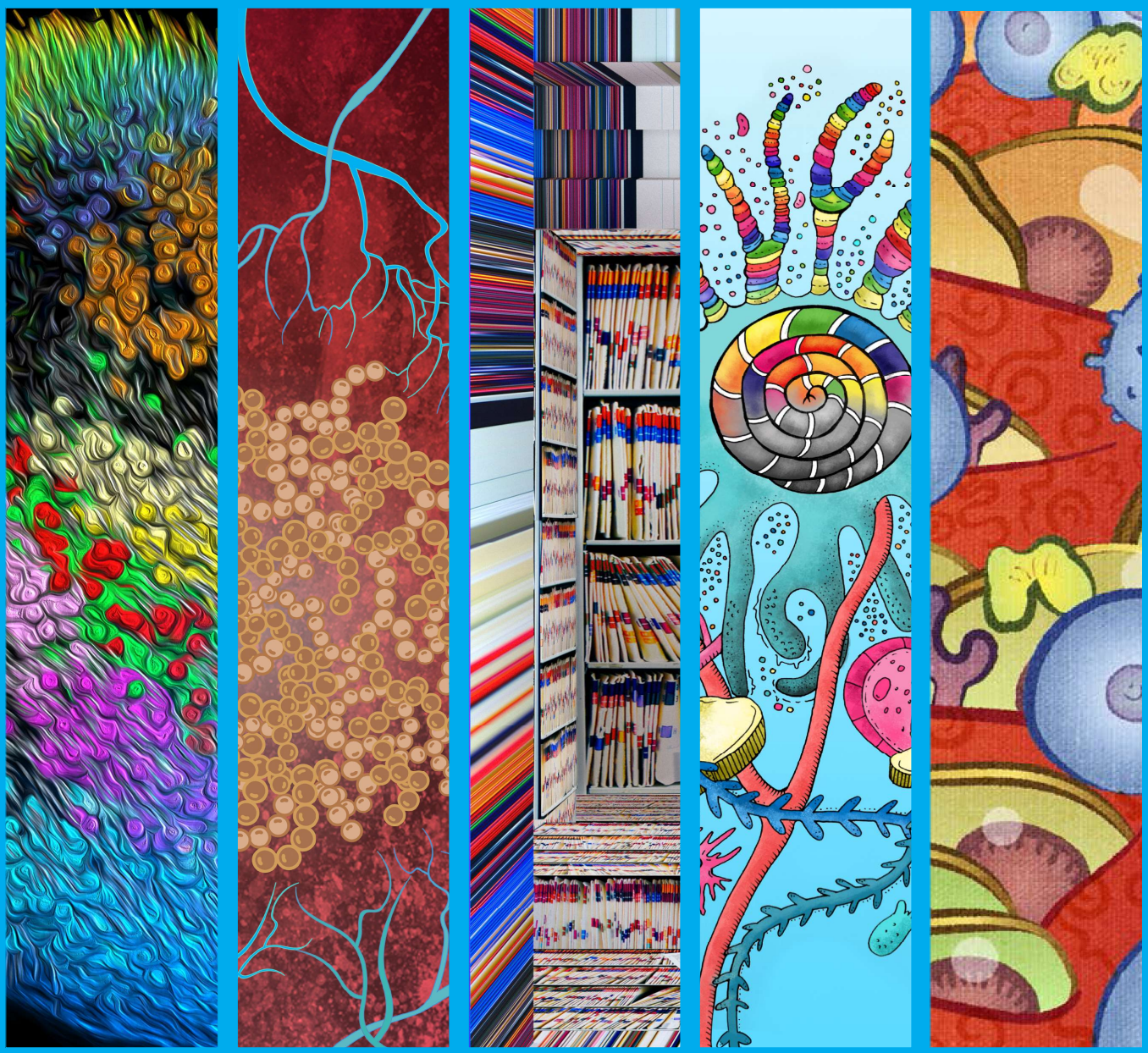
Cell

Best of 2018



Featuring a Collection of SnapShots

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Foreword

In 2012, Cell Press first launched the "Best of" reprint collections across a number of journals, most notably *Cell*. Now, we are happy to bring you a new *Best of Cell* that covers a broad selection of content published in late 2017 and 2018.

It's been an exciting and eventful year for *Cell*. Our scope is broader than ever before, reflecting the exciting interdisciplinary trajectories that the biomedical sciences are moving towards. This collection of research papers and reviews reflects this breadth, showcasing science that either captured our editorial interest or garnered wide attention for its conceptual impact. These topics include, among others, using AI to identify and diagnose medical diseases, visualizing mammalian development at the single-cell level, delving into the genetic adaptations of sea nomads, and cloning monkeys with state-of-the-art technologies. In addition, we've included a collection of SnapShots that present up-to-date, quick-read summaries to highlight several key areas of biology; we hope these SnapShots will be useful reference tools for the scientific community.

The research and reviews collected here are meant to give our readers and the scientific community a flavor of the kinds of studies that define a *Cell* paper. They come in all shapes and sizes and all topic areas and flavors—from molecular to translational, from fundamental to paradigm changing. We recognize that no single measurement can be indicative of the "best" papers over a given period of time, especially when the articles are relatively new and their true significance may still need time to be established. Regardless of citation indices, we believe these papers are noteworthy, and we hope you enjoy reading them.

Our team brings both editorial wisdom and fresh perspectives, and together under the new leadership of *Cell* Editor-in-Chief John Pham, we look forward to working with you to capture even more exciting science in 2019. Please feel free to contact us at cell@cell.com to tell us about your latest work or to provide feedback. You can access the entire "Best of" collection online at www.cell.com/bestof. Also visit www.cell.com/cell to learn about the latest findings that *Cell* has had the privilege to publish and www.cell.com to find other high-quality life science papers published in the full portfolio of Cell Press journals.

As a final note, we are grateful for the generosity of our sponsors, who helped to make this reprint collection possible.

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Best of 2018

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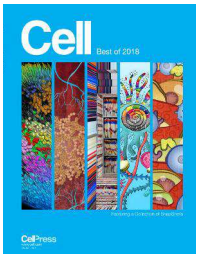

On the cover: Featured on the cover is a cross-section of visually striking images published on the cover of *Cell* in 2017 and 2018. Images are courtesy of (from left to right): McDole et al. (volume 175, issue 3), Erica Alana Alexandre (volume 173, issue 5), Polubriaginof et al. (volume 173, issue 7), Sullivan et al. (volume 175, issue 1), and Design and Creative Services, MSKCC (volume 174, issue 5). Cover design by Yvonne Blanco.

Cell

Article

Reconstituted Postsynaptic Density as a Molecular Platform for Understanding Synapse Formation and Plasticity

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SUMMARY

Synapses are semi-membraneless, protein-dense, sub-micron chemical reaction compartments responsible for signal processing in each and every neuron. Proper formation and dynamic responses to stimulations of synapses, both during development and in adult, are fundamental to functions of mammalian brains, although the molecular basis governing formation and modulation of compartmentalized synaptic assemblies is unclear. Here, we used a biochemical reconstitution approach to show that, both in solution and on supported membrane bilayers, multivalent interaction networks formed by major excitatory postsynaptic density (PSD) scaffold proteins led to formation of PSD-like assemblies via phase separation. The reconstituted PSD-like assemblies can cluster receptors, selectively concentrate enzymes, promote actin bundle formation, and expel inhibitory postsynaptic proteins. Additionally, the condensed phase PSD assemblies have features that are distinct from those in homogeneous solutions and fit for synaptic functions. Thus, we have built a molecular platform for understanding how neuronal synapses are formed and dynamically regulated.

INTRODUCTION

Postsynaptic densities (PSDs) of neuronal synapses were initially recognized by electron microscopy (EM) with observations of electron-dense thickenings beneath postsynaptic membranes (Palay, 1956). Subsequent biochemical and EM analyses of purified PSDs revealed that these structures are composed of densely packed proteins forming disc-shaped mega-assemblies with a few hundred nanometers in width and ~30–50 nm thick (Cohen et al., 1977; Harris and Weinberg, 2012). In response to diverse neuronal stimuli, PSD proteins undergo assembly/disassembly, which are tightly associated with various forms of synaptic plasticity.

A number of unique properties of synapses have hampered detailed understandings of synapse formation and regulation at the molecular level. First, it is hard to find two identical synapses in an entire brain, implying that there is no simple repeating structural unit within synapses. This has prevented detailed structural studies of synapses by existing biophysical methods. Second, synapses are highly compartmentalized, self-assembled reaction machineries with a spine head volume of ~0.1 μm^3 (Harris and Stevens, 1989; Kubota et al., 2007; Nishiyama and Yasuda, 2015). This elaborate and complex compartmentalization is necessary for intricate functions/wiring of neurons, but also creates practical difficulties in understanding each individual synapse. Third, the volume of a spine head is proportional to the size of PSD (Harris and Stevens, 1989; Matsuzaki et al., 2001). Thus, the morphology of synapses is closely coupled with synaptic functions, although with a poorly understood mechanism. Fourth, synapses are extremely plastic. A synapse can undergo chemical component changes on a timescale as fast as a few seconds. Two neighboring spines separated by <1 μm apart can undergo distinct morphology changes upon differential stimulations (Bartol et al., 2015; Matsuzaki et al., 2004; Nishiyama and Yasuda, 2015). There is no unifying theory to explain the molecular mechanisms underpinning the synaptic plasticity. Fifth, highly dense protein-rich assemblies in synaptic compartments (i.e., PSDs) are not enclosed by membrane bilayers and appear to form via certain self-assembling mechanisms (Zeng et al., 2016a). We do not understand how such dense synaptic assemblies can form and stably exist without physical barriers.

