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Vesicular neurotransmitter transporter trafficking in vivo

Moving from cells to flies

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During exocytosis, classical and amino acid neurotransmitters are released from the lumen of synaptic vesicles to allow signaling at the synapse. The storage of neurotransmitters in synaptic vesicles and other types of secretory vesicles requires the activity of specific vesicular transporters. Glutamate and monoamines such as dopamine are packaged by VGLUTs and VMATs respectively. Changes in the localization of either protein have the potential to up or downregulate neurotransmitter release, and some of the mechanisms for sorting these proteins to secretory vesicles have been investigated in cultured cells in vitro. We have used Drosophila molecular genetic techniques to study vesicular transporter trafficking in an intact organism and have identified a motif required for localizing Drosophila VMAT (DVMAT) to synaptic vesicles in vivo. In contrast to DVMAT, large deletions of Drosophila VGLUT (DVGLUT) show relatively modest deficits in localizing to synaptic vesicles, suggesting that DVMAT and DVGLUT may undergo different modes of trafficking at the synapse. Further in vivo studies of DVMAT trafficking mutants will allow us to determine how changes in the localization of vesicular transporters affect the nervous system as a whole and complex behaviors mediated by aminergic circuits.

Key words: VMAT, VGLUT, vesicular neurotransmitter transporter, membrane trafficking, tyrosine motif, synaptic vesicle (SV), large dense core vesicle (LDCV)

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The initiation and termination of synaptic transmission requires two types of neurotransmitter transporters. Plasma membrane transporters mediate the reuptake of transmitter from the synaptic cleft into either the presynaptic terminal or adjacent glia following exocytosis.[1-3] These proteins thereby help to determine the amount of neurotransmitter that is available for signaling at the synapse.[3] The structurally and bioenergetically distinct vesicular transporters are responsible for the storage of neurotransmitters in synaptic vesicles (SVs) as well as other types of secretory vesicles.[4,5] These include specific vesicular transporters for acetylcholine (VAChT), GABA and glycine (VGAT/VIAAT), glutamate (VGLUTs), purine nucleotides (VNUT)[6] and the monoamines serotonin, dopamine, noradrenalin, histamine and in invertebrates, octopamine (VMATs).[4,5] A variety of papers published over the past fifteen years have shown that vesicular transporter expression, and the number of transporters that localize to each vesicle, can regulate the amount of neurotransmitter that is stored and released during exocytosis.[7] Increasing the number of transporters on each vesicle may also influence the well-described electrophysiological parameter of quantal size, defined as the amplitude of a post-synaptic signal generated by a single vesicle's content of neurotransmitter.[7] Changes in quantal size have classically been considered to be strictly the domain of post-synaptic receptors and their attendant signaling machinery. The idea that quantal size might be regulated by vesicular transporters opens up a host of interesting possibilities including the possible presynaptic scaling of coordinated circuits in the central nervous system.[8,9]

In addition to increased expression, membrane trafficking represents another mechanism to regulate the number of transporters that localize to each vesicle.[10,11] Membrane trafficking also adds the possibility of regulated sorting of transporters to different types of secretory vesicles. In addition to synaptic vesicles (SVs), which cluster at specific sites in the nerve terminal, monoamines and perhaps other types of neurotransmitters can be released from the more diffusely localized large dense core vesicles (LDCVs), which also release peptide transmitters. It is possible that regulated events such as transporter phosphorylation could control the number of transporters that localize to LDCVs versus SVs.[12,13] In addition, it is possible that vesicular transporters distribute to different subclasses of SVs at the nerve terminal. It has been known for decades that VAChT localizes to at least two biochemically distinct populations of vesicles at the neuromuscular junction.[14,15] Other vesicular transporters may also sort to functionally distinct pools of SVs.[16] It is not yet clear whether distribution to subsets of SVs can be regulated via membrane trafficking, but there are a few tantalizing clues suggesting that this may indeed occur.[15,17]

The potential contribution of vesicular transporter trafficking to neurotransmitter release increases the importance of understanding the basic cellular mechanisms that sort the transporters to secretory vesicles. Some of the cellular machinery has been identified and appears to be identical to evolutionarily conserved proteins required for vesicle budding and fusion throughout the cell.[10,18] For "cargo," such as the vesicular transporters to be included in a particular budding vesicle, "sorting signals" must be encoded in the primary structure of the protein or added post-translationally. The signals required for protein trafficking in non-neuronal cells have been extensively characterized.[19] However, the sorting signals that allow vesicular transporters and other proteins to localize to secretory vesicles are less clearly defined. It also remains unclear whether different vesicular transporters use the same or different pathways to get to SVs at the nerve terminal. We are using the fly to help address these questions and in particular, to determine which signals are needed for sorting to secretory vesicles in vivo. In time, these studies will allow us to determine how vesicular transporter trafficking may influence normal and disease-related behaviors and the physiology of the nervous system as a whole.

