

## Review

# Liquid-Liquid Phase Separation in Neuronal Development and Synaptic Signaling

Xiandeng Wu,<sup>1</sup> Qixu Cai,<sup>1</sup> Zhe Feng,<sup>1,2</sup> and Mingjie Zhang<sup>1,3,\*</sup><sup>1</sup>Division of Life Science, State Key Laboratory of Molecular Neuroscience, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China<sup>2</sup>Institute for Advanced Study, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China<sup>3</sup>Center of Systems Biology and Human Health, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China\*Correspondence: [mzhang@ust.hk](mailto:mzhang@ust.hk)<https://doi.org/10.1016/j.devcel.2020.06.012>

## SUMMARY

Formation of biomolecular condensates that are not enclosed by membranes via liquid-liquid phase separation (LLPS) is a general strategy that cells adopt to organize membraneless subcellular compartments for diverse functions. Neurons are highly polarized with elaborate branching and functional compartmentalization of their neurites, thus, raising additional demand for the proper subcellular localization of both membraneless and membrane-based organelles. Recent studies have provided evidence that several protein assemblies involved in the establishment of neuronal stem cell (NSC) polarity and in the asymmetric division of NSCs form distinct molecular condensates via LLPS. In synapses of adult neurons, molecular apparatuses controlling presynaptic neurotransmitter release and postsynaptic signaling transmission are also likely formed via LLPS. These molecular condensates, though not enclosed by lipid bilayers, directly associate with plasma membranes or membrane-based organelles, indicating that direct communication between membraneless and membrane-based organelles is a common theme in neurons and other types of cells.

## INTRODUCTION

Compartmentalized distribution and subsequent local concentration of different molecular components allow eukaryotic cells to segregate various cellular processes into distinct sub-cellular regions. Such sub-cellular compartmentalization is vital for intra- and inter-cellular signaling as well as for development of animal tissues and organs. The best-known mode of cellular compartmentalization is to use lipid membrane bilayers as physical barriers to enclose a certain cellular space containing defined molecular components. By installing various proteins embedded in or associated with the surface of lipid membranes, these membrane-separated cellular compartments can interact and communicate with each other to support the wellbeing of cells.

Another distinct cellular compartmentalization mode has recently been recognized as a widely used strategy for diverse cellular functions including cell polarity establishment and maintenance, cell signaling, cell and organ development, cell survival, and aging, among others (Su et al., 2016; Zeng et al., 2016; Lin et al., 2015; Molliex et al., 2015; Murakami et al., 2015; Patel et al., 2015; Kim et al., 2013; Han et al., 2012; Kato et al., 2012; Brangwynne et al., 2011, 2009; and also see Chen et al., 2020; Banani et al., 2017; Shin and Brangwynne, 2017 for recent reviews). This cellular compartmentalization mode does not use membrane bilayers to enclose a certain molecular assembly, but instead harnesses the self-condensation properties of molecular networks formed by cellular biopolymers including proteins and nucleic acids in aqueous cellular milieu. Since this class of cellular compartments are not enclosed by lipid membranes

and are formed via a physical chemistry process known as liquid-liquid phase separation (LLPS), these compartmentalized molecular assemblies are often referred to as membraneless organelles or biological condensates in the literature (Banani et al., 2017; Shin and Brangwynne, 2017). A prominent feature of LLPS-mediated biological condensate formation is that sub-cellular compartments form autonomously (i.e., without requiring energy input) with defined molecular components at very high concentrations and without needing physical barriers to separate the condensed phase from the dilute bulk aqueous phase. Almost all demonstrated or inferred cellular functions of biological condensates reported in the literature are mechanistically rooted in the above feature.

Neurons are extremely polarized morphologically and functionally throughout their birth, growth, and adulthood. In the early brain development, neuroepithelial cells (or neuronal stem cells, NSCs) form polarized sheets with apical-basal polarity. Proper timing and balance of symmetric and asymmetric divisions of neuroepithelia determine the right number and the right types of different neurons in the animal brain (Holguera and Desplan, 2018; Doe, 2017; Taverna et al., 2014; Knoblich, 2010; Kriegstein and Alvarez-Buylla, 2009). A new-born neuron will migrate and grow into an extremely polarized cell with one single axon and multiple dendrites before integrating into a certain circuitry system. A mature neuron will need to keep its overall morphology intact throughout the entire lifespan of an organism for healthy living, but at the same time it will also need to constantly alter its local connections with target cells (i.e., synapses) to support functional plasticity of the animal brain. Due to their unique

polarity features, neurons can take cellular compartmentalization to an extreme with their soma occupying only a tiny fraction of the total cell volume and axonal and dendritic processes projecting centimeters or even meters away from their somas. Accordingly, neurons possess their own unique cellular compartmentalization strategies in addition to the ones shared among different cell types (Chen et al., 2020; Südhof, 2017; Silberer et al., 2016).

In this review, we will focus on the recent developments on the LLPS-mediated cellular compartmentalization of neurons during their development and throughout their adulthood. We summarize recent findings suggesting that the LLPS-mediated formation of molecular assemblies is critical for the polarity of neuroepithelial cells and for the asymmetric division of NSCs. We discuss how LLPS may be an ideal mechanism through which mature neurons form highly compartmentalized yet highly dynamic molecular assemblies critical for neurotransmitter release in presynaptic boutons and for signal processing in the postsynaptic densities (PSDs) of neuronal synapses. Due to the space limit, this review will not cover biological condensate formation during the aging and degeneration of neurons, but readers can turn to recent reviews on this topic including one in this issue of *Developmental Cell* (Nedelsky and Taylor, 2019; Ryan and Fawzi, 2019; Wolozin and Ivanov, 2019; Chuang et al., 2018; St George-Hyslop et al., 2018).

### PHASE SEPARATION IN NEURONAL DEVELOPMENT

In this section, we discuss the role of LLPS in neural development with a focus on the asymmetric division of neuroepithelial cells. Asymmetric cell division is a fascinating process in the context of neural development because in a single cell, multiple condensates of various types control cell polarity, orchestrate cell division, organize lateral interactions, and so on. Understanding how a cell organizes these phase-separated condensates in different subcellular regions of the cell to guide asymmetric cell division and how the different condensates interact with one another is a challenging problem.

