Journal Club

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How Do Synaptic Vesicles "Know" Which Pool They Belong to?

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Review of Cazares et al.

Presynaptic terminals contain hundreds to thousands of synaptic vesicles (SVs), specialized organelles that store neurotransmitter and fuse with the active zone plasma membrane for regulated transmitter release. Despite the fact that in electron micrographs these small clear vesicles appear morphologically similar, SVs are not all functionally identical: some vesicles are clearly more prone to be released than others. According to their recruitment by different patterns of electrical or chemical stimulation, SVs have been classified into three major functional pools: the readily releasable pool, the recycling pool, and the resting pool (Rizzoli and Betz, 2005; Denker and Rizzoli, 2010; Alabi and Tsien, 2012). The readily releasable pool comprises few vesicles that are docked at the plasma membrane and primed for immediate fusion upon stimulation. The recycling pool generally comprises 10%–20% of all vesicles in the terminal and is recruited after the readily releasable pool has been depleted during moderate physiological stimulation. The sum of readily releasable pool and recycling pool is referred to as the total recycling pool, thus including all SVs that can undergo fusion during moderate synaptic activity. The large majority of SVs present in a synapse belongs to the resting pool, which is only recruited upon intense high-frequency stimulation or prolonged low-frequency stimulation (Fernandez-Alfonso and Ryan, 2008; Ikeda and Bekkers, 2009). These three pools have been recognized in virtually all neuronal preparations, from the frog neuromuscular junction to synapses of mammalian hippocampal neurons in culture.

While readily releasable pool vesicles must be docked at the active zone membrane (even though not all docked SVs will be rapidly released upon stimulation) (Denker et al., 2009), recycling pool and resting pool vesicles appear to be largely intermixed and scattered in the presynaptic terminal (Denker and Rizzoli, 2010). This suggests that SVs might be partitioned by unknown molecular tags, likely soluble proteins that transiently associate with the SV membrane, that specify their identity and assign them to one pool or the other. One possible tag is the SV-associated protein synapsin I (SynI).

SynI has been proposed to dynamically organize the resting pool by reversibly tethering SVs to each other and to actin filaments in a phosphorylation-dependent manner (Cesca et al., 2010). Indeed, phosphorylation by protein kinase A (PKA), Ca^{2+}/calmodulin-dependent kinases (CaMKs), mitogen-activated protein kinase (MAPK), and cyclin-dependent protein kinase 1 (Cdk1) causes SynI detachment from SVs and actin, thus increasing the availability of SVs for fusion (Cesca et al., 2010). Conversely, phosphorylation by Src or Cdk5 increases the ability of SynI to bind SVs and actin, and favors stabilization of SVs in the resting pool (Messa et al., 2010; Verstegen et al., 2014). In Syn knock-out neurons, SVs disperse along axons and become highly mobile (Fornasiero et al., 2012; Orenbuch et al., 2012). However, ultrastructural analysis of presynaptic terminals reveals that SVs are normally interconnected by 30-nm-long filaments, which do not completely disappear upon Syn deletion, indicating that additional players participate in vesicle clustering (Siksou et al., 2007).

Recent work from Cazares et al. (2016) identified the SV-associated protein Tomosyn1 as a modulator of the resting pool. Tomosyn1 was previously shown to inhibit SV priming (a necessary step for vesicle fusion) at the readily releasable pool by interacting with the SNARE proteins syntaxin and SNAP25 and promoting the formation of a nonfusogenic SNARE complex (Hatsuzawa et al., 2003). Cazares et al. (2016) defined a novel role for Tomosyn1, demonstrating that this protein can prevent SV recruitment from the resting pool in the recycling pool for subsequent fusion.

Cazares et al. (2016) used primary rat hippocampal neurons deficient in or overexpressing Tomosyn1, and analyzed

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SV exo-endocytosis after 14–24 d *in vitro*, taking advantage of the pHluorin assay. This assay relies on the overexpression of a pH-sensitive green fluorescent protein fused with the vesicular glutamate transporter (vGpH) (Miesenbock et al., 1998). At rest, the acidic pH inside SVs quenches vGpH fluorescence, whereas upon electrical stimulation vGpH is exposed to the neutral extracellular space and its fluorescence is revealed. Varying the number of action potentials evoked allows the recruitment and visualization of either the readily releasable pool alone or the total recycling pool. Nonreleasable vesicles (resting pool) can then be visualized by performing ammonium chloride alkalinization at the end of the experiment.

