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## Synaptotagmins: mediators of $Ca^{2+}$ -regulated vesicle fusion. Focus on “Stable gene silencing of synaptotagmin I in rat PC12 cells inhibits $Ca^{2+}$ -evoked release of catecholamine”

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EXOCYTOSIS IS A GENERAL PROCESS by which intracellular vesicles containing cargo are transported to and then fuse with the plasma membrane. Exocytic vesicles either fuse constitutively or they accumulate under the plasma membrane, where they await fusion until an appropriate physiological signal is detected in a process called regulated exocytosis (4, 21). Examples of exocytic vesicles that undergo regulated exocytosis include discoidal vesicles that fuse with the apical plasma membrane of umbrella cells in response to stretch, synaptic vesicles that fuse with the presynaptic membrane in response to action potentials, and dense-core/secretory vesicles that fuse with the plasma membrane of neuroendocrine/endocrine cells downstream of hormonal stimuli (6, 21, 24). Dense-core granules are formed in the *trans*-Golgi network and then accumulate in the cell cytoplasm forming a reserve pool of vesicles. A fraction of this pool is delivered to the plasma membrane where vesicles are docked. The vesicles then undergo a  $Ca^{2+}$ - and ATP-dependent priming step(s) that is incompletely understood, but is thought to produce an asynchronous, slowly releasable pool of vesicles (21). Vesicle priming is likely to depend in part on the assembly of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) that reside on the vesicle SNARE (v-SNARE) and target membrane SNARE (t-SNARE), which drive the final steps in vesicle fusion and cargo release (6, 21). Upon priming, a synchronous rapidly releasable pool of vesicles is generated. Fusion of both pools of vesicles occurs in response to micromolar concentrations of intracellular  $Ca^{2+}$ , and as their names imply, the rapidly releasable pool is quickly exocytosed (in the range of 0.1–1 s), whereas the slowly releasable pool is exocytosed over a longer time scale (several seconds). The molecular basis of these distinct rates of vesicle fusion is only poorly understood.

$Ca^{2+}$ -regulated fusion is thought to depend on a family of proteins called the synaptotagmins (syts), the putative  $Ca^{2+}$  sensors that trigger exocytosis (6, 14, 23). The syt family in humans and mice is comprised of 16 members, but >90 syt members are present in the published plant and animal genomes (7). Some isoforms of this family, syt VII in particular, have multiple splice variants, but the functional consequence of these different forms is unknown (22). Syts are localized to secretory vesicles and the plasma membrane, and have a conserved domain structure that includes a short extracellular domain, a transmembrane domain, and a cytoplasmic domain that is folded into a membrane proximal C2A domain and a membrane distal C2B region (6). The C2 domains of most syt

isoforms bind  $Ca^{2+}$ , however, syts VI, VIII, XII, and XIII lack consensus  $Ca^{2+}$ -binding sites (22).

Both biochemical and genetic data point to an important role for syts in  $Ca^{2+}$ -dependent fusion. The C2A and C2B domains of syt I (a major component of synaptic vesicles) penetrate into the plasma membrane lipids in a  $Ca^{2+}$ -dependent manner and the C2B domain specifically binds to phosphatidylinositol 4,5-bisphosphate, a lipid primarily associated with the plasma membrane (2, 6). Significantly, syt I associates with the membrane-proximal region of the t-SNAREs syntaxin 1 and SNAP-25, and may regulate  $Ca^{2+}$ -dependent fusion by promoting SNARE assembly and fusion (5, 6, 21, 26). Synaptotagmins undergo  $Ca^{2+}$ -dependent homo- and heterooligomerization, which may potentially aid SNARE fusion by bringing opposing membrane and SNARE complexes in close proximity (3, 10, 22).  $Ca^{2+}$ -dependent fusion can be reconstituted *in vitro* by the addition of the cytoplasmic domain of syt I to an assay that measures SNARE-dependent fusion of liposomes (26). Strikingly, not only does the syt I cytoplasmic domain make the reaction  $Ca^{2+}$  dependent, it also stimulates the rate of vesicle fusion, consistent with a role for synaptotagmins in promoting the fusion reaction. Genetic experiments show that expression of mutant syt I in mice, *Caenorhabditis elegans*, or *Drosophila melanogaster* disrupts exocytosis of the rapidly releasable pool of synaptic vesicles (12, 15, 17, 29). Similarly, exocytosis of a release-ready pool of dense core vesicles is perturbed in chromaffin cells isolated from syt I-deficient mice (27). In all cases, significant  $Ca^{2+}$ -dependent exocytosis remains, but the rate of residual exocytosis is slower, and may reflect the utilization of other syt isoforms.

