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Previews

Clustering acetylcholine receptors in neuromuscular junction by phase-separated Rapsn condensates

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In this issue of *Neuron*, Xing et al. (2021) demonstrate that the multidomain scaffold protein Rapsn can form dense molecular condensates *in vitro* and *in vivo* via phase separation. The formation of Rapsn condensates is essential for clustering acetylcholine receptors on muscle membranes and for forming neuromuscular junctions.

Nerve cells extend their fibrous axons to innervate other neurons or muscles, causing neurotransmitter receptors on the surface of the postsynaptic membranes to form high-density clusters. Clustering of acetylcholine receptors (AchRs) beneath muscle membranes at the nerve-muscle junctions (NMJs) is essential for coordinated movements of muscles in all animals, including humans. AchRs alone, when expressed in cells, are diffused on plasma membranes. Rapsn, a 43 kDa scaffold protein, is absolutely required for the clustering of AchRs (Li et al., 2018). Numerous *Rapsn* mutations have been identified in patients suffering from congenital myasthenic syndrome, a genetic disorder caused by abnormal signaling at NMJs (Milone et al., 2009).

Rapsn was co-purified with AchRs at a comparable stoichiometry from membranes of *Torpedo* electrocyte (LaRochelle and Froehner, 1986). Extensive studies in the past 40+ years have revealed that Rapsn is a multi-domain scaffold protein capable of binding to many other proteins, including AchRs (Figure 1A). Rapsn was identified as an AchR clustering protein at least 20 years earlier than scaffold proteins such as PSD-95 or gephyrin that are required for clustering neurotransmitter receptors in brain synapses. However, our understanding of the molecular mechanism underlying Rapsn-mediated clustering of AchRs falls far behind those of glutamate receptors or glycine/$GABA_A$ receptors, in part due to a relatively poorer understanding of biochemical and biophysical properties of Rapsn.

In this issue of *Neuron*, Xing et al. (2021) report that purified Rapsn alone can spontaneously form dense molecular assemblies via liquid-liquid phase separation, a process that cells frequently use to form organelles not enclosed by lipid membranes. Unlike many proteins capable of forming condensed droplets via phase separation, Rapsn hardly contains any intrinsically disordered sequences. The authors showed that its 7 tetratricopeptide repeats (TPRs), which can self-associate with each other, are sufficient for Rapsn to phase separate. Interestingly, a number of Rapsn binding targets including AchRs can be recruited into the Rapsn condensates as client proteins, likely via direct binding to various domains of Rapsn (Figure 1A). The condensed Rapsn puncta formed beneath plasma membranes, when expressed in heterologous cells, myotubes, or muscles in mice, have liquid-like properties. Stimulating myotubes with Agrin, a factor released from motor neuron terminals, induces larger Rapsn condensate formation, indicating an activity-dependent Rapsn/AchR cluster formation via phase separation. Importantly, the authors showed that phosphorylation of Tyr86 in the 3rd TPR of Rapsn by muscle-specific kinase (Musk) promotes Rapsn phase separation, suggesting that AchR clustering by Rapsn condensates is regulated by Agrin-induced activation of Musk (Figure 1B). The authors investigated a panel of Rapsn mutations identified in myasthenic syndrome patients (Figure 1A) and found that some of the missense mutations in TPRs (e.g., L14P, N88K, and R164H) weaken Rapsn condensate formation and thus may impair clustering of AchRs, whereas most of the Rapsn mutations, either those with mutation sites within the TPRs or those in the C-terminal coiled-coil and RING domains, do not have an obvious impact on the phase separation of Rapsn in assays performed *in vitro*. Some of the mutations (e.g., E147K, L326P, 1177del2) may disturb the bindings of various target proteins such as AchRs, MACF1, and β-dystroglycan to Rapsn. Finally, the authors showed that one phase-separation-impairing Rapsn mutant (R164H) has significantly reduced AchR clustering in both embryonic and neonatal mouse muscles. It is noted that the R164H mutation specifically weakens the self-association of Rapsn but does not alter the expression level nor the target binding properties of the protein, indicating that impaired AchR clustering caused by the R164H missense mutation is likely due to impaired Rapsn phase separation.