Despite vast differences both in the number and in the morphology of cells in the nervous system, the operating mechanisms governing nervous system development are strikingly conserved throughout evolution. For example, the nervous systems of *Drosophila* and mice, which are representative of invertebrates and mammals, respectively, originate from simple polarized neuroepithelial sheets. These polarized epithelia can proliferate via symmetric divisions or by dividing asymmetrically to generate neuronal progenitors and eventually different types of neurons or glia (Figure 1) (Holguera and Desplan, 2018; Venkei and Yamashita, 2018; Taverna et al., 2014; Knoblich, 2010; Kriegstein and Alvarez-Buylla, 2009). Two cellular processes are vital during nervous system development: the development and maintenance of polarity of neuroepithelial cells and neuro-progenitors and the alignment of the mitotic spindle axis with the cell polarity axis during cell division. Extensive studies in the past several decades have revealed that distinct molecular complexes are required for these two processes (Wen and Zhang, 2018; Bergstralh et al., 2017; di Pietro et al., 2016; Taverna et al., 2014; Knoblich, 2010; Siller and Doe, 2009). Emerging evidence in the last few years indicate that some of these complexes may be assem-

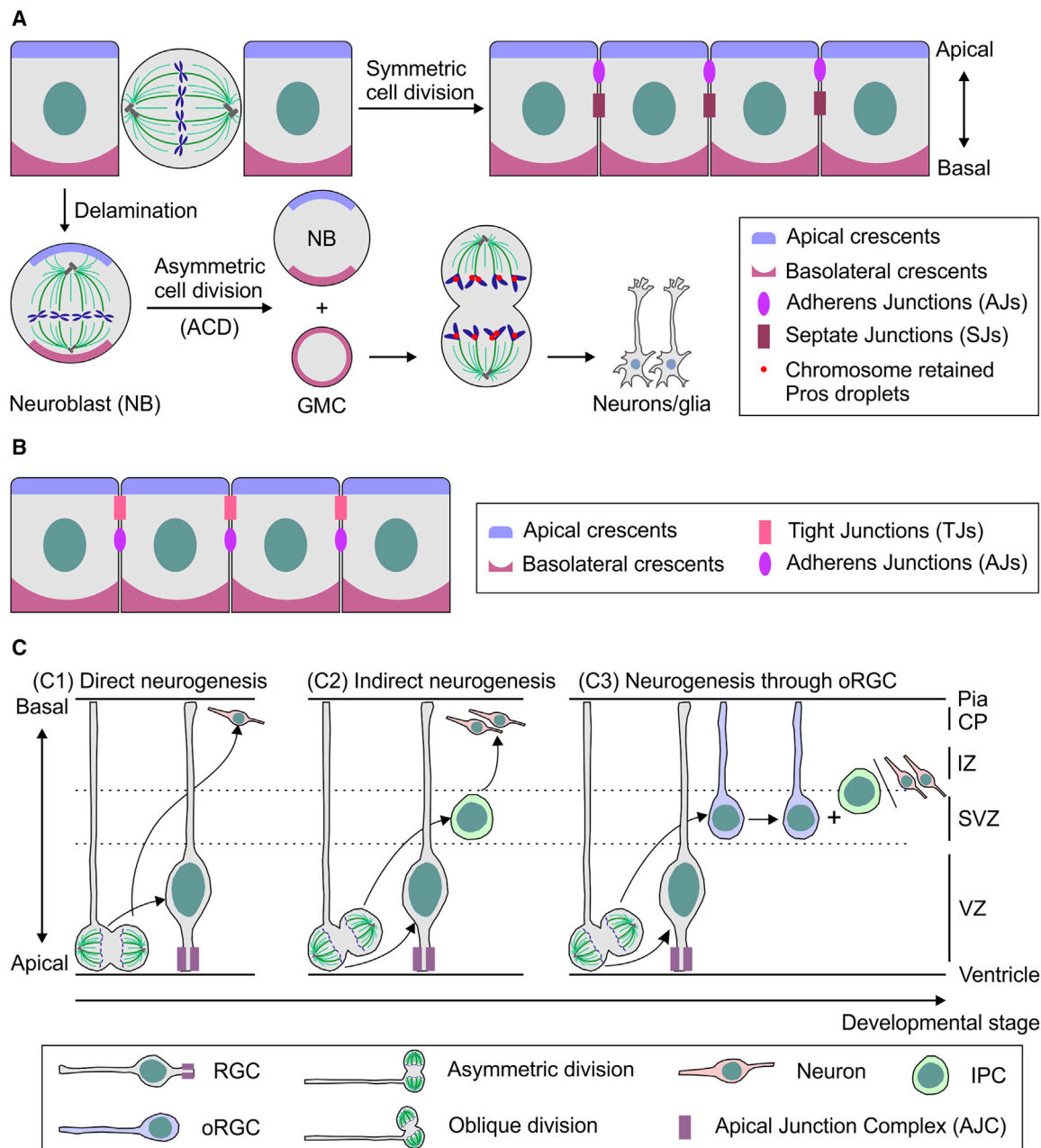
bled via LLPS to form condensed molecular networks in distinct cellular compartments (Liu et al., 2020a, 2020b; Kono et al., 2019; Shan et al., 2018).

Here, we take type I neuroblasts (NBs) as the example of NSCs for nervous system development in *Drosophila*. Neuroepithelial cells form a polarized sheet in the embryonic neuroectoderm with each cell having a cuboid shape and an apical-basal axis perpendicular to the cell sheet plane (Figures 1A and 1B). The apical and basolateral membrane domains of *Drosophila* neuroepithelia are demarcated by junctional protein complexes known as adherens junctions (AJs) and septate junctions (SJs, corresponding to tight junctions [TJs] in mammalian epithelia). A delaminated NB enters cell cycle and divides asymmetrically. During mitosis, this NB establishes another distinct mode of apical-basal polarity, where several sets of protein complexes become highly concentrated and unevenly distributed beneath either the apical or the basal cortical membrane forming crescent-shaped condensates under a fluorescence microscope (Figure 1A). The apical protein condensates directly interact with astral microtubules to align the mitotic spindle axis in parallel with the cell polarity axis. Therefore, the cleavage plane of the NB division lies in parallel with the neuroepithelial sheet, resulting into two distinct daughter cells: a larger NB capable of self-renewal and a smaller ganglion mother cell (GMC), which can divide once more to form neurons or glia (Figures 1A and 1C).

A hallmark of the asymmetric division of NBs is the formation of several sets of highly condensed protein complexes beneath the basal (the Numb-Pon and Mira-Pros complexes) (Lu et al., 1998; Ikeshima-Kataoka et al., 1997; Shen et al., 1997; Rhyu et al., 1994; Doe et al., 1991; Uemura et al., 1989) or the apical membrane (e.g., the Baz-Par6-aPKC, Pins-Mud-Gα<sub>i</sub> complexes) (Bowman et al., 2006; Schaefer et al., 2000; Wodarz et al., 2000; Yu et al., 2000; Kuchinke et al., 1998) (Figure 2A). The assembly and local condensation of these complexes are long known to be dynamic—they start to form at the onset of interphase, become most condensed during metaphase, and dissolve after telophase during mitosis (Taverna et al., 2014; Knoblich, 2010; Gönczy, 2008). Fluorescence recovery after photobleaching (FRAP) assays showed quite early on that Pon, Numb, and Mira concentrated at the basal membrane crescent, as well as Par6 condensed within the apical membrane crescent, can exchange with their counterparts in the dilute cytoplasm in living NBs or in *C. elegans* embryos (Goehring et al., 2011; Erben et al., 2008; Mayer et al., 2005; Lu et al., 1999). These observations indicate that the apical and basal protein complexes in NBs can form highly condensed assemblies that are not enclosed by membranes; a large protein concentration gradient exists between the condensate phase and the cytosolic phase, with an obvious boundary in between; proteins can freely diffuse between the two phases; and the formation or dispersion of each of these condensates is modulated in a cell cycle-dependent manner. All these features imply that these condensed protein assemblies may be formed via LLPS.