Here, we demonstrate that mixing purified postsynaptic scaffold proteins at physiological concentrations can form highly condensed, self-organized PSD-like assemblies via liquid-liquid phase separation (LLPS). Such PSD scaffold condensates can cluster glutamate receptors, enrich synaptic enzymes, and promote actin bundle formation. Importantly, the reconstituted PSD

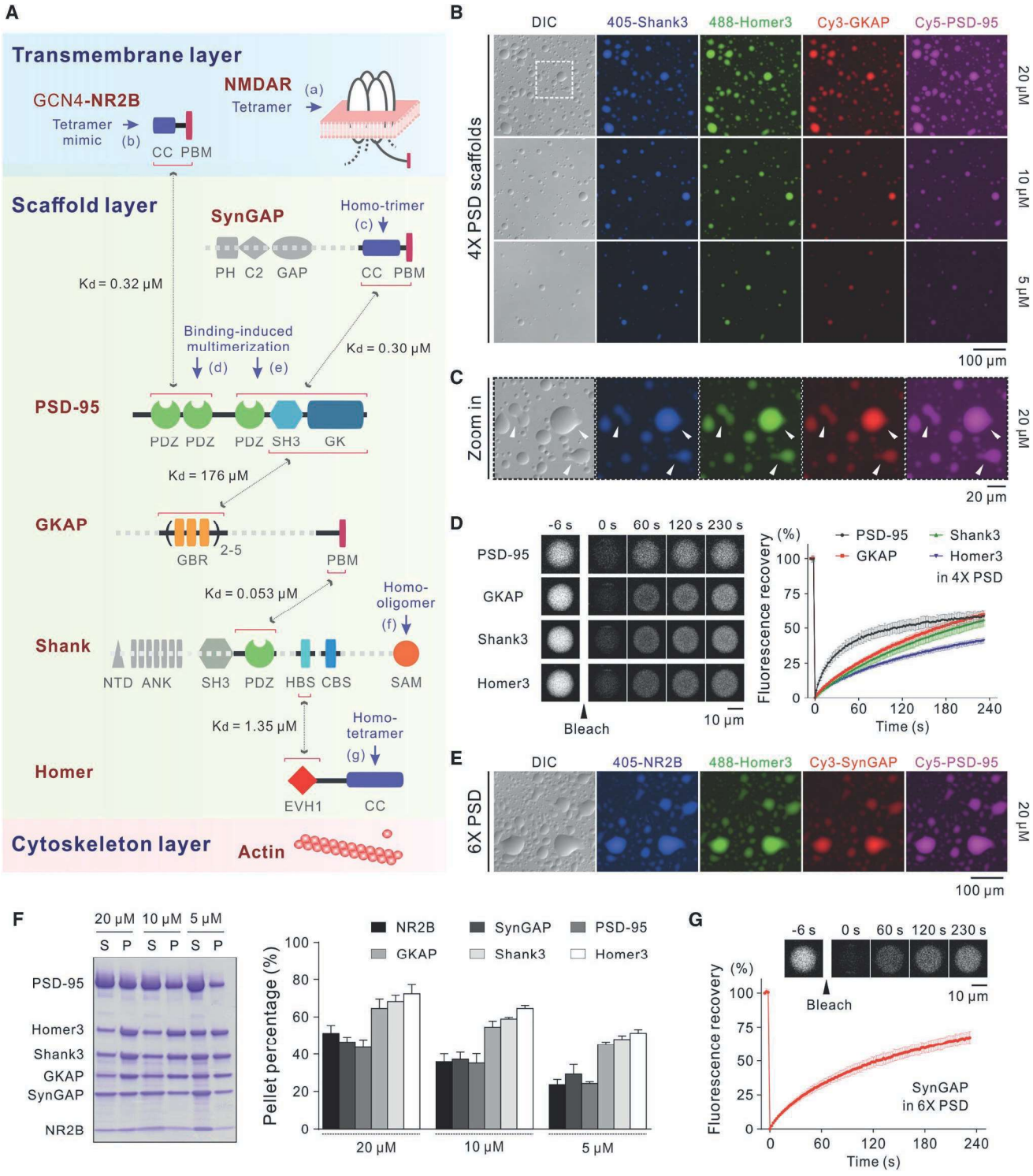


Figure 1. PSD Scaffold Proteins Form Phase Transition-Mediated Condensates Enriching Other Synaptic Proteins

(A) Schematic diagram showing the components and the interaction details of the PSD protein network used in the current study drawn in a spatial order. Solid black lines connecting protein interaction domains/motifs are flexible sequences. Domains drawn in gray and dashed lines are removed from the corresponding proteins for technical reasons. Black arrows indicate the measured binding affinities of the interactions (raw data in Figure S1). Dark blue arrows indicate the multimerization status of the indicated domains or complexes. (a) Lü et al. (2017); (c) Zeng et al. (2016a); (b and d) from Figure S1; (e) Zeng et al. (2018); (f) Baron et al. (2006); (g) Hayashi et al. (2009).

(legend continued on next page)

assemblies actively repel Gephyrin. The LLPS-mediated PSD assembly displays a series of distinct features that are not possessed by canonical protein interaction networks in homogeneous solutions. The reconstituted PSD assemblies may function as molecular platforms for understanding mechanisms governing synaptic formation and plasticity.

RESULTS

PSD Condensates Formation by Mixing Major PSD Scaffold Proteins

We began our reconstitution of PSD assembly using a group of highly abundant scaffold proteins including PSD-95, GKAP, Shank, and Homer. These four proteins, in the order of PSD-95, GKAP (aka SAPAP1 or DLGAP1), Shank, and Homer, serve to connect ion-channels/receptors on the postsynaptic plasma membrane with the actin cytoskeleton in the cytoplasm of PSDs (Figure 1A) (Dosemeci et al., 2016; Petralia et al., 2005; Valtschanoff and Weinberg, 2001; Zhu et al., 2016). We used the full-length PSD-95 and Homer3 for the reconstitution experiments. To obtain soluble and well behaving GKAP and Shank3, we removed part of the sequences of the two proteins (see Figure 1A and STAR Methods for details). The binding affinities between PSD-95 and GKAP, between GKAP and Shank3, and between Shank3 and Homer3 were essentially the same as the bindings investigated using the isolated domains as reported previously (Zeng et al., 2016b; Zhu et al., 2017) (Figures 1A and S1A–S1C). We also confirmed that Homer3 is a homo-tetramer (Hayashi et al., 2006, 2009). Shank3 had a concentration-dependent homo-oligomerization behavior, and both PSD-95 (Figure S1F) and GKAP were highly homogeneous monomers (data not shown).

When mixing fluorescently labeled PSD-95, GKAP, Shank3, and Homer3 at a 1:1:1:1 molar ratio at indicated concentrations (defined as the monomeric unit concentration of each protein throughout the entire study), we readily observed phase separation under light microscopy. Differential interference contrast (DIC) microscopic images revealed that micron-sized, phase-separated droplets had spherical shapes (Figure 1B), and some of the droplets were undergoing fusion process (Figure 1C). Fluorescence images showed each droplet was highly enriched with all four scaffold proteins. Each protein exchanged rapidly between the condensed phase and the surrounding aqueous solution, as revealed by fluorescence recovery after photobleaching (FRAP) analysis (Figure 1D; Video S1). The level of phase transition of the scaffold protein mixtures was correlated to their concentrations. When the concentration of each protein decreased, the amount of the condensed droplets also progressively decreased (Figure 1B). The above results demonstrate that mixing the four major PSD scaffold proteins leads to the formation of self-assembled, highly co-enriched protein networks that appear as liquid condensates.

The PSD Condensates Enrich Glutamate Receptors and Synaptic Enzymes

In addition to interacting with each other, the four scaffold proteins contain a number of domains/motifs capable of binding to the other PSD components. We tested whether the PSD scaffold condensates could incorporate other PSD proteins. We first tested NMDA receptor (NMDAR). To partially mimic the tetrameric nature of NMDAR, we fused the last 5 residues of NR2B to the tail of a tetrameric GCN4 coiled-coil domain (GCN4-NR2B, simply referred as NR2B hereafter) (Harbury et al., 1993). Isothermal titration calorimetry (ITC) assay confirmed that the purified NR2B directly binds to PSD-95 PDZ1-2 (Figures 1A and S1D). We demonstrated that GCN4-NR2B is a homo-tetramer and its binding can induce multimerization of the full-length PSD-95 (Figure S1F). SynGAP is a GTPase activating protein (GAP) highly enriched in PSD (Cheng et al., 2006). We used the C terminus of SynGAPα1 containing a trimeric coiled-coil and a PDZ-binding motif (CC-PBM) domain (referred to as SynGAP hereafter) for this study (Figures 1A and S1E) (Zeng et al., 2016a).

Upon mixing with the four PSD scaffold proteins, both NR2B and SynGAP underwent LLPS and were highly concentrated in the condensed droplets (Figure 1E). The distributions of all six proteins between the aqueous phase (represented by proteins in "supernatant") and the condensed droplets (the "pellet" fraction) were quantified by a sedimentation-based assay. Individually, all six PSD components were highly soluble and did not show any sign of condensed phase formation (Figure S2A). At the concentration of 20 μM (to maximize PSD-95 PDZ1-2 binding, two PSD-95 molar equivalent of NR2B was used here and

(B) DIC and fluorescence images showing that the mixtures of the four PSD scaffold proteins formed phase transition at indicated concentrations. The images were acquired at room temperature with 1% of each protein labeled by the indicated fluorophores. This protein labeling ratio was used throughout the study unless otherwise stated. The dashed box is selected for zoom-in analysis in (C).

(C) Zoom-in analysis of the droplet formation and fusions in the 4× PSD system. The arrow heads highlight those droplets in the process of coalescence into larger ones.

(D) FRAP analysis assaying the exchange kinetics of each protein between the 4× PSD condensates and dilute solution. The concentration of each protein was 20 μM. Related to Video S1.

(E) DIC and fluorescence images showing SynGAP and NR2B were concentrated into droplets in the 6× PSD system. The concentration of each component was 20 μM. GKAP and Shank3 were not labeled and thus not visible.

(F) Representative SDS-PAGE and quantification data showing the distributions of the six PSD components recovered in the dilute phase/supernatant (S) and condensed phase/pellet (P) at indicated protein concentrations. Results were from 3 independent batches of sedimentation assays and represented as mean ± SD.

(G) FRAP analysis showing that SynGAP enriched in the condensed 6× PSD droplets dynamically exchanges with those in the dilute phase. The concentration of each component was at 20 μM.

For all FRAP analyses in this figure, only the protein to be analyzed was Cy3-labeled. The curves represented the averaged signals from 12 droplets with a diameter ~17 μm. All data are presented as mean ± SD.

