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REVIEW

Liquid–liquid phase separation in neural development

Sara Surini[1] · Ivan de Curtis[1]

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Abstract
Liquid–liquid phase separation (LLPS) is a physicochemical process in which a homogeneous mixture of molecules separates into a denser phase originating from preferential self-assembly, driven by multivalent interactions, frequently mediated by intrinsically disordered protein regions, RNAs, or structured modular domains. LLPS produces a large variety of nuclear or cytoplasmic membrane-less biomolecular condensates with liquid-like properties. The importance of LLPS in several aspects of neural development and function is now supported by numerous studies. In this review we describe the findings supporting the importance of LLPS in different aspects of neural development, including asymmetric division of neural progenitors, chromatin remodeling and transcriptional/post-transcriptional control, localized translation and regeneration of neurites, and synaptic organization and function. We will focus especially on studies supporting the implication of LLPS in vivo. Finally, we will consider the important implication of LLPS-driven mechanisms as causes of neurodevelopmental and neurodegenerative diseases.

Keywords Asymmetric cell division · Local translation · Neural disease · Response to stress · Transcriptional/post-transcriptional regulation · Synaptic organization

Introduction

In recent years, the view on the way the intracellular environment is organized has been revolutionized by the interesting hypothesis that a physical process called *liquid–liquid phase separation* (LLPS) can be exploited by the cell to create dynamic microenvironments with specific functions [1–4]. LLPS is a physicochemical process in which a homogeneous mixture of molecules separates spontaneously into two liquid phases: the denser phase originates from the preferential partitioning of specific molecules. LLPS is driven by proteins and/or RNAs that self-assemble through well-defined interactions, or by intrinsically disordered regions (IDRs) (Fig. 1). Phase separation results in the formation of a condensed molecular assembly with liquid-like properties within a diluted aqueous solution. The mechanisms driving LLPS and the molecular groundworks of LLPS in biology have been presented in several reviews [5–7]. This conceptual framework allows to increase enormously the potential for distinct molecular assemblies dedicated to specific functions, at specific times and locations within the intracellular space. The hypothesis of the involvement of LLPS as a major player in the intracellular organization leads to postulate the possibility of an extraordinary increase in the complexity of the organization of both nucleoplasm and cytoplasm. The cytoplasm has been known for decades to be populated by several diverse membrane-bound organelles, while in the nucleoplasm a few relatively stable non-membranous organelles were known since long, first of all the nucleolus.

Increasing evidence has been supporting the importance of intracellular LLPS, leading to the discovery of the existence of many non-membranous assemblies, often referred to as *membrane-less organelles*. In many cases the identified supramolecular structures are extremely dynamic and limited in dimensions, leading to the use of the more comprehensive term *biomolecular condensates* to define LLPS-driven assemblies independently from their size [2].

Several reviews have appeared in recent years describing findings that supports the implication of LLPS-driven mechanisms during neuronal development, especially for

✉ Ivan de Curtis
decurtis.ivan@hsr.it

[1] Division of Neuroscience, Cell Adhesion Unit, Vita-Salute San Raffaele University and IRCCS San Raffaele Scientific Institute, Milan, Italy

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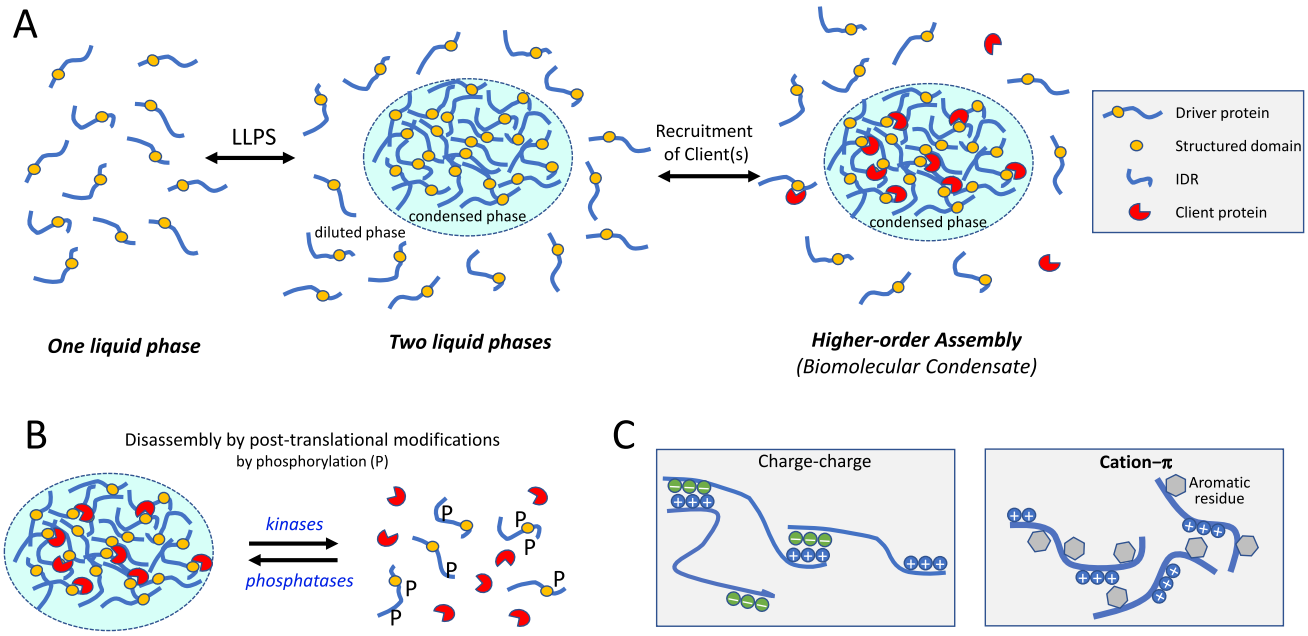


Fig. 1 (**A**) LLPS is promoted by *driver proteins* via *multivalent interactions* between *intrinsically disordered regions* (IDRs), or by specific interactions between *folded domains*. (**B**) Post-translational modifications like *phosphorylation* or *arginine methylation* can promote either the assembly or the disassembly (shown) of biomolecular condensates. (**C**) Different types of chemical interactions between IDRs can contribute to the formation of biomolecular condensates via LLPS, including *hydrophobic* interactions, *p-p* interactions, *cation-p* interactions between Arg and the electron-rich cloud (p system) of aromatic Phe, Trp, Tyr (left), or *charge-charge* interactions between oppositely charged amino acid residues (right)

the organization of functional synapses see, e.g., [8–12]. In this review, we discuss the role of LLPS in neural development with a particular focus on studies addressing the role of LLPS in vivo, inside living organisms.

Of note, the strength and amount of data supporting the role of LLPS in vivo are quite variable, depending on the protein considered. The experimental criteria supporting the evidence of LLPS in vivo include among others: concentration-dependent phase separation, dynamic exchange measured by FRAP, spherical morphology, dynamic behavior of the condensates including fusion/fission events, or sensitivity to 1,6-hexanediol treatment. On the other hand, care should be taken in considering the definite proofs for LLPS in vivo, that ideally should be based on a set of different criteria for each protein. As one example, although disruption of 1,6-hexanediol treatment is often used as an indication of LLPS driven by hydrophobic protein–protein interactions, but its effect may be non-specific. Also, the densely packed synaptic structures may cause difficulty in demonstrating liquid-like properties in the synapse.

We present evidence from the literature supporting the importance of LLPS-driven molecular events in distinct aspects of neural development, including: *Asymmetric cell division of neural progenitors; Chromatin remodeling and transcriptional regulation; Post-transcriptional regulation; Local translation in axons and dendrites; Response to stress; Axonal regeneration; Synaptic organization*; *LLPS and neural diseases*. Table 1 summarizes the

Asymmetric cell division of neural progenitors

Cell polarity is a fundamental property of many cell types [13], including embryonic cells that undergo asymmetric cell division to produce two cells with different fates to allow proper development. Biochemical and morphological cell polarity generates cells with distinct shapes for distinct functions [14]. The asymmetric distribution of polarity protein complexes on the cytoplasmic side of specific regions of the cell membrane is essential to establish and maintain cell polarity. Polarity complexes have been extensively studied, and how they concentrate at specific membrane sites remains an open question. A number of studies in the past years indicate that the concentration of the polarity complexes is driven by LLPS, to give rise to biomolecular condensates with compositional and functional specificities.

Asymmetric neuroblast division is required to start the differentiation of one of the daughter cells. It entails the uneven distribution and local concentration of specific signaling proteins that will partition differently between the two daughter cells. In *Drosophila* neuroblasts, the endocytic

Table 1 List of the proteins mentioned in this study that drive LLPS, with indication of their characteristics

LLPS drivers[1]	Protein function	Evidence for LLPS	RNA[2]	Associated neural disease	Neural process
Numb/Pon	Endocytic adaptors	in vitro, *in cellulo*, in vivo [20]	ND[3]		*Asymmetric cell division*
Par6/Par3	Adaptors	in vitro, in vivo [22]	ND		
Prospero/Prox1	Transcription factor	in vitro, *in cellulo*, in vivo [35]	ND		*Chromatin remodeling, transcriptional regulation*
Nde1	Nuclear scaffold protein	*in cellulo*, in vivo [39]	ND	Autism; mental retardation	
CBX (Polycomb)	Epigenetic regulator	in vitro, *in cellulo* [44, 45]	ND		
ATRX	Transcriptional regulator	in vitro, *in cellulo* [47]	ND	Intellectual disability (ATR-X syndrome)	
TBX3	Transcription factor	in vitro [48]	ND	Delayed onset of puberty	
YTHDF1	m^6A reader	in vitro, *in cellulo* [52]	mRNAs		*Post-transcriptional regulation*
Syncrip	RNA binding	in vitro, *in cellulo* [54]	ND	Autism spectrum disorder; intellectual disability	
FMRP	RNA-binding	in vitro, *in cellulo* [63], in vivo [58]	mRNAs	Fragile X Mental Retardation	*Local Translation*
IGF2BP1 (IMP1)	RNA-binding	*in cellulo*, in vivo [74]	mRNAs		
TDP-43	RNA/DNA binding	in vitro [197], *in cellulo* [81, 197], in vivo [101, 197]	mRNAs, lncRNAs	Amyotrophic lateral sclerosis	
G3BP1	RNA-binding	in vitro, *in cellulo* [84, 85, 200], in vivo [86, 200]	mRNAs		
Annexin A11	Membrane-binding	in vitro, *in cellulo,* in vivo [82]	ND	Amyotrophic lateral sclerosis	
Nucleolin	RNA-binding	*in cellulo* [90]	ND		
FUS	RNA-binding	in vitro, *in cellulo* [94], in vivo *(sections)* [183]	mRNAs	Amyotrophic lateral sclerosis; frontotemporal degeneration	
Abl2	Tyrosine kinase	in vitro, *in cellulo* [109]	ND		*Axonal regeneration*
TIAR-2	RNA binding	in vitro, in vivo [114]	ND	Amyotrophic lateral sclerosis; Welander distal myopathy	
Synapsin	Presynaptic	in vitro [128], in vivo [129]	ND	Epilepsy; Intellectual disability	*Presynaptic organization*
RIM/RIM-BP	Presynaptic scaffolds	in vitro [131]	ND		
ELKS/ERC	Presynaptic scaffolds	*in vitr* [132], *in cellulo* [201], in vivo [138]	ND		
Liprin-α/SYD-2	Presynaptic scaffolds	in vitro [138], *in cellulo* [143, 201], in vivo[138]	ND		
Tau	Microtubule-associated	in vitro [172, 173], *in cellulo* [151, 173]	ND	Frontotemporal Dementia; Alzheimer's disease	
Piccolo	Presynaptic scaffold	in vitro, *in cellulo* [152]	ND		
SynGAP/PSD-95	Postsynaptic scaffolds	in vitro, *in cellulo* [159, 163]	ND	PSD-95: Intellectual disability and others	*Postsynaptic organization*
Stargazin/PSD-95	Postsynaptic scaffolds	in vitro, in vivo *(sections)* [161]	ND	Stargazin: autism spectrum disorder (candidate)	
CaMKII	Serine/Threonine kinase	in vitro [164]	ND	Epilepsy and others	
Rabphilin-3A	Rab3A effector	in vitro, *in cellulo* [165]	ND		
nArgBP2	Adaptor	in vitro [167], *in cellulo* [167, 168]	ND	Intellectual disability (candidate)	
PSD-95/SAPAP	Postsynaptic scaffolds	in vitro, in vivo *(sections)* [169]	ND	SAPAP: Autism spectrum disorder and others	
Gephyrin	Postsynaptic scaffold	in vitro, *in cellulo* [145]	ND	Intellectual disability and others	
Shank3	Postsynaptic scaffold	in vitro, *in cellulo,* in vivo *(sections)* [171]	ND	Intellectual disability; autism spectrum disorders	
eIF4G	Translation initiation factor	in vitro, *in cellulo,* in vivo *(indirect)* [186]	ND		*LLPS and neural diseases*
α-/β-synuclein	Regulator of vesicle traffic	in vitro, in vivo [189]	ND	Parkinson's disease; dementia with Lewy Bodies	
CTTNBP2		in vitro, *in cellulo* [195]	ND	Autism spectrum disorder	

