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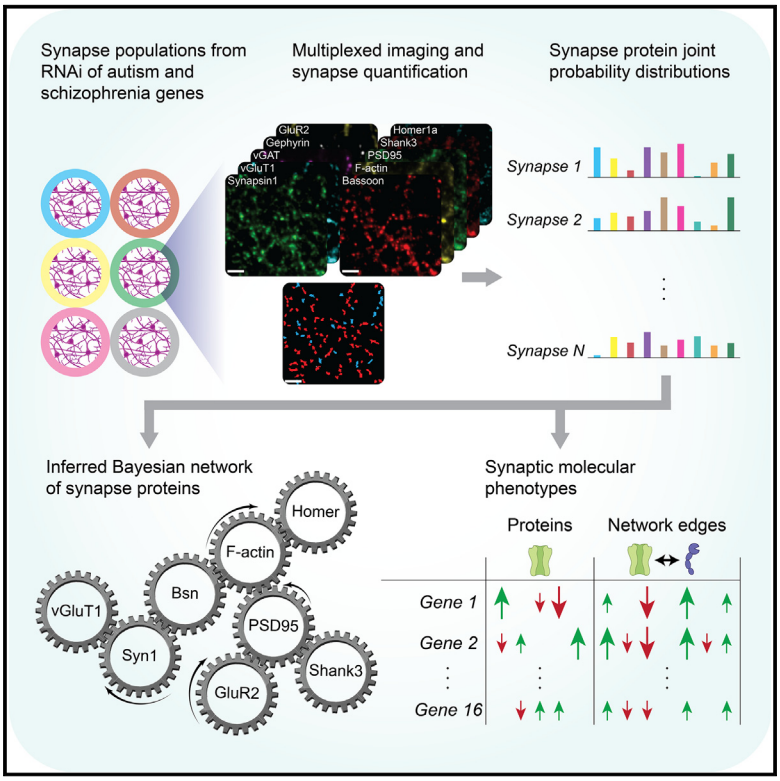
A synaptic molecular dependency network in knockdown of autism- and schizophrenia-associated genes revealed by multiplexed imaging

Graphical abstract



Highlights

- Single-synapse, multiprotein measurements under 16 ASD/SCZ gene knockdowns
- Identified diverse phenotypes in synaptic protein composition and distribution
- Inference of a predictive inter-dependency network among eight synaptic proteins
- Convergent changes in network structure driven by different gene knockdowns

Authors

Reuven Falkovich, Eric W. Danielson, Karen Perez de Arce, ..., Morgan Sheng, Jeffrey R. Cottrell, Mark Bathe

Correspondence

mark.bathe@mit.edu

In brief

To examine how the synaptic molecular system is affected in autism and schizophrenia, Falkovich et al. simultaneously measure multiple proteins across individual synapses in different gene knockdowns. They construct a predictive dependency network among these proteins and show diverse synaptic protein changes and convergent network structure changes across different genes.

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A synaptic molecular dependency network in knockdown of autism- and schizophrenia-associated genes revealed by multiplexed imaging

Reuven Falkovich,[1,6] Eric W. Danielson,[1,6] Karen Perez de Arce,[2,6] Eike-C. Wamhoff,[1] Juliana Strother,[1] Anna P. Lapteva,[1] Morgan Sheng,[2,3] Jeffrey R. Cottrell,[2] and Mark Bathe[1,4,5,7,*]

[1]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA
[2]Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA, USA
[3]Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA, USA
[4]Broad Institute of MIT and Harvard, Cambridge, MA, USA
[5]Harvard Medical School Initiative for RNA Medicine, Harvard University, Cambridge, MA, USA
[6]These authors contributed equally
[7]Lead contact
*Correspondence: mark.bathe@mit.edu
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SUMMARY

The complex functions of neuronal synapses depend on their tightly interconnected protein network, and their dysregulation is implicated in the pathogenesis of autism spectrum disorders and schizophrenia. However, it remains unclear how synaptic molecular networks are altered biochemically in these disorders. Here, we apply multiplexed imaging to probe the effects of RNAi knockdown of 16 autism- and schizophrenia-associated genes on the simultaneous joint distribution of 10 synaptic proteins, observing several protein composition phenotypes associated with these risk genes. We apply Bayesian network analysis to infer hierarchical dependencies among eight excitatory synaptic proteins, yielding predictive relationships that can only be accessed with single-synapse, multiprotein measurements performed simultaneously *in situ*. Finally, we find that central features of the network are affected similarly across several distinct gene knockdowns. These results offer insight into the convergent molecular etiology of these widespread disorders and provide a general framework to probe subcellular molecular networks.

INTRODUCTION

The functional complexity of the brain is enabled by trillions of chemical synapses that form connections among its $\sim 10^{11}$ neurons. Each synapse is capable of analog computation that integrates its activity history, chemical environment, and the state of its pre- and post-synaptic neurons to modulate communication through synaptic plasticity. This computation is achieved[1] in large part by the synaptic molecular network, a dynamic, compartmentalized biomolecular system of hundreds of proteins[2–4] that includes constantly varying levels and activity states of receptors, scaffolding proteins, kinases, and other protein types. This proteomic diversity likely underlies the remarkable cell- and context-specific functional diversity even among synapses of the same type.[5,6] Numerous studies have revealed mechanistic connections between two or three synaptic components at a time, providing the foundation to integrate these connections into a broader context of many-component networks. However, because of the high complexity and interconnectedness of such networks, this integration requires simultaneous single-synapse measurement of numerous proteins, a technique we developed and applied previously to analyze synapse compositions.[7]

The synaptic molecular network is tightly connected to cognitive disorders, with synaptogenesis and plasticity increasingly appreciated as molecular targets for psychiatric treatments.[8–10] Accumulating evidence also points to synaptic biochemistry as a focal point of the pathophysiology of psychiatric, neurodevelopmental, and neurodegenerative diseases.[11–17] Autism spectrum disorder (ASD) and schizophrenia (SCZ) are two such conditions that manifest in a range of specific higher cognitive symptoms that range in intensity from healthy neurodiversity to debilitating brain dysfunction. These latter conditions typically include changes in social and communication behavior;[18,19] altered perception and sensory habituation, including self-stimulatory behavior;[19–22] adherence to patterns and focused interests;[19] impaired language acquisition and use;[18,23] as well as general intellectual disability[24,25] and psychosis.[26] While divergent in symptom presentation, they are often studied genetically in the same context because of similarities in risk genes and possible functional and pathological associations.[27–29]

ASD and SCZ are highly heritable[30,31] and genetically heterogeneous,[32–34] with many identified risk genes, including rare, highly penetrant *de novo* mutations[35] as well as many common variants that contribute small increases in risk. Thus, a central

question is whether the genetic variants that increase risk for ASD or SCZ share similar downstream molecular etiologies and, if so, what their mechanisms are. Genome-wide association studies and rare-variant sequencing studies have revealed the prevalence of synaptic genes,[11,12,17,29,36–41] including adhesion, scaffolding, ion channel, and local translation control proteins, as well as transcription factors upstream of them, among those associated with ASD and SCZ.[42–46] Additional evidence points to consistent changes in synaptic structural and functional features, including dendritic spine morphology,[47] excitation/inhibition ratios,[48–51] and global features of gene expression and protein interaction networks,[11,17,52,53] which are common to different genetic models. Research that implicates perturbations in brain-wide connectivity patterns,[54,55] possibly related to deficits in predictive processing capacity,[56] supports the notion that a fundamental synaptopathology expressed variably throughout the brain may contribute to these disorders.[13] Physical protein-protein interaction approaches, such as yeast two-hybrid (Y2H) tests and co-immunoprecipitation (coIP),[52,53] have established interaction networks involving synaptic proteins and their changes *in vivo* in autistic individuals and autism models, demonstrating the promise of studying the synaptic molecular network as a focal point of autism pathogenesis. However, these studies fall short of measuring changes to joint distributions of protein expression levels or identifying perturbed causal connections between relevant proteins.

To characterize how synaptic molecular composition is affected across genetic perturbations associated with ASD and SCZ, we applied RNAi-mediated knockdown of 16 canonical, highly penetrant risk genes associated with either ASD, SCZ, or both at the onset of synaptogenesis. When a mature and stable synapse population was established in each genetic context, we measured the amounts of each of 10 synaptic proteins across individual synapses using probe-based imaging for sequential multiplexing (PRISM).[7,57,58] PRISM is a recently introduced multiplexed imaging technique that uses single-stranded DNA (ssDNA)-conjugated antibodies or peptides against desired targets that are confocally imaged sequentially using fluorescently labeled single-stranded locked nucleic acid (ssLNA) imaging probes. The affinity of imaging probes for their targets depends on ionic strength, allowing sequential rounds of imaging of multiple proteins in the same sample and fields of view by exchanging imaging strands using high- and low-salt buffers. Thus, this imaging method provides a combination of extensive multiplexing, moderate throughput, minimal disruption to delicate synapse structures, and single-synapse resolution.

The PRISM imaging output consists of images of the same synaptic puncta over numerous protein channels. Integrating fluorescence intensity over individual puncta and assigning puncta across channels to the same synapse yields individual protein measurements per synapse.[7,57] We refer to the total integrated fluorescence intensity per protein at a given synapse as the local synaptic protein level. With this approach, in a single experiment, we can generate a type-resolved systematic view of protein-level changes caused by different genetic knockdowns as well changes to distinct, compositionally defined synaptic populations, as performed previously in response to chemical perturbation.[57] In the current study, these changes included a global synaptic protein increase with knockdown of *Pten*, differential changes in response to *Cul1* knockdown, a potentially compensatory increase in synaptic PSD95 with knockdown of *Grin2a*, and several unique synaptic phenotypes resulting from *Dyrk1a* knockdown.

Leveraging the unique ability of single-synapse, multiprotein measurements from PRISM to provide a high-dimensional joint probability distribution (PD) of synapses in composition space, we additionally sought to infer the synaptic protein influence networks that generated the measured protein distributions. Toward this end, we used Bayesian network (BN) inference, a tool used previously to reconstruct entire signaling pathways from multiplexed single-cell data.[59] BNs are a framework to factor a joint PD into a product of individual conditional PDs. This can be represented by a directed acyclic graph between measured nodes, in which graph edges represent direct conditional dependencies between individual nodes (i.e., retaining only those connections that cannot be explained by mutual dependence on a third node) as well as the estimated causal direction of the pairwise dependencies. In this study, each node refers to the local synaptic level of a certain protein. Substructures in the resulting model generated testable predictions of causal connections (e.g., the hierarchy in which perturbing A affects B and C) between protein levels, some of which were consistent with known protein roles and interactions and others that were discovered anew, to the best of our knowledge.