### LLPS-Mediated Formation of the Basal Numb-Pon Complex

A recent study has provided direct biochemical evidence that the Numb-Pon complex can undergo phase separation *in vitro* (Shan et al., 2018). The authors discovered that the Numb PTB domain,



**Figure 1. Cell Polarity and Asymmetric Cell Division in Neuronal Development**

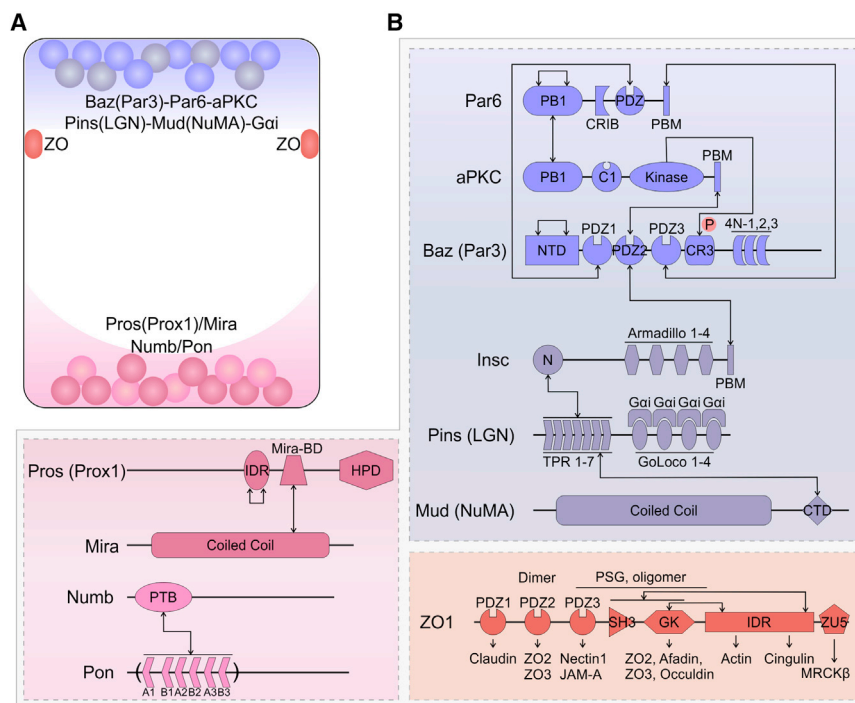
(A) Schematic diagrams showing the asymmetric cell division of a *Drosophila* NB. The apical and basolateral crescents, AJs, and SJs are likely organized through LLPs.

(B) Polarity of cuboidal mammalian epithelial cells.

(C) Schematic diagrams showing neurogenesis of the mouse neocortex. (C1) At the early developmental stage, each RGC divides asymmetrically to produce a self-renewing RGC and a neuron migrating to the basal cortical plate (CP). (C2) Each RGC indirectly generates neurons via first producing an intermediate progenitor cell (IPC) in the subventricular zone (SVZ) through oblique division. (C3) At the late developmental stage, the RGCs divide obliquely to generate outer RGCs (oRGCs) located at the SVZ. Each oRGC divides symmetrically to self-renew and asymmetrically to generate an IPC or two neurons.

using two distinct surfaces, binds to a bipartite motif containing the canonical “NxxF” sequence and a previously uncharacterized “NP[F/Y]E[V/I]xR” sequence arranged in tandem. Interestingly, Pon contains three such bipartite motifs at its N-terminal end (Figure 2B), suggesting the potential involvement of multivalent interactions in forming the Numb-Pon complex (Li et al.,

2012). When the Numb PTB domain was mixed with a Pon fragment containing the three bipartite motifs, the complex underwent phase separation, which was largely compromised when the protein interaction was weakened, or the valency was decreased. In larval brains, treatment with 1,6-hexanediol dispersed the basal condensation of Pon and Numb in dividing



**Figure 2. Molecular Assemblies form LLPS-Mediated Condensates in a Polarized Epithelial Cell**

(A) Schematic diagram showing protein condensates in a polarized epithelial cell.

(B) Domain organization and interaction network of protein complexes mediating the establishment and maintenance of cell polarity in specific subdomains. Interactions between proteins within each network and self-association of protein domains are denoted by two-way arrows. Phosphorylation and other types of bindings are indicated by one-way arrows.

due to the limited anchoring capacity of the basal Mira (Liu et al., 2020a). The chromosome retention behavior of Pros depends on a 59-residue fragment (aa812-870) at the center of the protein (Figure 2A), which is located outside the basal membrane localization domain. Interestingly, this 59-residue fragment by itself can undergo phase separation driven by the typical intrinsically disordered region (IDR) sequence. Deleting this fragment or replacing the five aromatic residues within the fragment impaired the

NBs. The condensates were restored after washing out the chemical. This hexanediol sensitivity provides evidence supporting possible LLPS of the Pon-Numb complex *in vivo*. In *Drosophila* larval NBs where WT Pon was replaced by mutants defective in phase separation with Numb, the basal localization of Numb was abolished whereas the basal condensation of these Pon mutants remained intact. This is consistent with earlier results that the C-terminal end of the protein is essential for Pon condensation at basal membranes (Lu et al., 1999). Thus, it appears that, different from the Numb and Pon binary system *in vitro*, Numb is not a driver but a client to co-condensate with Pon in NBs. The threshold concentration needed for the Numb PTB and Pon mixture to undergo LLPS *in vitro* was quite high (at least a few  $\mu$ M). This may be partly due to the fact that the majority of sequences located at the N- and C-terminal ends of the PTB domain were not included. In addition, the ~400-residue C-terminal part of Numb (and Numb1 in mammals) is predicted to be unstructured and particularly enriched with Pro and Gln, a sequence feature that is known to promote LLPS of proteins (Wang et al., 2018; Pak et al., 2016).

### LLPS-Mediated Basal Condensation of the Mira-Pros Complex

The localization and concentration of the Mira-Pros complex at the basal membrane cortex of NBs is critical for the smaller GMC to differentiate into neurons (Figure 1A). Mira is rapidly degraded in GMCs, and consequently, its binding partner Pros, a transcription factor, is released into nuclei to drive neuronal differentiation (Choksi et al., 2006; Ikeshima-Kataoka et al., 1997; Shen et al., 1997). A very recent study discovered that overexpression of GFP-Pros led to the formation of chromosome-localized puncta in addition to the basal crescent of Pros in mitotic NBs and these NBs prematurely differentiated into GMCs, perhaps

phase separation, eliminated the chromosome retention, and compromised the tumor suppression activity of Pros. Importantly, replacing this 59-residue fragment with another IDR sequence capable of LLPS or even with a leucine zipper dimerization domain could rescue all the above phenotypes. Pros, via its 59-residue chromosome retention fragment, can promote LLPS of HP1a and consequently drive the expansion of the trimethylated H3K9 heterochromatin domain and Pros-mediated suppression of target genes. One of the Pros targets is *Mira*, explaining that, once GMCs are committed to differentiation, a feedback loop is established to ensure a low level of Mira in GMCs (Choksi et al., 2006). It is possible that the Mira-Pros condensates at the basal membrane cortex of NBs may also be formed via LLPS, as both proteins contain hallmark features for phase separation (Banani et al., 2017; Shin and Brangwynne, 2017). The formation of different condensates of Pros in different cellular compartments, at various time points, and for distinct activities of Pros provides a nice example showing the functional roles of molecular assembly formation mediated by phase separation in neuronal development and likely in many other cellular processes.