[1]*The list of proteins is according to appearance in the main text*

[2]*RNAs involved in LLPS or included into biomolecular condensates induced by the corresponding LLPS driver protein*

[3]*ND, not described in the studies analyzed*

adaptor protein Numb is a cell fate determinant that interacts and colocalizes with the adaptor protein Pon (Partner of Numb) to the basal daughter cells originating from asymmetric cell division [15]. At the onset of neuroblasts mitosis determinants including the Numb/Pon complex, and the complex between the transcription factor Prospero and the scaffold protein Miranda form crescents on the basal cortex. The Par3(Baz)/Par6/aPKC protein complex concentrates to form a crescent at the apical cortex, thus establishing apicobasal polarity [16–19] (Fig. 2A).

A recent study has identified a multivalent interaction between Numb and Pon that causes the phase separation of the Numb/Pon complexes (Fig. 2B). Interfering with Numb/Pon complex LLPS impairs the localization of Numb at the basal cortex of cells, resulting in the suppression of Notch signaling during the asymmetric cell division of neuroblasts [20]. Structural analysis of the Numb/Pon complex reveals that the phospho-tyrosine-binding domain of Numb has two binding surfaces that recognize repeated motifs of Pon, thus allowing LLPS.

The conserved Par3/Par6/aPKC complex regulates the polarity of different types of cells. The mechanisms driving the concentration of the Par complex at the cell membrane to initiate cell polarization is unclear. Cell polarity has been rebuilt in non-polarized *Drosophila* S2 cells expressing the three endogenous core polarity complex components Par3 (called Bazooka in *Drosophila*), Par6, and aPKC [21]. High Par3 expression induces cortical localization of the Par-complex at interphase. Three steps have been recognized to establish the asymmetric distribution of the Par-complex: appearance of cortical spots, development of dynamic island-like assemblies with amorphous shape characterized by constant fusion and fission events, polarized clustering of these islands that contain a meshwork of units. Par-complex patches resembling Par-islands exist in Drosophila mitotic neuroblasts. The behaviour of the small cytoplasmic droplets formed by the Par-complex subsequently coalescing into spherical structures suggested that phase separation takes place for Par-complexes in the cytoplasm.

The Par complex has been shown to exhibit a cell cycle-dependent LLPS-driven condensation in *Drosophila* neuroblasts [22]. Par3 exists in two conformational states: a closed autoinhibited conformation induced by the interaction of the amino-terminal region including three PDZ (PSD-95, DLG, ZO-1) domains, with the carboxy-terminal region of the protein [23]. Upon activation, the open active conformation of Par3 undergoes LLPS likely driven by the amino-terminal conserved oligomerization domain (NTD)

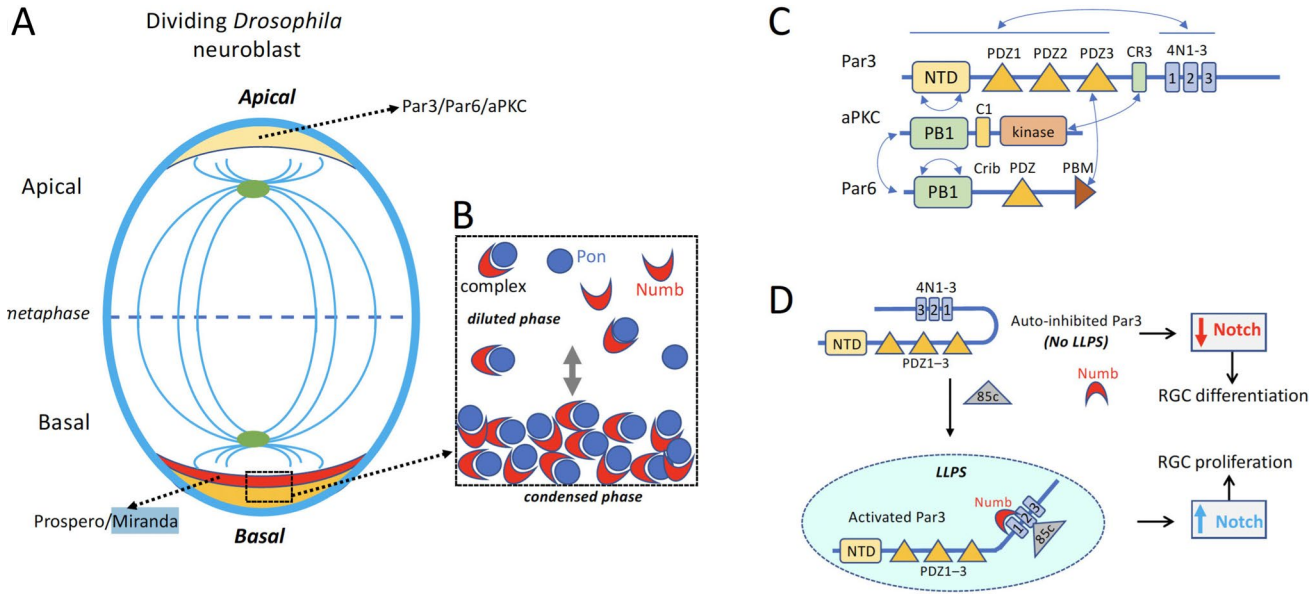


Fig. 2 (**A**) LLPS in asymmetric neuroblast cell division. (**A**) Scheme with the protein condensates in a polarized neuroblast. The Par3/Par6/aPKC complex localizes at the apical pole, while the Numb/Pon and Prospero/Miranda complexes concentrate at the basal pole of the dividing neuroblast. Localization occurs via LLPS guided by multivalent interactions. (**B**) Highly condensed complexes are dynamic in a space and time. Model for the Numb/Pon complexes that can dynamically exchange (double arrow) between the polarized liquid-like condensed phase and the diluted cytosolic phase. (**C**) Multivalent interactions within the Par3/Par6/aPKC complex: double arrows indicate the different self and intermolecular interactions within the trimeric complex. NTD, N-terminal domain; PDZ domain; CR, conserved region; PB1, Phox and Bem1; Crib, CDC42/Rac-interactive binding; PBM, PDZ-binding motif; PTB, phospho-tyrosine binding. (**D**) Model for the interaction of Ccdc85c (85c) with Par3. Under resting conditions, Par3 is auto-inhibited and can not recruit Numb. The free active Numb antagonizes Notch signaling, leading to *radial glia cell* (RGC) differentiation. The interaction of Ccdc85c with Par3 activates Par3 that undergoes LLPS and recruits Numb as a client into Par3 condensates. the released inhibition of Notch signaling promotes radial glia cell proliferation

of Par3 (Fig. 2C). The three PDZ domains of Par3 mediate distinct protein–protein interactions [24]. Par6 is recruited to the Par3 condensates as a client binding to PDZ3 of Par3. The interaction of Par6 with Par3 strongly promotes Par3-induced LLPS. The kinase aPKC is also concentrated to the Par3/Par6 condensates as a client via its specific binding to both Par3 and Par6. Post-translational modifications are important to regulate the dynamics of condensates formed by LLPS [25] (Fig. 1B). In particular, phosphorylation of protein driving phase separation events can prevent the multivalent interactions required for the formation of condensates. This is due to the addition of the negative charges of the phosphate groups covalently added on the surface of the driver protein that may interfere with the formation of the multivalent interactions needed to assemble the condensates. In this respect, the phosphorylation of Par3 by active aPKC can reverse the process of LLPS by Par3, and dissolve the Par3/Par6 condensates [22]. The physiological relevance of Par3 LLPS is supported by the finding that perturbation of LLPS by mutations of Par3 impairs the establishment of apico-basal polarity during the asymmetric division of neuroblasts, leading to defects in development [22].

Par3 is also involved in the control of the differentiation of mammalian radial glial cells, which is fundamental for correct mammalian brain development. Radial glial cells are neural progenitor cells that produce most cortical neurons [26]. Radial glial cells are highly polarized, connecting the ventricular zone with their apical process, and the pial surface with their basal process. At the onset of neurogenesis radial glial cells undergo proliferative divisions to expand the progenitor pool. Later on they divide asymmetrically to generate either neurons or intermediate progenitors undergoing a limited number of symmetric divisions to generate neurons [27]. Disrupting the balance between proliferation and neuronal differentiation causes abnormal neuronal production that results in cortical malformation [28, 29]. How Par3 is asymmetrically segregated into the daughter cells originating from the asymmetric division of radial glial cells is poorly understood. Recently, it has been shown that the scaffold protein Ccdc85c (coiled-coil domain-containing protein 85c) interacts with Par3 to regulate the proliferation of these cells [30]. The interaction with Ccdc85c releases the autoinhibition of Par3 to promote Par3 phase separation (Fig. 2D). If Ccdc85c is downregulated, mouse radial glia cells undergo differentiation. Importantly, Par3 activated by Ccdc85c recruits the Notch regulator Numb to the condensates, preventing the attenuation of Notch activity and resulting in radial glial cell proliferation. This study indicates that Ccdc85c-mediated phase separation of Par3 regulates Notch signaling to control radial glial cell proliferation during brain development.

Chromatin remodeling and transcriptional regulation

The eukaryotic nucleus is a center for information processing dedicated to genome organization and regulation. In the nucleus, gene expression is controlled by transcription factors that include DNA-binding domains and activation domains. Chromatin compaction is essential for chromosomal DNA packaging and to regulate gene expression. An intriguing question is how the genome is spatially and temporally organized to regulate cellular processes with high precision. LLPS has been implicated as a major player in the organization of the genome and in the regulation of transcriptional and post-transcriptional events [31]. Compact heterochromatin is prevalent in differentiated cells to act as a barrier to cellular reprogramming. An open question is how remodeling of heterochromatin is arranged during cell differentiation.