Rapsn condensate-mediated clustering of AchRs shown by Xing et al. is supported by earlier electron microscopic (EM) studies of AchR-containing membranes prepared from *Torpedo* ray (Sealock, 1982; Zuber and Unwin, 2013). Discrete patched Rapsn clusters right beneath membranes containing dense

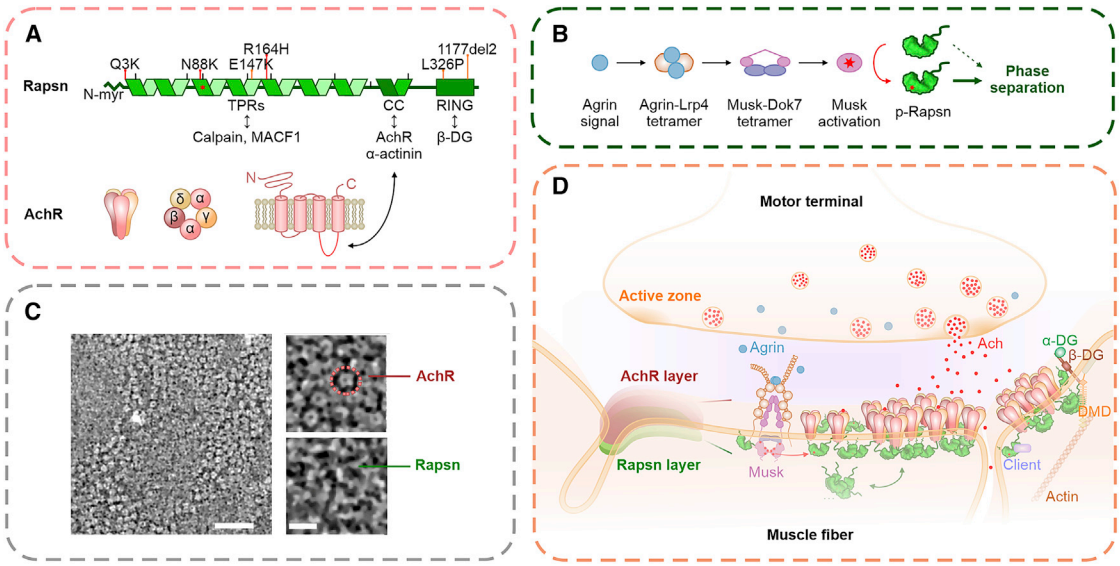

Figure 1. Clustering of AchRs in NMJ by phase-separated Rapsn condensates

(A) Domain organization of Rapsn and the pentameric assembly of AchR. Tyr86 in the 3rd TPR is marked by a red star. Mutations that weaken Rapsn condensate formation (red lines) or disturb the recruitment of various clients (orange lines) as shown by Xing et al. (2021) are indicated.

(B) Molecular mechanisms of Musk-promoted Rapsn phase separation by Agrin-Lrp4-Musk signaling.

(C) Tomographic slice cross-sectioning the extracellular domain of AchRs. (Upper right) Magnified view showing pentameric AchRs are densely packed with each other. (Lower right) A tomographic slice with the same *xy* coordinates as in the upper panel but taken 8.2 nm under it toward the cytoplasm. Scale bars: 50 nm (left panel); 10 nm (right panels). Reproduced from Zuber and Unwin (2013).

(D) Schematic diagram showing Agrin-induced and potential DAP-stabilized AchR clustering via phase separation of Rapsn.

AchRs were observed in negative EM staining of *Torpedo* membrane preparations (Sealock, 1982). Higher-resolution cryo-electron tomographic micrographs of *Torpedo* membrane preparations revealed that AchRs form condensed clusters with different sizes (Figure 1C), consistent with phase separation of the receptors on the plasma membranes. Interestingly, a layer of inter-connected Rapsn molecules condensed but rather amorphously distributed right beneath the dense AchR clusters via direct binding of Rapsn to the receptors (Figure 1C) (Zuber and Unwin, 2013).