Most current models of SV trafficking indicate that endocytosis from the cell surface is somehow required, thus predicting that endocytosis signals in vesicular transporters should have an important role in sorting the transporters to SVs.[20] Several studies have already identified signals on vesicular transporters that are important for both endocytosis and trafficking to SVs in cultured cells.[10] The neural isoform of the mammalian vesicular monoamine transporter (VMAT2) contains a dileucine motif that is necessary for both internalization from the cell surface and localization to synaptic-like microvesicles (SLMVs) in PC12 cells.[21,22] A similar motif and possibly an additional tyrosine-based signal allows VAChT to internalize from the cell surface and localize to SLMVs in neuroendocrine cells.[23-25] A variant of the dileucine motif (FV), and in some cases a polyproline motif may be important for recycling mammalian VGLUTs to SVs in cultured cells.[26] These studies notwithstanding, the trafficking of vesicular transporters in vivo has remained relatively obscure.

As a first step toward determining how transporter trafficking might affect the function of the nervous system as a whole, we set out to identify trafficking motifs in the Drosophila orthologs DVMAT and DVGLUT.[27-29] For DVMAT, we focused on DVMAT-A, the splice variant expressed in all aminergic neurons.[28,30] (Another splice variant, DVMAT-B, is expressed in a small subset of glia in the visual system and contains a distinct trafficking domain).[31] We find that a tyrosine in the carboxy terminus of DVMAT-A (Y600) is necessary for efficient endocytosis in vitro.[29] This signal is likely to be similar to the canonical tyrosine-based motif YXXΦ, where Φ represents a bulky hydrophobic residue.[19] To determine whether this signal would affect targeting of DVMAT-A to synaptic vesicles in vivo, we generated transgenes containing the wild type protein and others containing mutations in the putative trafficking domain. Using biochemical fractionation of adult head homogenates, we showed that mutation of Y600 or deletion of the entire carboxy terminus dramatically reduces the localization of DVMAT-A to SVs. To our knowledge, these findings are the first to show that mutation of a trafficking motif in a vesicular transporter or any vesicular protein can disrupt localization to secretory vesicles in vivo. We are now investigating whether additional motifs might function as back-up signals for trafficking of DVMAT-A to SVs via alternative pathways, or for sorting to LDCVs.

In parallel with our studies on DVMAT, we have used both biochemical and genetic methods to study the trafficking of DVGLUT. The Drosophila genome contains one VGLUT ortholog, thus facilitating the genetic analysis of glutamate release and signaling.[27,32] Overexpression of DVGLUT can increase quantal size and the resultant increase in glutamate release is lethal (Daniels RW and DiAntonio AD, unpublished). Screening for intragenic mutations that suppressed lethality led to the isolation of a number of DVGLUT variants with missense or nonsense mutations in potential trafficking domains. In addition, we used in vitro mutagenesis to more directly disrupt proposed trafficking domains. Using mutants derived from both in vitro mutagenesis and in vivo screening, we then showed that large deletions of the DVGLUT C-terminal trafficking domain had very modest effects on either internalization in cultured cells in vitro, or on DVGLUT's localization to SVs in vivo. Previous studies have shown that mutation of proposed trafficking domains in mammalian VGLUTs similarly result in relatively subtle effects on internalization in cultured cells.[26,33,34] Based on these differences, we speculated that the pathways that sort VMATs and VGLUTs to SVs at the synapse might be significantly different.[29] This idea is supported by a recent demonstration that mammalian VMAT2 and VGLUT1 may undergo endocytosis to different populations of SVs in mammalian neurons.[16]

The relative resistance of VGLUTs to the disruption of canonical trafficking motifs raises the possibility that VGLUTs could employ novel

trafficking mechanisms. Lumenal and transmembrane domains help sort proteins to the apical surface of polarized epithelia,[35] and it is conceivable that similar signals play a role in transporter trafficking at the synapse. In addition, a subset of mutations in a cytosolic loop domain of DVGLUT appear to disrupt exit from the soma.[29] Further study of these mutants may help disentangle non-specific changes in protein structure from potentially more interesting effects on trafficking.

In ongoing studies, we are testing the behavioral affects of disrupting transporter trafficking. We have recently shown that *dVMAT* mutants exhibit several deficits in monoamine-linked behaviors.[36] To determine how DVMAT trafficking may influence behavior, we are genetically rescuing the *dVMAT* mutant with a panel of transgenes containing altered trafficking signals. As described for DVGLUT,[29] forward genetic screens may be used to study trafficking of DVMAT or other vesicular transporters such as DVGAT[37] and DVAChT.[38] In addition to identifying important motifs within the transporters themselves, future genetic screens may also be used to identify interacting proteins possibly important for trafficking. This type of screen was recently used to identity a binding partner for the *C. elegans* ortholog of VAChT.[39]

Since the mechanisms of neurotransmitter transport and release are highly conserved, our studies in the fly may provide important clues about the potential contribution of transporter trafficking to human behavior and disease. Several recent papers suggest that mutations in plasma membrane neurotransmitter transporters may be linked to specific neuropsychiatric syndromes.[40] Alterations in VMAT2 expression have also been tentatively linked to neuropsychiatric illness[41] and Parkinson's disease[42] and the overexpression of both VMAT2 and DVMAT-A can have neuroprotective effects.[43,44] The ability of VMATs to confer neuroprotection against the toxic effects of amphetamines has been proposed to depend on the localization of VMAT2 to SVs.[45] We are currently investigating whether mutations in DVMAT trafficking motifs ablate its ability to confer neuroprotection in the fly. More generally, we speculate that our studies of transporter trafficking in Drosophila may help determine how changes in neurotransmitter storage and release contribute to the pathophysiology and treatment of a variety of disease states.

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