### The Par Complex Condensates

The Par complex (Baz-Par6-aPKC in *Drosophila* and Par3-Par6-aPKC in mammals), originally discovered in *C. elegans* while screening for genes required for asymmetric division of worm embryo development (Hung and Kemphues 1999; Tabuse et al., 1998; Etemad-Moghadam et al., 1995), is highly evolutionarily conserved and known to be required for essentially all aspects of cell polarity events (St Johnston, 2018; Lang and Munro, 2017; Goehring, 2014; Rodriguez-Boulant and Macara, 2014; Knoblich, 2010; Goldstein and Macara, 2007). The apical-basal polarity axis of NBs is set up by the Par complex, which in turn interacts with the Pins-Mud complex to orientate the mitotic



spindle and determines the basal localization of the Numb-Pon, Mira-Pros complexes via aPKC-mediated phosphorylation (St Johnston, 2018; Knoblich, 2010; Gönczy, 2008) (Figure 2). The apical localization of the Par complex itself is likely directly inherited from the neuroectoderm when NBs delaminate. Imaging studies of fixed or living fly embryos with endogenous or ectopically expressed Baz have shown that the Par complex typically forms a crescent-shaped condensate beneath the apical membrane of NB. Most of these Par complex images were acquired via confocal microscopes with the focal plane near parallel to the apical-basal axis of NBs. Interestingly, when the imaging focal plane is perpendicular to the apical-basal axis and focused on the apical side of NBs, each component of the Par complex showed a patch-like distribution with clearly visible individual puncta within the patches (Liu et al., 2020b). Such patch-like Par complex formation has also been observed when *Drosophila* S2 cells were induced to polarize with overexpression of Baz, and the small puncta are dynamic and can fuse into larger ones (Kono et al., 2019). All these observations suggest that the Par complex in NBs or in induced polar S2 cells may be formed via LLPS.

The Par complex formation involves extensive intermolecular interactions among the three proteins (Holly et al., 2020; Wang et al., 2012; Hirano et al., 2005; Joberty et al., 2000; Lin et al., 2000; Wodarz et al., 2000; Izumi et al., 1998) and oligomerization of the N-terminal domain (NTD, also known as CR1) of Baz or Par3 (Feng et al., 2007; Benton and Johnston, 2003) (Figure 2B). The multivalent network of interactions suggests that phase separation may contribute to the formation of the Par complex (Li et al., 2012). Indeed, Liu et al. (Liu et al., 2020b) showed that the N-terminal half of Par3 containing NTD-PDZ1-3 (Par3N) could undergo LLPS, although at fairly high concentration (a few  $\mu$ M). Formation of the Par6-Par3N complex lowered the phase separation threshold concentration to a sub-micromolar level. The authors showed that the Par3 NTD-mediated complex multimerization and the direct binding between Par6 and Par3 are critical for the complex to undergo LLPS *in vitro* and in heterologous cells. Replacing endogenous Baz or Par6 in *Drosophila* NBs with Baz or Par6 mutants that are defective in LLPS compromised the apical condensation of Baz and Par6 and led to defects in neuronal differentiation of the larval brain. The monomeric mutant of Baz/Par3 NT showed reduced phase separation with Par6, and deletion of its NTD disrupted the formation of condensed droplet-like structures in *Drosophila* S2 cells (Kono et al., 2019). Interestingly, when the Baz NTD was replaced with the IDRs of fused in sarcoma (FUS; aa1-141 and aa1-214), the resulting Baz chimeras could rescue phase separation of the Par3-Par6 complex in heterologous cells (Liu et al., 2020b). The *FUS*<sub>1-214</sub>-baz transgenic flies were not viable, presumably due to the very strong LLPS of the Baz chimera arising from the *FUS*<sub>1-214</sub> fragment (Murakami et al., 2015; Patel et al., 2015). Instead, a shorter FUS fragment (aa1-141) with a lower LLPS capacity was used. Replacing the endogenous Baz with *FUS*<sub>1-141</sub>-baz led to the apical condensate formation of the Baz chimera, restored the apical crescent formation of Par6 and aPKC as well as the basal localization of Mira in NBs, and largely rescued the smaller brain phenotype caused by the NTD deletion of Baz. These findings strongly indicate that LLPS, instead of the classical protein-protein interactions, is critical for the apical condensation of the Par complex in NBs and the Par complex-regulated brain development in *Drosophila*.