See also Figures S1, S2, and S3.

throughout the rest of the study), ~50% of PSD-95/SynGAP/NR2B and ~70% of GKAP/Shank3/Homer3 proteins were recovered from the condensed phase (Figure 1F). Consistent with the imaging-based analysis (Figure 1B), the amount of proteins in the condensed droplets gradually decreased with lowered protein concentrations (Figure 1F). After photobleaching, the Cy3-SynGAP intensities in the condensed droplets recovered within minutes (Figure 1G). It indicates that SynGAP enrichment in the condensed droplets is also dynamic, as observed for the enzyme in PSDs of living neurons (Araki et al., 2015). We have also tested phase transition properties of the 6× PSD system with its component stoichiometry matching those derived from proteomic studies and from fluorescence imaging studies (Cheng et al., 2006; Sugiyama et al., 2005) and confirmed that all six components underwent phase separation under these conditions (Figure S2B). In summary, the condensed phase formed by the four PSD scaffold proteins can dramatically enrich NMDA receptors and SynGAP.

Multivalent and Specific Protein-Protein Interactions Drive PSD Condensates Formation via LLPS

Specific and multivalent protein-protein interactions among PSD components are thought to promote PSD formation (Hayashi et al., 2009), although direct experimental evidences supporting this conclusion are difficult to acquire in living neurons. Therefore, we tested such hypothesis using our reconstituted PSD system.

First, we used Cy3-labeled full-length PSD-95 to investigate concentration- and network complexity-dependent phase transition of the PSD assemblies (red panels, Figure 2A). At 40 μM, PSD-95 alone was highly homogeneous and did not form any condensed droplet in solution. Both NR2B and SynGAP are capable of inducing PSD-95 multimer formation (Figure S1F) (Zeng et al., 2018). Upon addition of NR2B and SynGAP, condensed droplets appeared though with small sizes and low in numbers. Next, we added GKAP, Shank3, and Homer3 in a stepwise manner following their order along the axonal-dendritic axis of PSD (from right to left in Figure 2A). Addition of GKAP, which contains three PSD-95 GK domain-binding repeats (GBRs), dramatically increased the condensed droplet formation and lowered the threshold concentration required for LLPS. Addition of Shank3, which contains a self-oligomerizing SAM domain, further promoted phase transition and lowered threshold concentration of LLPS. This trend was further manifested by the addition of the tetrameric Homer3 (Figure 2A). With all of the six components present, formation of the condensed droplets was readily observed at individual protein concentration of 1 μM or lower (Figure 2A), indicating that PSD condensates could form at the physiological concentrations of PSD proteins in dendritic spines.

We next reversed the assembly order of the six PSD proteins using Alexa 488-labeled tetrameric Homer3. Homer3 alone was uniformly distributed in the solution (Figure 2B). The threshold for the Homer3 and Shank3 mixture to undergo LLPS was ~80 μM (Figure S2C). Stepwise additions of GKAP, PSD-95, SynGAP, and NR2B progressively increased the phase transition efficiency and lowered the threshold concentration to undergo LLPS (Figure 2B).

Material Properties of the Reconstituted PSD Condensates

PSD assembly *in vivo* may have semi-solid and gel-like features instead of being a totally fluid-like dilute solution. However, direct measurement of the material properties of PSDs in living neurons is difficult. Additionally, there are limited methods available to study the material properties of biological condensates formed via LLPS, although LLPS is increasingly recognized to be important in diverse cell biology-related processes (Alberti, 2017; Banani et al., 2017; Brangwynne et al., 2009; Feric et al., 2016; Shin and Brangwynne, 2017). Here, we used atomic force microscopy (AFM) to characterize the mechanical properties of PSD droplets. A colloidal probe with a glass bead was adhered under the tip of a triangular AFM cantilever. The AFM probe was gradually lowered with a constant speed toward a condensed droplet in solution (Figure 2C). AFM measured the force generated by the condensed droplet deformation after the probe compressed the droplet. By fitting the slope of the approach curves using the Hertz model, an elastic modulus (E) of the contacted condensed droplet was measured (Figure 2D).

We measured the elastic modulus of droplets formed by 6× PSD and by 2× PSD (SynGAP CC-PBM&PSD-95 PDZ3-SH3-GK) (Zeng et al., 2016a). The droplets formed by 6× PSD showed a sharper approach curve (Figure 2D) and hence a larger measured elastic modulus (E = 3.5 kPa) than the droplets formed by 2× PSD (E = 2.5 kPa) (Figure 2E), indicating that the 6× PSD droplets are more elastic. We also compressed the droplets with different probe indentation speeds. The measured elastic modulus of droplets formed by 6× PSD exhibited a weak power law dependence on the speed (Figures S3A and S3B), which is characteristic of soft glassy materials as a consequence of disorder and metastability of their internal structures (Kollmannsberger and Fabry, 2011). The power law exponent of α = 0.345 indicates that the droplets formed by 6× PSD are more toward solid-like material, as the α value for pure-elastic solid and totally fluid solution is 0 and 1, respectively. The measured elastic modulus was not related to the size of droplets measured (Figure S3C). These results indicate that the expanded multivalent protein-protein network not only reduces the threshold concentration required for LLPS (Figures 2A and 2B), but also changes the material properties of the formed condensates. Because the valency of PSD assemblies in real synapses are higher than our *in vitro* system, the PSDs in neurons are likely to be more toward gel- or glass-like structures as implied by EM observations (Petersen et al., 2003). Finally, we found that the 6× PSD system displays obvious aging and hardening over the time (see Figures S3D–S3G and legends for details).

Quantification of Protein Concentration in the Condensed Phase

Although formation of condensed phase can dramatically concentrate molecules in biological condensates, determining the exact concentrations of components in the condensed droplets has been difficult. Here, we developed a simple confocal microscope imaging-based method for accurately quantifying protein concentrations within the PSD droplets. We illustrate the method using Homer3 concentration quantification in the 4× PSD system as the examples (Figure 3A). Droplets formed

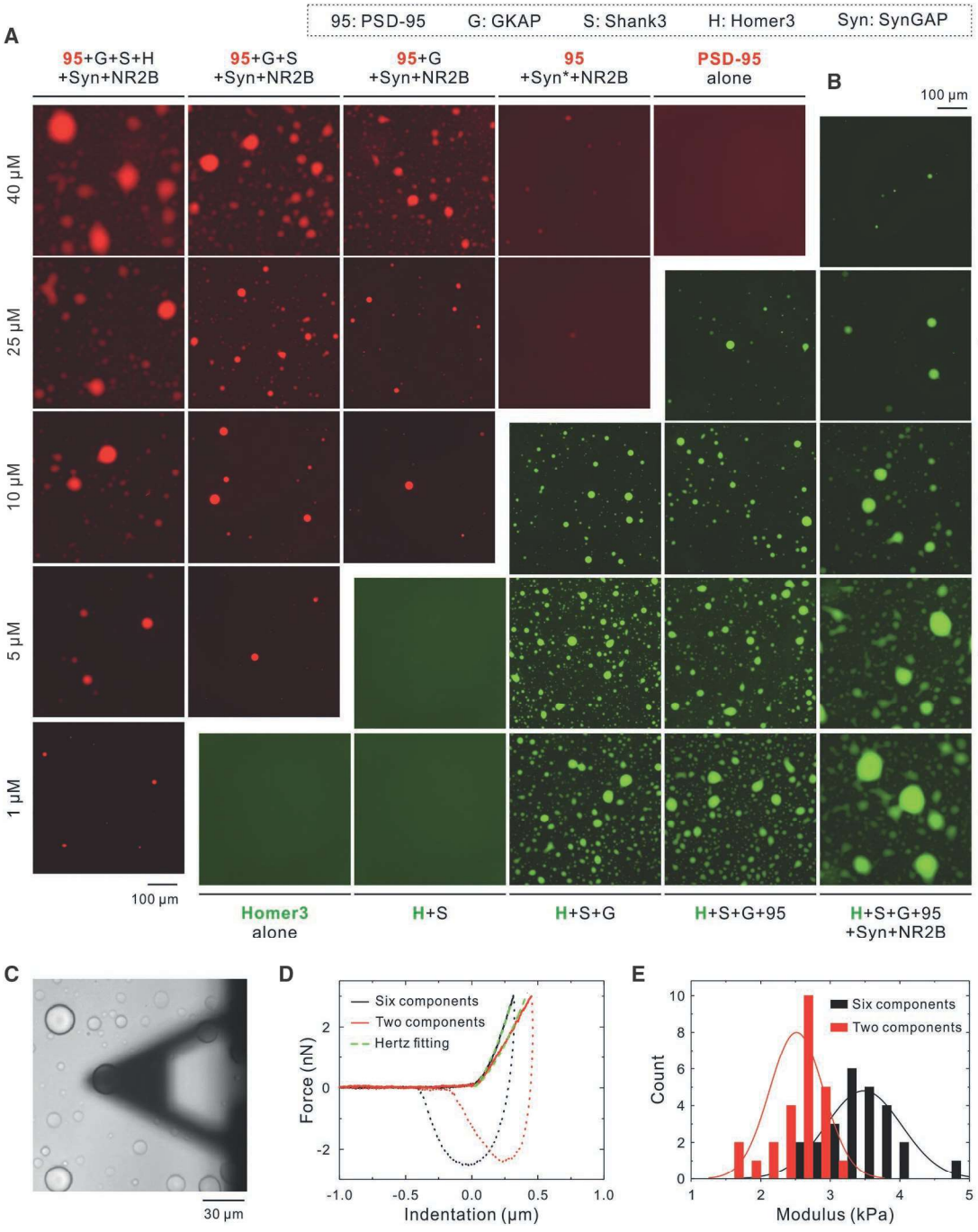


Figure 2. Multivalent Interactions between Scaffold Proteins Drive PSD Condensates Formation