In particular, the causal chain by which F-actin determines post-synaptic PSD95 levels, which, in turn, determines post-synaptic SHANK3 levels, we validated independently via direct perturbations, thereby establishing a new causal hypothesis that shapes synaptic protein distributions and, by confirming a novel prediction presented by the model, generating confidence in the new model as a whole. Finally, we present evidence of convergent changes in the inferred synaptic molecular network that are caused by distinct genetic knockdowns, specifically in the strengths of transsynaptic and intra-postsynaptic edges, offering evidence of a convergent molecular etiology across ASD/SCZ-associated genes.

RESULTS

Effects of ASD- and SCZ-associated gene knockdowns on the synaptic molecular system

The following core synaptic proteins were characterized using PRISM to provide snapshots of the synaptic molecular subnetwork (Figure 1A). Synapsin1 was used to define all synapses, with vGluT1 and vGAT used to differentiate glutamatergic from GABAergic synapses. Other proteins included Bassoon, a central presynaptic scaffolding protein that served as a proxy for active zone size,[60] and the glutamate receptor subunit GluR2, which served as an indicator of synapse strength.[61] Filamentous β-actin (F-actin), measured via phalloidin, was included as the core of the dendritic spine cytoskeleton that is locally regulated by several ASD/SCZ-associated genes (e.g., *Trio*, *Pten*, and *Dyrk1a*) and whose dysregulation is implicated in various synaptopathologies.[62,63] Finally, four scaffolding proteins were included that have crucial roles in shaping the post-synaptic

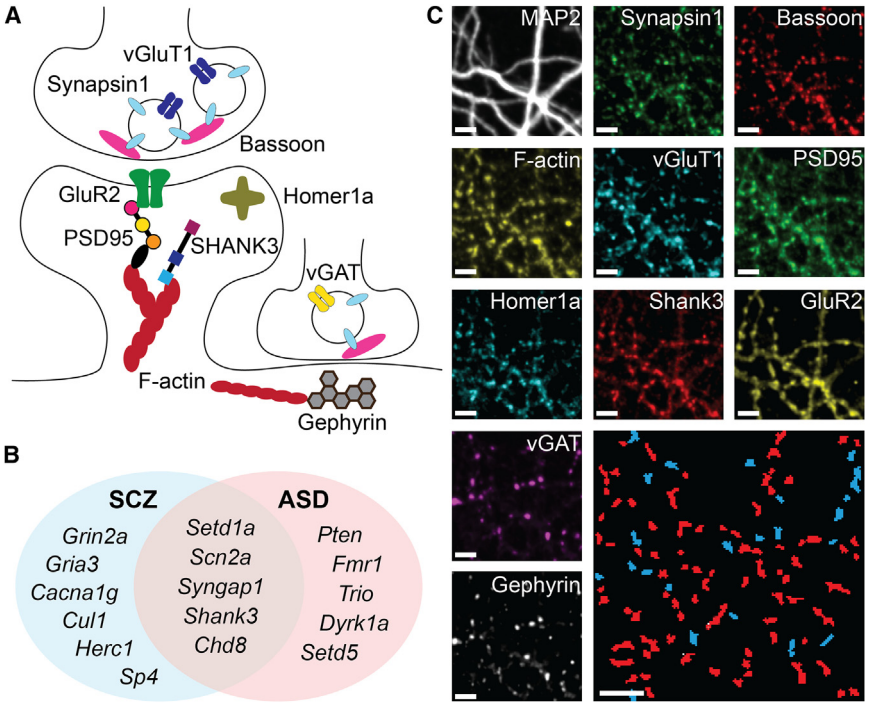

Figure 1. Genes and targets

(A) Schematic summarizing the approximate synaptic context of the 10 imaged targets.

(B) Venn diagram of gene knockdowns.

(C) Representative images of the same neuronal culture in different imaging rounds, showing colocalized puncta of each protein. Bottom right: automatically identified and segmented excitatory (red) and inhibitory (blue) synapses. Scale bars, 5 μm.

density: PSD95, Homer1a, and SHANK3 in excitatory synapses and Gephyrin in inhibitory synapses. MAP2 staining via conventional immunofluorescence was also used to trace dendrites to the degree feasible in dense cultures and constrain punctum assignments to synapses as well as to align images from different imaging probe exchange rounds.[7,57]

For genetic perturbations, 10 autism genes (Figure 1B) were chosen as the best-scoring targets in the Simons Foundation Autism Research Initiative database[64,65] (Simons Foundation Autism Research Initiative [SFARI] score), half of which are implicated in SCZ as well. Six additional SCZ-specific genes were chosen from the highly penetrant *de novo* mutations identified by Singh et al.[35] A mixture of four small interfering RNA (siRNA) reagents was used for each gene, and each siRNA treatment was separately validated by qRT-PCR for reduction in mRNA levels in cultured hippocampal neurons (Figures S1A–S1C). The siRNA reagents appeared to act more similarly to a small-molecule treatment than a transfected gene, with transfection universal (>99% of cells) and concentration dependence occurring even at low doses (Figures S1D–S1F). A non-targeting siRNA mix (NonT) was included as a negative control and used for treatment comparisons throughout results.

To measure the effect of each gene knockdown on synaptic protein distribution, we treated hippocampal neuronal cultures on day *in vitro* (DIV) 6 with one of the corresponding siRNA reagent mixes and fixed the cultures at DIV 19 to image using PRISM (STAR Methods). We integrated an automated liquid-handling platform for probe exchange to complete seven imaging rounds of the same 60 cultures (3–4 per treatment group) in under 12 h. The resulting fluorescent images of the same synapses across imaging rounds (Figures 1C and 2A) were automatically segmented, classified, and quantified using CellProfiler[66,67] (STAR Methods). We combined data from 4 such experiments (plates), where each used embryonic day 18 (E18) neurons from a different pregnant rat for a total of 3.5×10^6 11-protein synaptic measurements across 220 separate neuronal cultures in 18 treatment groups. To account for variability in staining and imaging conditions between plates, fluorescence intensity values of each protein channel were normalized by the average intensity of that channel in untreated cultures of that plate.

Automatically segmented puncta, identified as above-threshold intensity peaks within a size range (STAR Methods) in each protein channel, were assigned to specific individual synapses based on overlap with Synapsin1 puncta (STAR Methods). The existence of a punctum in a protein channel assigned to a specific synapse was taken as the presence of that protein in the synapse, and the integrated fluorescence intensity of a certain protein channel across its synapse-associated punctum was assumed to be proportionate to the total level of that protein in the synapse. Based on these data, we first examined the individual effects of different siRNA treatments on three global parameters: excitatory:inhibitory (E:I) synapse ratio (Figure 2B), fraction of GluR2− excitatory synapses[68] (Figure 2C), and dendrite growth, measured as overall area stained by MAP2 (Figure 2D). E:I synapse ratio, implicated previously to be dysregulated in ASD and SCZ,[48] was calculated as the ratio of (+vGluT1, −vGAT) to (−vGluT1,+vGAT) synaptic puncta. This ratio was, on average, ~5:1 and was significantly increased in knockdown of *Dyrk1a*, consistent with reports of inverse correlation of *Dyrk1a* expression to E:I ratio *in vivo*.[49] It also increased in knockdown of *Grin2a*, *Shank3*, and *Chd8*.

Next, by examining the average levels of each measured protein across synapse populations, we created a map of how the synaptic levels of each protein were, on average, affected by each treatment (Figure 2E). Beyond this overall characterization, we observed several novel synaptic phenotypes, the strongest of which included (1) a 2-fold increase in Homer1a under knockdown of *Setd1a*, a nuclear regulatory lysine methyltransferase, while knockdown of *Setd5*, a gene of the same family, decreased Homer1a; (2) an 80% increase in synaptic F-actin after knockdown of *Cul1* (other proteins including Synapsin1 are decreased); (3) knockdown of *Grin2a* leading to an ~70% increase in PSD95 as well as increases in other proteins, including Homer1a, GluR2, and Bassoon; (4) a 2-fold decrease in synaptic

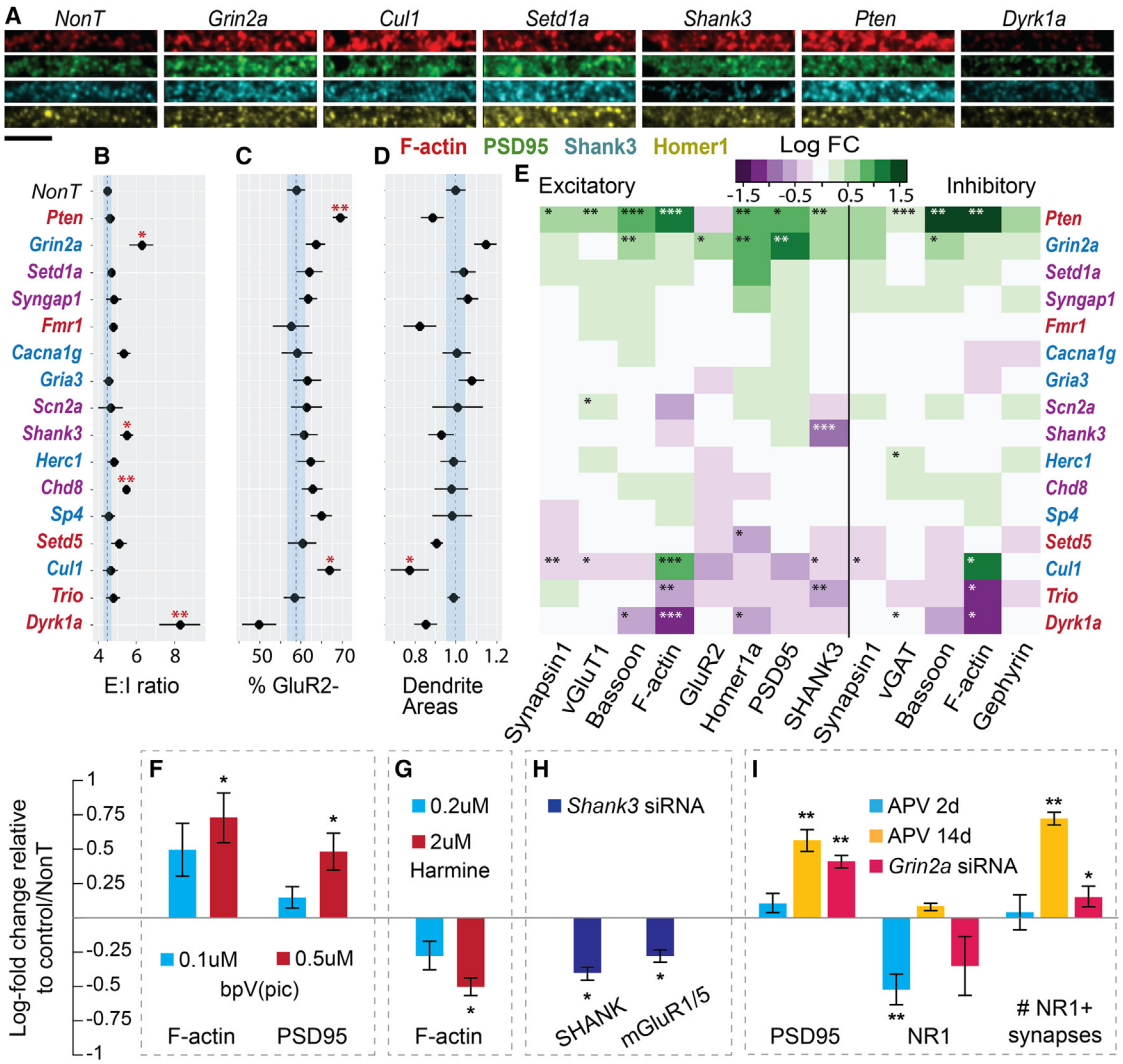

Figure 2. Synapse effects of siRNA knockdowns

(A) Representative images from medium-sized dendrites across 4 channels. Bottom left scale bar, 5 μm.