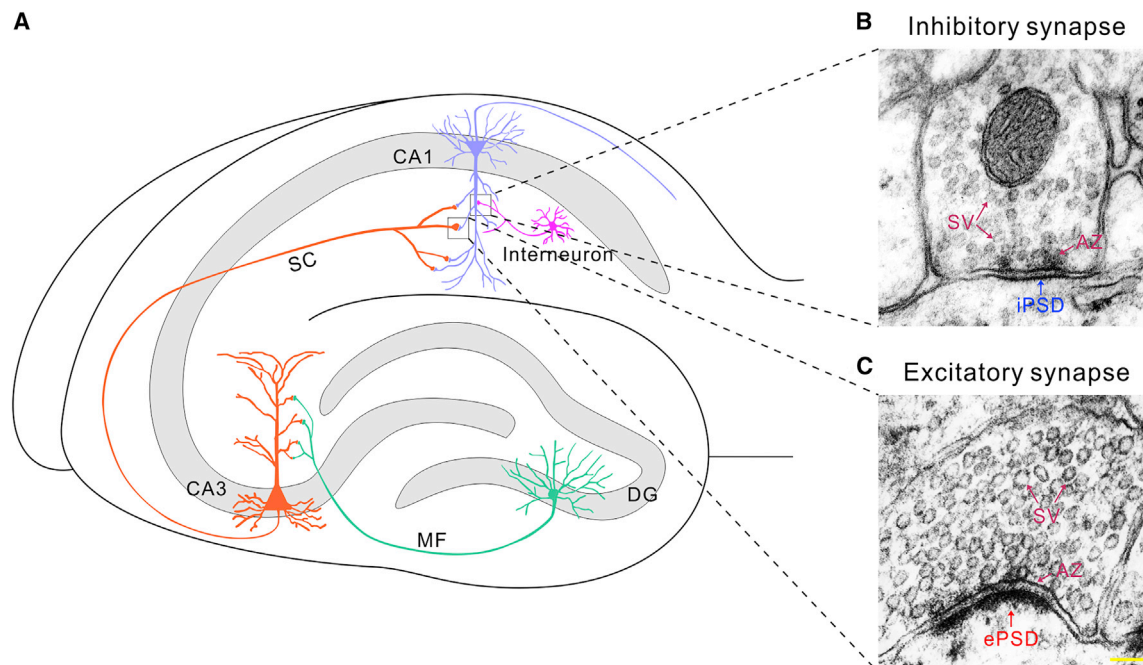
### Formation of TJ Condensates

The apical and basolateral membrane domains of polarized mammalian epithelia (e.g., neuroepithelia in ectoderm) are separated by TJs and AJs (Figure 1B). Zona occludens (ZO) proteins ZO1-3 are scaffold proteins belonging to the membrane-associated guanylate kinase (MAGUK) family that are critical for the assembly of TJs by forming dense clusters right beneath the apical membrane domain (Figure 2B) (Zhu et al., 2016; Fanning and Anderson, 2009; Umeda et al., 2006; Stevenson et al., 1986). ZO1 is also a critical component in the assembly of the apical junction complex (AJC) at the endfeet of radial glial cells (RGCs, which are NSCs in mammalian cortex development, Figure 1C). Beutel et al. (Beutel et al., 2019) showed that purified ZO proteins can undergo phase separation via multivalent inter- and intra-molecular interactions. The ZO1 condensate can selectively enrich ZO1 TJ-binding partners, such as afadin and claudins. The ZO1 condensate formation is negatively regulated by ZO1 phosphorylation. By tagging the endogenous ZO1 with a fluorescent tag, the authors showed that ZO1 in polarized MDCK cells appears to phase separate using the continuous TJ belts as the template. Depolymerization of TJ actin filaments by latrunculin-A (LatA) in polarized MDCK cells led to the formation of many droplet-like puncta, which were co-condensed with cingulin and actin. Washing LatA out restored the continuous ZO1 condensation on the TJ belts. In 3D MDCK cysts grown in Matrigel, ZO1 mutants defective in LLPS displayed leaky TJ barriers. In a back-to-back paper with Beutel et al. (Beutel et al., 2019), Schwyer et al. (Schwyer et al., 2019) showed that during early development of zebrafish embryos ZO1 could form condensed assemblies via phase separation and these ZO1 condensates were transported by actomyosin flow toward and incorporated into TJs via binding to actin filaments. Together, these two studies revealed that formation of highly concentrated ZO1 condensates via LLPS is critical for organizing large TJ assemblies, which are in turn critical for polarity development and maintenance of epithelial cells. LLPS-mediated molecular assembly formation may even be a general mechanism for forming other inter-cellular junctional structures, such as AJs in epithelial cells and trans-synaptic adhesion assemblies in neurons.

### PHASE SEPARATION IN DEVELOPED NEURONS

In this section we focus on LLPS in the assembly and organization of synapses in developed neurons. We summarize recent findings on the phase separation-mediated formation of PSDs in excitatory synapses as well as on LLPS in synaptic vesicle clustering and on active zone formation in presynaptic boutons. These examples highlight the exciting implications of LLPS in highly polarized and extremely compartmentalized neurons.

Asymmetric division of NSCs and subsequently the combined asymmetric division and symmetric division of RGCs, in spatially and temporally defined manners, generate billions of neurons with diverse morphologies and functions in mammalian brains (Figure 1C). These neurons grow and migrate into specific regions and form elaborate networks in the adult brain (Figure 3A). In order to keep its morphological and functional polarity, a neuron must solve a basic logistic challenge of localizing different molecular components to and concentrating them in distinct compartments (e.g., dendritic protrusions or axon terminal boutons) that are far



**Figure 3. Complex Morphologies and Intricate Connections of Neurons**

(A) Schematic drawing showing an example of circuitry formation in mouse hippocampal neurons. Dentate gyrus (DG) granule cells project to CA3 pyramidal neurons via the mossy fibers (MFs). The CA3 pyramidal neurons then project to CA1 pyramidal neurons via the Schaffer collateral (SC). The axons of CA1 pyramidal neurons project to other brain regions. Interneurons project to the soma or dendritic shafts of the pyramidal neurons.

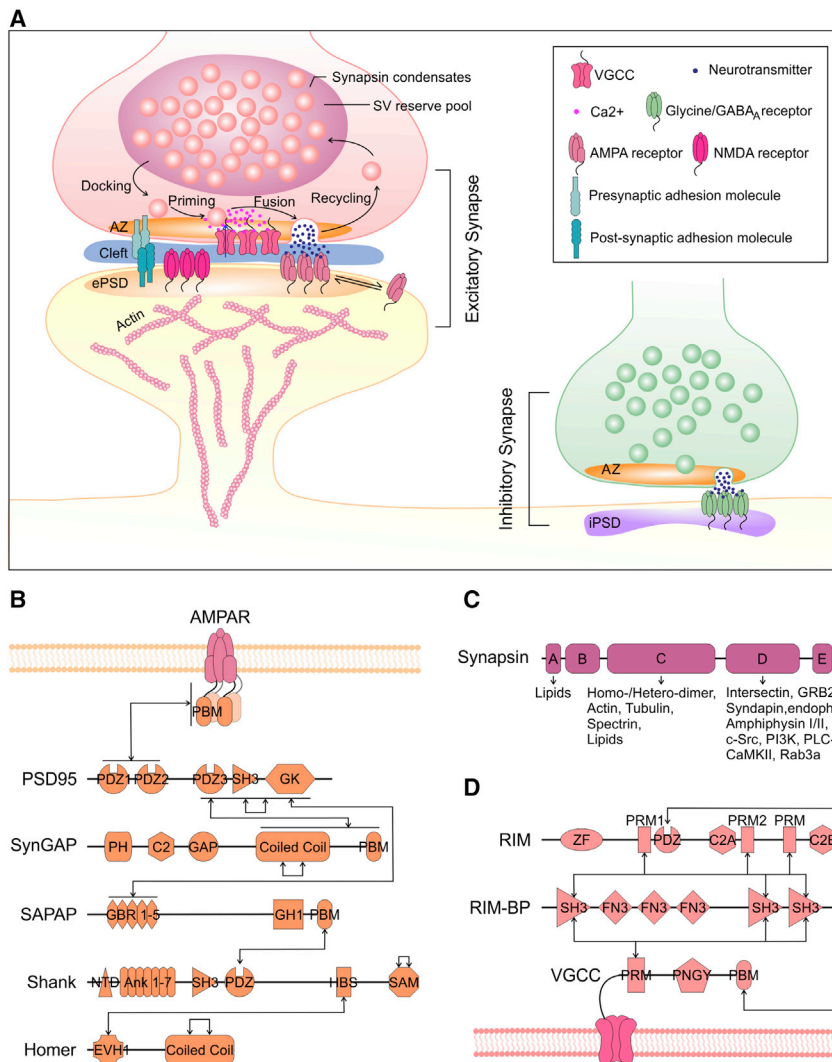
(B and C) Electron microscope images of an inhibitory and an excitatory synapse observed in a hippocampal pyramidal neuron. Scale bar, 200 nm. Images are adapted from SynapseWeb (<https://synapseweb.cim.utexas.edu/>).