LLPS of Prospero to guide terminal differentiation

The conserved transcription factor Prospero warrants neuronal differentiation by driving the condensation and expansion of heterochromatin (Fig. 3A). In this context, the term *mitotic retention* is referred to transcription factors or histone post-translational modifications remaining on chromosomes during mitosis. It is believed that mitotic retention is important to preserve the cellular identity and function through cellular generations [32]. Heterochromatin domains are marked by histone H3 lysine-9 trimethylation (H3K9me3) and are refractory to transcription. These chromatin regions become condensed and expanded during cell differentiation [33]. The formation of H3K9me3-positive heterochromatin depends on a *reader* of H3K9me3, the heterochromatin protein 1 (HP1), and the H3K9me3 *writer* SUV39H1, a histone H3K9 methyltransferase [34]. Cycles of H3K9 trimethylation and recruitment of HP1 and SUV39H1 drive heterochromatin formation and spreading [34]. During the mitotic cycles of *Drosophila* neural precursors, Prospero is held at pericentromeric H3K9me3-positive heterochromatin regions via LLPS [35]. During the exit of neural precursors from mitosis, Prospero recruits and concentrates HP1 into these condensates, to promote heterochromatin compaction, thus creating a repressive chromatin environment that favours cell-cycle exit and neuronal differentiation (Fig. 3B). Analysis by deletion mutants has identified the region of Prospero required for LLPS and for the mitotic retention of Prospero: a Prospero mutant unable to undergo phase separation abolishes its retention on the mitotic chromosomes of dividing neural stem cells. Restoring phase separation by fusing the LLPS-deficient Prospero mutant with the C-terminal IDR of hnRNPA1 that is known to drive

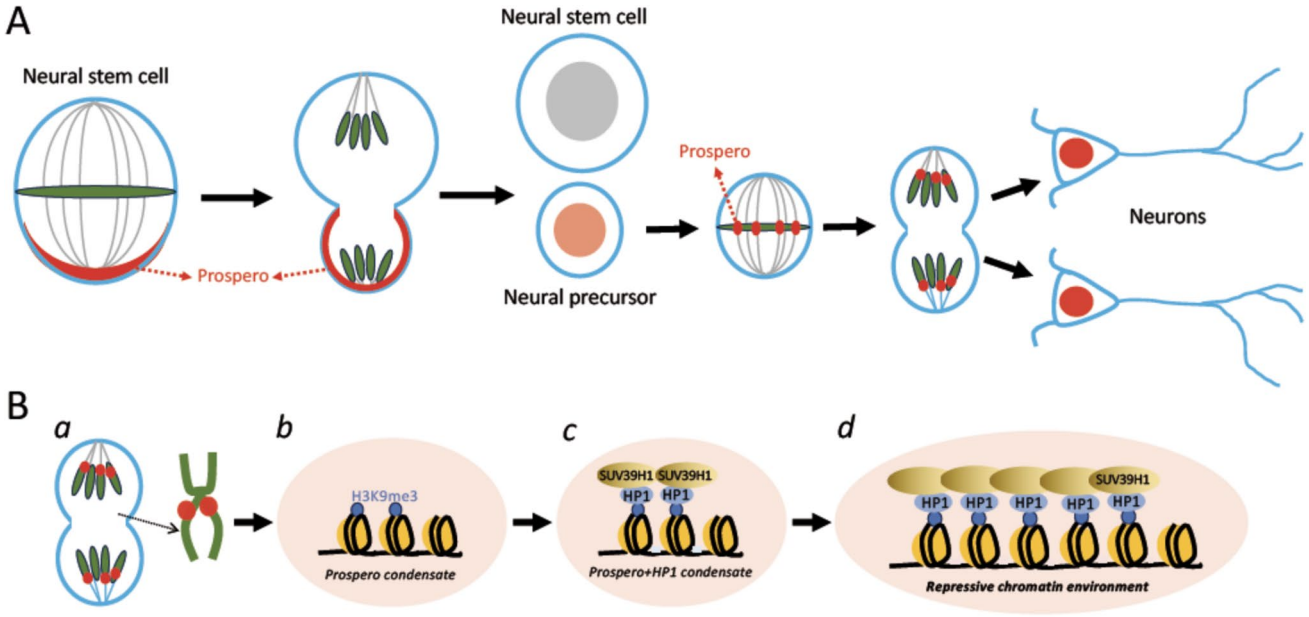


Fig. 3 (**A**) Schematic drawings of the distribution of the transcription factor Prospero (red) during the division of a neural stem cell, and then of a neural precursor to produce differentiating neurons. (**B**) In the proposed model [35], Prospero supports neuronal differentiation by two sequential processes. In dividing neural precursors *(a),* LLPS-driven Prospero condensates (shaded area) associate to H3K9me3-positive regions of heterochromatin *(b).* The *H3K9me3 reader* HP1 is recruited by Prospero into the condensates, reinforcing the expansion of H3K9me3+heterochromatin regions by the "writer " SUV39H1 *(c),* thus creating a repressive environment and gene silencing *(d*) that leads to cell-cycle exit and neuronal differentiation

LLPS, can rescue chromosome retention and the differentiation-promoting function of Prospero [35]. Interestingly, Prox1, the mammalian homologue of Prospero, expressed in *Drosophila* neuroblasts employs a similar strategy to promote neuronal differentiation. The results from this study highlight an important new mechanism by which mitotic retention of a transcription factor via LLPS guides heterochromatin remodeling and terminal differentiation.

Role of Nde1 in heterochromatin organization

Nde1 is a nuclear scaffold protein involved in the organization of the heterochromatin, and essential for mammalian brain development. Nde1 loss of function causes microcephaly and mental retardation [36, 37], while mutations in the *NDE1* gene are associated to neuropsychiatric disorders [38]. The origin of the diverse phenotypes from *NDE1* abnormalities is unknown. Cortical neurogenesis is coupled to compaction of heterochromatin marked by the trimethylation of the DNA packaging protein Histone H4 (H4K20me3). It has been shown that the mammalian Nde1 protein controls neurogenesis by facilitating heterochromatin compaction marked by H4K20me3. This mechanism stabilizes the heterochromatin of neocortical neurons. A recent study combining the use of mutant mice, human neural stem/progenitor cultures, and Cos7 cells has shown that the protein Nde1, which is predicted to be largely disordered, undergoes LLPS in Cos7 cells. Nde1 localizes in the nucleus of neucortical cells in mouse cortical sections, and interacts with pericentromeric and centromeric satellite repeats, i.e. repetitive DNA sequences near the chromosomal centromeres [39].

In mice, loss of function of Nde1 leads to altered nuclear architecture, DNA damage, and instability of the pericentromeric satellite repeats in neocortical neurons. Although this study does not directly link LLPS to Nde1 function, it raises the interesting possibility that the formation of localized nuclear condensates by this protein may underlie the role of Nde1 in protecting neuronal heterochromatin. One interesting hypothesis that remains to be explored is whether disruption of Nde1 phase separation may cause the heterochromatin instability that is known to cause brain dysfunction.

Regulation of polycomb function during neural cells differentiation

The development of multicellular organisms requires that different cell types develop and preserve their identity. For this, in each cell type specific genes must be expressed for proper function, while genes associated to other cell types must be repressed. During differentiation Polycomb proteins

maintain properly repressed their cell type-specific target genes to allow cell differentiation to proceed, thus playing a critical role during development [40]. The mammalian CBX (chromobox) family includes proteins with the conserved chromodomain whose function is to bind methylated histone residues. During development the CBX proteins maintain the repression by Polycomb. Polycomb forms two repressive complexes, PRC1 and PRC2 (Polycomb Repressive Complex 1 and 2). PRC2 catalyzes the methylation of lysine 27 in histone H3 (H3K27me3) to recruit both PRC1 and PRC2 complexes [41–43]. The PRC1 complex compacts chromatin and can drive LLPS via its CBX subunit to maintain chromatin in the repressed state [44]. Undifferentiated pluripotent embryonic stem cells express the CBX7 family member that lacks the domain required for chromatin compaction and phase separation. Interestingly, the addition of this domain to CBX7 induces a switch in the ability of the chimeric CBX7 to compact chromatin and to phase separate [45]. Embryonic stem cells expressing the chimeric form of CBX7 are unable to form neural progenitor cells, show reduced activation of lineage-specific genes for differentiation, and an improper maintenance of Polycomb binding at neural-specific loci during differentiation. This study suggests that CBX-driven LLPS contributes importantly to Polycomb function during the shift from pluripotency to differentiating neural cells.

Regulation of neural progenitor cells by ATRX

A recent study supports a model in which LLPS of the chromatin remodeling protein ATRX regulates gene networks needed for human neural progenitor cells identity. The IDR of ATRX is essential to form LLPS-induced dynamic biomolecular condensates that recruit transcriptional co-activators. In human neural progenitor cells the ATRX condensates are required to localize the protein at super-enhancers [46], which are clusters of regulatory elements of the genome with a prominent role in cell-type-specific processes [47]. Interestingly, deletion of the poly-glutamine sequence within the central disordered region of ATRX required for LLPS does not only disrupt the formation of ATRX condensates, but causes also a sustained inhibition on the neurogenic potential of the neural progenitor cells, as revealed by the reduction of neuronal markers and by the concomitant upregulation of neural crest cell and mesenchymal stem cell markers. Therefore this study shows that ATRX condensates connect ATRX localization to the regulation of transcription, and that disruption of ATRX biomolecular condensates impairs neuronal differentiation [47]. These results support the concept that LLPS is a key regulatory mechanism to support transcription during neural development.

LLPS in the control of puberty

Hypothalamic neuroendocrine KNDy (Kisspeptin, neurokinin B, dynorphin) cells are a subtype of neurons that control the onset of puberty. The cellular mechanisms working to specify this subtype of neuroendocrine neurons remains unclear. The human transcription factor TBX3 establishes and maintains the identity of KNDy neurons to trigger puberty. Genetic studies have established a link between mutations in the gene encoding the human transcription factor TBX3 and the delayed onset of puberty. It has been found that mutations of the human *TBX3* gene affect the phase separation of the TBX3 protein and impair the regulation of the transcription of key neuropeptides, providing a link between LLPS and the pathological mechanism underlying *TBX3*-associated puberty disorders [48].

Post-transcriptional regulation

N^6-methyladenosine (m^6A) is the most common post-transcriptional modification of the mammalian mRNAs [49]. The m^6A modification is recognized by reader proteins to modulate mRNA splicing, the efficiency of translation, and the stability of the mRNA [50], thus facilitating cell fate determination and embryonic development [51] (Fig. 4A).

A recent study shows that phase separation plays an essential role in cell fate transition by influencing the post-transcriptional modification of the mammalian mRNA [52]. m^6A is formed by a methyltransferase that reversibly methylates the 6th nitrogen atom of adenine. The m^6A reader proteins binds to m^6A-modified transcripts to regulate gene expression and control cell fate determination [49]. LLPS of the central m^6A reader protein YTHDF1 (YTH N6-methyladenosine RNA binding protein 1) promotes the trans-differentiation of spermatogonial stem cells into neural stem cells. YTHDF1-driven LLPS inhibits the translation of the mRNAs for IkBα/β proteins, which are inhibitors of NF-kB activation [52]. The inhibition of IkBα/β translation occurs by recruiting the mRNAs for IkBα/β into the YTHDF1 condensates (Fig. 4B): the formation of biomolecular condensates of these *protein-mRNA complexes* act as a barrier that prevents the interaction of the IkBα/β mRNAs with the machinery required for their translation. Therefore inhibition of IkBα/β by YTHDF1-driven LLPS is a key event to activate the NF-kB–Cyclin D1 axis. The relevance of YTHDF1 LLPS for trans-differentiation was confirmed by showing that disrupting YTHDF1 phase separation inhibits trans-differentiation: incubation of the cells with the LLPS inhibitor 1,6-hexanediol significantly reduced the rate of formation of Nestin-positive neural stem cells.