(B) Excitatory (Syn+, vGluT1+, vGAT−) to inhibitory (Syn+, vGluT1−, vGAT+) count ratios.

(C) Percentage of GluR2− synapses from all excitatory synapses.

(D) Estimates of total dendrite proliferation from MAP2 staining, normalized to NonT.

(B–D) Blue lines indicate mean and SEM of NonT measurements.

(E) Log fold change (relative to NonT) of mean levels of each protein in excitatory and inhibitory synapses. Coloring of genes in (B)–(E): red, only ASD; blue, only SCZ; purple, both.

(F–I) Validation experiments with chronic treatments (DIV 6–19) measured by conventional immunofluorescence (IF). Mean synaptic protein levels or other measurements are depicted as Log_2 fold change from control-treated (for chemical treatment experiments) or NonT siRNA-treated (for RNAi) wells.

(F) Treatment with bpV(pic), a PTEN inhibitor.

(G) Treatment with harmine, a Dyrk1a inhibitor.

(H) SHANK and mGluR1/5 after knockdown of *Shank3*.

(I) PSD95, NR1, and density of NR1+ synapses after short and chronic NMDAR blockade with D-APV or chronic RNAi knockdown of *Grin2a*.

Error bars are mean ± SEM across wells. *p < 0.05, **p < 0.01, ***p < 0.001, two-sided t test.

F-actin following knockdown of *Dyrk1a*, accompanied by decreases in other proteins, including Bassoon and Homer1a; and (5) knockdown of *Trio* leading to a decrease in synaptic F-actin and SHANK3. We did not identify significant differential effects on the same protein in the context of excitatory versus inhibitory synapses.

The same images could also be used to assess changes in global protein expression (Figures S2A–S2C), either somatic (i.e., fluorescence in synapse-excluded neuronal bodies) or total (averaged fluorescence over the entire image). A few notable differences from synapse-specific phenotypes appeared. For example, *Cul1* knockdown showed a significant

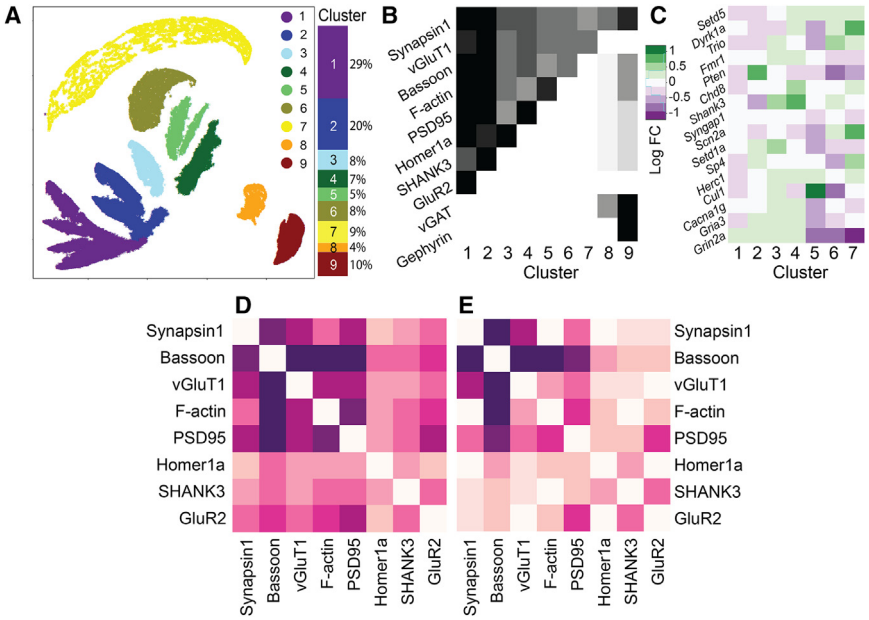

Figure 3. Synaptic multiprotein distributions

(A–C) Composition-defined synaptic subtypes.

(A) UMAP projection of scaled synaptic measurements. Right: overall composition of the synapse population by cluster.

(B) Row-normalized (across all clusters) mean levels of each specific protein in specific clusters identified in (A).

(C) Log fold change of excitatory cluster populations under each treatment.

(D) Direct correlations between pairs of proteins in excitatory synapses.

(E) Correlations in each pair, controlling for all other 6 proteins.

increase in synaptic F-actin, but not in global somatic levels, in parallel with synaptic and (stronger) somatic reduction in other proteins, raising the possibility of a compensatory connection, in either direction, between an increase in F-actin and reduction in other postsynaptic proteins via an unknown mechanism, possibly related to the role of Cul1 in regulating SPAR turnover.[69]

Some treatment effects in Figure 2E have been reported previously or were expected based on pre-existing knowledge of mechanisms involved. For example, we confirmed that siRNA knockdown of *Shank3* led to a marked reduction in SHANK3 levels at synapses. Knockdown of *Pten* led to a broad increase in nearly all synaptic markers, consistent with its role as a negative regulator of phosphatidylinositol 3-kinase (PI3K)-dependent neurite and synapse proliferation.[70]

To support the validity of RNAi with PRISM to reveal phenotypes directly connected to gene function, we performed chemical inhibition and knockdown experiments, measuring the synaptic response using conventional immunofluorescence (Figures 2F–2I). These showed that (1) treatment with bpV(pic), a PTEN phosphatase inhibitor, increased synaptic F-actin and PSD95, mimicking *Pten* knockdown in a dose-dependent manner (Figure 2F); (2) treatment with harmine, a Dyrk1a inhibitor, decreased F-actin, mimicking *Dyrk1a* knockdown in a dose-dependent manner (Figure 2G) and also increased the fraction of GluR2+ synapses (Figures S2D–S2F); (3) knockdown of *Shank3* reduced synaptic SHANK as well as mGluR1/5, as reported previously[71] (Figure 2H); and (4) knockdown of *Grin2a* increased PSD95 levels and density of NMDAR-containing synapses (Figure 2I). We also noted that the effects of Dyrk1a inhibition on synaptic F-actin and the fraction of GluR2+ synapses were similar with and without application of heat-induced antigen retrieval (Figures S2D and S2E), suggesting that the observed phenotypes are likely due to changes in protein content and not antigen accessibility.

Finally, we noted that the effect on the GluR2+ fraction was similar whether staining for total or only externalized GluR2 (Figure S2F), indicating that, although GluR2− synapses identified in our screen are not necessarily silent[68] and vice versa (because GluR2 may be present but below the threshold or present but not externalized), they may act as a reasonable proxy for changes in silent synapse populations.

To test the hypothesis that PSD95 levels respond to mitigate decreased NMDAR presence or activity, we treated cultures for either a short time (48 h) or chronically (DIV 6-19) with the NMDAR blocker D-aminophosphovalerate (D-APV)[72] (Figure 2I). After the short treatment, NMDAR levels per synapse decreased ~30%. This effect disappeared after 2 weeks, accompanied by an increase in PSD95 and excitatory synapse density that mimics *Grin2a* knockdown, consistent with a compensatory response.

Multiplexed imaging reveals clusters of hierarchical synaptic protein compositions

We applied uniform manifold approximation and projection (UMAP)[73,74] to the 10-dimensional dataset of synapse protein levels, yielding the 2D projection in Figure 3A of different synapse compositions. The distribution of synapses shows distinct clusters defined combinatorially by the presence or absence of certain proteins, similar to our previous observations.[57] These included two inhibitory clusters and several excitatory clusters. Protein absences that defined certain clusters may have resulted from "true" complete absences or merely from levels below threshold. However, we observed similar distributions when changing threshold levels for synapse identification (to 75% and 133% of defined levels; Figure S3), indicating that the clusters arose at least in part from qualitatively different synapse compositions.

All treatment groups had synapses in all 9 clusters (Figure S4), with some population changes between clusters observed as a result of different gene knockdowns (Figures 3C and S4). These changes were consistent with the changes observed in mean protein levels in Figure 2E. For example, *Pten* knockdown, which increased all excitatory proteins save for GluR2, was seen here

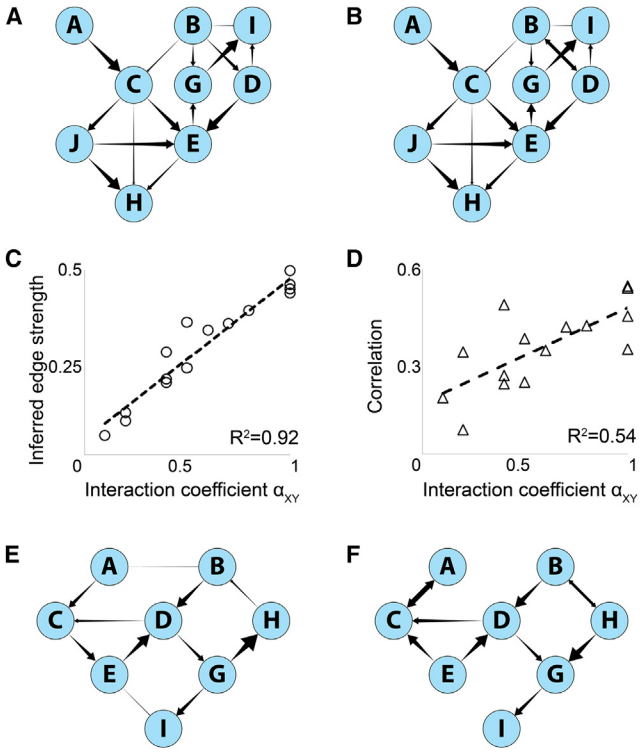

Figure 4. BN inference on simulated networks

(A) Example BN used to simulate PRISM-like data.

(B) Network reconstructed from simulated data produced by the network in (A).

(C and D) Calculated edge strengths of the edges in (B) versus defined interaction coefficients in (A). (C) Edge strengths calculated by parent-controlled correlations. (D) Total (uncorrected) correlations.

(E) Example non-Bayesian network with cycles (C-E-D and B-D-G-H) used to simulate PRISM-like data.

(F) Reconstructed BN from simulated data produced by the network in (E). Cycles cannot be represented, but the overall structure and relative edge strengths are preserved.