away from the soma where most of these molecules are made or degraded. Each neuron takes thousands of both excitatory and inhibitory inputs from other neurons (and occasionally from itself) via the formation of micron-sized physical connections known as synapses, which are the most basic units enabling neurons to communicate with each other. Regardless of whether it is excitatory or inhibitory, each synapse is asymmetric and contains three distinct sub-compartments: a presynaptic terminal responsible for neurotransmitter release, a postsynaptic apparatus for receiving and interpreting the signals carried by neurotransmitters, and a synaptic cleft enriched with cell adhesion molecules to connect pre- and postsynaptic plasma membranes and to align molecular machineries responsible for neurotransmitter release (Figures 3B and 3C) (Chen et al., 2020; Südhof, 2018; Biederer et al., 2017). Under the electron microscope (EM), the bulk of neurotransmitter-containing synaptic vesicles (SVs) are clustered densely near the presynaptic membrane (the so-called reserve pool of SVs). Only a few SVs are docked to the presynaptic active zone for rapid transmitter release induced by an action potential. The depleted SVs in the active zone after transmitter release can be rapidly replenished by SVs from the reserve pool (Alabi and Tsien, 2012; Südhof, 2012). At the postsynaptic side of synapses, a thick electron-dense molecular layer known as the PSD is formed beneath the synaptic membrane. Excitatory synapses tend to have thicker PSDs than inhibitory synapses (Figures 3B and 3C). PSDs are molecular assemblies formed by numerous proteins with very high concentrations. Both the volumes and molecular constituents of PSDs are dynamically modulated by synaptic activities. A series of recent studies have provided evidence

that LLPS is directly involved in the formation of SV clusters and active zone molecular assemblies in the presynaptic boutons as well as the assembly and activity-dependent modulation of PSDs (Pechstein et al., 2020; Wu et al., 2019; Milovanovic et al., 2018; Zeng et al., 2019, 2018, 2016).

### LLPS in PSD Formation and AMPA Receptor Synaptic Transmission

Activity-dependent modulation of synapse formation and elimination and synaptic strength are regarded as one of the most important steps in learning and memory. The physical size of a PSD is positively correlated with the strength of a synapse (Berry and Nedivi, 2017; Nishiyama and Yasuda, 2015). For example, excitatory PSDs (ePSDs) are assembled by a set of highly abundant multidomain scaffold proteins including PSD-95 (Kornau et al., 1995), synapse-associated protein 90/postsynaptic density-95-associated protein (SAPAP) (Kim et al., 1997), Shank (Naisbitt et al., 1999), and Homer (Xiao et al., 1998), forming highly dense molecular condensates attached to but not enclosed by the postsynaptic membranes (Figures 4A and 4B) (Chen et al., 2020; Zhu et al., 2016; Feng and Zhang, 2009). Via PSD-95-mediated binding to transmembrane AMPA receptor (AMPA) regulatory proteins (TARPs), which are AMPAR auxiliary subunits, ePSDs can promote the number and density of AMPARs on PSD membranes and thus strengthen synapses (Nicoll et al., 2006). Via Homer- and Shank-mediated binding to the actin cytoskeleton away from the postsynaptic membrane, ePSD can support or even promote structural stability of a synapse. Additionally, different enzymes (e.g., SynGAP [Chen



**Figure 4. Phase Separation at the Synapse**

(A) Schematic diagram of LLPS-mediated condensates at synapses. Synapsin condensates, active zone condensates, hypothetical synaptic cleft condensates, ePSD condensates, and speculated iPSD condensates are indicated.

(B–D) Domain organization and interaction network of major scaffold proteins mediating phase separation of the ePSD (B), synapsins (C), and the active zone (D). Interactions between proteins within each network and self-associations of domains are denoted by two-way arrows. Other types of binding are indicated by one-way arrows.

simplified two component system (i.e., PSD-95 and SynGAP), the authors proposed that PSDs may be autonomously assembled by major scaffold proteins via LLPS. The authors went on to test their hypothesis by trying to reconstitute PSDs using purified scaffold proteins as the core organizers (PSD-95, SAPAP1, Shank3, and Homer3; Figure 4B). They found that mixing the four-scaffold proteins, either at the molar ratio of 1:1:1:1 or at molar ratios derived from quantitative mass spectrometry- or quantitative imaging-based studies of PSDs, led to the formation of phase-separated condensates with all four proteins colocalized together (Zeng et al., 2018). Phase separation of the four-scaffold PSD system is driven by highly specific and multivalent interactions and occurs at a threshold concentration of below 1  $\mu$ M for each protein, which is lower than their physiological concentrations at the synapse (Sheng and Hoogenraad, 2007; Sugiyama et al., 2005). These condensates can recruit and enrich SynGAP and cluster the cytoplasmic tails of

et al., 1998; Kim et al., 1998) can enrich themselves into the small but spatially stable of PSDs by interacting with PSD scaffold proteins (e.g., specific binding of SynGAP to the PDZ3-SH3-GK tandem of PSD-95; Zeng et al., 2016). Upon synaptic stimulation, SynGAP is dispersed from the PSD condensates in living neurons—an example highlighting the dynamic nature of PSD assemblies (Araki et al., 2015).

In an effort to try to understand how SynGAP can be specifically and abundantly incorporated into PSDs, Zeng et al. (Zeng et al., 2016) discovered that mixing SynGAP (the C-terminal fragment containing the coiled coil and PDZ-binding motif to be precise) with the full-length PSD-95 led to the formation of condensed droplets via LLPS. Both proteins alone were highly soluble and monodispersed in solution. Disruption of the high-affinity binding between SynGAP and PSD-95 or converting the SynGAP trimer into a monomer completely abolished LLPS between SynGAP and PSD-95 and impaired PSD-95-mediated synaptic clustering of SynGAP, indicating that the highly specific and multivalent interaction between PSD-95 and SynGAP is critical for the complex to phase separate. Based on the observations from the highly

NR2B, indicating that the reconstituted PSDs have the capacity to cluster glutamate receptors and enrich synaptic enzymes. By tethering the NR2B tail tetramer to supported lipid membrane bilayers (SLBs) mimicking postsynaptic membranes, the phase separation threshold for the four-scaffold PSD system was further lowered. During the study, the authors also developed methods to measure the absolute concentration or density of each component of PSD condensates in solution or on SLBs and found that phase separation could concentrate proteins into the tiny volume of a condensed phase by up to over 1,000-fold (see also Wu et al., 2019). An interesting and likely physiologically relevant observation is that Homer 1a, a monomeric splicing variant of tetrameric Homer 1c, can disperse the reconstituted PSDs in solution or on SLBs in a dose-dependent manner, due to the monomeric Homer 1a directly competing with the tetrameric Homer 1c for binding to Shank3. Sleeping induces a massive increase in the expression of Homer 1a without obvious changes in Homer 1c, accompanied by a global down-scaling of the size of PSDs in cortical neurons of mice (de Vivo et al., 2017; Diering et al., 2017). Gephyrin, which is the key scaffold protein of the inhibitory PSD (iPSD), was not



only not enriched in the ePSD condensate, it was in fact actively excluded from the ePSD. This observation indicates that the LLPS-mediated formation of PSD condensates is highly specific and suggests that an ePSD condensate and an iPSD condensate may co-exist within a tiny dendritic spine protrusion in neurons. This reconstituted PSD system, though still much simpler than the PSDs in living neurons, not only provides strong evidence supporting the model of LLPS-mediated PSD formation but may also function as an experimental platform for understanding molecular mechanisms underlying synapse formation and regulation *in vitro*.