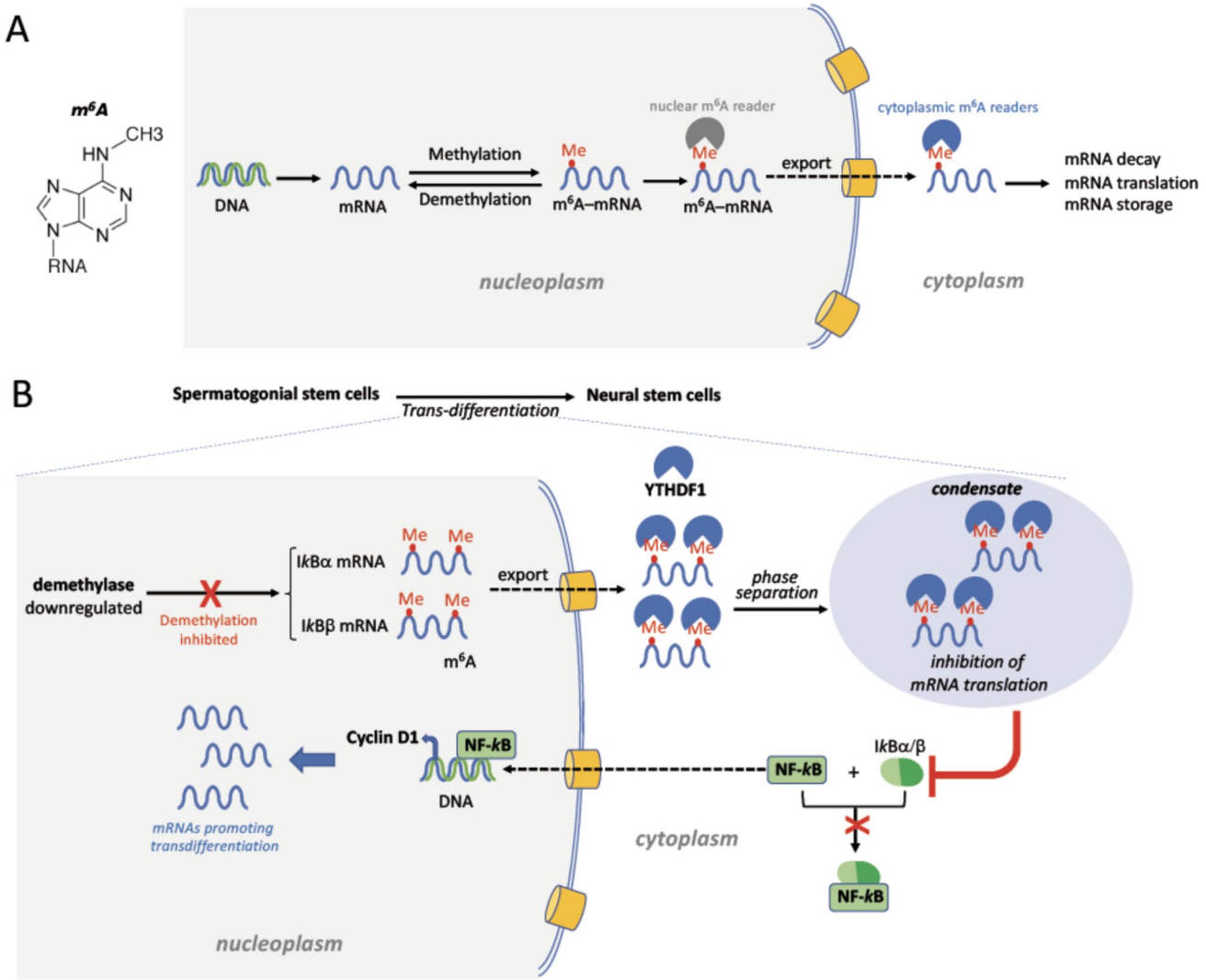


Fig. 4 (**A**) Pathway of m6A in nuclear RNAs. m6A methyltransferases and demethylases control m6A methylation of the mRNA in the nucleus. Different m^6A readers bind to methylated mRNA and mediate specific functions. m^6A affects alternative splicing and mature mRNA storage and export. In the cytoplasm different m6A readers can bind to the m^6A-containing mRNAs to mediate their decay, translation, or storage [49]. (**B**) LLPS of the m^6A reader YTHDF1 promotes the trans-differentiation of spermatogonial stem cells into neural stem cells by activation of the NF-kB–Cyclin D1 axis [52]. Inhibition of the translation of the mRNAs for IkBa/b by m6A-mediated YTHDF1 LLPS activates the NFkB–Cyclin D1 axis required for transdifferentiation

The development of the mammalian neocortex is regulated by gene expression. Radial glial cells give rise to six neuronal layers at different times during neocortical development. This process relies on different transcriptional and post-transcriptional mechanisms that ensure proper development [53]. It is not clear how radial glial cells generate specific types of neurons at distinct times. A recent study identifies the ribonucleoprotein Syncrip as a key modulator of superficial neuronal differentiation in neocortical neurogenesis. Syncrip knockout in radial glial cells of mice disrupts late-stage neurogenesis, resulting in learning/memory impairment [54]. Syncrip maintains the time-dependent transcription by forming stabilizing ribonucleoprotein condensates via LLPS, to control the fate of radial glial cells. Human mutations in Syncrip destroy its ability to undergo phase-separation that is needed to stabilize and protect bound RNAs from degradation. Thus, Syncrip acts as a post-transcriptional regulator to govern the temporal programs linked to fate progression of radial glial cells.

Local translation in axons and dendrites

Axonal and dendritic growth cones navigate in response to extracellular directional and inhibitory signals to reach their specific targets for the formation of synaptic terminals. The possibility to supply new proteins by local translation far from the cell body is needed to allow high motility of

growth cones and vesicles delivery to the presynaptic sites. During transport along axons to the growth cone, RNA-binding proteins and mRNAs form neuronal membrane-less transport granules formed by LLPS. The final peculiar morphology of mature neurons requires that protein synthesis is regionalized from the cell body to sustain the activity in the mature dendritic and axonal compartments [55]. Specific mRNAs need to be distributed by a transport apparatus including regulatory elements within the RNA molecules, RNA-binding proteins to generate ribonucleoprotein granules, and motor proteins that link these granules for long-distance RNA trafficking and local protein synthesis [56].

Ribonucleoprotein granules include transport granules, stress granules, and P-bodies to exert a variety of functions [57]. Ribonucleoprotein granules include specific sets of mRNAs and RNA binding proteins that regulate translation in two ways: by suppressing translation when not needed, and by promoting translation upon activation at specific times and sites along the developing axon. The contribution of LLPS to the transport along axons is still an open question, although several studies support now the hypothesis of LLPS involvement in the formation of transport granules [58]. The analysis of the molecular mechanisms linking the transport mechanisms to local protein synthesis and to LLPS is important to build a thorough representation of the complex mechanisms regulating axonal extension and synaptic development. We present here several examples of studies linking phase separation to the assembly, function, and modulation of ribonucleoprotein granules in neurons. The evidence is based on studies showing that purified ribonucleoproteins components can phase separate to form liquid-like biomolecular condensates in vitro, and studies that show the presence of membrane-less structures with liquid properties in cells.

Ribonucleoprotein granules are not conventional molecular complexes, but due to the dynamic multivalent interactions between their components, they consist in liquid-like biomolecular assemblies that may form by demixing from the surrounding cytosol via LLPS [59] (Fig. 5A). In response to extracellular signals, ribonucleoprotein granules can rapidly and reversibly assemble/disassemble via post-translational modifications including phosphorylation and arginine methylation [5, 25, 58]. Several studies have addressed the mechanisms driving the formation of ribonucleoprotein granules in vitro and *in cellulo*. In the context of ribonucleoprotein granules, multivalency has been proposed to originate from intrinsically disordered, low complexity regions, which are frequently found in RNA-binding proteins [60].

The Fragile X Mental Retardation Protein (FMRP) is implicated in the Fragile X syndrome, a neurodevelopmental

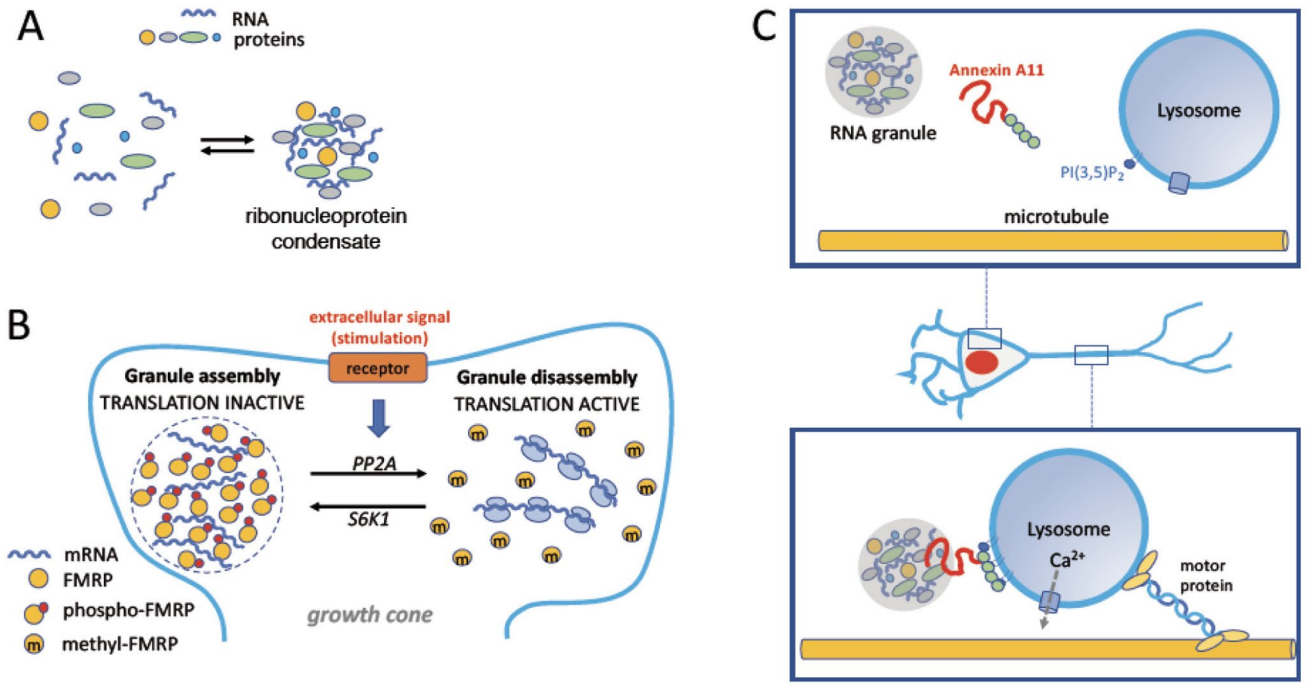


Fig. 5 (**A**) mRNAs associate with RNA-binding proteins to form membrane-less ribonucleoprotein condensates. (**B**) Model for the reversible assembly or ribonucleoprotein granules. During neuronal stimulation, dephosphorylation of FMRP by the protein phosphatase PP2A induces the disassembly of the neuronal granule with activation of translation. FMRP methylation reduces its propensity to form condensates. At the end of stimulation, the kinase S6K1 phosphorylates FMRP, which promotes neuronal granule assembly and prevents translation. FMRP demethylation promotes LLPS [63]. (**C**) RNA granules "hitchhike" on motile lysosomes for long-distance transport [82]. Annexin A11 binds to RNA and lysosomes with an amino-terminal IDR mediating LLPS, and 4 carboxy-terminal Ca^{2+}–dependent membrane binding domains. The tethering of the RNA granules via Annexin A11 allows the transport of mRNAs along the axon