Arrowhead sizes represent predefined interaction parameters α_{XY} in (A) and (E) and inferred edge strengths in (B) and (F) (see Methods).

to enrich cluster 2 (GluR2−, positive for all others) at the expense of clusters 1 (GluR2+) and 6 (F-actin−). Nevertheless, they provided more detailed information about the specific synapse population changes that occurred as a result of gene knockdown.

In addition, we were able to use spatial information to aid in interpreting protein combinations. A small fraction of puncta was identified as vGluT1+, vGAT−, and Gephyrin+ (Figures S5A and S5B), despite Gephyrin being well established as an inhibitory synaptic protein.[75] However, upon closer examination, we observed that Gephyrin-Synapsin puncta distances in that subset were 50% greater than expected (Figure S5C), leading us to infer that these puncta were probably not associated physically with the other excitatory markers and to exclude them from future analyses.

Finally, we observed that not every combination of proteins was present (Figure 3B). For example, synapses that were negative for Bassoon or β-actin typically lacked or had very low levels of other post-synaptic proteins, indicating a hierarchy in protein dependencies on one another, which we sought to characterize systematically using BN inference, as previously applied to signaling networks.[59]

BN inference of the glutamatergic synapse

All excitatory synaptic proteins were positively correlated with one another (Figure 3D). However, when we examined each pair individually by measuring their correlation controlled via stratification for all other six proteins (STAR Methods), some correlations disappeared (Figure 3E). This revealed protein pairs that were not directly connected but only correlated through their inter-dependence on a third protein, which could be either a common effector (A←C→B) or an intermediate (A→C→B). To systematically map which causal connections among excitatory synaptic proteins were direct versus indirect and to establish the directionality of their inter-dependence, we derived a BN from the 8-dimensional distribution of protein levels in excitatory synapses.

This approach was first tested on simulated PRISM-like BNs—high-dimensional distributions that mirrored real PRISM data but with the distributions of variables conditional on one another in predetermined ways (Figures 4A and 4E; see STAR Methods for details on network simulation).

To derive the BNs (Figures 4B and 4F), we applied the "tabu" algorithm of the BNLEARN package[76] in R that searches model space in a Monte Carlo-like manner, maximizing an overall score that is based on the likelihood of the data given the model.[76–79] Additional costs to the score were imposed on each edge to prefer simpler, more parsimonious models.[76,79] To estimate confidence levels on the presence of edges and their directions, we applied a bootstrapping method that re-derived separate BNs for 50 independent samplings of 10,000 points in the simulated distribution. An edge was considered present (and is shown in Figures 4B and 4F) when it appeared in more than 80% of bootstrapped networks, and a direction was indicated (as a unidirectional arrow in Figures 4B and 4F) when that direction appeared in more than 60% of bootstrapped networks where the edge was present. To derive edge strengths, shown as arrowhead sizes in Figures 4B and 4F, we calculated the correlations between the parent and child node when controlling by stratification for all other parents of that node.[79,80] In bidirectional edges, both nodes were considered child nodes for this purpose.

This method reconstructed predefined networks with high fidelity. Notably, even when a distribution was not generated by a BN (i.e., it contained cycles, which cannot be reflected in a directed acyclic graph but can occur in reality as feedback loops), the inference algorithm still reconstructed the general network structure and edge strengths with reasonable fidelity, reversing some intra-cycle edges to avoid loops but preserving extracyclical edges. Edge strength calculation recovered the original interaction parameters (Figure 4E) better than using uncontrolled, total correlations (Figure 4F).

We applied the above approach to the entire 3.5-million synapse dataset across all treatments, yielding the network shown in Figure 5A. This network exhibited several features that were anticipated given our knowledge of the function and connectivity of these proteins. For example, the presynaptic proteins Bassoon, Synapsin1, and vGluT1 (the latter two co-localized in

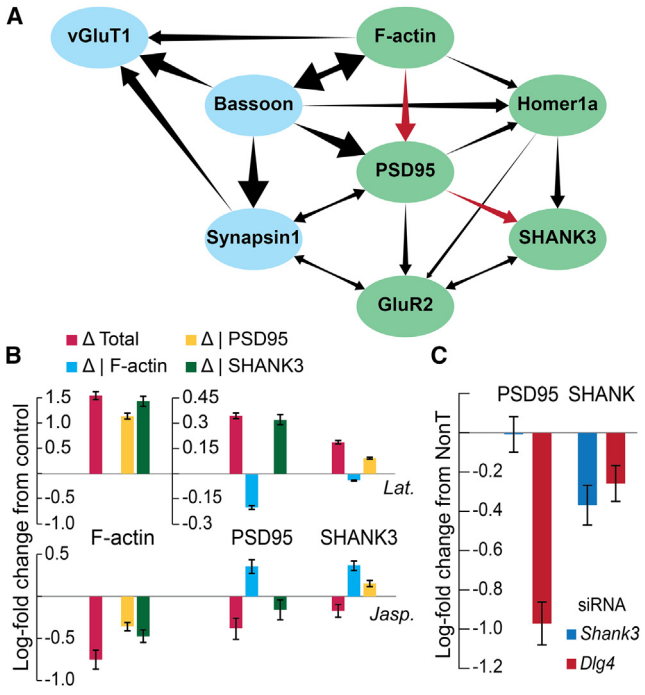

Figure 5. BN of eight excitatory synaptic proteins

(A) The inferred network. Presynaptic proteins are shown in blue and postsynaptic proteins in green. Red arrows indicate the substructure probed in (B) and (C).

(B) Log fold change in F-actin, PSD95, and SHANK3 after treatment with two actin polymerization perturbations, total and controlled for each protein.

(C) Log fold change in PSD95 and Shank3 after treatment with 3 siRNAs: NonT, against *Dlg4* (PSD95), and against *Shank3*.

Error bars are mean ± SEM across wells.

the same synaptic vesicles) appear tightly interconnected, and their correlation was independent of the postsynaptic proteins. Of the three presynaptic proteins, Bassoon levels were most directly connected to those of the postsynaptic proteins, possibly by acting as a proxy for the size of the active zone. Of the postsynaptic proteins, GluR2 was directly downstream of PSD95, in accordance with the latter's role as a dynamic anchor for the receptor whose levels dictate the number of sites that can capture diffusing AMPARs on the postsynaptic density.[81] Finally, levels of synaptic F-actin were upstream determinants of all other postsynaptic proteins, likely because of the cytoskeletal protein acting as a proxy for the size of the dendritic spine, which may limit other protein amounts. Additional, unanticipated features included presynaptic Bassoon appearing to influence levels of postsynaptic F-actin, PSD95, and Homer1 more than the latter influenced one another, as inferred from network edge strengths as defined above.

Importantly, our model also lends itself to testable hypotheses of causal connections derived from sub-structures of the network. For example, the position of SHANK3 downstream of the other components predicted that they would not be affected significantly by direct perturbation of SHANK3; e.g., by siRNA-induced knockdown. Our screen confirmed this (Figures 2E, *Shank3* line, and 2H), consistent with a previous study that used shRNA knockdown of *Shank3* to show that mGluR5 levels were reduced but other postsynaptic proteins were unaffected.[71] To prevent circular logic (i.e., the presence of the Shank3 knockdown data in the network generating this prediction), we derived the same BN when excluding the Shank3 treatment group (Figure S6), showing that BN inference can predict the effects of perturbations that are excluded from the training data.

A more complex, predictive sub-structure of the network is the inter-dependence chain: F-actin → PSD95 → SHANK3. This structure predicts that (1) perturbing F-actin should affect PSD95 and SHANK3, but the effect on SHANK3 should decrease or disappear when controlling for PSD95, while the effect on PSD95 should be relatively independent of SHANK3, and (2) perturbing PSD95 should affect SHANK3 but not vice versa. To test the first prediction, we treated hippocampal cultures with jasplakinolide (Jasp) or latrunculin B (Lat) at DIV 6, fixing at DIV 19 and staining for Synapsin1, F-actin, PSD95, and SHANK3 (Figure 5B). Interestingly, although Jasp and Lat B are generally inhibitors of F-actin depolymerization and polymerization, respectively, their effects on synaptic F-actin after chronic treatment were reversed. Effects on PSD95 and SHANK3 were consistently in the same direction as the effects on F-actin (decreased for Jasp and increased for Lat) and completely disappeared or even reversed when controlling for F-actin. In addition, controlling for PSD95 greatly diminished or reversed the effect on SHANK3, as predicted, while the reverse was not true. To test the second prediction, we treated DIV 6 cultures with siRNA mixes against *Shank3*, *Dlg4* (the gene coding for PSD95), or NonT and fixed and stained at DIV 21 for MAP2, Synapsin1, SHANK3, and PSD95 (Figure 5C). Synapsin-controlled SHANK3 levels were reduced compared with NonT in *Dlg4* and *Shank3*-treated synapses, but PSD95 was reduced only in *Dlg4*-treated synapses, establishing the PSD95 > SHANK3 hierarchy. Taken together, these observations support the conditional dependency chain predicted by the network.

Network inference on real PRISM data may be sensitive to experimental and image analysis artifacts, such as thresholding, image quality, and rules for synapse identification, because these may impose artifacts on correlations between protein measurements. We therefore performed several quality controls to ensure that our model did not result in such artifacts (Figure S6). In one, we limited network inference only to synapses positive for all protein components. In another, we varied the thresholds for punctum identification in CellProfiler to 75% or 133% of their values used in the primary analysis. In a third, we used puncta of postsynaptic proteins (F-actin and PSD95) to assign synapse identity instead of Synapsin1 and vGluT1. All of these manipulations yielded network structures that were largely similar to that in Figure 5A, especially in presence, relative strengths, and directionalities of transsynaptic and intra-postsynaptic edges. The few inconsistencies included interchanges in the relative positions of Synapsin1 and vGluT1 in the network. Finally, we derived a network based on different eight-protein measurements in a previous study[57] (Figure S6). Although that dataset was smaller, with fewer perturbations than the current one, and, thus, with reduced confidence in edge presence and