A widely used model system to study syt function is the rat pheochromocytoma PC12 cell line. These cells have a population of dense core granules that contain catecholamines, and express multiple syt isoforms including I, III, IV, VII, and IX (9, 16, 28). Syt I and IX are thought to be expressed at relatively high levels, whereas expression of the other isoforms is low and somewhat variable depending on the PC12 cell line. Evidence from mechanically perforated PC12 cells show that both syt III and syt VII C2A domains inhibit exocytosis (25). Syt IX may also play a significant role in dense-core vesicle exocytosis as antibodies to the syt IX C2A domain inhibits exocytosis in permeabilized PC12 cells (11), and downregulation of syt IX by interfering RNA (RNAi) impairs dense-core granule exocytosis (9). The function of syt I in PC12 cells is controversial because earlier studies indicate that it may be dispensable. Naturally occurring variant PC12 cell lines, which have low levels of syt I expression, still undergo significant  $Ca^{2+}$ -dependent exocytosis (9, 20). Furthermore, knockdown of syt I by transient RNAi expression has no impact on

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exocytosis (9). In contrast to these findings, antibodies to syt I or isolated cytoplasmic domains of syt I block exocytosis of dense-core granule content (8, 11, 25).

The article by Moore et al. (Ref. 16, see p. C270 of this issue) revisits the question whether syt I expression is important for exocytosis of dense core granules in PC12 cells. The approach taken in this study was to generate stable PC12 cell lines that homogeneously expressed short hairpin RNA (shRNA) to block expression of syt I by RNAi. The previous analysis by Fukuda (9) generated syt I knockdowns using transient transfection, which effected knockdown in only a fraction of the cells. The cell line used by Moore et al. has no detectable levels of Syt I and there is no effect on the expression of syts III, VII, or IX or of the SNAREs synaptobrevin 2, syntaxin 1, or SNAP-25. The knockdown cells show a normal rise in  $\text{Ca}^{2+}$  in response to KCl-mediated depolarization. With the use of amperometry, a sensitive tool that measures catecholamine release from individual cells by quantifying changes in voltage across a carbon-fiber electrode, the authors observed that the number of exocytic events and the amplitude of these events are significantly decreased. Furthermore, other parameters that measure catecholamine release (peak amplitude, rate of rise, quantal content, half width, and falling phase) are all significantly reduced in cells expressing syt I shRNA, consistent with changes in fusion pore opening. The decrease in exocytic events is rescued if cDNA encoding human syt I, which differs in 4/19 nucleotides encoded by the shRNA target region, is coexpressed in the syt I knockdown cells. Finally, the authors observe that the stimulated release of catecholamines, as measured by HPLC analysis, is also significantly reduced in syt I shRNA-expressing cells.

Besides prompting a reexamination of the role of syt I in PC12 cells, the study by Moore et al. (16) suggests an approach whereby stable cell lines could be made lacking each of the known syts expressed in PC12 cells. The systematic downregulation of individual syts, coupled with careful amperometric analysis, would allow for a more defined analysis of syt function in dense-core secretory granule exocytosis. While other studies (9, 28) have used a similar approach, the use of stable cell lines may increase the likelihood of success. Furthermore, by reintroducing expression of the downregulated protein, or mutant versions of the protein, one can define the functional domains of syts in the absence of an endogenous pool of the wild-type protein. Such an approach has been successfully used in *Drosophila* (29). The work by Moore et al. is consistent with previous studies in that syt function is likely to be somewhat redundant, as residual  $\text{Ca}^{2+}$ -dependent exocytosis is always observed when individual syts are knocked down or inhibited by biochemical approaches. In the case of PC12 cells, syt IX is likely to be functional in the absence of syt I expression (9, 11). Silencing the expression of multiple syts may allow for a clearer picture of the involvement or redundancy of function of various syt proteins.

What is the purpose of having multiple syts expressed in one cell type? Beyond a simple mechanism that allows for redundancy, different syts may allow the cell to refine the cellular response to extracellular stimuli or regulate exocytosis of different populations of secretory vesicles. The C2 domains of syt VII bind divalent cations ( $\text{Ba}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Sr}^{2+}$ ) with a higher affinity than those of syt I or IX (28). While an increase in syt VII expression increases the metal sensitivity of exocytosis, a

decrease in endogenous syt VII expression by RNAi results in a decrease in metal sensitivity (28). These results indicate that syt VII may modulate that metal requirement for exocytosis. The rates of syt dissociation from membranes may also have functional significance. There are three classes of syts that are characterized by distinct rates of dissociation from membranes when  $\text{Ca}^{2+}$  is chelated (13). Consistent with a role in fast synchronous release, syt I C2 domains release quickly, whereas syt VII C2 domains dissociate slowly (13). It is possible that syt members that are slow to release  $\text{Ca}^{2+}$  may account for the asynchronous slow release that occurs after the initial  $\text{Ca}^{2+}$  burst has subsided because they are slow to release  $\text{Ca}^{2+}$ . Localization of syts to different intracellular compartments may allow syts to regulate multiple exocytic events. For example, syt VII is not only localized to secretory granules, but also to secretory lysosomes, which undergo  $\text{Ca}^{2+}$ -regulated fusion in response to various extracellular stimuli, including cell damage (1). Finally, beyond their role in regulating exocytosis, syts also play an important role in the endocytic recovery of secretory vesicle content (18, 19). Thus syts are multifunctional proteins that may play divergent roles in multiple trafficking steps and pathways, and the use of RNAi, combined with techniques such as amperometry, allow for the function of syts to be carefully dissected and explored in a range of cell types.

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