The study by Xing et al., together with a series of recent studies in brain synapses (Bai et al., 2021; Zeng et al., 2018), has provided compelling evidence showing that phase-separation-mediated clustering of neurotransmitter receptors and formation of postsynaptic molecular assemblies are common mechanisms for both brain and muscle synapses. Via phase separation, scaffold proteins beneath the postsynaptic plasma membranes can concentrate a large amount of neurotransmitter receptors within a small area of membrane surface for specific and effective signaling. These scaffold proteins, often lacking intrinsically disordered sequences, interact with each other and with neurotransmitter receptors forming highly condensed postsynaptic molecular assemblies via specific and multivalent molecular interactions. Various signaling proteins in each type of these synapses can be enriched into the postsynaptic condensates as clients via binding to scaffold protein(s). The scaffold-protein-mediated clustering of neurotransmitter receptors via phase separation in brain synapses and NMJs are regulated processes, as the cluster sizes and densities can be bidirectionally regulated via enhancing or dispersing the phase separation processes. The postsynaptic scaffold protein condensates formed in glutamatergic synapses are very different from the ligand-gated pentameric receptors in NMJs and inhibitory synapses in the brain. In glutamatergic synapses, multiple scaffold proteins such as PSD-95, SAPAP, Shank, and Homer interact with each other, forming a layered organization beneath the receptor-containing membranes. Therefore, each glutamatergic synapse contains a highly condensed postsynaptic density with a thickness of 40–50 nm. Rapsn and gephyrin appear to be the only key scaffold proteins required for clustering receptors in NMJs and inhibitory synapses, respectively. Accordingly, postsynaptic densities in NMJs and inhibitory synapses each form a thin layer of sheet-like condensates beneath the postsynaptic plasma membranes with a thickness of only ~5 nm (Liu et al., 2020). Thus, neither NMJs nor inhibitory synapses contain obvious electron-dense postsynaptic thickenings under conventional EM.

The study by Xing et al. has provided compelling evidence showing the molecular mechanism underlying Rapsn-driven AchR clustering in NMJs (Figure 1D). The study also leaves some questions that remain to be answered. One pressing question is the role of AchRs in the formation of the AchR/Rapsn condensates. Each AchR contains 2–3 Rapsn binding sites (Zuber and Unwin, 2013). Thus, each AchR pentamer can increase the valency of the AchR/Rapsn complex. Additionally, AchRs can further dimerize via

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its δ subunit. Therefore, although AchRs alone on plasma membranes are not sufficient to form condensed clusters via phase separation, the receptors are likely to promote phase separation of Rapsn upon formation of the AchR/Rapsn complex via augmentation of the valency of the molecular network. Consistent with this hypothesis, AchRs are required for clustering Rapsn in myotubes (Bruneau et al., 2008). The role of the dystrophin-associated protein (DAP) complex, a multiprotein complex assembled on muscle membranes and essential for maintaining muscle strength, in the AchR/Rapsn cluster formation is also a worthy question. The RING domain of Rapsn specifically interacts with the cytoplasmic tail of β-dystroglycan. The DAP complex is a highly condensed molecular network formed by multiple scaffold proteins including dystrophin, syntrophin, utrophin, dystrobrevin, and transmembrane protein β-dystroglycan. It will be interesting to investigate how the AchR/Rapsn condensates and the DAP condensates may interact and regulate each other in NMJs. Furthermore, AChR/Rapsn clusters are concentrated at the shoulder areas of NMJ fold crests and aligned with presynaptic active zones (Figure 1D). Such a nanoscale organization has also been reported in synapses within the central nervous system, thus representing a conserved mechanism for effective synaptic transmission. Adhesion molecules are believed to play essential roles in *trans*-synaptic alignment and restricting the diffusion of receptors. Whether the DAP complex or other *trans*-synaptic adhesion molecules function to align the active zone condensates and AchR/Rapsn condensates in NMJs needs to be evaluated in future research.

The study by Xing et al. (2021) represents a new starting point for studying NMJs through the lens of phase separation. Clustering of the AchR/Rapsn complex via Rapsn-driven phase separation also provides a new paradigm for elucidating mechanisms of neurological disorders caused by mutations of NMJ proteins.

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Aligning one's sights: The pulvinar provides context for visual information processing

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The pulvinar (lateral posterior [LP]), like other higher-order thalamic nuclei, receives input from—and sends output to—multiple neocortical structures. In this issue of *Neuron*, Blot et al. (2021) demonstrate that LP integrates multimodal inputs to put visual information in context.

Converging data from studies of the visual system have supported a new interpretation of thalamic function. Rather than simply relaying information between sensing structures and the neocortex, thalamic neurons can transform this information in important ways. For example, neurons in the lateral geniculate nucleus (LGN), once thought to be simple relays between retinal ganglion cells and primary visual cortex (V1; Figure 1B), have response modulation by attentional state (Schneider, 2011; McAlonan et al., 2008; Dhruv and Carandini, 2014) and past visual experience (Durkin et al., 2017; Huh et al., 2020). These