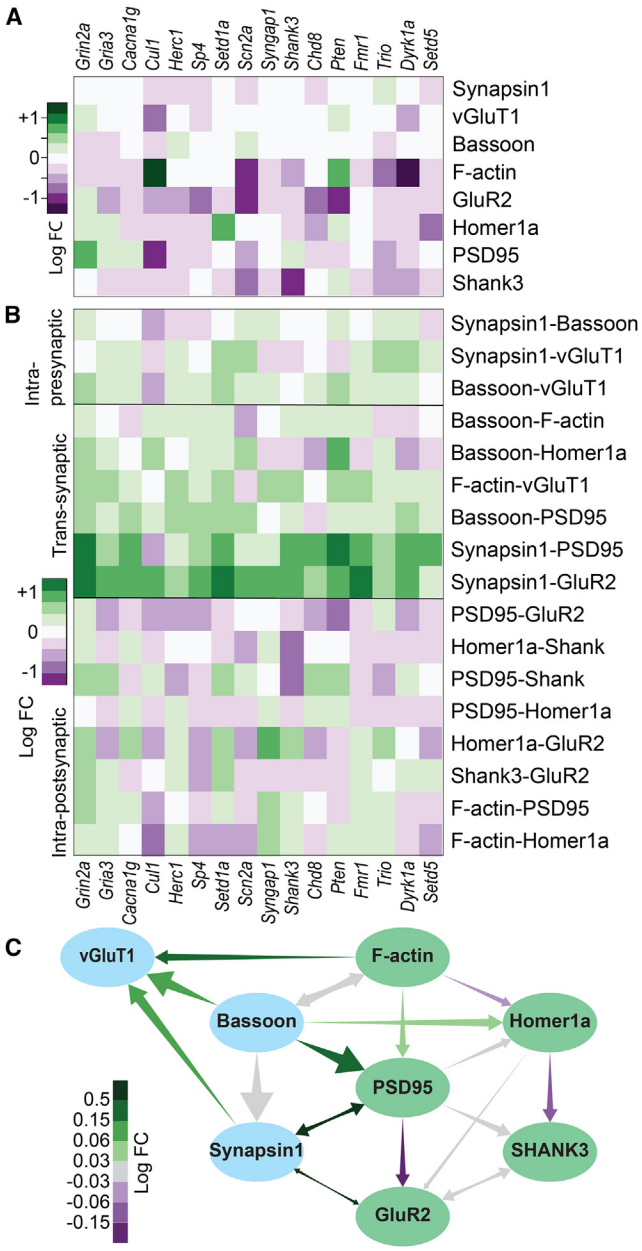

Figure 6. Network-informed analysis of the genetic screen

(A) "Direct" effects of each treatment on each protein separately; as in Figure 2F but controlled for the parent nodes of each protein.

(B) Effect of each treatment on the strength of each network edge.

(C) Network from Figure 4A, with each edge colored by the average change in strength across treatments. All colors are $\log_2$ fold change relative to the NonT siRNA control.

directionality, we observed that the six proteins common to both studies exhibited similar connectivity patterns. That the network features were robust against these manipulations, replicated across experimental conditions, and include substructures that were directly validated in perturbation experiments strengthened our confidence that these features represent real underlying biology.

Convergent effects of siRNA treatments on network structure

Next, we sought to use the causal network structure identified above for a more in-depth exploration of gene knockdown effects on the synaptic molecular system as a whole, including possible convergent phenotypes resulting from distinct genes. First, we used the simultaneous measurements to isolate direct treatment effects from secondary or downstream ones, by measuring the log-fold change in each protein under each siRNA treatment, when controlling for all parent nodes of that protein in the network, thus distinguishing direct effects from those mediated by a parent node (Figure 6A). Isolating direct effects revealed that *Pten* knockdown affected all post-synaptic protein levels that we examined nearly exclusively through increased F-actin within dendritic spines, in accordance with what is known of its regulatory role.[70] This also allowed us to discover direct effects that were hidden under second-order effects in the opposite direction; for example, that knockdown of *Pten* caused a strong specific decrease in synaptic GluR2 in parallel to a general increase of all post-synaptic proteins.

Finally, we looked for quantitative effects of siRNA gene knockdowns on the BN structure itself, particularly on edge strengths that indicate inter-dependencies of synaptic protein components. We quantified the strengths of the 17 edges in the network for each treatment group by treating each culture as an individual joint PD (Figures 6B and 6C). We discovered that some edges, particularly centered around PSD95 and GluR2, were uniformly strengthened or weakened by nearly all treatments, regardless of direct effects on the proteins themselves, even in treatments that had weak, if any, effects on the protein levels. For example, Synapsin1-PSD95 and Synapsin-GluR2 edges were strengthened in nearly all gene knockdowns compared with the NonT groups and not weakened in any, even though different treatments increased or decreased each protein on its own. Conversely, the PSD95-Homer1, Homer1-GluR2, or PSD95-GluR2 edges were weakened in most treatments compared with NonT.

Independent treatment effects on different proteins were likely to reduce edge strengths indiscriminately, indicating that that there might be an underlying molecular process by which knockdowns of many different genes all serve to, for example, weaken the extent by which PSD95 determines GluR2 and strengthen the extent by which PSD95 and Synapsin1 influence each other, where these effects are superimposed on any direct effects they may also have on these proteins.

To the best of our knowledge, this is the first time that a direct measurement of synaptic protein networks has yielded molecular phenotypes common to many different ASD- and SCZ-associated mutations, which may be reflective of shared synaptic pathogenesis.

DISCUSSION

Detailed parallel phenotyping of synaptic biochemistry in ASD and SCZ models

We used simultaneous measurement of multiple proteins at single-synapse resolution to infer causality relationships among

protein numbers in the synaptic molecular network and systematically map how it is affected by perturbation of ASD- and SCZ-associated genes. PRISM, supported by automated probe exchange, image analysis, and synapse segmentation and quantification, can be used to measure multiple synapse-specific phenotypes across many treatment groups in a single experiment as well as to identify synapse types and population changes that are more intricate than bulk effects on a protein because of its ability to resolve synapse-to-synapse heterogeneity in multiple protein levels.

We observed phenotypes that were anticipated based on previous studies or their known activity, such as *Pten*[70] and *Shank3*.[71] We also observed phenotypes that, to the best of our knowledge, have not been reported previously, such as *Cul1* on F-actin, *Setd1a* on Homer, and *Grin2a* on PSD95. Follow-up on the latter yielded tentative evidence of a long-term compensatory mechanism that responds to NMDAR deficiency (by gene knockdown) or reduced activity (by APV blockade, which also reduces NMDAR levels), with increased excitatory synapse density and levels of the NMDAR anchoring protein PSD95, possibly a compensatory effect. Of the overall molecular signatures, *Dyrk1a* exhibited the most unusual pattern, combining a markedly higher E:I ratio and lower levels of F-actin and downstream postsynaptic proteins but not of GluR2 (and generally a lower percentage of silent synapses). This is congruent with reports of *Dyrk1a*-associated autism presenting a unique neurological character.[82,83]

Another important consequence of the ability to measure multiple proteins simultaneously is that it can facilitate deconvolution of direct causal relationships from those mediated by other processes. Instead of having to experimentally constrain any possible confounding variables, multiplexed imaging allows measuring them simultaneously, while moderate throughput offers a large enough number of data points (>3 × 10^6 in this study) to directly control for possible confounding variables via stratification. The results of such intra-dataset controls must be interpreted carefully because of potential artifacts arising from comparing different synapse populations. Nevertheless, when using information about causal connections between variables, either from prior knowledge or, as in this case, inferred directly from the PDs, such a controlled analysis can help identify when a certain effect is entirely mediated by other variables (as with *Pten* or *Dyrk1a* on postsynaptic proteins, mediated by F-actin) or when a certain variable deviates significantly because of a hidden direct effect from what is expected given its upstream network connections (as with *Pten* on GluR2). The network-based prediction that *Dyrk1a* effects are mediated via F-actin is supported by an in-depth investigation[84] of changes to dendritic spine formation in heterozygous *Dyrk1a* truncation mutants.

It is important to note that, although parallel measurements of many proteins may serve as a hypothesis generator for synapse-level molecular mechanisms, this study is fundamentally a phenotypic assay and does not purport to definitively test or establish any specific mechanism. Some gene perturbation effects may be the result of transcriptional changes in upstream gene expression networks (for example, by histone-modifying enzymes), to which our assay is agnostic.

Interpreting inter-protein dependencies from BN structure and edge strengths

In the inferred BN, an edge from node A to B indicates that the PD of B depends on A even after accounting for all other measured components. This dependence can be direct or via a hidden (unmeasured) component. Conversely, lack of a direct edge between two correlated nodes indicates that any correlation between them can be explained by the other known components. Thus, while the overall structure is dependent on the set of proteins measured, we expect "non-edges," such as F-actin-SHANK3, to be a conserved feature even as the network expands to include more proteins. The structure we observed hints at some general rules governing synaptic molecular composition, which have also been observed in the literature: relative independence of the presynaptic active zone assembly and molecular composition[85,86] and receptor levels driven by scaffolding proteins rather than the reverse.[81,87,88]

The interpretation of changing edge strengths is not mechanistically obvious. As a rule, injecting perturbative noise into a system weakens correlations by default. Thus, a weakened edge may correspond to a loss of correlation that can occur when only one protein of a pair is perturbed or when they are perturbed in different directions. However, this is not always the case; for example, knockdown of *Cul1* reduces PSD95 and GluR2 to a similar extent but also weakens the PSD95-GluR2 edge. In general, an edge from a parent node to a child node is considered in the context of all the other parents of that node. Thus, an effect that perturbs the mechanism by which one parent node affects the distribution of the child will weaken the corresponding edge but may strengthen the edge from another parent and vice versa. It is thus interesting to observe that transsynaptic edges are strengthened by the genetic knockdowns in this study, while intra-postsynaptic edges are generally weakened.

Comparison with other protein network models

Our results add to a growing body of genomic and proteomic observations of convergent changes at the protein network level in ASD. These are based on physical interaction networks derived from Y2H tests[52] or bulk quantitative multiplexed coimmunoprecipitation (QMI).[53] It is important to note differences between physical interaction networks as obtained from Y2H screens or coIP and our network, which does not provide information about direct biochemical protein-protein associations but rather provides constraints on the multiprotein joint PD that reports on synaptic-level protein co-localizations. When many parallel biochemical interaction pathways exist, the BN enables us to infer those that causally determine synapse protein levels. For example, the F-actin → PSD95 → SHANK3 causal chain we established implies that it is likely an interaction chain from actin to PSD95 (possibly via ARPC4[89]) that drives PSD95 (and therefore SHANK3) levels. It must be noted that, for F-actin, our measurement mixes together signal from pre- and post-synaptic β-actin filaments. However, because postsynaptic F-actin is thought to be more abundant, we believe that the observed conditional dependencies are mostly due to postsynaptic F-actin levels.

Causal correlations may also show up when a certain target is the best available proxy for a different measure that drives protein levels, such as Bassoon for the active zone or F-actin for

the dendritic spine. For example, knockdown of Bassoon itself does not seem to affect postsynaptic protein localization and ultrastructure. Rather, we hypothesize that the edges from Bassoon to F-actin, PSD95, and Homer represent different paths, through unmeasured targets, by which the size of the active zone affects levels of different post-synaptic proteins. Finally, our network so far only measured protein abundances and not phosphorylation activation or other modification changes (or splice isoforms), which are often influenced by interaction partners.