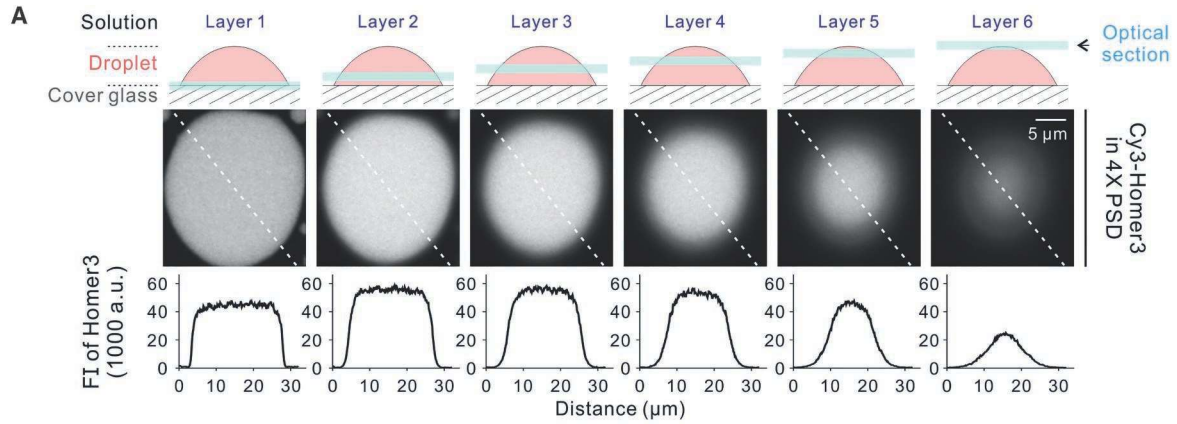
(A and B) Valency- and protein concentration-dependent PSD condensates formation. In (A), only PSD-95 was Cy3-labeled. In (B), only Homer3 was Alexa 488-labeled. In the "95+Syn*+NR2B" group in (A), SynGAP:PSD-95 ratio was set at a molar ratio of 3:1 as one SynGAP trimer to one full-length PSD-95. In all other groups, components were mixed at equimolar concentration with the exception that NR2B was at doubled molar ratio of the rest of the components. For each group of the mixtures at a given protein concentration, the fluorescence imaging settings were identical for easy comparison. Images were acquired at ~5 min after injecting the mixture into the chamber.

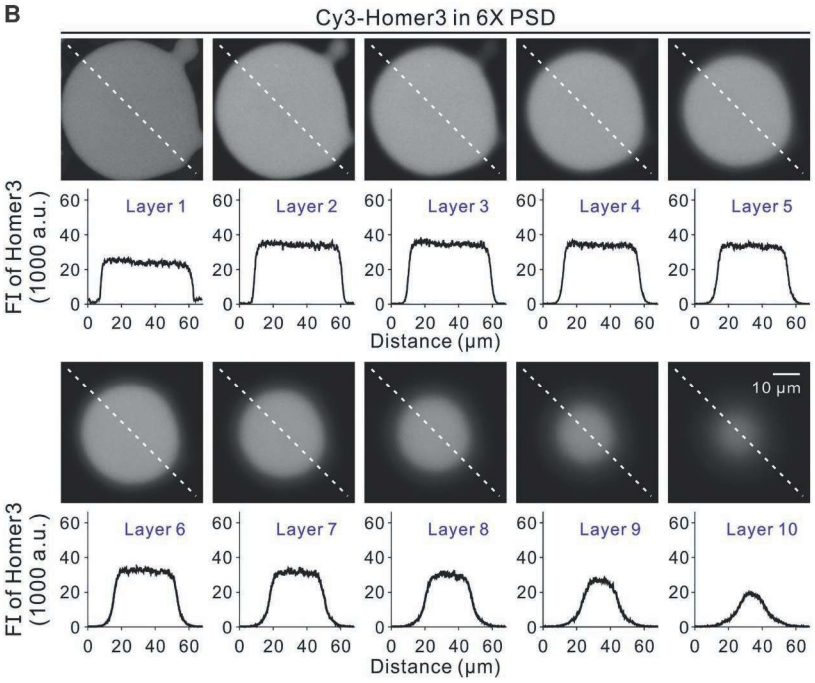
(C) Top-on view image of the condensed droplets under the indentation of an AFM probe.

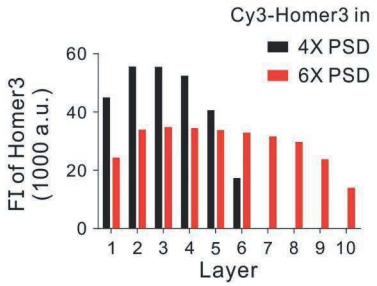
(D) The force-indentation curves measured on the PSD droplets with the approach curves in sloid lines and the retraction curves in dotted line. The green dashed lines show the fitting of the Hertz model to the approach curves.

(E) The distribution of the measured elastic modulus *E* of the PSD droplets. The total number of the measured droplets (n) is 25 for each type of PSD droplets. The solid lines show the fit to the normal distribution with the peak value E = 3.5 kPa for the 6× PSD droplets and E = 2.5 kPa for 2× PSD droplets.

See also Figures S2 and S3.

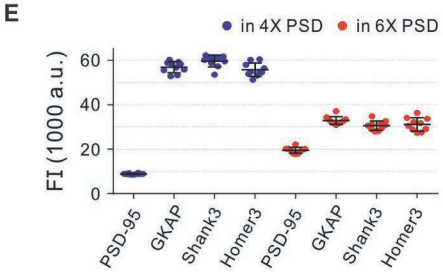


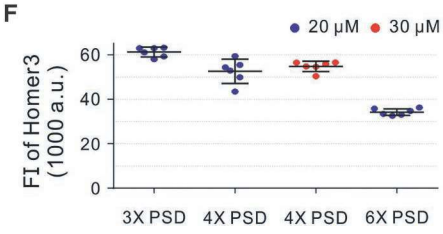




D

	4X PSD	6X PSD
Layer with I_{max}	2	3
Layers with I ($I > 0.9\ I_{max}$)	2, 3, 4	2, 3, 4 5, 6, 7
Mean intensity of layers with I (1000 a.u.)	54.4	33.5





G

Cy3-protein	Composition & conc. (μM)	Droplet Cy3 intensity (1000 a.u.)	Droplet Cy3-protein conc. (μM)	Droplet total protein conc. (μM)
PSD-95	4X PSD & 20	8.9± 0.2	4.1 ± 0.5	410 ± 50
GKAP		56.9 ± 2.5	24.3 ± 1.4	2430 ± 140
Shank3		59.7 ± 2.6	25.5 ± 1.5	2550 ± 150
Homer3		55.8 ± 3.0	23.9 ± 1.6	2390 ± 160
PSD-95	6X PSD & 20	19.5 ± 1.3	8.6 ± 0.9	860 ± 90
GKAP		32.9 ± 1.8	14.2 ± 1.1	1420 ± 110
Shank3		30.6 ± 2.1	13.2 ± 1.3	1320 ± 130
Homer3		30.9 ± 3.0	13.4 ± 1.6	1340 ± 160
Homer3	3X PSD & 20	61.3 ± 2.2	26.2 ± 1.3	2620 ± 130
	4X PSD & 20	52.6 ± 5.5	22.5 ± 2.7	2250 ± 270
	4X PSD & 30	54.8 ± 2.3	23.4 ± 1.3	2340 ± 130
	6X PSD & 20	34.2 ± 1.4	14.8 ± 1.0	1480 ± 100

(legend on next page)

in flow chambers were imaged by a confocal microscope in a z stack mode with a step interval of 1.0 μm and an optical section thickness of ~0.9 μm. The peak fluorescence intensities of optical layers that are completely within the z-dimension of a droplet (e.g., layers 2–4 in Figure 3A and layers 2–7 in Figure 3B) were averaged for determining the concentration of Homer3 in the 4× PSD and 6× PSD condensates (Figures 3B–3D). A standard calibration curve using a Cy3-labeled protein was constructed to convert the Cy3 fluorescence intensity into absolute protein concentration (Figure S4A; see STAR Methods for details).

Using this method, we quantified concentrations of each component (PSD-95/GKAP/Shank3/Homer3) in the 4× 20 μM PSD droplets. In each measurement, only one component was Cy3-labeled to avoid possible signal cross-talk (Figure 3E). The concentrations for GKAP, Shank3, and Homer3 in the condensed phase are similar and at ~2.5 mM, whereas PSD-95 has a ~6-fold lower concentration (Figures 3E and 3G). We also quantified the intensities for these four scaffold proteins in the 6× 20 μM PSD droplets. The concentrations for GKAP, Shank3, and Homer3 in the 6× PSD condensed phase are still similar (~1.3 mM) but reduced to around half of that as those in the 4× PSD droplets (Figures 3E and 3G). The PSD-95 concentration in the 6× PSD droplets is approximately double of that in the 4× PSD droplets (Figures 3E and 3G).

We next compared Homer3 concentrations in various droplets composed of (1) 3× 20 μM PSD (H + S + G), (2) 4× 20 μM PSD (H + S + G + 95), (3) 4× 30 μM PSD (H + S + G + 95), and (4) 6× 20 μM PSD (H + S + G + 95 + Syn + NR2B) (Figures 3F and 3G). This quantification data showed: (1) Homer3 concentration in 3× PSD droplets is slightly higher than that in 4× PSD droplets and is about twice as that in 6× PSD, and (2) higher initial concentration of each component (30 μM versus 20 μM) does not change the final concentration in the droplets in 4× PSD. This means that once a system reaches the phase separation threshold, the component concentration in the condensed phase does not increase but the volume will grow when the initial material concentration further increases, (3) combining the data in Figure 2, the phase separation threshold concentration became lower and lower when the components in the PSD system gets more complicated. It is noted that the final concentration of each component in a more complicated system does not increase continuously. It appears that the total material concentration in a condensed phase will have a limit, and this limit may be determined by the solubility of the assembled condensates, (4) the concentrations of each protein component in droplets with different sizes in a given system are same (Figure S4B).