disease causing intellectual disability. Loss of FMRP causes Fragile X Syndrome and Autism Spectrum Disorder (ASD) in humans [61]. FMRP-knockout in mice down-regulates the translation of many genes associated with ASD. FMRP is a RNA-binding protein and a key regulator of translation in neurons through the formation of neuronal ribonucleoprotein granules [62, 63] that traffic from the soma to dendrites [64]. FMRP binds to the mRNA of several presynaptic proteins [65], and is therefore also considered a key regulator of local presynaptic protein synthesis. FMRP undergoes phosphorylation-dependent LLPS to generate membrane-less RNA transport granules: while phosphorylation of FMRP increases its LLPS, methylation of FMRP decreases LLPS [63]. The formation of RNA transport granules results in the suppression of translation until extracellular signals promote the translation of mRNAs at specific sites in neurons. The release of the mRNAs to promote local translation of specific subsets of mRNAs may involve the dispersion by dephosphorylation of the FMRP granules formed by LLPS [63] (Fig. 5B). In this direction, it has been shown that the signal triggered by extracellular Semaphorin 3A promotes dephosphorylation of FMRP to allow the local translation of specific mRNAs in growth cones [66, 67]. In vivo FMRP granules localize at presynaptic terminals and in axons in different regions of the developing brain, including the olfactory sensory neurons and hippocampal neurons [68]. FMRP-containing granules include ribosomal RNA, proteins, and specific mRNAs [69, 70], in support of the role of FMRP granules in the regulation of local translation of mRNAs in the brain. Interestingly, a recent bioinformatic analysis of published ribosome profiling studies has revealed that the proteins encoded by these genes are enriched in predicted IDRs and are prone to LLPS, suggesting that FMRP regulates the translation of proteins involved in the formation of biomolecular condensates [71].

The IGF2 mRNA-binding protein IGF2BP1/IMP1 is a post-transcriptional regulator of the localization and translation of RNAs [72]. Knockout of IGF2BP1/IMP1 in mice causes the disorganization of the developing neocortex: neurons from embryonic knockout animals show decreased dendritic β-actin ribonucleoprotein transport along dendrites [73]. An interesting study provides evidence for the modulatory role during development of the low complexity prion-like domain of the *Drosophila* protein Imp, a component of neuronal ribonucleoprotein granules and a homologue of mammalian IGF2BP [74]. Imp forms granules at the leading edge of migrating cells and at the tips of neuronal processes to help the concentration and release of specific mRNAs important for axon growth and migration [75, 76]. The prion-like domain of Imp is essential for the developmentally-controlled localization of Imp ribonucleoprotein granules to axons and for axonal remodeling in vivo. Interestingly, the prion-like domain of Imp is not required for granule assembly, but is needed to modulate the size and number of granules: rather than triggering phase separation as described for the prion-like domains of other proteins [77–79], these results are consistent with a modulatory function of the prion-like domain for the homeostasis of Imp ribonucleoprotein granules [60, 80].

Axonal transport granules including mRNAs and the TDP-43 (TAR-DNA binding protein 43) protein have also been reported to display liquid-like properties [81]. TDP-43 granules in axons of primary cortical neurons display liquid-like properties, characterized by fusion events, deformation, and rapid fluorescence recovery after photobleaching. Analysis by super-resolution microscopy of the biophysical properties of ribonucleoprotein granules formed by the wildtype TDP-43 protein suggests a maturation process towards more stable structures linked to the aging of the granules. Ribonucleoprotein granules formed by the TDP-$43M^{337}V$ and TDP-$43G^{298}S$ mutant proteins identified in human ALS patients are more viscous and show disrupted transport dynamics, suggesting a possible toxic gain of function by the ALS mutants [81].

G3BP1 is a stress granule protein that is also used to store mRNAs in granules in adult peripheral nerves. G3PB1 granules mitigate the translation of their bound mRNAs in axons, while disassembly of the G3BP1 granules increases protein synthesis and axon extension [83]. G3BP1 granules form by LLPS driven by the IDRs of this protein; LLPS by G3BP1 is amplified by RNA binding, and decreased by phosphorylation on Ser^{149} [84, 85]. In vivo, LLPS by axonal G3BP1 is increased soon after axotomy in rats, which induces new synthesis of casein kinase 2α that phosphorylates G3BP1, causing a decrease in the number of axonal G3BP1 granules [86]. In this way G3BP1 phosphorylation releases axonal mRNAs for translation to support nerve regeneration.

Annexin A11 is a member of the multigene family of Ca^{2+}-regulated phospholipid-dependent membrane-binding annexins. Rare mutations in the gene for Annexin A11 cause amyotrophic lateral sclerosis (ALS). Annexin A11 plays an important role in cell division, Ca^{2+} signaling, membrane trafficking and apoptosis. Unlike most annexins, Annexin A11 has a ≈180 residue long amino-terminal prion-like low-complexity region. Low-complexity regions are known to facilitate the formation of phase-separated assemblies associated to RNAs [1]. The amino-terminal low-complexity region of Annexin A11 drives LLPS to form RNA granules, and allows the protein to bind to lipid membrane [82]. The intrinsic membrane-binding and phase separating properties of Annexin A11 can tether membrane-less RNA granules to motile lysosomes, thus promoting the hitchhiking of RNAs to distal regions of the neuron (Fig. 5C). Interestingly, ALS-associated mutations of Annexin A11 alter the dynamic

properties of the RNA granules and disrupt the link between RNA granules and lysosomes, preventing RNA granule transport in cultured rat neurons and in ganglion axons of zebrafish embryos [82].

Nucleolin is an abundant RNA binding protein found in the nucleolus but also in the cytoplasm, and implicated in many cellular processes [87], including the axonal trafficking of mRNAs that regulate neuronal growth. The carboxy-terminal *glycine–arginine rich domain* of nucleolin drives the interaction with plasma membrane and subcellular localization via kinesin [88]. The same domain drives LLPS [89]. Heterozygotic deletion of the glycine/arginine-rich domain of nucleolin in mice causes reduced axonal localization of nucleolin cargo mRNAs and enhanced sensory neurites outgrowth [90]. Thus, the glycine-arginine rich domain of nucleolin oversees axonal transport of a growth controlling ribonucleoprotein complex, which may depend also on the ability of this protein to undergo LLPS. Interestingly mutants in the glycine–arginine rich domains of different RNA binding proteins are associated with neurodegenerative diseases, again underlying the possible link between perturbed LLPS and neural disorders. For example, mutations in the glycine–arginine rich domains of FUS, EWSR1, and TAF15 are associated with ALS [91].

Post-translational modifications like phosphorylation and arginine methylation govern the assembly or disassembly of several biomolecular condensates [25]. The multivalent interactions needed for LLPS can be enhanced by arginine methylation. *In cellulo* studies using HeLa cells [92] and primary neurons [93] have shown that the proteins FUS (FUsed in Sarcoma) and SMN (Survival of Motor Neuron) collaborate in the distribution of mRNAs for local translation. These proteins form ribonucleoprotein condensates by LLPS. The asymmetric dimethylation of arginine creates extra binding sites on the FUS protein that contribute to multivalent protein–protein interactions, thereby lowering the threshold for the formation of ribonucleoprotein FUS/SMN condensates by phase separation in primary cortical neurons [94]. Accordingly, hypomethylation of FUS or knockdown of SMN disrupts the formation and transport of neuronal granules in axons. Interestingly, the neuronal defect caused by SMN knockdown can be rescued by expression in neuroblastoma C6 cells of wildtype SMN, but not of the SMN-D7 mutant associated to spinal muscular atrophy that lacks the carboxy-terminal domain required for oligomerization [95].

Response to stress

Stress granules are membrane-less RNA-containing cytoplasmic organelles formed by LLPS in response to exposure to stress [96]. Responding to external stress is crucial for neurons: defective stress granule dynamics has been proposed to facilitate early death of motor neurons in ALS. The RNA-binding protein TDP-43 plays essential roles in different cellular processes, including the modulation of stress granule dynamics [97]. In ALS patients TDP-43 accumulates frequently as aggregates in the cytoplasm [98]. Motor neurons from mice carrying the TDP-43^{M337V} mutation linked to ALS are defective in the assembly of stress granules [99]. Evidence indicates that stress granules are transient structures, but chronic stress associated to ageing directs to persistent stress granules that may act as a nest for aggregation of disease-related proteins [100]. It has been hypothesized that stress granules may seed the TDP-43 inclusions observed in neurons of many ALS and frontotemporal dementia patients. This hypothesis has been recently tested in vivo by exposing wildtype and transgenic mice carrying the TDP-43^{M337V} mutation to full body heat stress to trigger the formation of stress granules in the central nervous system [101]. An age-dependent decline in the formation of heat-induced stress granules is observed in control mice, while stress granules were mostly absent in age-matched TDP-43^{M337V} mice. In these mice acute hyperthermia induces TDP-43 cytoplasmic localization without aggregation, indicating that TDP-43^{M337V} expression is associated to defective stress granules in the nervous system [101]. TDP-43^{M337V} mice show decreased levels of the RNA-binding proteins TiaR and G3BP1 that are essential to initiate stress granule formation [84]. In these mice the levels of cytoplasmic polyadenylated mRNA were decreased, while the RNA chaperone eIF4A1 preventing RNA–RNA condensation was increased, thus limiting the recruitment of mRNAs to stress granules [102]. Altogether these findings indicate alteration of key components contributing to the threshold for LLPS in the neurons of TDP-43^{M337V} transgenic mice in response to hyperthermia, which could be relevant to the pathogenesis of ALS and frontotemporal dementia.

Axonal regeneration

Several microtubule-binding proteins undergo LLPS that is often driven by specific IDRs [103–108]. Genetic studies in *Drosophila* indicate that tyrosine kinases of the Abl family interact with microtubules *to regulate axon guidance and neuronal morphogenesis*. By combining cell-free assays and *in cellulo* analysis it has been shown that the Abl2 kinase co-condensates with tubulin to form a compartmentalized reactor that may contribute to the nucleation of microtubules and to promote the incorporation of tubulin dimers to repair damaged microtubules [109], two processes required for proper axonal development and growth cone guidance [110].

The TIA RNA binding proteins are involved in RNA splicing and regulation of translation. The family member TIAR-2 is an intrinsic inhibitor of axon regeneration and has a carboxy-terminal prion-like region. Mutations in this region are associated with several diseases such as ALS and Welander distal myopathy [111–113]. Work in *C. elegans* demonstrates an in vivo function for phase-separated TIAR-2. In *C. elegans* TIAR-2 functions cell autonomously to inhibit axonal regeneration. TIAR-2 undergoes LLPS in vitro and forms liquid-like granules in vivo. TIAR-2-positive granules increase transiently upon axonal injury of posterior lateral mechanosensory neurons [114]. The dynamic liquid-like behaviour of the neuronal granules in vivo measured by quantifying fission and fusion events, changes following axonal injury. The prion-like domain is needed for the formation of granules and to inhibit axonal regeneration after injury. The same study identifies molecular features important for TIAR-2 function in axon regeneration, revealing that both tyrosine and serine residues in the prion-like domain are critical for the formation of granules and for axon regeneration [114]. The study shows that the role of the tyrosine residues is independent from their phosphorylation, and may instead be relevant for the formation of weak interactions via their aromatic ring with other aromatic rings (π-π interactions) or cationic interactions (cation-π interactions) that are known to be fundamental to promote LLPS of other disordered proteins like FUS [115–117].