The two frameworks thus complement each other. Physical interaction networks provide mechanistic biochemical associations, albeit, in some cases, under unphysiological conditions, such as in yeast, and, by casting a wider, less biased net, identify new targets to measure, whereas BN analysis integrates these details into a global picture of what shapes the overall synapse protein composition. This, in turn, provides insight into possible dynamical processes in the synaptic molecular network, which are tested by again returning to mechanistic connections between components. In the future, BN analysis could be directly combined with single-synapse-level knowledge of physical protein organization from automated, multiplexed superresolution imaging such as maS^{3}TORM.[90]

The steady state of the synaptic molecular network in ASD and SCZ

We propose a theoretical framework for future exploration of the convergence of phenotypes of different disease-associated mutations at the level of the synaptic molecular network. A dynamic system like the synapse, with many components, generally exists in a high-dimensional space of component abundances and activation states. However, with sufficient interactions and feedback loops, such systems invariably settle into a much lower-dimensional space of allowable stable or metastable states. In the case of the synapse, these are guaranteed by the high interconnectivity among synaptic proteins and feedback constraints such as homeostatic plasticity. In other words, because synaptic structural dynamics (e.g., LTP and LTD) occur at longer timescales than the biochemical feedback interactions that establish the allowed states, the former navigate a comparatively narrow landscape of only those states that the dynamical system can consistently sustain, and the synapse population distribution reflects this landscape.

A similar constraint-induced dimensionality reduction is considered to stabilize symmetric phenotypes in genotype-phenotype maps.[91] In the context of ASD- and SCZ-associated genotypes mapping to a synaptic phenotype space with reduced dimensionality caused by interaction-based constraints, this means that a multitude of seemingly unrelated disease-associated mutations can drive similar perturbations to the lower-dimensional stable/allowed-state landscape, as measured in synaptic protein networks.

Thorough investigation of such a mechanism in ASD and SCZ will require characterization of this space of allowed synaptic states and how it changes under different mutations, a characterization for which this work and others provide initial outlines. This characterization should also provide additional starting points to investigate the downstream effects of convergent synapse structure phenotypes on the architecture of the synaptome and specific neuronal circuits.

Given the potential of PRISM with automated probe exchange for increasingly higher throughput, along with the theoretically unlimited multiplexing capacity, our method of multiplexed imaging with single-synapse analysis is well poised to investigate such hypotheses as well as any other processes in the synaptic molecular network. Specifically, the properties of BN inference mean that additional measured nodes would increase the scope of and confidence in network structures as well as help establish differences in network structure underlying qualitatively different functionality in excitatory synapses connecting different neuron types.[6] Finally, the tool presented here does not need to be limited to synapses and can be applied to any subcellular structure that can be identified individually in fluorescence microscopy, including mitochondria, phagosomes, nuclear compartments, etc. Thus, PRISM, supported by BN analysis, may come to serve as a general hypothesis-generating tool for understanding complex protein networks *in situ* in cells, organelles, and subcellular structures.

Limitations of the study

The objective of this study was to perform high-dimensional *in situ* synaptic protein phenotyping in an ASD- and SCZ-associated genetic screen and search for similarities in synaptic protein compositions and convergent synapse phenotypes across disease-associated genes. One major limitation of this study was the use of self-transfecting siRNA knockdown in 2D rodent neuronal culture as a model for ASD and SCZ genotypes. While this model system often reproduces qualitative effects of haploinsufficiency or partial loss of function (PLOF), they may be fundamentally different from, for example, complete knockout or gain-of-function models, some of which have observed different synaptic phenotypes.[92,93] Even for the former, gene dosage differences may produce qualitatively different results, with our treatments variably reducing mRNA levels by 40%–80%, which may be different from the gene dosage produced by heterozygous mutants or PLOF. Therefore, this study should be treated as a starting point to investigate convergent gene-proteome connections rather than a definitive description of protein changes in the disease itself.

Another potential limitation is the exclusive use of immunofluorescence for protein quantification, which offers the possibility of artifacts because of variability in epitope accessibility as well as non-specific binding that may confound results. While we have performed extensive validations in this and previous studies to establish the quantitative validity of PRISM, the possibility of such artifacts remains. Finally, the network inference approach presented is based on one imaging dataset and may additionally contain artifactual edges and substructures despite care to account for possible statistical artifacts. Future work that incorporates additional protein nodes and a larger variety of cellular conditions will help to further establish the biological validity of the present work.

STAR★METHODS

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

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.112430.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.W.D., K.P.d.A., J.R.C., M.B., and R.F.; investigation, E.W.D., K.P.d.A., and R.F.; software and formal analysis, R.F. and E.W.D.; resources, E.-C.W. and K.P.d.A.; validation, R.F., K.P.d.A., A.P.L., and J.S.; writing – original draft, R.F. and M.B.; writing – review & editing, R.F., M.B., M.S., E.W.D., K.P.d.A., and J.R.C.; funding acquisition and supervision: M.B. and J.R.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-MAP2, chicken polyclonal	Novus Biologicals	Cat#NB300-213 RRID:AB_2138178
anti-PSD95, rabbit monoclonal	Cell Signaling Technology	Cat#3450 Clone: D27E11 RRID:AB_2292883
anti-Gephyrin, rat monoclonal IgG1	Synaptic Systems	Cat#147208 Clone: rtmAb7a RRID:AB_2619836
anti-GluR2, N-terminus, guinea pig polyclonal	Synaptic Systems	Cat#182105 RRID:AB_2619875
anti-NR2A, mouse monoclonal IgG2a	Neuromab	Cat#75-288 Clone: N327/95 RRID:AB_2315842
anti-Shank3, mouse monoclonal IgG1	Synaptic Systems	Cat#162311 Clone: 144b12 RRID:AB_2713972
anti-vGAT, mouse monoclonal IgG3	Synaptic Systems	Cat#131011 Clone: 117G4 RRID:AB_887872
anti-Synapsin1, mouse monoclonal IgG1	Synaptic Systems	Cat#106011 Clone: 46.1 RRID:AB_2619772
anti-Homer1, mouse monoclonal IgG1	Synaptic Systems	Cat#160011 Clone: 2G8 RRID:AB_2120992
anti-vGluT1, camelid sdAb, FluoTag X2	Synaptic Systems	Cat#N1602-AF568, RRID:AB_2737074
anti-mGluR5, rabbit polyclonal	Sigma Aldrich	Cat#AB5675 RRID:AB_2295173
anti-Shank1/2/3, mouse monoclonal IgG2a	Synaptic Systems	Cat#162111 Clone: 151E3 RRID:AB_2636900
anti-NR1, mouse monoclonal IgG2a	Millipore	Cat#MAB363 Clone: 54.1 RRID:AB_94946
anti-GluR2, C-terminus, mouse monoclonal IgG2a	Millipore	Cat#MAB397 Clone: 6C4 RRID:AB_2113875
anti-Synapsin1, goat polyclonal	Santa Cruz biotechnologies	Cat#sc-7379 RRID:AB_677474
anti-Rabbit, goat polyclonal	Invitrogen	Cat#A16126
anti-Mouse IgG1, goat polyclonal	Abcam	Cat#ab98689
anti-Mouse IgG2, goat polyclonal	Novus Biologicals	Cat#NB7513
anti-Guinea Pig, goat polyclonal	Invitrogen	Cat#A18777
anti-Rat, goat polyclonal	Invitrogen	Cat#A18873
anti-Goat 405, donkey polyclonal	Abcam	ab175665
anti-Goat 488, donkey polyclonal	Invitrogen	A-11055
anti-Chicken 405, goat polyclonal	Abcam	ab175674
anti-Chicken 488, goat polyclonal	Invitrogen	A-11039
anti-Mouse 488, goat polyclonal	Abcam	ab150113
anti-Mouse 568, donkey polyclonal	Invitrogen	A10037
anti-Mouse 647, donkey polyclonal	Abcam	ab150107
anti-Rabbit 488, goat polyclonal	Abcam	ab150077
anti-Rabbit 568, donkey polyclonal	Abcam	ab175470
anti-Rabbit 647, donkey polyclonal	Invitrogen	A-31573
Biological samples		
E18 Rat Hippocampal Neurons	Privately sourced at the Broad Institute	
Chemicals, peptides, and recombinant proteins		
4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid N-hydroxysuccinimide (SMCC)	Sigma Aldrich	Cat#573115 CAS 92921-24-9
Phalloidin 7-Ornithine	Bachem	Cat#H7643
Harmine	Tocris	Cat#5075 CAS 442-51-3
bpV(pic)	Sigma Aldrich	Cat#SML0885 CAS 148556-27-8
D-AP5	Alomone Labs	Cat#D-145 CAS 79055-68-8
RNase A	Invitrogen	Cat#EN0531
RNase T1	Invitrogen	Cat#EN0542

(*Continued on next page*)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hibernate E	Thermo Fisher	Cat#A1247601
NbActiv1	Brainbits LLC (now Transnetyx)	Cat#NB1
NbActiv4	Brainbits LLC (now Transnetyx)	Cat#NB4
B27 Supplement	Thermo Fisher	Cat#17504044
Arabinofuranosyl Cytidine (AraC)	Sigma Aldrich	Cat#C6645 CAS 69-74-9
Atto 565 NHS	Atto-Tec GmbH	Cat#AD 565-31
Atto 655 NHS	Atto-Tec GmbH	Cat#AD 655-31
Critical commercial assays		
LightCycler 480 Probes Master	Roche	Cat#04707494001
TaqManTM Gene Expression Assays	Life Technologies	See Table S3
SiteClick Azido Antibody Modification Kit	Thermo Fisher	Cat#S10900
Experimental models: Organisms/strains		
Rattus Norvegicus, Sprague-Dawley, wild-type	Broad Institute	
Oligonucleotides		
Thiol-modified ssDNA docking strands	Integrated DNA Techologies	See Table S2
DBCO-modified ssDNA docking strands	Qiagen	See Table S2
Amine-modified LNA imaging strands	Qiagen	See Table S2
Software and algorithms		
CellProfiler version 3.0	McQuin, C. et al. CellProfiler 3.0: Next-generation image processing for biology. PLoS Biol. 16, (2018).	
R package: umap, version 0.2.7.0	Tomasz Konopka. umap: Uniform Manifold Approximation and Projection. R package version 0.2.7.0 https://CRAN.R-project.org/package=umap. (2020).	
R package: bnlearn, version 4.7	Scutari, M. Learning Bayesian networks with the bnlearn R Package. *J. Stat. Softw.* 35, 1–22 (2010).	
CellProfiler Synapse Analysis Pipeline	This study	https://doi.org/10.5281/zenodo.7753915
R markdown document: Workup and analysis of PRISM data	This study	https://doi.org/10.5281/zenodo.7753915
R markdown document: Network simulation and inference	This study	https://doi.org/10.5281/zenodo.7753915
Dataset, CSV file: Normalized single-synapse multiprotein measurements extracted by CellProfiler	This study	https://doi.org/10.17632/cjyzby7z82.1

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mark Bathe (mbathe@mit.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Single-synapse measurements as processing output of CellProfiler applied on imaging data have been deposited to Mendeley Data as a CSV file under the paper title. https://doi.org/10.17632/cjyzby7z82.1. Raw and processed imaging data are locally stored due to excessive file sizes and will be delivered upon request.

