Neurotransmitter release: **Variations on a theme**

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Similarities between the ways that synaptic vesicles and large dense-core vesicles release their contents have been emphasized, but recent studies have revealed important mechanistic differences between these two exocytotic processes.

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Neurotransmitter release involves the regulated fusion of specialized secretory vesicles, such as synaptic vesicles, with the plasma membrane. Variations in the properties of this regulated exocytosis affect the kind of information processing performed by different types of synapse. Regulated transmitter release is also thought to make an important contribution to neural plasticity, increased release probability being associated with increased synaptic efficacy. Although the molecular basis for this variation remains largely uncharacterized, it has been known for decades that two morphologically distinct types of secretory vesicle mediate transmitter release with different physiological properties. These are the synaptic vesicles and the large dense-core vesicles, and recent studies have revealed important mechanistic differences between their respective exocytotic mechanisms.

Synaptic vesicles have a diameter of 30–40 nanometers, appear clear by electron microscopy and contain classical neurotransmitters, such as acetylcholine, γ-amino-butyric acid (GABA) or glutamate. They cluster over the 'active zone' at a synapse, respond to high micromolar calcium concentrations and release their contents very rapidly — within a millisecond — which presumably contributes to the brain's efficient information processing [1]. In contrast, large dense-core vesicles have a diameter of 80–200 nanometers and exhibit a characteristic electron-dense core that contains neural peptides. Large dense-core vesicles occur in the cell body and dendrites of a neuron as well as the axonal terminals, and release their contents relatively slowly — taking more than 50 milliseconds — in response to low micromolar calcium concentrations.

This slow release is consistent with the view that the neural peptides stored in large dense-core vesicles have a modulatory role in neurotransmission, in contrast to the more direct role of the transmitters stored by synaptic vesicles. Despite these marked differences in both the structure and function of synaptic vesicles and large dense-core vesicles, recent focus on the role of fusion proteins — 'SNAREs', see below — in transmitter release has emphasized the similarities between these two types of secretory vesicle.

The membrane docking and fusion processes of vesicles involve interactions between complementary 'soluble NSF attachment receptors' on the vesicle and target membranes — v-SNARE and t-SNARE, respectively [2]. Synaptic vesicles and large dense-core vesicles use the same v-SNAREs: vesicle-associated membrane proteins (VAMPs) 1 and 2, otherwise known as synaptobrevins. These v-SNAREs interact specifically with the t-SNAREs syntaxin-1 and SNAP-25. Synaptic vesicles and large dense-core vesicles also both contain Rab3a, a small GTPase that has been implicated in exocytosis. The two types of vesicle thus do not differ in core aspects of their fusion machinery.

They do differ in other respects, however. Synaptic vesicles, but not large dense-core vesicles, contain the integral membrane protein synaptophysin [3]. Furthermore, multiple isoforms of the calcium-sensing protein synaptotagmin have been described that vary substantially in their affinity for calcium, and these might be responsible for the differential responsiveness of synaptic vesicles and large dense-core vesicles to cytoplasmic calcium [4]. The subcellular location of these isoforms is not known, however, and their contribution to the physiological differences between synaptic vesicles and large dense-core vesicles is uncertain. Three recent papers [5–7] have now reported functional differences between the exocytotic mechanisms of synaptic vesicles and large dense-core vesicles.

The assays used in these studies all relied on the ability of both synaptic vesicles and large dense-core vesicles to accumulate classical transmitters through specific vesicular transport proteins [8]. After preloading the vesicles with the radiolabelled transmitter, exocytosis was monitored by the release of radioactivity into the medium. Although large dense-core vesicles generally contain neural peptides, rather than classical transmitters, the large dense-core vesicles in PC12 pheochromocytoma cells do contain a vesicular monoamine transporter (VMAT1), enabling the analysis of norepinephrine secretion. In monoamine neurons, the central transporter VMAT2 is present on both large dense-core vesicles and synaptic vesicles [9], and serotonin release in the leech occurs from both large dense-core vesicles and synaptic vesicles [10].

Using semi-permeabilized neuroendocrine PC12 cells, Martin and colleagues [11] have characterized two steps in the exocytosis of large dense-core vesicles. Both presumably occur after vesicle docking; the first involves priming by ATP, and the second calcium-dependent triggering of release. Martin's group has used this simple but powerful assay to purify soluble proteins required for each of the two steps. In their recent work [5], they focussed on the 'calcium-dependent activator for secretion', or CAPS, a 145 kDa calcium-binding protein that has been reported to contribute to the second, triggering step of exocytosis [12]. Martin and colleagues [5] have extended the analysis of regulated exocytosis from cultured endocrine cells to synaptosome preparations, and shown that CAPS is required for exocytosis of large dense-core vesicles by neurons. Further support for the view that CAPS has a role in exocytosis of large dense-core vesicles comes from genetic studies in the nematode *Caenorhabditis elegans*, where the CAPS homologue Unc-31 has been implicated in the secretion of serotonin [13,14], a transmitter known to be stored in large dense-core vesicles [9,10].

To determine directly whether CAPS has a role in the exocytosis of synaptic vesicles as well as large dense-core vesicles, Balch and colleagues [6] used semi-permeabilized synaptosomes. Synaptic vesicles, but not large dense-core vesicles, contain glutamate, so that release of glutamate into the medium should reflect only the exocytosis of synaptic vesicles. Although the spontaneous leakage of glutamate from synaptic vesicles has in the past complicated the analysis of synaptic vesicle exocytosis from permeabilized systems, this assay can detect calcium-activated glutamate release. In support of the assumption that the assay can distinguish exocytosis of synaptic vesicles from that of large dense-core vesicles, activated glutamate release was only detected when the calcium concentration was as high as 250 μM, whereas release of norepinephrine — considered a marker for exocytosis of large dense-core vesicles — was detected at a calcium concentration of about 1 μM [6].