Given that the strength of a synapse is positively correlated with the ePSD size and the number of AMPARs in the synaptic membranes (Nishiyama and Yasuda, 2015; Hugarir and Nicoll, 2013), LLPS-mediated ePSD formation may be directly linked to AMPAR synaptic clustering. In a recent study, Zeng et al. (Zeng et al., 2019) discovered that the entire cytoplasmic tail of TARPs was involved in binding to PSD-95 with the TARP PBM binding to PDZ2 via the canonical PDZ-PBM recognition mode and an upstream Arg-rich motif bound to a negatively charged surface away from the PBM-binding groove of PDZ1. Such multivalent TARP-PSD-95 binding not only renders a strong interaction between the two proteins but also triggers LLPS of the TARP/PSD-95 complex. Membrane-tethered TARP tails can be clustered via LLPS by the ePSD mixture, which comprises PSD-95, SynGAP, SAPAP, Shank, and Homer at physiological concentrations—a finding that is reminiscent of the clustering of the AMPAR-TARP complex at PSDs in living neurons. Mutation of TARP tails impairing the multivalent interaction between TARP and PSD-95 disrupted or weakened the phase separation of the TARP and ePSD mixture. Importantly, such TARP mutants (TARP  $\gamma$ -8 to be specific) impaired AMPAR synaptic transmission and long-term potentiation in hippocampal neurons, indicating that LLPS-mediated PSD formation is functionally connected with the AMPAR synaptic clustering and transmission.

### Clustering of SVs by Synapsin Condensates

Synapsins are extremely abundant (in the order of 100  $\mu$ M or more) in presynaptic boutons (Takamori et al., 2006). Knockout of synapsins or injection of an antibody against synapsin led to the dispersion and disappearance of the reserve pool of SVs without affecting the docked SVs in active zones (Pieribone et al., 1995; Rosahl et al., 1995, and reviewed in Milovanovic and De Camilli, 2017). If an individual SV is treated as a protein molecule, SVs contain signature features of protein condensates. These features include forming dense clusters without physical barriers, SVs within and outside clusters are mobile and can exchange with each other, and SV associated proteins, such as synapsins and intersectin, are capable of forming multivalent interaction-mediated molecular networks (Milovanovic and De Camilli, 2017). Indeed, Milovanovic and colleagues (Milovanovic et al., 2018) found that synapsin I alone at a concentration of few  $\mu$ M could undergo phase separation driven by its C-terminal IDRs (Figures 4A and 4C). Intersectin, which contains five SH3 domains and binds to Pro-rich sequences in synapsin IDRs, can promote synapsin phase separation. Remarkably, when small and negatively charged synthetic liposomes resembling SVs were mixed with synapsin I, liposomes and synapsin I formed co-condensate via phase separation. EM images of a glutaraldehyde-fixed mixture containing liposomes and synapsin I showed

liposome clustering. Phosphorylation of synapsin I by CaMKII could disperse the condensates formed by synapsin I alone or by the synapsin-liposome mixture. This *in vitro* study suggests that, via LLPS, synapsins can form dense clusters and subsequently recruit SVs into the condensed phase. The formation of the synapsin I-SV co-condensate serves to cluster SVs but at the same time do not prevent the clustered SVs from exchanging with surrounding pools such as those in active zones. A very recent published paper (Pechstein et al., 2020) showed that injection of antibodies specific to the IDR region of synapsin I into living lamprey giant reticulospinal axons selectively dispersed the reserve pool of SVs, providing another evidence that synapsin I IDR-driven LLPS plays a critical role in the SV clustering *in vivo*.

### Organizing Presynaptic Active Zones by the RIM and RIM-BP Condensates

Docking and fusion of SVs in active zones require a distinct set of multidomain scaffold proteins including Rab3-interacting molecule (RIM), RIM binding protein (RIM-BP), and ELKS (Acuna et al., 2016; Wang et al., 2016; Südhof, 2012; Ohtsuka et al., 2002; Wang et al., 2000, 1997). EM images of active zones obtained from synapses under chemically fixed conditions revealed grid-like electron-dense structures known as dense projections, which largely disappeared when both RIM and RIM-BP or RIM-BP and ELKS were removed (Acuna et al., 2016; Wang et al., 2016). Super-resolution optical imaging study has also shown that RIM forms dense clusters in active zones (Tang et al., 2016). Thus, analogous to PSDs, the presynaptic active zones may also be formed by a set of scaffold proteins via LLPS. A study by Wu et al. (Wu et al., 2019) provided evidence to support this hypothesis. The study showed that a RIM and RIM-BP mixture at a concentration  $\sim 1$   $\mu$ M readily started to form condensates via LLPS. Interactions between the three SH3 domains of RIM-BP and multiple Pro-rich motifs as well as several IDRs of RIM are critical for the formation of the RIM and RIM-BP condensates (Figures 4A and 4D). Interestingly, the RIM/RIM-BP condensates can recruit and massively cluster the cytoplasmic tails of voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs) in solution or tethered to SLBs, indicating that clustering of VGCCs near the vesicle release sites is also mediated by phase separation. The study by Wu et al. indicated that presynaptic active zones may be formed by major scaffold proteins including RIM, RIM-BP, and likely others, such as ELKS and Liprins via LLPS and anchored to the presynaptic membranes via binding to proteins, such as VGCCs and LAR phosphatase. However, currently it is not clear how docked SVs are physically connected to active zones.