With these methods, Cazares et al. (2016) showed that Tomosyn1 downregulation increased the readily releasable pool size, whereas Tomosyn1 overexpression had the opposite effect, consistent with its role as an inhibitor of SV recruitment into the readily releasable pool. Importantly, the authors also showed that Tomosyn1 knockdown increased the size of the total recycling pool at the expense of the resting pool, thus clearly demonstrating a role for Tomosyn1 in regulating the balance between the total recycling pool and the resting pool. Overexpression of full-length Tomosyn1 or a deletion mutant lacking the SNARE-binding motif resulted in a comparable reduction of total recycling pool size, indicating that the roles of Tomosyn1 at the resting/recycling pool interface and at the readily releasable pool are independent and rely on different protein domains.

As is the case for SynI, Tomosyn1 function might be rapidly modulated by phosphorylation. Cazares et al. (2016) thus addressed the possibility that Tomosyn1 is a target for Cdk5 phosphorylation. Indeed, Cdk5 is known to play a primary role in setting the balance between the resting and total recycling pools (Kim and Ryan, 2010). SynI was proposed to be the main effector of Cdk5 because SynI deletion or the expression of a dephospho-mimetic mutant of SynI phenocopied inhibition of Cdk5 in terms of SV partitioning (Verstegen et al., 2014). Cazares et al. (2016) demonstrated that Tomosyn1 and Cdk5 coimmunoprecipitate in neuronal lysates and that Cdk5 can phosphorylate Tomosyn1 *in vitro*. They also showed that knockdown of Tomosyn1 and inhibition of Cdk5 activity (either pharmacologically or by overexpressing a dominant-negative Cdk5 mutant) led to comparable increases in total recycling pool size. The inhibition of Cdk5 in Tomosyn1-deficient neurons did not further increase total recycling pool size, suggesting that the two proteins work in the same pathway to regulate the number of SVs in the total recycling pool and resting pool. Further characterization would be needed to univocally demonstrate that Cdk5 phosphorylation is needed for Tomosyn1 to alter the recycling/resting pool balance upon neuronal stimulation. To this aim, Cdk5 phosphorylation sites should be identified and the vGpH assay should be performed in neurons overexpressing Tomosyn1 phosphomimetic and phospho-depleted mutants.

To identify the molecular context in which Tomosyn1 exerts its functions, Cazares et al. (2016) performed coimmunoprecipitation and proximity-ligation assays. They showed that Tomosyn1 interacts with the GTPase Rab3A, preferentially in its GTP-bound (active) form. SynI is also recruited to this complex, via interaction with Rab3A. Notably, increasing the phosphorylation level, and thus the activity, of Cdk5 significantly increased the formation of the Tomosyn1/Rab3A/SynI complex. Whether increased formation of this complex negatively modulates total recycling pool size remained unaddressed, but this would be an important demonstration of the relevance of this interaction in the regulation of recycling/resting pool balance.

Finally, the authors showed that chronic incubation with the $GABA_A$ inhibitor picrotoxin increased Tomosyn1 phosphorylation and that Tomosyn1 knockdown abolished the homeostatic increase in SV release that occurs with chronic inhibition of neuronal activity. This form of synaptic plasticity, known as synaptic scaling, is exploited by neurons to adjust the strength of excitatory synapses to restore normal firing upon chronic perturbation of their activity (Turrigiano, 2008). The presynaptic mechanisms mediating synaptic scaling are largely unknown, but a role for Cdk5 and SynI has been reported (Kim and Ryan, 2010; Verstegen et al., 2014). The contribution of Tomosyn1 to presynaptic scaling is difficult to interpret because Tomosyn1 downregulation per se significantly increased SV recycling, but the idea is intriguing and needs further in-depth analysis.

In conclusion, Cazares et al. (2016) identified the soluble Tomosyn1 protein as a novel regulator of SV partitioning in total recycling and resting pools. This function seems to be regulated by Cdk5 phosphorylation (although the contribution of other kinases cannot be excluded) and to rely on the formation of a ternary complex composed of Tomosyn1, Rab3A, and SynI. A speculative model of how these proteins regulate vesicle pools and influence synaptic strength and scaling is the following: increased activity of Cdk5, which may be driven by a chronic rise in neuronal activity, leads to phosphorylation of Tomosyn1 and SynI; this may stabilize the Tomosyn1/Rab3A/SynI complex on SV membranes, leading to clamping of vesicles in the resting pool. Conversely, chronic inhibition of neuronal activity may lead to decreased Cdk5-dependent phosphorylation, destabilization of the ternary complex, and increased recruitment of SVs in the recycling pool. Identifying other molecules that target SVs to specific functional pools will improve our understanding of the mechanisms regulating neurotransmitter release in physiology and pathology. The work of Cazares et al. (2016) provides a valuable contribution in this direction.

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