"Drivers" versus "Clients" in Forming PSD Condensates

We adopted the sedimentation assay to further evaluate the contribution of each protein to the condensate formation in the 6× PSD system. To do this, we dropped out one component at a time and measured the dilution phase versus condensed phase distributions (or S/P ratios) of the rest five components (Figures 4A and 4B). The normalized decreases of PSD components resulted from dropout of one protein in each experiment were plotted in Figure 4C. Deletion of any component in the 6× PSD system resulted in decreases of one or more other proteins, with direct binder(s) as the largest affected component. For instance, the removal of PSD-95 resulted in obvious decreases of its direct binders (NR2B, SynGAP, and GKAP) from being incorporated into the condensed phase; whereas little changes were detected for Shank3 and Homer3 (Figures 4A–4C). When Shank3 was deleted, the alterations on the GKAP and Homer3 were very obvious (larger than 50%), but the changes to NR2B, SynGAP, and PSD-95 were smaller.

Overall, removal of a "riding" protein such as SynGAP had smaller impact on the rest five PSD proteins from entering the condensed phase. In contrast, removing a scaffold protein tends to have larger and overall changes on the PSD condensate formation. The most dramatic example is the removal of GKAP, which led to the overall reduction of all other five components in the condensed phase (Figure 4C). This may be related to the organization feature of the PSD assembly in that GKAP is in the middle layer serving to connect the NR2B/SynGAP/PSD-95 sub-group and the Shank3/Homer3 sub-group in forming the large PSD network (Figure 1A). We further tested the role of GKAP in synergizing the phase transition of the rest 5 PSD proteins by keeping the 5 PSD components at 4 μM and gradually increased GKAP concentration from 0 μM to 8 μM. Increasing GKAP concentration increased the PSD condensates formation in parallel (Figure 4D). Correlated with what was observed, *Sapap3* (the major member of the GKAP/SAPAP family expressed in mouse striatum) knockout mice showed prominently reduced PSD thickness in synapses from striatal neurons (Welch

Figure 3. Confocal Imaging-Based Method to Quantify Concentrations of PSD Components in the Condensed Droplets

(A) Fluorescence intensity analysis of Cy3-Homer3 in each layer of a 4× PSD droplet (20 μM, as in Figure 1C). The top of the panel shows a schematic diagram of moving the confocal plane from near the cover glass to the surface of the droplet (i.e., layer 1 to layer 6). Cy3 fluorescence intensity along the dashed line in each layer is plotted below each image layer.

(B) Fluorescence intensity analysis of Cy3-Homer3 in each layer of a 6× PSD droplet (20 μM, as in Figure 1E).

(C) Plot of measured peak intensity as a function of the layer number for the images from (A) and (B).

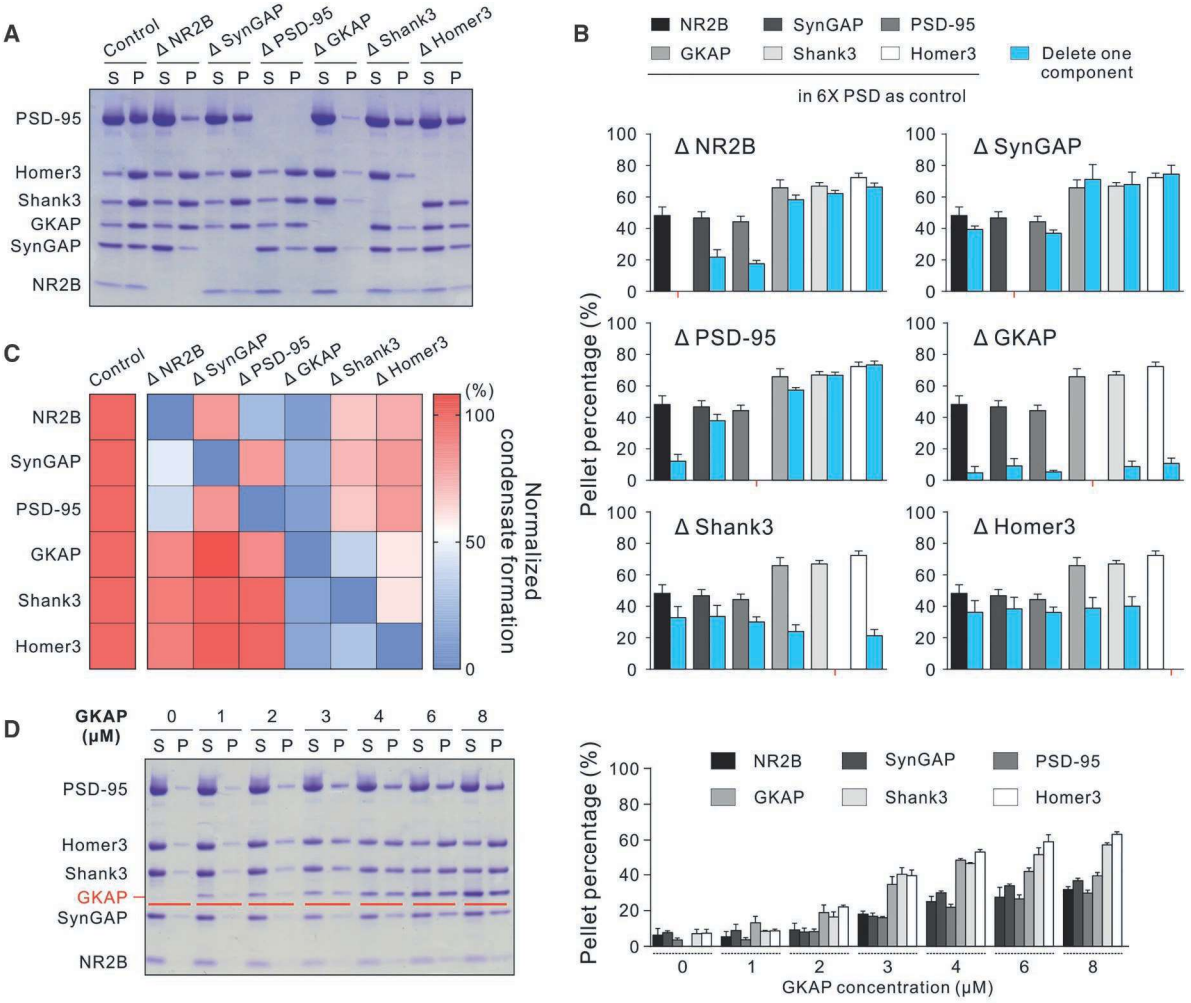
(D) Illustration of the droplet intensity quantification. In the 4× PSD system, layers 2–4 are used to calculate the mean peak intensity of Homer3. In the 6× PSD system, layers 2–7 are used to calculate the mean peak intensity of Homer3.

(E) Cy3 fluorescence intensity of each PSD scaffold protein in 4× or 6× PSD droplets. Data for each protein were collected from 10 droplets in each group and presented as mean ± SD. Both the composition and concentrations of 4× PSD droplets (in blue) and 6× PSD droplets (in red) are the same as in Figures 1C and 1E, respectively.

(F) Cy3-Homer3 fluorescence intensity analysis from four different groups (with different composition and concentration between each groups). Data for each protein were collected from 6 droplets in each group and presented as mean ± SD.

(G) Summary of the fluorescence intensities in each group in (E) and (F) and the corresponding protein concentrations calculated by converting fluorescence intensities to molar concentrations using the calibration curve in Figure S4A. Values are represented as mean ± SD.

See also Figure S4.



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et al., 2007). Based on the above observations, we propose that the four scaffold proteins are key "drivers" for the condensed PSD assemblies, whereas SynGAP is a "client" protein recruited by the scaffold proteins.

We also investigated the roles of direct interactions between paired components or valency of selected proteins in PSD condensate formation. Homer1c shares a similar domain organization with Homer3 and also exists as a tetramer (Hayashi et al., 2009). Accordingly, the phase transition efficiency of the 6× PSD system with Homer1c replacing Homer3 is highly similar to that containing Homer3 (Figure 4E). Homer1a is a short splice variant of Homer1 that contains the N-terminal EVH1 domain followed by a ~60-residue Homer1a-specific sequence with no defined structure but lacks the tetrameric CC domain (Hayashi et al., 2006; Sala et al., 2003) (Figure S5F). Replacing Homer3 by Homer1a dramatically decreased the phase transition efficiency of all components in the 6× PSD system (Figure 4E). It is noted that replacing Homer3 with Homer1a caused more dramatic decrease of condensed phase formation than just removing Homer3 (Figures 4A and 4E), indicating a dominant-negative effect of Homer1a on the phase transition of the 6× PSD system. We found that the 60-residue Homer1a-specific sequence following its EVH1 domain plays an important role in preventing the PSD-95/SynGAP/NR2B subgroup from entering the condensed phase (see Figure S5 for more details). Homer3 LR&LE is a Homer3 mutant in which the Leu338 and Leu343 in its CC region were replaced by Arg and Glu, respectively. Homer3 LR&LE binds to Shank3 as the WT protein does, but the mutant became a dimer (data not shown, also see Hayashi et al., 2009). Replacing WT Homer3 with the LR&LE mutant also reduced phase transition efficiency of the 6× PSD system (Figure 4E), underscoring the critical role of Homer tetramerization in promoting LLPS.

Another set of manipulations was made on the "client" protein SynGAP. Deleting the last 4-residue PBM of SynGAP (d4) abolished its interaction with PSD-95 *in vitro* and resulted in reduced synaptic enrichment in neurons (Zeng et al., 2016a). Mirroring this observation, SynGAP d4 exhibited dramatically reduced enrichment in the condensed phase in the 6× PSD system (Figure 4F). An LD&KD mutant of SynGAP, which converted the protein from trimer to monomer but had minimal impact on the PSD-95 binding, has a weaker synaptic localization in living neurons (Zeng et al., 2016a). This LD&KD mutant of SynGAP also had reduced enrichment in the condensed phase (Figure 4F).

Reconstituted PSD Assemblies Undergo Phase Separation on Lipid Membrane Bilayers

In living neurons, one side of the condensed PSDs is attached to the postsynaptic plasma membranes and the other side is exposed to the cytoplasm of dendritic spines, creating a directional PSD network assembly vital for synaptic signal transmissions. To mimic the semi-open PSD assembly, we reconstituted the 6× PSD system on a 2D membrane system using supported lipid bilayers with a defined lipid composition (Figure 5A) (Banjade and Rosen, 2014).