- Original code for data analysis, network simulation, and network inference, as well as original CellProfiler pipelines, have been deposited to github.com/lcbb and on Zenodo. https://doi.org/10.5281/zenodo.7753915
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experiments were performed on dissociated hippocampal neurons of embryonic day 18 Sprague-Dawley rats. Each experiment included neuronal cultures of pooled neurons from 1-3 embryos of a single pregnant rat, and each experiment used a separate pregnant rat. Embryos were not sexed prior to dissection and culturing.

Culturing conditions (for detailed conditions see method details section): Embryo hippocampi were dissected in 4°C Hibernate E supplemented with B27. Hippocampal tissues were digested in Hibernate E using papain at 37°C. Neurons were plated at 15,000 cells/well in NbActiv1 supplemented with 25mM glutamate on poly-d-lysine coated 96-well-plates, treated at DIV 2 with AraC and at DIV 6 with the siRNA/chemical treatment and left to develop in NbActiv4 until fixation and staining at DIV 19.

Procedures for rat neuronal culture were reviewed and approved for use by the Broad Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

METHOD DETAILS

Antibody details

Table S1 details the antibodies used for multiplexed imaging, including the conjugation strategy used for each. The key resources table provides additional details, and information on all other antibodies used for follow-up validation experiments.

Antibody-docking strand conjugation – SMCC

Most antibodies (see Table S1) were conjugated using 4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid N-hydroxysuccinimide (SMCC), a heterobifunctional molecule which binds exposed amines on the antibody via an N-hydroxysuccinimide (NHS) moiety on one end and binds thiol-oligonucleotides via a maleimide on the other end. Antibodies were purchased as formulations without serum proteins as listed below (Table S1), 0.1-1 mg of antibody were purified into PBS using Zeba spin columns (7 kDa, Thermo Fisher Scientific). Subsequently, antibodies were concentrated to 1 mg/ml using Amicon Ultra centrifugal filters (100 kDa, 4000 g, EMD Millipore). The initial concentration of anti-vGAT was 2 mg/ml. From a freshly prepared stock at 2 mM in DMF, SMCC (Sigma Aldrich) was added to the antibody at 7.5x molar excess. The reaction mixture was protected from light and incubated for 3 h at 4°C on a shaker. Excess SMCC was removed by purification into PBS using Zeba spin columns (7 kDa, Thermo Fisher Scientific). In parallel, 25 nmol 5' thiol-modified ssDNA (Integrated DNA Technologies, modification catalog no. /5-ThioMC6-D/) was dissolved in 25 ul water and 55 ul PBS with 2 mM EDTA at pH 8.0 (Table S2). After the addition of 20 ul of a freshly prepared stock of 500 mM DTT in PBS with 2 mM EDTA at pH 8.0, the reaction mixture was protected from light and incubated for 2 h at 25°C on shaker. The reduced 5′ thiol-modified ssDNA was purified into water using NAP-5 columns (GE Life Sciences). Fractions containing ssDNA were identified using absorbance measurements at 260 nm and DTT was monitored calorimetrically using bicinchoninic acid. The reduced 5' thiol-modified ssDNA was immediately added to the antibody-SMCC conjugate at 15x molar excess, the reaction mixture was protected from light and incubated overnight at 4°C on a shaker. Antibody-ssDNA conjugates were purified into PBS using Amicon Ultra centrifugal filters (50 kDa, 4000 g, EMD Millipore). Amino-modified phalloidin (Bachem) was conjugated using the procedure described above, but with the following changes: the molar excess of SMCC was 10x and the molar excess of reduced 5' thiol-modified ssDNA was 1x. HPLC purification was employed to remove unreacted SMCC and 5' thiol-modified ssDNA, respectively (Waters, BEH C18 column, gradient for phalloidin-SMCC: from 80% TFA in water and 20% acetonitrile to 20% TFA in water and 80% acetonitrile over 10 min, gradient for phalloidin-ssDNA: from 90% 0.1 M TEAA in water and 10% acetonitrile to 60% 0.1 M TEAA in water and 40% acetonitrile over 10 min). Antibody concentration were determined by absorbance measurements at 280 nm. Conjugation efficiency was estimated by MALDI-TOF mass spectrometry and ranged from 1 to 3, depending on the antibody. Antibody-ssDNA conjugates were stored at -20 C in PBS with 50% glycerol.

Antibody-docking strand conjugation - SiteClick

For Homer1, ssDNA-antibody conjugates were synthesized using the SiteClick™ (Invitrogen) conjugation technique following the manufacturer's protocol. This technique replaces the Fc galactoses on the antibody with azide-modified sugars, which then react with a DBCO-modified oligonucleotide. 200 μg of the anti-Homer 1 was concentrated to 2 mg/ml in 1x Tris buffer and incubated with β-galactosidase. Azide-modified, terminal galactosides were attached using β-galactosyltransferase. Azide-modified antibody was purified into 1x Tris buffer using Amicon Ultra centrifugal filters (50 kDa, 4000 g, EMD Millipore). 5' DBCO-modified ssDNA (Integrated DNA Technologies, modification catalog no. /5-DBCON/) was dissolved in water, added to azide-modified antibody at a molar excess of 30 and incubated overnight at 25°C (Table S2). Antibody-ssDNA conjugates were purified into PBS using Amicon Ultra centrifugal filters (50 kDa, 4000 g, EMD Millipore). Antibody concentrations were determined by absorbance measurements

at 280 nm. Conjugation efficiency was estimated by MALDI-TOF mass spectrometry and ranged from 1 to 2, depending on the antibody batch. Antibody-ssDNA conjugates were stored at -20°C in PBS with 50% glycerol.

Imager strands

25 nmol of 5'/3' diamino-modified ssLNA (Qiagen) was dissolved in 500 ul PBS with 10% DMSO at pH 8.3 and 250 nmol of NHS-Atto 565 or NHS-Atto 655 (Sigma Aldrich) were added from a 15 mM stock in DMSO (Table S2). Following immediate vortexing, the reaction mixture was protected from light and incubated overnight at 25°C on a shaker. Excess dye was removed using NAP-5 columns (GE Life Sciences). Fractions containing ssDNA were identified using absorbance measurements at 260 nm. Subsequently, 0.1 M TEAA was added ssLNA-dye conjugates and conjugates bearing two dyes were purified by HPLC (Waters, BEH C18 column, gradient for Atto 565: from 80% 0.1 M TEAA in water and 20% acetonitrile to 70% 0.1 M TEAA and 30% acetonitrile over 10 min, gradient for Atto 655: from 90% 0.1 M TEAA in water and 10% acetonitrile to 75% 0.1 M TEAA in water and 25% acetonitrile over 10 min). Peaks corresponding to ssLNA conjugates bearing two, one or no dye were assigned based on absorbance spectra. Solvents were removed in vacuo and ssLNA-dye conjugates were dissolved in water at 10 to 100 μM, depending on the yield. Yields were determined by absorbance measurements using 565 nm or 655 nm wavelengths.

Neuronal culture and treatment

Procedures for rat neuronal culture were reviewed and approved for use by the Broad Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In each of N=4 biological repeats, 1-2 Embryonic Day 18 embryos were collected from a separate pregnant Sprague Dawley rat killed by CO_2 (Taconic). Embryo hippocampi were dissected in 4°C Hibernate E supplemented with 2% B27 supplements and 100 U/ml penicillin/strep (Thermo Fisher Scientific). Hippocampal tissues were digested in Hibernate E containing 20 U/ml papain, 1 mm L-cysteine, 0.5 mm EDTA (Worthington Biochem), and 0.01% DNase (Sigma-Aldrich) for 8 min. Neurons were centrifugated at 1000rpm by 5min, pellet with cells were then resuspended into NbActiv1 (BrainBits LLC, now TransnetYX) supplemented with 25mM glutamate, and plated at a density of 15,000 cells/well onto poly-d-lysine-coated, black-walled, thin-bottomed 96-well plates (Corning BioCoat). After 48 hours, AraC was added to each culture at a concentration of 1uM, to suppress glia proliferation and minimize well-to-well variability resulting from it. At DIV 5, the media was entirely replaced with warm NbActiv4. At DIV 6, each culture was treated with Accell SMARTpool (Dharmacon/Horizon from Perkin Elmer), a mix of four chemically modified self-transfecting siRNAs, against the relevant gene (Table S3) to a total siRNA concentration of 1uM in NbActiv4. Cultures were then left undisturbed until fixation on DIV 21. Each plate included 60 wells/separate cultures, 3-4 in each treatment group. Across 4 plates, one for each biological repeat, this results in a total of n=11-18 technical repeats in each treatment group. For validation experiments, cultures were treated at DIV 6 with 0.1uM/0.5uM bpV(pic), 0.2uM/2uM Harmine, 20uM/50uM D-AP5, and Nontargeting, *Shank3* or *Grin2a* Accell SMARTpool siRNA, and left undisturbed until fixation on DIV 8 or 19 as described.

RTqPCR knockdown validation

RTqPCR was performed using Fast Advanced Cells-to-CT kit (Ambion) according to the manufacturer's protocol. In short, cells were prepared for lysis by washing them with cold PBS 1x, then Stop solution was added following lysis buffer with DNAse I. RT Master Mix using Cells-to-Ct lysate was prepared and reverse transcription was done on a thermal cycler. Lastly, qPCR was done using LightCycler® 480 Probes Master (Roche) with TaqManTM Gene Expression Assays designed for each target (see Table S3 for catalog numbers) and performed on a LightCycler® 480 Instrument. Two TaqManTM Gene Expression Assays (Life Technologies). *Actb* was used as a reference gene to normalize the results (Life Technologies). For relative quantification of gene expression, the $2-\Delta\Delta Ct$ method was used.

Staining and imaging

Cells were fixed and stained as described previously.[7,57,58] Cells were fixed in fixation solution (4% paraformaldehyde and 4% sucrose in PBS) for 20 min at RT, then permeabilized with 0.25% Triton X-100 in PBS for 10. They were then incubated in a mixture of RNases A and T1 to reduce the fluorescent background caused by ssLNA-RNA binding and blocked with 5% Bovine Serum Albumin (BSA). The first round of primary staining was performed using unconjugated primary antibodies (Table S1 rows 1-6) diluted in the regular blocking buffer. Cells were blocked with nuclear blocking buffer [5% BSA and 1 mg/mL salmon sperm DNA (Sigma-Aldrich) in PBS] and then incubated with conjugated secondary antibodies (Table S1 rows 7-11) diluted in the nuclear blocking buffer. After post-fixation, cells were stained in the third round with conjugated primary antibodies (Table S1 rows 12-16) in nuclear blocking buffer and then with DAPI.