### COMMUNICATION BETWEEN MEMBRANELESS AND MEMBRANE-BASED ORGANELLES

Except for the condensation of Pros on chromosomes, the LLPS-mediated molecular condensates discussed above all share a common feature. Although not enclosed by lipid membranes, these condensed molecular assemblies directly interact with membrane-based compartments, such as plasma membranes or SVs. The interaction between molecular condensates and lipid membranes can occur in different ways, including direct binding of condensates to transmembrane proteins (e.g., binding of the TARP tail to PSD-95 in PSDs and the VGCC tail



to the RIM/RIM-BP complex) or binding of a protein in the condensates to lipids in membranes (e.g., synapsins binding to negatively charged lipids in liposomes). Direct interactions between membraneless compartments and membrane-based subcellular organelles or plasma membranes significantly expand modes of operations for many cellular processes in cells including neurons. For example, limited amounts of molecules will be able to concentrate into a small volume via LLPS and subsequently be localized at defined subcellular regions (e.g., beneath pre- or postsynaptic plasma membranes). It is conceivable that membraneless molecular condensates may utilize elaborate lipid membrane-based sorting and trafficking mechanisms for intracellular distribution or secretion to the extracellular space. It is also possible that certain extracellular protein assemblies may form condensates and interact with the outer plasma membranes of cells (e.g., highly condensed proteinaceous molecular assemblies in the synaptic junctions). Emerging evidence shows that membraneless molecular condensates can communicate with membrane-based compartments to regulate the functions of both types of organelles.

For example, the N-terminal half of annexin11 co-phase separates with RNA granule condensates and the C-terminal annexin repeats directly bind to PI(3)P-enriched lysosomal membranes. By doing so, annexin11 serves as an adaptor that connects membraneless RNA granules and membrane-delimited lysosomes for long-distance RNA transport in cellular processes, such as neuronal axons (Liao et al., 2019). Noda and colleagues (Yamasaki et al., 2020) showed that, in yeast selective autophagy, cargo protein Ape1 could spontaneously form condensates via LLPS. Atg19 bound to Ape1 and Atg8 via its central coiled-coil domain and C-terminal “LIR” motif, respectively, serving as an adaptor linking autophagy cargo Ape1 condensates to the autophagosome membranes. Curiously, the N-terminal 153-residue fragment, together with the following Ape1-binding coiled-coil domain, of Atg19 formed a membrane-like coat on the Ape1 condensate. The “LIR” motif of Atg19 then tethered the coated Ape1 condensates to Atg8-containing membranes. Thus, the study uncovered a clear molecular mechanism underlying the direct association between a membraneless protein condensate and a membrane-based organelle. The authors also showed that the pre-autophagosomal structure (PAS) composed of Atg1, Atg13, Atg17, Atg29, and Atg31 was formed via LLPS driven by highly specific and stoichiometric interactions between the Atg proteins (Fujioka et al., 2020). The PAS condensates, via the Atg13-mediated binding to Vac8 on the vacuolar membranes, directly associated with the membrane-based vacuole. In another example, yeast TIS11, an AU-rich element specific RNA binding protein, could form liquid or gel-like condensates attached to the rough endoplasmic reticulum (ER) membrane surface, thereby forming a unique subcellular compartment that could selectively enrich transcripts with 3'UTR AU-rich elements and recruit a specific set of proteins to promote translation of the proteins encoded by these transcripts (Ma and Mayr, 2018). Interaction between the TIS11B-containing membraneless organelle and the membrane-based ER generated a unique subcellular compartment that allows genetic information encoded by 3'UTR to be transmitted in a spatially defined manner.

From the evolutionary perspective, it is perhaps not surprising that membraneless molecular condensates can associate and communicate with membrane-based organelles. Membraneless

molecular condensates likely emerged earlier than membrane-based organelles during the evolution of life, as the synthesis of membrane-forming lipids needs a host of different enzymes. If this logic holds, there is no reason to think that cells do not also make use of these two types of organelles for their wellbeing.

## CONCLUSIONS AND FUTURE PERSPECTIVE

With the fast-growing list of molecular condensates observed *in vitro*, one pressing question is what their biological functions are *in vivo*. Various molecular condensates formed via LLPS discussed in this review appear to play critical roles in modulating molecular processes vital for nervous system development and in orchestrating signal transduction between neuronal synapses. Although the significance of phase separation has been increasingly appreciated in the past few years, researchers should always ask whether or not the observations made can simply be explained by canonical biomolecular interactions. As exemplified by the studies discussed here, one might introduce mutations that specifically disrupt the interface involved in phase separation or replace a critical region with another typical sequence capable of driving phase separation to test the role of LLPS in cellular functions. Nevertheless, direct observation and characterization of phase separation *in vivo* can be challenging in many biological systems. For instance, neuronal synapses are minuscule in size and hence far beyond the resolution limit of many existing live imaging techniques. Therefore, the majority of studies on the formation of synaptic biomolecular condensates mediated by phase separation are carried out *in vitro*. Development of new methods is, thus, required to overcome current technical barriers. Another fertile research direction is how the regulation of condensates formation and dispersion mediated by phase separation might be coupled with cellular activities. In neuronal development, the formation of protein condensates beneath the apical or basal membranes of NBs is tightly synchronized with cell-cycle stages. Cell-cycle regulators, such as various cell-cycle-dependent protein kinases are likely involved in the assembly or dispersion of various protein condensates. In developed neurons, the size and molecular constituents of PSDs are closely correlated with synaptic strength. How is the synaptic condensate formation spatially and temporally coordinated? How do regulatory proteins such as kinases and small GTPases function in the condensed phases? Calmodulin-dependent kinase II (CaMKII) is a particularly important synaptic structure and activity regulator and an extremely abundant ePSD component. CaMKII may simultaneously act as an ePSD scaffold and an assembly regulator. But little is known about the kinase's role in the formation and regulation of the ePSD condensates. These questions and more await answers.

Molecular condensates formed via LLPS are not enclosed by lipid membranes and such membraneless subcellular organelles have many unique properties not found in membrane-based organelles (Chen et al., 2020; Feng et al., 2019; Banani et al., 2017; Shin and Brangwynne, 2017). These membraneless organelles, either alone or together with various membrane-based organelles, greatly expand the means by which cells orchestrate diverse functional processes with the limited amounts of materials they possess. Thus, communications among membraneless condensates and between membraneless and membrane-bound organelles represent another research direction in the field of phase separation.

One topic that has not been touched on in this review is the potential involvement of phase separation in neurodevelopmental, neuropsychiatric, and neurodegenerative diseases. Studies discussed here have indicated that LLPS mediates the formation of protein condensates involved in determining the polarity of NSCs and their fate. It is therefore possible that alteration of these condensates may cause developmental defects in the human brain. Understanding biological condensate formation in synaptic organization might help in elucidating mechanisms underlying the broad spectrum of genetic mutations, which are associated with neuropsychiatric disorders, in various synaptic proteins. We should certainly take the concept of phase separation into consideration when studying the occurrence and progression of various human brain diseases. Doing so might offer new therapeutic opportunities.

### WEB RESOURCES

SynapseWeb, <https://synapseweb.clm.utexas.edu/>

### ACKNOWLEDGMENTS

Work in our laboratory is supported by grants from the Research Grants Council of Hong Kong (AoE-M09-12 and C6004-17G) and a grant from the Simons Foundation Autism Research Initiative (SFARI) (510178). M.Z. is a Kerry Holdings Professor of Science and a Senior Fellow of IAS at HKUST. We apologize for leaving out many of the excellent papers relevant to the topic covered in this review due to the space limit.

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