Synaptic organization

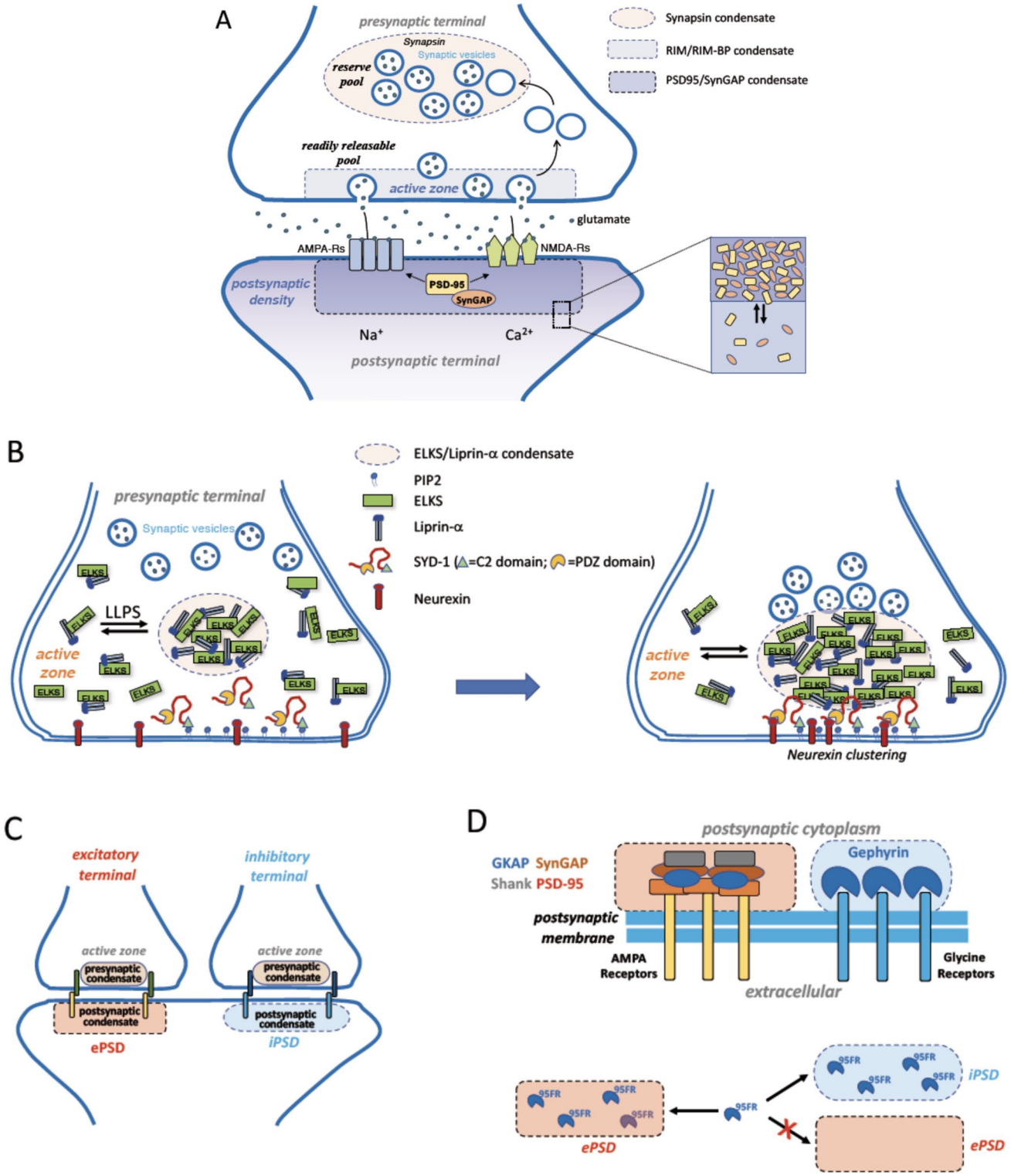
Neuronal synapses are asymmetric compartments transmitting signals between neurons [118]. Each synapse includes a pre- and a postsynaptic terminals separated by a narrow extracellular gap (Fig. 6A). The presynaptic terminal includes the synaptic vesicles carrying the neurotransmitter, and the membrane-associated active zone with the machinery for the recruitment, docking and fusion of the synaptic vesicles for neurotransmitter release. On the postsynaptic side, the membrane-associated postsynaptic density is needed to concentrate and organize the molecular machinery including the transmembrane receptors that bind to neurotransmitters. In the case of the excitatory synapses, ligation of the neurotransmitter to the receptor will start the series of events leading to the depolarization of the postsynaptic plasma membrane needed to trigger the propagation of the electric signals through the neuronal circuits. For this, the postsynaptic density includes several molecular components required for the efficient reception of the presynaptic signal. In recent years, several studies have directed the attention to the role played by LLPS in the organization of both pre- and postsynaptic terminals. Since several reviews have revised exhaustively the literature on this topic [9, 10, 12, 119–122], we will mention here studies in vitro and in cultured neurons, while focussing mainly on the fewer studies on living organisms supporting the evidence of the role of LLPS in synaptic organization and function in vivo.

Fig. 6 Protein phase separation at the synapse. (A) Schematic diagram of an excitatory synapse. In the presynaptic compartment distinct pools of synaptic vesicles are detectable: the *reserve pool* associated to the synapsin condensate, the *readily releasable pool* of vesicles that can rapidly fuse with the cell membrane and release the neurotransmitter (glutamate), and the *recycling pool*. Binding of glutamate to the AMPA and NMDA glutamate receptors on the surface of the postsynaptic membrane triggers the influx of Na^+ and Ca^{2+}, respectively, which is essential for the induction of the action potential. In the presynaptic terminals different types of biomolecular condensates have been identified, including a liquid phase driven by the protein synapsin that entraps the vesicles of the *reserve pool*, and a liquid phase of the scaffolds RIM and RIM-BP to position Ca^{2+} channel clusters near Ca2+sensors on the surface of the docked vesicles. (B) Model of presynaptic assembly in *C. elegans* [144]: the scaffold protein SYD-1 coordinates synapse assembly via interactions with phosphatidylinositol 4,5-bisphosphate (PIP2), to then recruit the presynaptic transmembrane cell adhesion molecule Neurexin. Early during synaptic development, SYD-2/liprin- α and ELKS-1 form LLPS-driven condensates that are able to concentrate downstream proteins at the active zone (left). SYD-1 accumulation contributes to the recruitment of the SYD-2/liprin-α and ELKS-1 condensates at the plasma membrane, thanks to the interactions of SYD-1 with the PIP2 phospholipids (C2 domain). SYD-1 binds and recruits Neurexin to presynapses via the PDZ domain (right). (C) Model of a dually innervated synapse: an excitatory and an inhibitory presynaptic terminal interact with the surface of a spine to form an excitatory (ePSD) and an inhibitory (iPSD) postsynaptic density, respectively. (D) Top: diagram of molecular components specific for the excitatory and inhibitory postsynaptic condensates, respectively. Bottom: a Gephyrin protein tagged with an intrabody with high affinity for PSD-95 (95FR) is recruited as a client into postsynaptic excitatory condensates (left). In the presence of inhibitory postsynaptic condensates the Gephyrin.^{95FR} construct is preferentially recruited to the inhibitory condensate (right) [145]

LLPS at the presynaptic terminal

Organization of the presynaptic vesicles

Synaptic transmission is fundamental to transfer information within the nervous system. Vesicles docked at the active zone fuse with the membrane in response to Ca^{2+} influx upon neural stimulation [123–125], driven by complex molecular events [126, 127]. The first indication of LLPS involved in the presynaptic organization came from the finding that synaptic vesicles cluster in proximity of the presynaptic active zone by inclusion into a liquid phase driven by the protein synapsin (Fig. 6A). This finding has helped explaining how synaptic vesicles remain clustered without binding to scaffolds. The entrapped vesicles can be rapidly released by phosphorylation of synapsin, to be efficiently recruited at the active zone for neurotransmitter release [128].



The in vitro studies indicating that LLPS may underlie the organization of synaptic vesicle clusters have guided a study that has tested this possibility in vivo. Microinjection into living lamprey giant reticulospinal synapses of compounds that perturb the IDR of synapsin that is critical for LLPS in vitro, cause the dispersion of the synaptic vesicles from the clusters, while reagents perturbing the intermolecular interactions mediated by the SH3 domain of synapsin were ineffective [129]. These results support the conclusion that a liquid phase maintained by the

IDR of synapsin is required to organize synapses in living organisms.

It is known that the synaptic activity entails the reorganization of synaptic vesicles, synapsin and actin, but the details of the interactions among these players is still unclear. Super-resolution imaging and cryo-electron tomography has been recently used to dissect the role of synapsin condensates in the organization of the actin cytoskeleton [130]. The condensation of synapsin-1 triggers actin polymerization to facilitate the clustering of synaptic vesicles, which is similar in the native presynaptic organization observed in lamprey spinal cord and in mammalian synapses.

Tethering of synaptic vesicles to the presynaptic active zone governs synaptic strength: the molecular mechanisms guiding vesicle tethering is still undefined. In this direction, it has been shown that components of the presynaptic active zone can undergo LLPS: the scaffold proteins RIM and RIM-BP undergo multivalent interactions to form dynamic assemblies, where they concentrate via direct binding the client voltage-gated Ca^{2+} channels [131]. RIM/RIM-BP LLPS forms microdomains to position Ca^{2+} channel clusters near Ca^{2+} sensors on the surface of the docked vesicles, to guide efficient synaptic transmission.

According to a current view, the organization of the synaptic spaces entails the interaction among distinct biomolecular condensates. An in vitro reconstitution study has shown that isolated synaptic vesicles can bind to the surface of LLPS-induced condensates made by the active zone proteins RIM, RIM-BP, and ELKS [132]. The bound vesicles are encircled by a second synapsin condensate. In this way, two distinct pools of synaptic vesicles are organized in vitro that are reminiscent of the two distinct synaptic vesicle *reserve pool* associated to the synapsin condensate [133, 134], and the *readily releasable pool* [135, 136] associated to the RIM/RIM-BP/ELKS condensate.

The presynaptic active zone

The nematode *Caenorhabditis elegans* has been used for decades to explore and uncover the machinery underlying the formation and function of synapses in vivo [137]. Several conclusions on the nematode synaptic organization have been later confirmed for the organization of mammalian synapses. The active zone scaffold proteins Liprin-α/SYD-2 and ELKS-1 undergo LLPS to concentrate other components at nascent synapses in vivo [138]. The IDR of Liprin- α/SYD-2 drives its LLPS that is needed to recruit at the presynaptic site other active zone components like ELKS-1 and RIM/UNC-10 [138]. Liprin- α/SYD-2 proteins are critical regulators of synaptic structure and function in *C. elegans, Drosophila* and mammals [139–142]. Liprin- α's LLPS mediates their activity also in mammalian Liprin- α proteins. In particular, mammalian Liprin-α3 reversibly undergoes LLPS triggered by protein kinase C-induced phosphorylation at Ser^{760}. Liprin-α3 condensates are needed to recruit presynaptic adaptor proteins RIM and Munc13 at the membrane [143]. In hippocampal neurons from Liprin-α2/α3 double knockout mice, the pool of synaptic vesicles is decreased and is rescued by re-expression of wildtype Liprin-α3, but not of the phospho-null mutant Ser^{760}Gly unable to undergo LLPS.

At the presynaptic active zone, clusters of ion channels are required to trigger the fusion of synaptic vesicles for neurotransmitters release [123]. The composition of the active zones is well characterized, but the mechanisms to assemble these structures are unclear. Recently the implication of LLPS in the organization of the active zone has helped explaining how the active-zone proteins assemble in vivo the machinery required for vesicle release. In *C. elegans* the active-zone scaffold proteins Liprin-α/SYD-2 and ELKS-1 undergo LLPS during early synaptic development.