His-NR2B alone captured by the Ni^{2+}-NTA-DGS-containing lipid bilayers was uniformly distributed (Figures 5B and 5C, at 0 min) and freely diffusing on the bilayers as revealed by FRAP analysis (Figure S4C). Upon the addition of other five PSD components (premixed at a concentration of 2 μM each), submicron-sized clusters appeared within a few minutes. Small clusters gradually grew on the entire membrane surface and fused into larger ones with irregular shapes, and finally the majority of clusters coalesced into a mesh-like network (Figure 5C). Meanwhile, the fluorescence intensity in the dilute phase progressively reduced (Figures 5B–5D; Video S2). The phase separation of the 6× PSD system on the lipid bilayers follows the spinodal decomposition mode as observed recently (Banjade and Rosen, 2014; Su et al., 2016). The irregular shaped laminar structures of the condensed PSD assemblies on the lipid bilayers are actually reminiscent of the shapes of PSDs observed in neurons. If the premixed five PSD components contained 2 mM EDTA in the buffer, no mesh-like NR2B could be observed. Instead, tiny spherical shaped condensed droplets were observed in solution (Figure 5E), indicating that tethering PSD scaffold proteins to lipid membranes via His-NR2B is essential for the spinodal decomposition to occur.

Confocal imaging revealed that PSD-95, Shank3, and Homer3 all colocalized within the NR2B-containing clusters (Figure 5F). FRAP analysis showed that proteins in the condensed clusters exhibited dynamic exchanges with those in the aqueous solution (Figure 5G). Mirrored the behavior of the condensates formed in 3D solutions, cluster formation on lipid bilayers also depended on the specific and multivalent interactions between PSD

Figure 4. Contributions of Each Component to the Phase Transition Efficiency

(A) Representative SDS-PAGE and quantification data showing the distributions of the rest of five PSD components recovered in the dilute phase (S) and condensed phase (P) after removing one component at a time in the sedimentation-based assays.

(B) Quantifications of the dropout experiments shown in (A).

(C) Heatmap plot showing the normalized changes of each protein recovered in the condensed phase in the component dropout experiments quantified in the (B). The percentage in 6× PSD group was normalized as 100%. The depleted protein was set as 0%.

(D) Addition of GKAP into other five PSD protein mixture triggers phase separation and formation of PSD condensates. SDS-PAGE and quantitative analysis show the S/P distribution of each component upon addition of increasing amount of GKAP when the rest of the proteins are kept at 4 μM.

(E) Sedimentation-based assay showing the S/P distributions of six PSD components when the tetrameric Homer3 was replaced by the tetrameric Homer1c, the dimeric Homer3 LR&LE mutant, and monomeric Homer1a, respectively.

(F) Sedimentation-based assay showing the S/P distributions of six PSD components when the wild-type SynGAP was replaced by binding deficient SynGAP d4 or monomeric SynGAP LD&KD mutant.

In all sedimentation assays, protein concentrations were set at 20 μM except in (D). Statistic data were presented as mean ± SD with results from 3 independent batches of sedimentation experiments. ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ using one-way ANOVA with Dunnett's multiple comparisons test.

See also Figure S5.

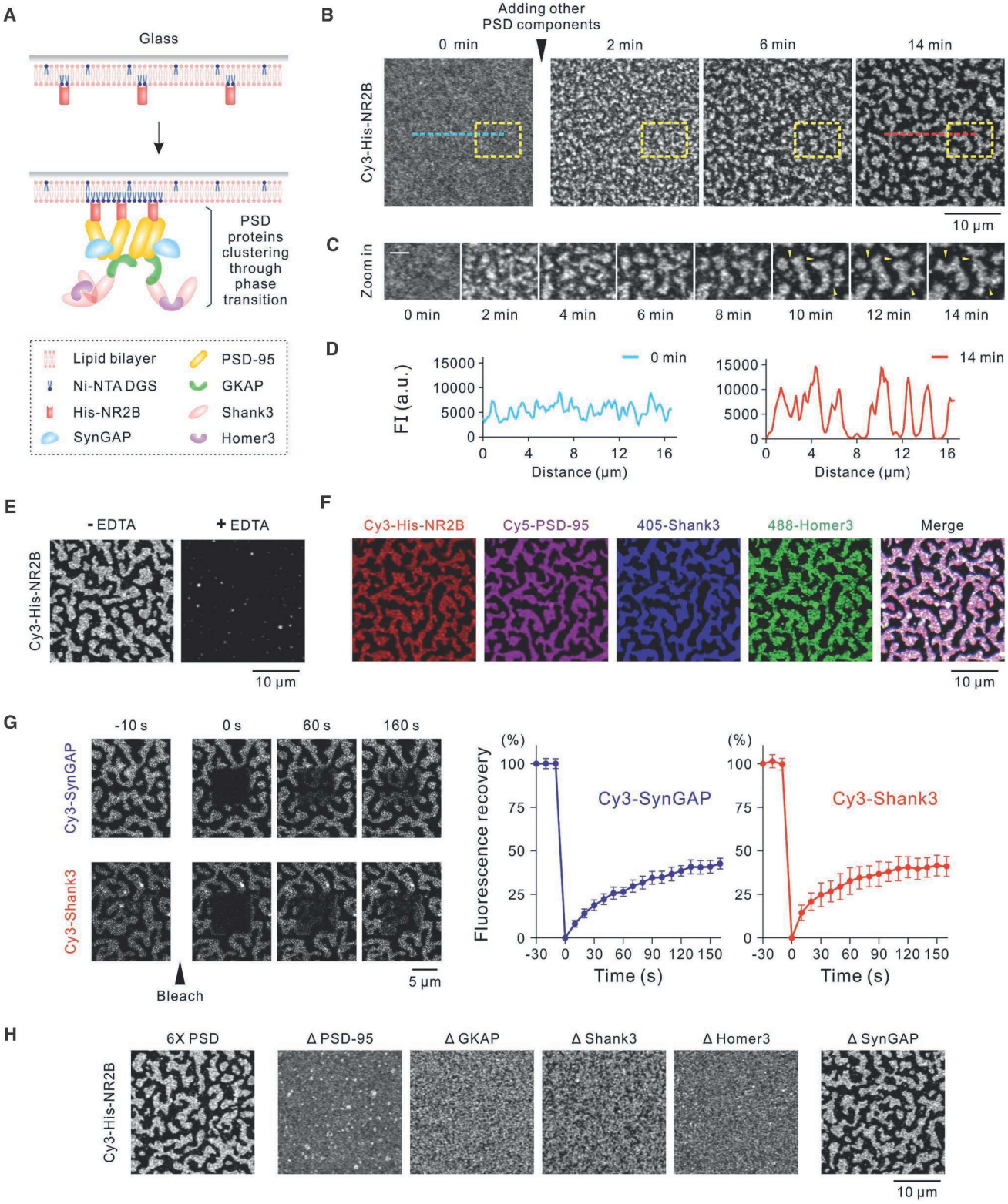

Figure 5. PSD Condensates Formation and NR2B Clustering on Lipid Membrane Bilayers

(A) Schematic diagram showing the principle of PSD phase transition and NR2B clustering on supported lipid membrane bilayers.

(B) Time-lapse confocal images showing that homogeneously distributed Cy3-His-NR2B on membrane bilayers gradually clustered upon addition of the rest of five unlabeled PSD proteins. Related to Video S2. Dashed box and lines are analyzed in (C) and (D) below.

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proteins. Under the assay condition shown in Figure 5B, removal of any one of the four scaffold proteins completely prevented the formation of PSD clusters. In contrast, removal of SynGAP had little impact on the cluster formation of the NR2B (Figure 5H). The above results reveal that the formation of PSD condensates on the lipid bilayers also occur via LLPS. Additionally, formation of the PSD condensates by the major scaffold proteins can massively cluster NR2B receptor on the membrane bilayers and enrich enzymes such as SynGAP.

Network-Level Scaling of the PSD Assembly in the Condensed Phase

We next explored whether the LLPS-mediated PSD assemblies could be modulated by regulatory mechanisms mimicking neuronal activity events, and whether the PSD condensates might contain properties that are distinct to the protein interaction networks commonly studied in dilute aqueous solutions. We picked Homer proteins to study these questions. Overexpression of Homer1a exhibited broad inhibitory effects on dendritic spine morphogenesis, synaptic targeting of PSD scaffold proteins, surface expression of AMPARs, and synaptic transmissions (Sala et al., 2003). Recently, it was shown that sleeping induced massive elevation of Homer1a in excitatory synapses (Diering et al., 2017). Interestingly, EM studies observed global down-scaling of PSD sizes of motor and sensory cortical synapses in mice during sleep (de Vivo et al., 2017).

We tested whether Homer1a might be able to regulate PSD condensate formation. ITC assay showed that Homer1a and Homer1c displayed the same Shank3 binding affinity as Homer3 does (Figures 6A and S1C). In the solution system, we observed progressive droplets dispersions accompanied by gradually increased Cy3-PSD-95 signal in the dilute aqueous phase upon injection of Homer1a into a chamber containing pre-formed 6× PSD droplets (Figure 6B; Video S3). In the sedimentation-based assays, addition of increasing amounts of Homer1a into a fixed amount of Homer1c-containing 6× PSD system (20 μM) progressively prevented all six components from entering the condensed phase (Figure 6C). On the 2D lipid bilayers, addition of Homer1a also disassembled the condensed 6× PSD clusters (Figure 6D; Video S4). An interesting feature is that, although Homer proteins only specifically interact with Shank3 in the 6× PSD system, the formation of the condensed PSD assembly via LLPS allows the entire PSD network to be regulated by alterations of a single protein Homer1a (see Figure S5 for more details). Our observation nicely correlates with the global down-scaling of the PSD sizes in mice induced by sleep (de Vivo et al., 2017). It also indicates that the formation of PSD condensates may allow the PSD network to have distinct properties that are not achievable by the dilute homogeneous solutions.

Reconstituted PSD Condensates Promote Actin Polymerization

Shank is localized in the deep layer of PSD facing dendritic cytoplasm (Petersen et al., 2003; Petralia et al., 2005). It is also known to directly interact with actin cytoskeleton regulatory proteins like cortactin (Naisbitt et al., 1999) and Arp2/3 complex subunits (Han et al., 2013). In Shank3 overexpression transgenic mice, F-actin levels of excitatory synapses increased significantly compared to the wild-type littermates (Han et al., 2013). We next explored whether the reconstituted PSD condensates could also recruit actin-related proteins.

