous equilibrium sucrose gradient (0.6–1.6 *M*). The distribution of chromogranin B (CgB), synaptophysin (p38), rat VMAT1, and hVAChT or hVMAT2 were analyzed by Western blot. hVMAT2 is found in heavy fractions containing the LDCV

ACh and neuropeptides are differentially released, reflecting the fact that they are stored in SSVs and LDCVs, respectively (Agoston et al., 1988; Matteoli et al., 1988). It will be of considerable interest to examine the biogenesis of these different classes of regulated vesicles in neuronal cell lines or primary cultures, which are purely cholinergic or monoaminergic, in addition to neuroendocrine PC12 cells to determine how the cell background influences the trafficking of these proteins and the biogenesis of cholinergic and monoaminergic secretory organelles.

Future Perspectives

The recent cloning of the mammalian VMAT isoforms and VAChT has enabled the characterization of their functional and pharmacological properties and further description of their relative distributions in cells and, in particular, their synapses throughout the central and peripheral nervous systems and the diffuse neuroendocrine system. Future mutagenic work altering the molecular structures that determine vesicular transporter specificity will be important to establish a relationship between the activity of vesicular neurotransmitter transporters and vesicular storage and levels of neurotransmitter available for regulated neurosecretion. Understanding the molecular basis for the selective targeting of VAChT to SSVs and VMATs to LDCVs and the biogenesis of monoaminergic and cholinergic synaptic vesicles are important areas of cell biology that can be studied now using specific antibodies directed against these proteins. Understanding the regulation of the expression the VMAT genes and of the "cholinergic" gene locus will be important not only to determine which factors govern development of monoaminergic vs cholinergic cell types, but also to assess whether expression of these genes is coupled to the transcription of the genes encoding the respective neurotransmitter biosynthetic enzymes, coordinately regulating the level of vesicular storage and the releasable pool of neurotransmitter. Finally, understanding how vesicular sequestration of neurotransmitters affects neurotoxicity and neurotransmitter release will be important for a more complete understanding of human diseases of monoaminergic and cholinergic neurotransmission, such as Parkinson's and Alzheimer's disease.

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markers CgB and rVMAT1. hVAChT is largely recovered in light fractions containing the SSV marker p38, but is also observed in heavier fractions containing LDCV markers. Immunocytochemical EM analysis of hVMAT2 (left) and hVAChT (right) distribution in NGF-treated stably transfected PC12 cell lines. hVMAT2 immunoreactivity (silver-enhanced immunogold particles) is confined to the membrane of LDCVs and is rarely found on SSVs. hVAChT immunoreactivity is found predominantly on SSV clusters but is also observed on LDCVs, particularly along processes.

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