Later on the active zone matures into a solid structure [138], but the mechanism mediating the transition of Liprin-α/SYD-2 condensates from liquid to solid is still unknown. Regions of the two proteins required to drive LLPS have been identified; their mutation induces defects in active-zone assembly and in synaptic function. Interestingly, the addition of an LLPS-prone motif from an unrelated protein to the LLPS-defective mutants is able to rescue these defects [138].

The mechanism triggering LLPS by Liprin- α/SYD-2 has been recently disclosed. Phosphorylation by the kinase SAD-1 activates Liprin- α/SYD-2 phase separation by releasing an intramolecular autoinhibition mediated by the Liprin- α SAM (Sterile Alpha Motif) domains [146]. This mechanism is different from the activation of LLPS triggered by phosphorylation of mammalian Liprin-α3 described before [143].

In the hierarchical model proposed for presynaptic organization (Fig. 6B), the cytosolic scaffold protein SYD-1 accumulates at nascent presynapses via phosphatidylinositol 4,5-bisphosphate (PIP2) to then recruit the transmembrane cell adhesion molecule neurexin that stabilize nascent presynaptic sites [144]. SYD-1 also recruits Liprin- α/SYD-2 and ELKS that accumulate at the plasma membrane as phase-separated condensates, to then mediate the recruitment and clustering of synaptic vesicles for efficient neurotransmitter release [138, 147–150]. The emerging picture suggests that presynaptic assembly is initiated by multiple intracellular interactions of cytosolic active zone scaffold proteins able to undergo LLPS, followed by the trans-synaptic stabilization prompted through interactions with extracellular trans-synaptic partners.

The reserve and recycling pools of synaptic vesicles co-cluster in the presynaptic terminal (Fig. 6A). A recent study has tested the hypothesis that biomolecular condensates regulate the nanoscale distribution of the two different pools within a presynaptic terminal. Activity-dependent phosphoproteomic analysis from hippocampal neurons has been used to identify structurally disordered synaptic proteins whose phosphorylation changes in response to stimulation of hippocampal synapses [151]. This analysis has revealed the phosphorylated disordered protein Tau as a candidate involved in the activity-dependent presynaptic reorganization. Single-molecule super-resolution microscopy has shown that Tau undergoes LLPS to form presynaptic nanoclusters reorganized by synaptic activity. Tau moves and forms biomolecular condensates in the presynaptic terminal to control the mobility of the recycling vesicles [151].

To move between different presynaptic pools, synaptic vesicles need to move short distances without involving molecular motors. LLPS can facilitate directional synaptic vesicle transport between different presynaptic subcompartments. In response to activity-dependent increased concentration of intracellular Ca^{2+}, a coiled-coil region of the presynaptic scaffold protein Piccolo interacts with the active zone scaffolds RIMBP and ELKS1 to extract synaptic vesicles from the synapsin-organized *reserve pool condensate* for delivery to the *active zone condensate* [152].

Liprin-α and RIM play a central role in scaffolding the presynaptic architecture. The determination of the crystal structure of the Liprin-α2/RIM1 complex has revealed a complex intermolecular interaction driving the Liprin-α/RIM assembly, which is blocked by disease-associated mutations impairing the formation of the complex, as well as synaptic transmission by reducing the readily releasable pool of synaptic vesicles in human neurons [153]. Structural and biochemical analyses have highlighted the relevance of this interaction in assembling Liprin-α2/RIM1 condensates: disrupting the Liprin-α2/RIM interface via point mutations reduces the size and number of condensates, directly linking the integrity of the Liprin-α2/RIM complex to LLPS, which may concentrate other active zone components like ELKS, Munc13, and the $Ca.^{2+}$ channels required for efficient neurotransmitter release [153]

The requirement for LLPS at the synapse has been tested directly by disrupting liquid condensates in rat hippocampal neurons. Treatment with the aliphatic alcohol 1,6-hexanediol is a common way to dissolve biomolecular condensates [154]. This treatment does not affect spontaneous neurotransmission, but disturbs action potential-dependent neurotransmission [155].

The intracellular concentration of ATP may impact the formation of condensates in the cytoplasm of mammalian neurons by regulating their axoplasmic viscosity [156]. The decrease in intracellular concentration of ATP by inhibition of the mitochondrial activity has consequences on the functional organization of mouse glutamatergic synapses, causing condensation of axonal cytosol, of synaptic vesicles, and of proteins involved in active zone organization (synapsin, α-synuclein) and neurodegeneration (parkin). Increased ATP concentrations in vitro could reverse the condensation of these proteins. Human glutamatergic neurons and human motor neurons from pluripotent stem cells from patients with Parkinson's disease (glutamatergic neurons) or ALS (motor neurons) show reduced axonal cytosol fluid phase and intracellular ATP compared to neurons derived from healthy donors. Interestingly, treatment with the neuroprotectant agent nicotinamide mononucleotide (a NAD+precursor) reversed the decrease in axonal cytosolic fluidity detected in these neurons, and increased the intracellular levels of ATP. Based on the findings that ATP is an effective hydrotropic agent that regulates protein aggregation in vitro [157, 158], the results from this study suggest that the hydrotropic activity of ATP contributes to the regulation of neuronal homeostasis under physiological and pathological conditions.

LLPS at the postsynaptic terminal

The postsynaptic terminal of excitatory synapses includes clustered glutamatergic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate) ionotropic glutamate receptors that are essential for synaptic transmission and plasticity (Fig. 6A). Several studies have highlighted the relevance of LLPS in controlling the dynamic organization of the postsynaptic terminals, and in the remodelling of the intermolecular connections required to establish neuronal activity-dependent LTP.

The first report of the involvement of LLPS at the synapse has shown that the multivalent interaction between the Ras/Rap GTPase-activating protein SynGAP and the scaffold PSD-95, two important components of the postsynaptic density, induces phase separation of the complex that forms highly concentrated liquid-like condensates reminiscent of the postsynaptic density [159] (Fig. 6A). The biochemical reconstitution in vitro with phospholipid bilayers supports the hypothesis of the formation of the postsynaptic density by LLPS [160]. The same group has shown by in vitro reconstitution that the membrane-associated AMPA receptor regulatory protein stargazin, a central component of the postsynaptic density, binds to PSD-95 via a multivalent interaction that directs the formation of stargazin/PSD-95 condensates by LLPS, a process that is critical for the synaptic targeting of AMPA receptors [161]. Of the different SynGAP isoforms, SynGAP- α1 undergoes LLPS with PSD-95, is highly enriched in synapses, and is

required for LTP (long-term potentiation) [162]. A mutation in SynGAP- α1 disrupting its phase separation and synaptic targeting, abolishes the ability of SynGAP-α1 to regulate plasticity [163].

N-methyl-D-aspartate (NMDA) glutamate receptors are essential for excitatory neurotransmission and synaptic plasticity. GluN2A and GluN2B are major subunits of the NMDA receptors in hippocampus and cortex. CaMKII is a central player of synaptic plasticity that undergoes Ca^{2+}-induced LLPS with the NMDA receptor subunit GluN2B [164]. Binding of activated CaMKII to GluN2B co-segregates AMPA receptors and the synaptic adhesion molecule neuroligin to condensates. This activity-dependent mechanism has been proposed to reorganize the postsynaptic terminal for synaptic plasticity. The persistence of CaMKII/GluN2B condensates through autophosphorylation of threonine-286 provides a mechanism linking transient Ca^{2+} signals to lasting synaptic changes. Finally, the reversibility of these condensates by the endogenous inhibitor suggests a mechanism for bidirectional regulation, which is relevant to synaptic development as well as synaptic plasticity.

Rabphilin-3A, a downstream effector of the synaptic small GTPase Rab3A, undergoes LLPS to cluster GluN2A by binding to its carboxy-terminal domain. Rabphilin-3A phase separation is promoted by a complex including PSD-95 [165]. Disrupting phase separation by Rabphilin-3A suppresses its GluN2A clustering activity, synaptic localization, and synaptic response of GluN2A in hippocampal neurons.

A possible function of LLPS is to mediate the storage of molecules in condensates until needed at specific times during neuronal activity. One example is provided by the partially disordered postsynaptic scaffold protein nArgBP2 that is critical for dendritic spine development. nArgBP2 links to the actin cytoskeleton by interacting with the WAVE regulators of actin polymerization [166]. nArgBP2 knockdown prevents the enlargement of spines induced by LTP [167]. In resting neurons the nArgBP2 protein forms liquid-like condensates in the spine that are dispersed by LTP, coincident with spine head enlargement. CaMKII inhibition inhibits both LTP-induced dispersion of nArgBP2 condensates and spine enlargement. LLPS occurs via multivalent interactions of the SH3 domains with proline-rich regions of nArgBP2 molecules. These condensates recruit CaMKII, and are disassembled by Ca^{2+}/CaMKII-dependent phosphorylation. Binding of the actin-organizing protein WAVE1 to nArgBP2 competes with nArgBP2 LLPS, while blocking nArgBP2-WAVE1 interaction prevents spine enlargement during LTP [167]. These results support the hypothesis that storage and release of nArgBP2 from condensates contribute to appropriate spine head enlargement. More recently, super-resolution imaging has shown the differential intra-spine distribution of nArgBP2 with respect to other postsynaptic components, and has suggested its involvement in the formation of the layered molecular organization observed in the dendritic spine [168].

A recent study has shown that altering the complexity of the postsynaptic network by targeting different interactions, alters the mobility of proteins within the network. The experimental setup in this study includes for example the block of the interaction between scaffold proteins PSD-95 and SAPAP/GKAP by a high affinity inhibitory peptide. Without altering the interaction between AMPA receptor and PSD-95, this perturbation of the network results in the increased mobility of the receptor within synapses, with negative consequences on synaptic transmission and plasticity in rat hippocampal neurons analyzed ex vivo in hippocampal organotypic slice cultures [169].

Another important question underlying the complexity of the nervous system is how excitatory and inhibitory postsynaptic terminals are segregated in dually innervated synapses (Fig. 6C). In vitro and *in cellulo* reconstitution has shown that excitatory and inhibitory terminals spontaneously segregate into distinct molecular assemblies by LLPS. Tagging the inhibitory scaffold protein gephyrin with a high affinity PSD-95 intrabody [170] leads to mistargeting of gephyrin to excitatory postsynaptic condensates [145] (Fig. 6D). Interestingly, the formation of inhibitory postsynaptic condensates drives the intrabody-tagged gephyrin out of excitatory condensates, showing that LLPS can create subcellular compartments through specific multivalent interactions with intrinsic organelle-like structures.

Different biomolecular condensates may show a wide-range of material properties, from fluid to solid. The regulation of the material properties of a biological condensate is poorly understood. A recent study indicates that the material properties of the postsynaptic condensates are critical for postsynaptic function, learning and memory. The oligomerization of the postsynaptic scaffold protein Shank3 contributes to the soft glass-like material properties of the postsynaptic condensate. Defects in Shank3 oligomerization obtained by introducing a single aminoacid substitution in the Shank3 SAM domain ($Met^{1718}Glu$) results in softer postsynaptic condensates, leading to impairment of the synaptic transmission, and causing autism-like phenotypes in mice carrying the Shank3-$Met^{1718}Glu$ mutation [171].

LLPS and neural diseases

LLPS of a number of partially disordered proteins within the nervous system is thought to be critical for their pathological aggregation and toxicity. These proteins include the microtubule-binding protein Tau involved in Alzheimer's disease [172, 173], the RNA/DNA binding proteins TDP-43

and FUS involved in ALS [77, 174, 175], the scaffolding protein huntingtin involved in microtubule-based axon trafficking and in Huntington's disease [176], the α-synuclein protein regulating trafficking and neurotransmitter release [177], and β-amyloid precursor proteins involved in Parkinson's disease [178, 179].