Cortactin has an N-terminal acidic domain that binds to and stimulates Arp2/3 complex, followed by tandem cortactin repeats (CR) that associate with F-actin and a C-terminal SH3 domain binding to the Shank3 (Figures 7A and 7B) (Naisbitt et al., 1999; Weed et al., 2000). Purified cortactin interacted with Shank3 with a K_d ~4.4 μM (Figure 7A). As shown by both sedimentation-based assay and fluorescence imaging assay, cortactin could also be enriched to the condensed phase formed by the 6× PSD system (Figures 7B and 7C). We then added cortactin, Arp2/3 complex together with G-actin into the 6× PSD system assembled on the lipid bilayers. Both G-actin and His-NR2B were initially homogeneously distributed. After adding the other five PSD components, His-NR2B clusters appeared within a few minutes. At ~15 min after phase transition started, polymerized F-actin structures started to emerge in the condensed PSD assemblies. The actin bundles co-localized with the PSD condensates gradually became thicker and longer (Figure 7D; Video S5). Phalloidin staining confirmed that the fluorescence signals of actin represent bundled actin filaments (Figure 7E). Without adding the five PSD components but with the presence of cortactin, Arp2/3 complex, and G-actin, neither His-NR2B clusters nor actin bundles were observed (Figure S6A). We further showed that the PSD can also promote actin bundle formation on the lipid bilayers when Arp2/3 was removed (Figure S6B) and G-actin can be enriched in 6× PSD droplets (Figure S6C), suggesting that PSD condensates can promote F-actin assembly through enriching G-actin and cortactin.

(C) Zoomed-in images of the dashed yellow box in (B) showing the time-dependent clustering of His-NR2B during the spinodal decomposition. Small yellow arrow heads in images at 10–14 min highlight the clusters that were undergoing fusion process. Scale bar, 2 μm.
(D) Fluorescence intensity line-plots showing that the NR2B underwent from originally evenly diffused state (0 min, blue curve) to clustered state (14 min, red curve) after the addition of the PSD scaffold proteins.
(E) Confocal images showing that in the presence of EDTA no NR2B clusters were formed on the lipid bilayers, but instead spherical shaped small droplets were observed in solution.
(F) Co-localization of the PSD scaffold proteins with the NR2B clusters on lipid bilayers (GKAP and SynGAP were not labeled and thus invisible).
(G) FRAP analysis showing the dynamic nature of PSD clusters on lipid bilayers as both Shank3 and SynGAP rapidly exchanged with their counterparts in the dilute phase. The FRAP curves represent averaged results from 20 bleached regions with a squared-shape size of 7.6 μm^2. Data were presented as mean ± SD.
(H) Representative images showing that NR2B failed to be clustered when any one of the four scaffold proteins (PSD-95, GKAP, Shank3, and Homer3) was dropped out from the assay. The clustering of NR2B did not appear to change when SynGAP was omitted. In this assay, only His-NR2B was Cy3-labeled.
The starting concentration of the proteins in solutions (PSD-95, GKAP, Shank3, Homer3, and SynGAP) was at 2 μM each. Cy3-labeled His-NR2B:unlabeled His-NR2B was at 2:100. Experiments were performed in room temperature.
See also Figure S4.

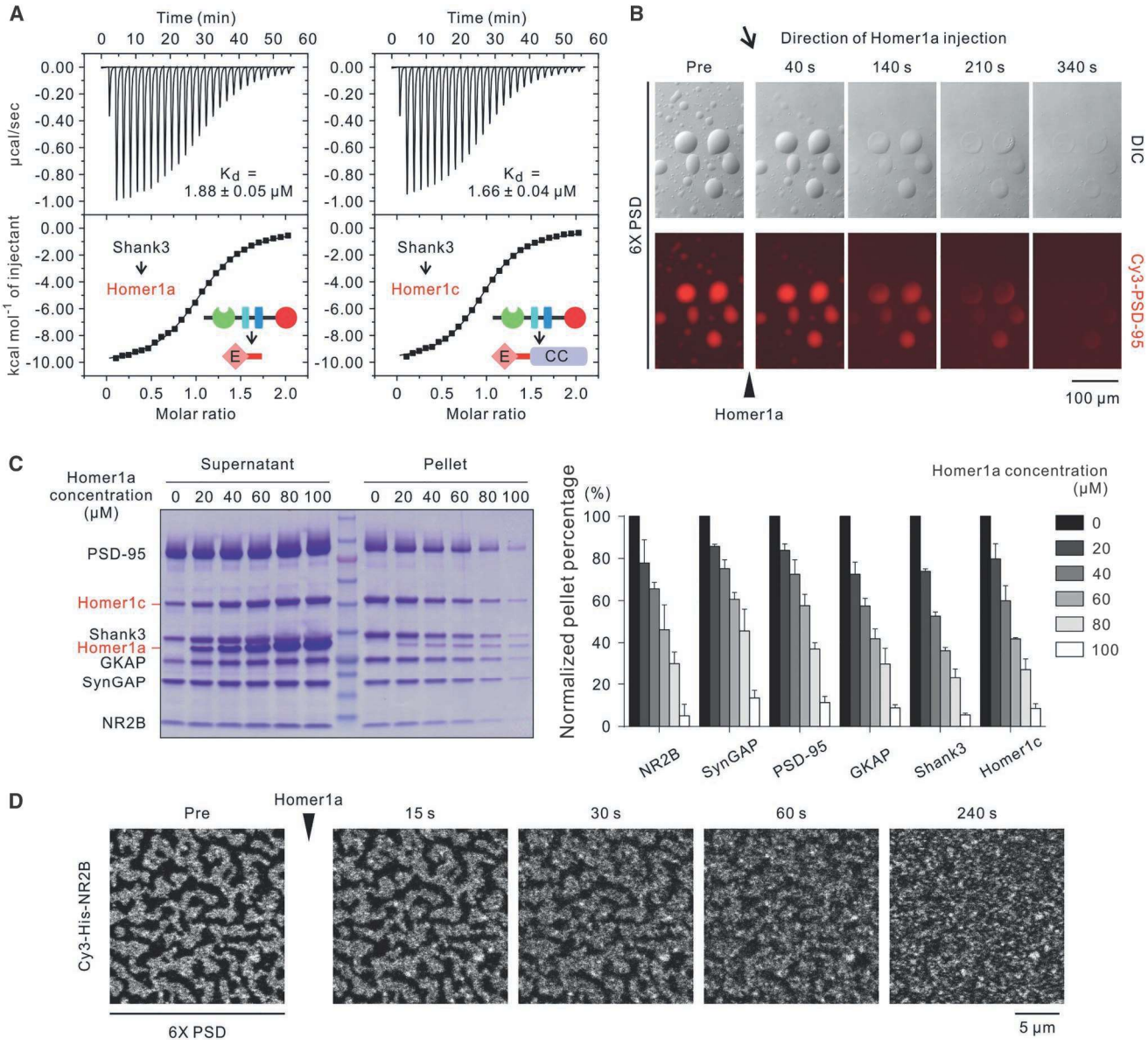


Figure 6. Homer1a Can Globally Scale down PSD Condensates Formation

(A) ITC-based measurement comparing Shank3 binding to Homer1a and Homer1c. Shank3 at 300 µM was titrated into Homer1a or Homer1c at 30 µM in the cell.

(B) Time-lapse DIC and fluorescence images showing that spherical droplets in solution gradually diminished and Cy3-labeled PSD-95 dispersed back to aqueous solution after the injection of purified Homer1a into the chamber. The direction of injection was indicated with an arrow. Related to Video S3.

(C) Sedimentation assay showing that Homer1a prevented the PSD condensates formation in a dose-dependent manner. Indicated concentrations of Homer1a were added into the 6× PSD mixture with each component at the 20 µM concentration. Pellets recovered under the condition without Homer1a were normalized to 100%. Data are presented as mean ± SD with results from 3 independent batches of sedimentation experiments.