High-throughput spinning disk confocal LNA-PRISM imaging was performed using the Opera Phenix High-Content Screening System (PerkinElmer) as described before[7,57,66] with the following main changes: first, two colors were used for PRISM in each round, and second, probe introduction, wash and exchange was performed automatically using a Bravo automated liquid handling system. In each round, a pair of imaging probes in two colors (see Table S2 for sequences) was freshly diluted to 10 nM in imaging buffer (500 mm NaCl in PBS, pH 8) immediately before imaging. Neurons were incubated with imaging probes for 5 min and then washed twice with imaging buffer to remove unbound probe. The plates were then imaged in 4 wavelengths: 405nm (DAPI), 488nm (MAP2), 561nm (orange probe) and 647nm (red probe). For each field of view, a stack of five images was acquired with an axial step-size of

1 μm. Either four (in one plate) or nine (in the other three plates) lateral fields of view were imaged in each culture. Following each round of imaging, cells were washed two times with wash buffer (0.01 × PBS) for 3 min per round, and then re-imaged to ensure that all PRISM fluorescent signal was removed before introducing the next probe pair. After all imaging rounds, neurons were stained with a 568nm fluorescent nanobody against vGlut1 (Table S1 row 17) for 1 hour and imaged again. Although the combined staining with multiple antibodies simultaneously may reduce primary PRISM signal due to crowding, we observed this reduction to be less than 15%, and should be identical across all treatment groups.

For staining externalized GluR2 (Figure S2), an N-terminal-specific Guinea Pig anti-GluR2 antibody (see Table S1) was used before permeabilization, and a C-terminal-specific Mouse anti-GluR2 antibody was used after permeabilization, along with a labeled anti-vGluT1 nanobody and a chicken anti-MAP2 antibody. For heat-induced antigen retrieval, wells were incubated after permeabilization with pre-heated antigen retrieval buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) at 95°C for 30min, and then stained as usually. We also attempted proteolysis-induced epitope retrieval (0.05% Trypsin, 20min at 37°C) which completely abolished Synapsin1 and F-actin signals.

Automated image analysis using CellProfiler

CellProfiler was used to automatically correct images for uneven illumination, align images across channels, and segment and quantify synapses in images. This tool allows for modular construction of pipelines for image analysis.[66,67] The pipeline used here is similar to a previous study[57] and is available on github.com/lcbb/PRISM-CellProfiler-Analysis-Pipelines. The main steps in the image analysis pipeline are as follows:

1) By-pixel maximum projections of confocal Z-stacks of all images in each round are calculated separately and loaded into CellProfiler.
2) MAP2 (488nm) images in each round are used to align images of other channels between rounds.
3) An illumination profile correction is applied to all images based on background averages across all wells.
4) For each round and wavelength, the average intensity in untreated wells of a plate is calculated and used to normalize the images in all other wells. This is used to account for between-plate differences in exogenous brightness (staining strength, laser strength, exposure time etc.)
5) The DAPI image is used to identify nuclei objects. All other images of the same field are then masked by the nuclei to prevent artifacts from non-specific nuclear localization of the antibodies.
6) The MAP2 image is used to identify dendrite objects.
7) A white top hat filter with a radius of 4px is applied to all synaptic protein images across all rounds to enhance puncta.
8) For synapse counting analysis (Figures 2B–2D), synaptic objects were segmented and identified in images of each channel by applying the RobustBackground tool, which calculates an optimal threshold value for each window individually based on the intensity histogram. For all other analyses, we calculated a per-channel global threshold from the average threshold calculated by RobustBackground across all imaged fields in untreated wells. We then applied this value as a uniform threshold to all images of that channel to ensure that all images are segmented identically.
9) Synapsin1 puncta are then masked using the dendrites previously identified, to retain only puncta which are within 12px of a dendrite. These are then defined as synapses.
10) Puncta in all other channels are assigned to synapses if they overlapped with Synapsin1 puncta more than 6.25% (for postsynaptic proteins) or more than 50% (for presynaptic proteins).
11) Finally, levels of each protein per synapse are calculated as the intensity integral of that protein's image across its punctum. If a certain protein did not have an identified puncta associated with a synapse, its level was marked as 0.

Synapses were identified as excitatory if they contained only vGlut, inhibitory if they contained only vGAT, and otherwise excluded from further analysis (positive or negative for both vGlut and vGAT). Excluded synapses were 20-30% of all identified synapses. We also performed the same analysis with uniform threshold values of 75% and 133% of the calculated average, which yielded more and less synaptic puncta, respectively, but similar observations in treatment effects, clusters, Bayesian networks and edge strengths. In controls for network inference by alternative synapse identification, instead of Synapsin1 puncta for synapse definition and assignment of all other proteins, we used postsynaptic puncta defined by merging of F-actin, PSD95 and Shank3 puncta.

Network simulation

Data points for simulated networks were sampled from a modified gamma distribution, which contains a separate probability for a value of 0 (see Equation 1). To simulate dependency of one variable on others, the distribution from which the child variable is sampled has p_0 and scale parameter θ modified based on the values of the parent variables according to Equation 2.

$$P(X = x) = \begin{cases} p_X^0, x = 0 \\ \Gamma_X(k, \theta), x > 0 \end{cases} \quad B \rightarrow A \leftarrow C \qquad \text{(Equation 1)}$$

$$p^0_{A,modified} = p^0_{A,base} \cdot \tilde{b}^{-\alpha_{AB}} \cdot \tilde{c}^{-\alpha_{AC}}$$
$$\theta^0_{A,modified} = \theta^0_{A,base} \cdot \tilde{b}^{-\alpha_{AB}} \cdot \tilde{c}^{-\alpha_{AC}} \quad \text{(Equation 2)}$$

Where X, A, B, C are variable names, p^0_X is the probability for node X to be 0, $\Gamma(x|k,\theta) \propto x^{k-1} \cdot e^{-\frac{x}{\theta}}$ is the gamma distribution with shape parameter k and scale parameter θ, $\tilde{b} = \frac{b}{median(B)}$ and $\tilde{c} = \frac{c}{median(C)}$ are the values of B and C for a specific datapoint relative to their median, and α_{AB} and α_{AC} are the predefined interaction coefficients of A with B and C, respectively (edge strengths in the B → A ← C subgraph).

20 such networks with 6-12 nodes were generated, sampled, and reconstructed to determine the optimal Bayesian network inference algorithm. To simulate a network with a cyclic dependence, e.g. A→B→C→A, A is separated into two variables A_1 and A_2 to create a directed acyclic graph which is sampled as above, and the final value of A is $A_1 + A_2$.

Bayesian network analysis and controlled edge calculation

Bayesian network inference was performed on a pooled random sample of synapses from each well, while limiting only to excitatory-labeled synapses and 8 excitatory synaptic proteins. Measurements in each protein were discretized into 51 bins in the following way: all measurements of 0 (no puncta of that protein associated with the synapse) were assigned to bin 0, and bins 1-50 were assigned by equal-frequency discretization.

The discretized dataset was then sampled for 3000 points which were used to construct a Bayesian network using the likelihood-score-maximizing 'tabu' algorithm.[76] 50 such samplings and rederivations of the network were used to establish confidence in the presence and direction of edges. Network derivation was done using the tabu and boot.strength functions in the R package bnlearn,[76] A similar procedure was applied to simulated datasets, data from a previous synaptic scaling study,[57] and adversarially modified datasets.

Given a network, we define the strength of an edge between two nodes as the average correlation of the two variables across strata where the other parents of the daughter node are held constant.[79,80] That is, the strength of an edge from A to B, where B also has edges leading to it from n other variables, for example C and D with n = 2, as the correlation between A and B when controlling for C and D. To estimate that, we repeated the following algorithm to calculate average correlations between A and B across strata of equal C and D:

- Sample a point (A_0, B_0, C_0, D_0)
- Find set of all points (A, B, C, D) such that $\sqrt{(C - C_0)^2 + (D - D_0)^2} < \varepsilon \cdot \sqrt{n}$ where n is the number of variables to control for (2 in this example) and ε is a predetermined tolerance level set at 0.5 (smaller tolerances did not yield significantly different measures)
- If the set contains more than 5 points, calculate Pearson's correlation coefficient $cor(A, B)$ across that set.
- Average the resulting correlation measure across $20 \cdot 2^n$ such samplings.

A similar stratification procedure was done to assess the conditional effect of a certain treatment on protein A when controlling for proteins B and C. The treatment and NonT groups were pooled together, a point was sampled at random and a set of all points with similar B and C was found, and the $\log_2$-fold difference between the mean levels of A in treated vs NonT synapses was calculated and averaged across many samplings.

QUANTIFICATION AND STATISTICAL ANALYSIS

Software

All statistical analysis was performed in R (versions 4.0.0-4.3.0), using the base, stats, umap (version 0.2.7.0), and bnlearn (version 4.7) packages.

Biological and technical repeats

Each experiment includes pooled neurons from 1-3 embryos of one pregnant rat, split into wells of a 96-well-plate. The wells constitute separately grown, treated and stained cultures. Each well is subsequently considered a technical repeat. All images in different wells from a single experiment are analyzed using the same CellProfiler pipeline for consistency in synapse determination and quantification. The main RNAi screen pools 4 experiments into one dataset by dividing all synaptic protein values of a certain experiment by the average values for untreated wells in that experiment.

Nature of repeats and values per repeat

For statistical analysis, relevant values are calculated for synapses in individual wells: (i) Average protein intensity integral over synaptic puncta. (ii) Total number of synapses or synapses conforming to a certain condition, (iii) Fraction of synapses conforming to a certain condition, (iv) Total area of identified dendrites, (v) Average protein intensity over whole image, (vi) Average protein intensity integral over soma, (vii) Network edge strengths (i.e., controlled correlation between nodes). By-well values are then used for calculating standard error and significance testing. Significance testing was done using a two-tailed Student's t test. The number of wells

per treatment group is 11-18 for the main RNAi screen and 4-7 for each validation experiment. All heatmaps and bar graphs present average values across wells, all error bars are standard error of mean across wells.

Data exclusion

The following subsets of images from the main RNAi screen were excluded from subsequent analysis:

- One row in plate #1, for which one of the imaging rounds was out of focus.
- All images of NR2A, for which staining was very diffuse and very few puncta could be identified.
- All images of cultures treated with siRNA against Xpo7, which exhibited highly irregular staining patterns for Homer1 that could not be reproduced with other batches of that siRNA. We attributed the effect to an issue with the specific siRNA batch used.

UMAP and clustering

Uniform Manifold Approximation and Projection (UMAP) was performed using the umap R package (version 0.2.7.0). A combined sample was used with 200 points randomly sampled from each well. Each variable was scaled to a standard deviation of 1, and UMAP was applied with min_dist=0. Density-based clustering on the 2D layout was done automatically using HDBSCAN[94,95] with parameters that yielded 14 clusters, after which small clusters that accounted for <1% of all points were manually merged into the nearest (by centroid distance) large cluster, resulting in the 9 main clusters shown in Figure 3.

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