Using this assay, Tandon *et al.* [6] found that an antibody against CAPS impaired the release only of norepinephrine, and not of glutamate. This argues strongly that CAPS has a selective role in the exocytosis of large dense-core vesicles. However, synaptic vesicles and tubulovesicular structures also store monoamines and express the transporter VMAT2 [15]. It is therefore surprising that low concentrations of calcium are sufficient to trigger release of all of the stored norepinephrine, including that stored in synaptic vesicles, which usually require relatively high calcium concentrations for exocytosis. However, the synaptic vesicles in monoamine neurons may have some of the characteristics of large dense-core vesicles, and depending on the fixation procedure, are sometimes seen to have a dense core [16]. It is therefore possible that these so-called small dense-core vesicles may also differ from more typical synaptic vesicles in their calcium-dependence and mechanism of exocytosis, including their dependence on CAPS.

C. elegans unc-31 mutants, which as mentioned above lack function of a CAPS homologue, may facilitate investigation of the specificity of CAPS. Does the 'uncoordinated' phenotype of these mutants result from a defect in the release of peptides as well as serotonin, which would suggest a disturbance in the exocytosis of large dense-core vesicles, or from a defect in the release of other, more classical transmitters? Interestingly, *unc-31* mutants also show a defect in the release of acetylcholine [17]. This may simply reflect a defect in the activation of motor neurons by neural peptides released from large dense-core vesicles, but it could also reflect the storage of acetylcholine in large dense-core vesicles as well as in synaptic vesicles.

Another recent study has identified another variable property of the exocytotic mechanisms of vesicles carrying different neurotransmitters. Khvotchev and Sudhof [7] have used the drug phenylarsine oxide to inhibit the phosphorylation of phosphoinositide lipids in intact synaptosomes. Strikingly, they found that phenylarsine oxide inhibits the depolarization-evoked, calcium-dependent release of preloaded norepinephrine but not of preloaded glutamate. Phenylarsine oxide also inhibited norepinephrine release induced by the calcium ionophore α-latrotoxin and hypertonic sucrose, suggesting that it interferes with a step after calcium entry. The transmitter release observed with this assay may, however, involve repeated rounds of vesicle docking and fusion, raising the possibility that phosphoinositide lipids act earlier in the exocytotic pathway.

The precise phosphorylated lipid species affected by phenylarsine oxide have not been identified, but newly synthesized phosphoinositide lipids appear to contribute to the release of monoamines, and not of other classical transmitters. If norepinephrine release reflects the exocytosis of large dense-core vesicles, the results implicate the phosphorylation of phosphoinositide lipids in the exocytosis of large dense-core vesicles and not on synaptic vesicles, with the same caveats about monoamine release from synaptic vesicles described above for the studies with CAPS. But how does phosphorylation of phosphoinositide lipids contribute to the selective exocytosis of large dense-core vesicles? Observations on CAPS suggests an explanation.

Consistent with the view that CAPS has a selective role in the exocytosis of large dense-core vesicles, the protein appears to associate specifically with large dense-core vesicles and the plasma membrane, but not with synaptic vesicles [5]. Interestingly, trypsin does not abolish the

association of this peripheral membrane protein with large dense-core vesicles, suggesting an interaction with lipid. Indeed, CAPS binds specifically to phosphatidylinositol 4,5-bisphosphate (PIP_2) in artificial membranes [18]. It does not simply interact with any anionic phospholipid, as it fails to bind the even more negatively charged phosphatidylinositol 3,4,5-trisphosphate (PIP_3).

This specific interaction would seem likely to be physiologically important, as the ATP-dependent priming step in exocytosis requires such phospholipid-modifying enzymes as the phosphatidylinositol transfer protein, phosphatidylinositol 4-kinase and phosphatidylinositol 4-phosphate 5-kinase [19–21], which may thus serve to recruit CAPS for the subsequent calcium-dependent triggering step. That the binding of CAPS to PIP_2 plays a part in the triggering step of exocytosis is supported by a couple of further observations: the change in conformation of CAPS that, as assayed by proteolytic sensitivity, is induced on binding to PIP_2 [18], and the specific inhibition of CAPS binding to PIP_2 by calcium. Although some of the recent data raise questions about the association of CAPS with PIP_2 in native vesicles [5], the selective effects of both CAPS and the phosphorylation of phosphoinositide lipids on the exocytosis of large dense-core vesicles, but not synaptic vesicles, suggest they have closely related, rather than independent roles, in vesicle fusion.

Three new studies [5–7] thus imply that there are significant differences between the exocytotic mechanisms used by large dense-core vesicles and synaptic vesicles. The differential dependence on CAPS may simply reflect the difference in size between large dense-core vesicles and synaptic vesicles — CAPS might be required for the exocytosis of larger, but not smaller, vesicles, primarily because of their differences in membrane curvature. Together with different synaptotagmin isoforms, CAPS may also contribute to the increased calcium sensitivity of large dense-core vesicles. The CAPS-independence of synaptic vesicle exocytosis suggests, however, that the difference with respect to large dense-core vesicles is qualitative, rather than simply quantitative. Phosphorylation of phosphoinositide lipids and CAPS recruitment appear to act in a biochemical pathway unique to the exocytosis of large dense-core vesicles.

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