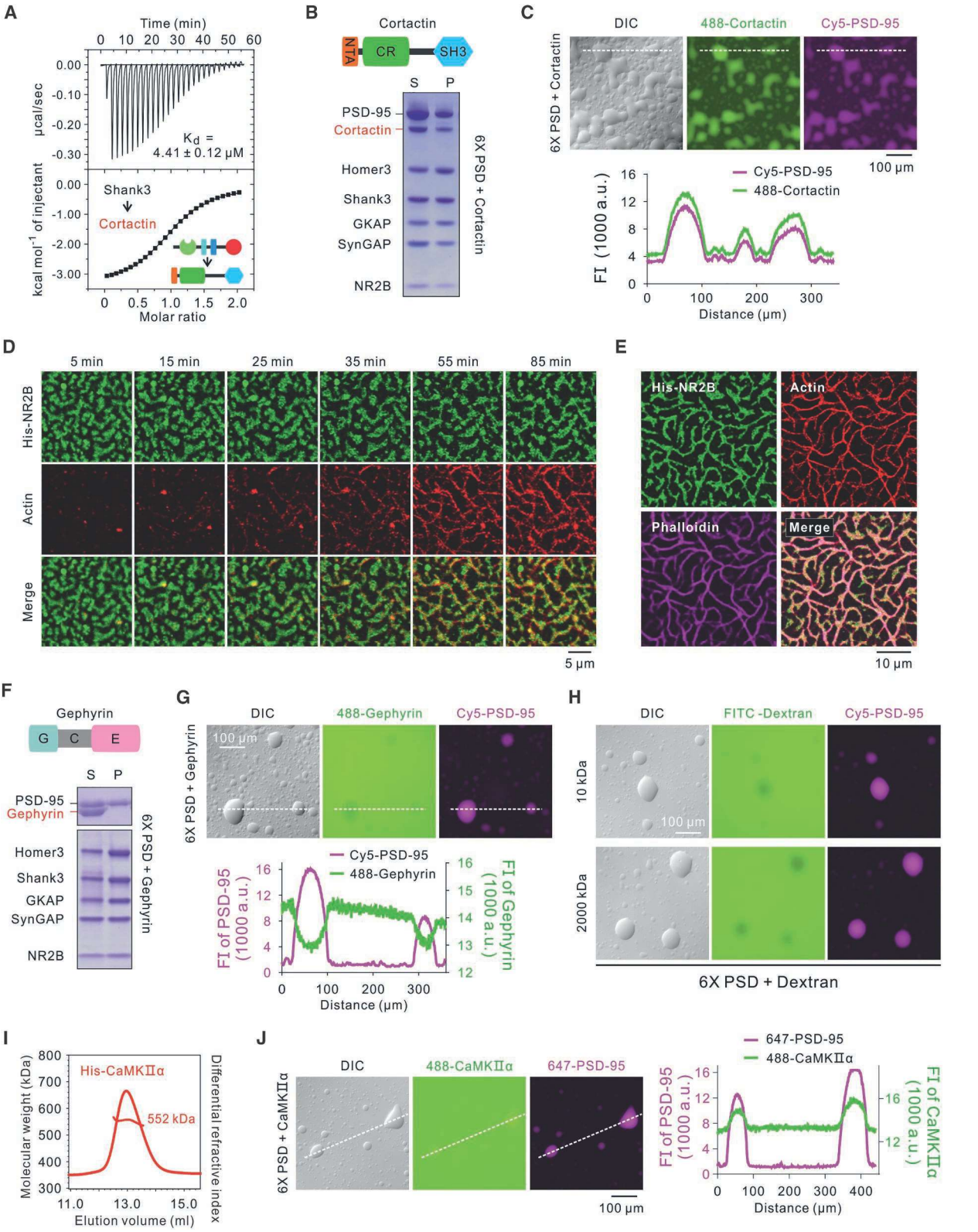
(D) Time-lapse confocal images showing that PSD condensates on lipid bilayers dissolved after addition of purified Homre1a. In this 6× PSD de-clustering assay, only His-NR2B was Cy3-labeled and the PSD condensates were pre-formed as indicated in Figure 5B. Related to Video S4.

See also Figure S5.

Reconstituted PSD Condensates Repel Inhibitory Postsynaptic Protein

Finally, we asked whether the reconstituted PSD condensates are driven by a specific mechanism for building up a functional synaptic assembly or a non-specific protein concentration phenomenon capable of gathering any proteins.

The PSD protein condensates should allow small and non-interacting proteins to diffuse in and out freely. We first assayed thioredoxin (TRX), a synapse unrelated protein, on its possible enrichment in PSD condensate. When included at a doubled concentration as the rest of the 6× PSD components, a faint band of TRX existed in the condensed phase (Figure S7A).



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Fluorescence imaging-based assay also showed that the PSD condensates is permeable to TRX (Figure S7B). We next investigated the distribution of gephyrin, a master scaffold protein of inhibitory PSDs capable of forming homo-oligomers by itself (Tyagarajan and Fritschy, 2014). Very interestingly, full-length gephyrin showed no condensed phase enrichment at all in the 6× PSD system (Figure 7F), suggesting that it is actively excluded by the 6× PSD condensates. We investigated this possibility further using fluorescence microscopy. It was striking to observe that gephyrin was indeed excluded from the condensed 6× PSD droplets (Figure 7G). It is further noted that such mutual exclusion appeared to only occur in the condensed PSD assembly, as gephyrin and the six excitatory PSD proteins in the dilute phase inter-mixed homogenously (Figure 7G).

A trivial explanation for the gephyrin exclusion from the 6× PSD condensates is that the matrix pore of the 6× PSD network in the condensed phase is too small for gephyrin to enter. To test this possibility, we used a series of FITC-labeled dextran with different molecular weights (10–2,000 kDa). We found by surprise that all forms of dextrans were excluded from the droplets (Figures 7H and S7C), likely due to the highly hydrophilic nature of dextrans. We then used a major excitatory PSD component CaMKIIα for subsequent study. Purified CaMKIIα holoenzyme exists as a dodecamer (Figure 7I). It does not directly interact with any of the six PSD components and thus serves as a "client" to the system. We found that the PSD condensates is also permeable to CaMKIIα (Figure 7J), indicating that the 6× PSD droplet pore size is larger than the diameter of the CaMKIIα holoenzyme. The above data indicate that the exclusion of gephyrin from the 6× PSD condensates is due to the specific molecular features of gephyrin.

DISCUSSION

Via a biochemical reconstitution approach, we have demonstrated here that four major scaffold proteins (PSD-95, GKAP, Shank, and Homer) can form PSD protein condensates at physiological concentrations. The results indicate that the highly condensed PSD assemblies in living neurons might autonomously form and stably exist via LLPS. It provides a likely answer to the mechanism of synapse-based cellular compartmentalization of neurons, a striking morphology that is critical for the functions of neurons. Because the reconstituted PSD condensates are simple and chemically defined, the system could be used to investigate the roles of other PSD proteins in regulating PSD assembly formation and dynamics. The information derived from such a reconstitution system, when combined with experiments performed in living neurons, may offer valuable insights in understanding roles of these proteins in synaptic formation and functions. Additionally, given that a very large proportion of brain disorders may be caused by mutations on genes encoding synaptic proteins, the reconstitution system may also be attractive in investigating how such mutations may alter PSD structure and function.

We observed that the ePSD condensates selectively expel inhibitory postsynaptic (iPSD) scaffold protein gephyrin from entering the condensates. This observation may have implications in understanding the mutual exclusive organization of excitatory and inhibitory synapses on dendrites. A large proportion of inhibitory synapses are localized on dendritic spines (Kubota et al., 2007; Villa et al., 2016). Among those inhibitory synapses, a considerable proportion co-exist with excitatory synapses. However, iPSD and ePSD within a single spine do not inter-mix (Kubota et al., 2007). We speculate that gephyrin and ePSD proteins weakly repel each other. Such weak repulsion forces, when manifested in the highly concentrated condensed phase, causes active exclusion of gephyrin. Such active exclusion of one type of molecule(s) from another functionally distinct type of molecular assembly via formation of biological condensates highlights important roles of weak repulsions in organizing specific functional compartments in living cells.

In summary, the *in vitro* reconstituted ePSD condensates studied here recapture many of the hallmark features observed for these major postsynaptic scaffold proteins in organizing

Figure 7. Reconstituted PSD Condensates Promote Actin Polymerization and Repel Gephyrin

(A) ITC-based measurement showing the binding between cortactin and Shank3. Shank3 at 400 μM was titrated into cortactin at 40 μM in the cell.

(B) Sedimentation assay showing the S/P distribution of cortactin (10 μM) in the 6× PSD (with each component at 30 μM).

(C) DIC and fluorescence images showing that cortactin (10 μM) was recruited to the 6× PSD condensed droplets (with each component at 20 μM). Cortactin and PSD-95 were labeled by Alexa 488 and Cy5, respectively. The rest of the proteins were not labeled. Fluorescence intensities along the dashed line are plotted.

(D) Time-lapse images showing that PSD condensates (indicated by Alexa 488-labeled His-NR2B) on lipid bilayers promoted actin (5% Rhodamine-labeled) polymerization. Time points indicate the time after the rest of 5 unlabeled PSD components (2 μM) together with G-actin (0.5 μM), cortactin (0.2 μM), and Arp2/3 complex (0.03 μM) were added to the NR2B-anchored lipid membrane bilayers. Related to Video S5.

(E) Co-localization of the bundled F-actin with NR2B cluster and the PSD condensates. Bundled F-actin were stained by Alexa 633-phalloidin, and actin was labeled with Rhodamine as in (D). Images were acquired at ~100 min after addition of PSD proteins as indicated in (D).

(F) Sedimentation assay showing that gephyrin (20 μM) was not recovered in the pellet fraction in the 6× PSD (20 μM) phase transition assay. To maximize the resolution, PSD-95 and gephyrin were resolved by 8% SDS-PAGE, and the rest of the proteins were resolved by 4%–15% gradient SDS-PAGE.

(G) DIC and fluorescence images showing that gephyrin (20 μM) was excluded from the 6× PSD (20 μM) condensed droplets. Instead, gephyrin was evenly distributed in the dilute phase. Gephyrin and PSD-95 were labeled by Alexa 488 and Cy5, respectively. Other proteins were unlabeled. Fluorescence intensities along the dashed lines are plotted below.

(H) Both 10 kDa and 2,000 kDa dextrans were excluded by the 6× PSD droplets. 2 μM FITC-labeled dextrans were added to 6× PSD mixtures (20 μM, only PSD-95 was labeled by Cy5). Droplet morphologies were captured by DIC imaging.

(I) FPLC-coupled static light scattering analysis showing the column behavior and detected molecular weight of the purified CaMKIIα holoenzyme. 25 μM purified CaMKIIα holoenzyme was loaded to a Superose 6 size-exclusion column.

(J) PSD condensates are permeable to and can slightly enrich CaMKIIα holoenzyme. 10 μM Alexa 488-labeled CaMKIIα holoenzyme was added to 6× PSD mixtures (20 μM, only PSD-95 was labeled by Alexa 647). Fluorescence intensities along the dashed lines are analyzed at right.

See also Figures S6 and S7.

synaptic signaling complex, promoting synaptic development and maturation, and regulating synaptic plasticity. Although still vastly simplified, this well-defined biochemically traceable system provides a platform and a new paradigm for studying excitatory PSD formation and regulation as well as for elucidating mechanisms of a range of brain disorders caused by mutations of synaptic genes in the future. In a broader sense, principles and mechanisms revealed in the reconstituted PSD condensates here, together with methods developed in this work, may also be applied to other biomolecular condensates.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- METHODS DETAILS
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- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and five videos and can be found with this article online at https://doi.org/10.1016/j.cell.2018.06.047.

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AUTHOR CONTRIBUTIONS

M. Zeng, X.C., D.G., J.X., and H.W. performed experiments. M. Zeng, X.C., D.G., J.X., H.W., P.T., and M. Zhang analyzed data. M. Zeng, X.C., and M. Zhang designed the research. M. Zeng, X.C., and M. Zhang drafted the paper. All authors commented the paper. M. Zhang coordinated the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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