Ribonucleoproteins, altered translation, and disease

As seen earlier in this review, reversible interactions between specific RNAs and RNA-binding proteins like FUS and TDP-43 result in the formation of ribonucleoprotein condensates critical for RNA processing, mRNA transport, and translation. In neurons, ribonucleoprotein condensates promote long-range mRNA transport and local translation in dendrites and axon, that are essential for spatio-temporal regulation of gene expression and synaptic function. Mutation, mislocalization, and aggregation of RNA-binding proteins are hallmarks of several neurodegenerative diseases, including ALS, frontotemporal dementia, and Alzheimer's disease. In these diseases, aberrant ribonucleoprotein condensates are detrimental for mRNA stability, localization, and translation, and compromise axonal integrity and synaptic function [180, 181]. The DNA/RNA-binding protein FUS plays a role in various processes including transcription regulation, RNA splicing, RNA transport, and DNA repair. A FUS mutant with enhanced propensity for LLPS suppresses local translation in the growth cones, potentially linking the formation of granules by LLPS to the suppression of local translation in the axonal terminals [116, 174]. Along the same line, in axons of mouse and human neurons carrying endogenous FUS with ALS-associated mutations, condensates formed by mutant FUS sequester the RNA binding protein FMRP that regulates translation in neurons through the formation of neuronal granules [62, 63]. This causes repression of translation in FUS-ALS motor neurons and in vitro. Accordingly, the translation of FMRP-bound RNAs is reduced in vivo in FUS-ALS embryonic spinal cord motor neurons [182].

Mutations and hypomethylation of arginine residues of the DNA/RNA-binding protein FUS produce pathological irreversible LLPS-induced condensates that cause frontotemporal lobar degeneration and familial ALS in humans [77, 116, 174]. A recent work has indicated that the formation of irreversible condensates by LLPS of hypomethylated FUS may underlie synapse dysfunction in frontotemporal lobar degeneration. Constructs mimicking hypomethylated FUS cause the formation of aberrant FUS condensates in the dendrites of CA1 neurons in rat organotypic hippocampal slices [183]. The aberrant condensates show spontaneous and activity-induced movements within dendrites. Neurophysiological defects dependent on the dendritic localisation of the condensates include the altered expression of postsynaptic PSD-95, and impaired excitatory synaptic transmission and dendritic spine plasticity.

The function of wildtype TDP-43 can be indirectly altered by disease-associated mutations in other genes. Misregulation of the RNA-binding protein TDP-43 and polyglutamine expansions of the RNA binding protein Ataxin-2 are associated to ALS [98, 184]. A recent study supports a model in which Ataxin-2 polyglutamine expansion disrupts the stability, localization, and translation of critical axonal mRNAs important for motor neuron integrity. Analysis in rat primary cortical neurons and in mouse motor neurons in vivo shows that Ataxin-2 polyglutamine expansions aberrantly sequester TDP-43 within ribonucleoprotein condensates, disrupting its axonal motility and liquid-like properties [185], thus perturbing the spatial localization of mRNA and suppressing local translation.

Microexons within the mRNA coding for the disordered prion-like domain of the translation initiation factor eIF4G are involved in eIF4G alternative splicing, and are frequently disrupted in autism. Deletion of the microexons upregulates synaptic proteins that control neuronal plasticity. Mice lacking the eIF4G1 microexons display social behaviour, learning and memory deficits, and altered hippocampal plasticity [186]. Prion-like domains are predisposed to undergo phase separation. As predicted, the inclusion of the microexons increases the propensity of eIF4G to undergo phase separation and to be included into phospho-FMRP condensates, suggesting that eIF4G microexons promote neuronal granule formation leading to ribosomal stalling and downregulation of the translation of synaptic proteins required for proper cognitive functions.

Liquid-to-solid transition and disease

Parkinson's disease and Dementia with Lewy Bodies are neurodegenerative disorders characterized by abnormal accumulation of α-synuclein aggregates [187, 188]. α-synuclein first forms liquid-like condensates by LLPS, then undergoes liquid-to-solid phase transition to form insoluble amyloids. β-synuclein partitions into α-synuclein condensates to promote LLPS and to slow down the transition to the solid state of α-synuclein [189]. These effects are lost by disease-associated β-synuclein mutations. Analysis in vivo in *C. elegans* shows that the expression of wildtype β-synuclein, but not of disease-associated β-synuclein mutants, improves the lifespan and the defect in movement of a worm strain overexpressing α-synuclein [189]. Single-point mutations associated with familial Parkinson's disease cause α-Synuclein aggregation [190] that leads to the formation of solid α-synuclein-positive Lewy bodies [191]. The $Glu^{46}Lys$ mutation of α-Synuclein identified in

Parkinson's disease increases the formation of α-Synuclein condensates compared to the wildtype protein, and accelerates the formation of solid amyloid aggregates within condensates [192].

Tau forms dynamic droplets in vitro at physiological protein levels, and droplets formation is enhanced by disease-associated modifications. Tau droplet dynamics is reduced for the mutated forms of Tau [193], and prolonged LLPS promotes time-dependent development of toxic conformations, suggesting that Tau LLPS facilitates the formation of pathogenic Tau conformations.

Tau LLPS plays a critical role in controlling kinase nanoclustering and downstream signalling. The Tau mutant Pro^{301}Leu from frontotemporal dementia patients is associated to increased LLPS. Expression of Pro^{301}Leu Tau promotes aberrant nanoclustering of the Src family tyrosine kinase Fyn in hippocampal dendrites [194]. Single-particle tracking microscopy shows that kinase activated Fyn nanoclusters increase its downstream signalling that is known to control mechanisms implicated in learning and memory. it is interesting to observe that postsynaptic enrichment of Fyn has been shown to underline synaptic toxicity in Alzheimer's disease and frontotemporal dementia.

Synaptic dysfunction and disease

Several synaptic proteins form biological condensates by LLPS. Autism spectrum disorders (ASD) are conditions characterized by damage and dysfunction of the synapse, which is likely due to impaired LLPS of ASD-linked synaptic proteins. It is known that LLPS and zinc-induced liquid-to-gel transition regulate the synaptic distribution of CTTNBP2 (cortactin-binding protein 2), an ASD-linked protein. CTTNBP2 self-assembles into condensates via a carboxy-terminal IDR, improving SHANK3 recruitment at dendritic spines. Zinc binds the amino-terminal coiled-coil of CTTNBP2 promoting higher-order assemblies by liquid-to-gel phase transition [195]. The reduced mobility of CTTNBP2 causes synaptic retention of CTTNBP2 condensates. ASD-linked mutations that reduce the density and size of dendritic spines [196] alter the formation of condensates and synaptic retention of CTTNBP2, and impair the social behaviour of mutant mice, that is improved by zinc administration [195].

Altered response to stress and disease

TDP-43 forms protective nuclear bodies in response to stress, which is mediated by LLPS of the long non-coding RNA NEAT1 [197]. Defects in the assembly of stress-mitigating TDP-43 nuclear bodies may contribute to ALS pathogenesis. By using transgenic flies expressing either WT or mutant forms of human TDP-43, it has been shown that various cellular stresses trigger the formation of dynamic, reversible TDP-43 nuclear bodies in mouse primary neurons, and in vivo in the brain of *Drosophila*. Toxicity is more evident when the ALS causing mutation TDP43-Asp-^{169}Gly is expressed in fly motor neurons: the age-dependent decline in the climbing ability of the flies was more evident compared to flies expressing wildtype TDP-43: the Asp-^{169}Gly mutation makes cells more prone to stress, which may underlie the pathogenesis of ALS.

Charcot-Marie-Tooth type 2 neuropathies (CMT2) are a group of genetically heterogeneous disorders. Principles underlying LLPS have helped clarifying how mutations in the diverse genes underlying different forms of CMT2 may give rise to a similar neuropathology. Upon environmental stress, different CMT2-causing mutant proteins enter stress granules and interact aberrantly with G3BP, a key protein driving LLPS for stress granule assembly [84]. A CMT2-linked mutant form of the enzyme glycyl-tRNA synthetase [198, 199] is translocated upon stress from the cytoplasm into stress granules, where it binds aberrantly to G3BP, disrupting stress granule-mediated stress responses [200]. The abnormally strong interaction of mutant glycyl-tRNA synthetase is detected both in vitro and in the spinal cord of CMT2 mutant mice. As a consequence, motoneurons become more vulnerable to stress, and CMT2D mutant mice show motor deficits. Preventing the aberrant interaction with G3BP by a point mutation in the domain of glycyl-tRNA synthetase required for binding to G3BP rescues stress granule abnormalities and motor deficits in the CMT2D mice [200].

Conclusions

Increasing evidence supports the role of LLPS in the organization of the developing and mature nervous system. The diversity of events where LLPS is implicated reflects the diversity of cellular processes involving the concentration of specific molecular components and molecular events at specific intracellular sites. LLPS-derived membraneless assemblies embrace different kinds of nucleoplasmic or cytosolic membrane-less structures, including a variety of ribonucleoproteins and stress granules; as well as networks built at specialized membrane regions, as in the case of the pre- and postsynaptic terminals. The variety of biomolecular condensates involved in neural development and function is expected to increase as research proceeds. At the same time other important open questions remain that need to be further investigated. One interesting raising issue is the interplay among molecularly distinct condensates, as those recently identified in the synapse, where

dynamic interactions between condensates are expected to finely regulate the intricate events occurring during the normal functioning of the synapse. Linked to this is the role of post-translational regulation in fine-tuning the assembly and function of the different biomolecular condensates at different places and different times. Both issues add layers of complexity in the organization of the supramolecular signaling and scaffolding networks forming at specific sites of the developing and mature neuron. The distinct roles that have been postulated for the large variety of biomolecular condensates identified so far include: the sequestration of components that need to be removed from the intracellular environment; the effects of biomolecular condensates on the kinetics and efficiency of specific reactions; the buffering of the cellular concentration of specific proteins or RNAs; the regulation of the specificity of biochemical reactions. For example, condensates may confine and concentrate specific signaling enzymes to enhance post-translational events that contribute to their own maintenance or turnover. In this direction, one relevant issue to be addressed is the functional role of the interplay between condensates with distinct molecular composition.

Condensates formed by LLPS of distinct drivers may merge or remain distinct to respond to functional requirements, increasing the functional plasticity of condensates regulating for example either translation, or synaptic assembly and plasticity. In this direction, the effects of Liprin-α/ELKS condensates on RIM/RBP condensates at the presynapse suggest that Liprin-α may control the compartmentalization of different active zone components [201].

The compartmentalization of the membrane is a way to confine specific transmembrane receptors that may act as ligands to concentrate cytoplasmic protein partners undergoing LLPS, thanks to their increased concentration near the plasma membrane [202]. The organization of membrane regions limiting the free diffusion of specific proteins by LLPS allows the formation of signaling and scaffolding assemblies to efficiently carry on specific cellular processes, as observed at the pre- and postsynaptic terminals dedicated to synaptic exocytosis and to reception and signaling, respectively. On the other hand, LLPS of cytoplasmic components may favour the clustering of transmembrane proteins, as in the case of the clustering of ion channels at the synapse [144].

Given the several examples linking altered phase separation events to neurodevelopmental and neurodegenerative diseases, it is evident that unraveling the complexity and variety of the molecular mechanisms underlying the organization and regulation of LLPS-driven biomolecular condensates will greatly help not only the understanding of the pathogenetic mechanisms of these diseases, but also the development of potential therapeutic strategies [203, 204].

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Declarations

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