Multiple signaling pathways are essential for synapse formation induced by synaptic adhesion molecules

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Little is known about the cellular signals that organize synapse formation. To explore what signaling pathways may be involved, we employed heterologous synapse formation assays in which a synaptic adhesion molecule expressed in a nonneuronal cell induces pre- or postsynaptic specializations in cocultured neurons. We found that interfering pharmacologically with microtubules or actin filaments impaired heterologous synapse formation, whereas blocking protein synthesis had no effect. Unexpectedly, pharmacological inhibition of c-jun N-terminal kinases (JNKs), protein kinase-A (PKA), or AKT kinases also suppressed heterologous synapse formation, while inhibition of other tested signaling pathways—such as MAP kinases or protein kinase C—did not alter heterologous synapse formation. JNK and PKA inhibitors suppressed formation of both pre- and postsynaptic specializations, whereas AKT inhibitors impaired formation of post- but not presynaptic specializations. To independently test whether heterologous synapse formation depends on AKT signaling, we targeted PTEN, an enzyme that hydrolyzes phosphatidylinositol 3-phosphate and thereby prevents AKT kinase activation, to postsynaptic sites by fusing PTEN to Homer1. Targeting PTEN to postsynaptic specializations impaired heterologous postsynaptic synapse formation induced by presynaptic adhesion molecules, such as neurexins and additionally decreased excitatory synapse function in cultured neurons. Taken together, our results suggest that heterologous synapse formation is driven via a multifaceted and multistage kinase network, with diverse signals organizing pre- and postsynaptic specializations.

synapse formation | adhesion molecules | Pten | signal transduction | c-jun N-terminal kinase

Synapse formation is the universal process that underlies construction of all of the brain's circuits, but little is known about its mechanisms. Unknown signaling pathways presumably organize synapses, but what pathways are involved remains unclear. Synapse formation likely requires interactions between pre- and postsynaptic neurons via adhesion molecules that transmit bidirectional signals to pre- and postsynaptic neurons and organize pre- and postsynaptic specializations (reviewed in refs. 1–3). Synapses exhibit canonical features that include a presynaptic side that releases neurotransmitters rapidly and transiently and a postsynaptic side that recognizes these neurotransmitters. Interestingly, only the presynaptic side of a synapse harbors canonical features that are shared by all synapses, such as synaptic vesicles and active zones with the same components in excitatory and inhibitory synapses. In contrast, the postsynaptic sides differ dramatically between excitatory and inhibitory synapses. Even excitatory and inhibitory neurotransmitter receptors exhibit no homology, and few if any molecular components are shared among excitatory and inhibitory postsynaptic specializations.

At present, it is unknown what intracellular signaling pathways are involved in the assembly of pre- and postsynaptic specializations, whether different types of signaling pathways exist for pre- vs. postsynaptic specializations, and how excitatory vs. inhibitory synapses are organized. In the present study, we chose the heterologous synapse formation assay as an approach in order to begin to address these fundamental questions (4). In the heterologous synapse formation assay, nonneuronal cells, such as HEK293T cells, express a synaptic adhesion molecule that then induces pre- or postsynaptic specializations when these nonneuronal cells are cocultured with neurons (5–9). For example, if a postsynaptic adhesion molecule, such as neuroligin-1 (Nlgn1) or latrophilin-3, is expressed in HEK293T cells, and the HEK293T cells are cocultured with neurons, these neurons form presynaptic specializations on the HEK293T cells (5, 10). If, conversely, a presynaptic adhesion molecule, such as a neurexin or teneurin, is expressed in HEK293T cells, postsynaptic specializations are induced in cocultured neurons (8, 9, 11).

Many adhesion molecules have been shown to induce heterologous synapse formation, including neurexins, neuroligins, latrophilins, teneurins, SynCAMs, neuronal pentraxin receptors, SALMs, LAR-type PTPRs, and others (5, 6, 8–15), suggesting that there are common "synapse signaling" pathways and that the heterologous synapse formation assay nonspecifically transduces different adhesion molecules signals into a response that organizes pre- or postsynaptic specializations. Even engagement of neuronal AMPA-type glutamate receptors by the neuronal pentraxin receptor, when expressed in HEK293T cells, causes organization of postsynaptic specializations in the heterologous synapse formation assay, testifying to the broad nature of the signals that mediate heterologous synapse formation (12). Strikingly, any given adhesion molecule triggers only either pre- or

Significance

Formation of synapses is thought to be mediated by transsynaptic adhesion molecules, but the intracellular signaling pathways involved are largely unknown. Here we studied synapse formation using as an approach heterologous synapse formation assays, in which synaptic adhesion molecules expressed in a nonneuronal cell induce formation of pre- or postsynaptic specializations in cocultured neurons. Aided by pharmacological inhibitors and genetic tools, we found that c-jun N-terminal kinases (JNK) and protein kinase-A signaling contributes to the formation of both pre- and postsynaptic specializations during heterologous synapse formation, whereas the PI3 kinase/AKT signaling pathway is required only for formation of post- but not of presynaptic specializations. Our results suggest that a multistage kinase network is essential for heterologous synapse formation.

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Competing interest statement: T.C.S. and T.B. are coauthors together with 71 other principal investigators on a summary article (Koopmans F, et al. [2019] SynGO: An Evidence-Based, Expert-Curated Knowledge Base for the Synapse. Neuron 103: 217-234) that discusses functional assignments of synaptic proteins.

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postsynaptic specializations, but not both, indicating signaling specificity. Most adhesion molecules—with the exception of teneurin splice variants (11)—induce both excitatory and inhibitory synaptic specializations at the same time. Heterologous synapses resemble real synapses and are functional (6, 7). Overall, these observations suggest that specific signaling pathways regulate synapse formation and that the heterologous synapse formation assay provides a plausible and practical paradigm to dissect such signaling pathways, even though it represents an artificial system that lacks much of the specificity of physiological synapse formation.

In the present study, we have employed pharmacological inhibitors and molecular interventions to probe the nature of the signals mediating heterologous synapse formation. Our data reveal that multiple parallel protein kinase signaling pathways are required for heterologous synapse formation. We identified a role for both JNK and PKA signaling in the formation of pre- and postsynaptic specializations and found that the PI3 kinase pathway is specifically required for the formation of post- but not presynaptic specializations. Thus, our data provide initial insight into the signaling mechanisms underlying heterologous synapse formation that may be relevant for synapse formation in general.

Results

Heterologous Synapses Are Formed from Presynthesized Components by a Mechanism Involving the Cytoskeleton. In the heterologous synapse formation assay, HEK293T cells expressing a particular adhesion molecule are cocultured with cortical neurons cultured

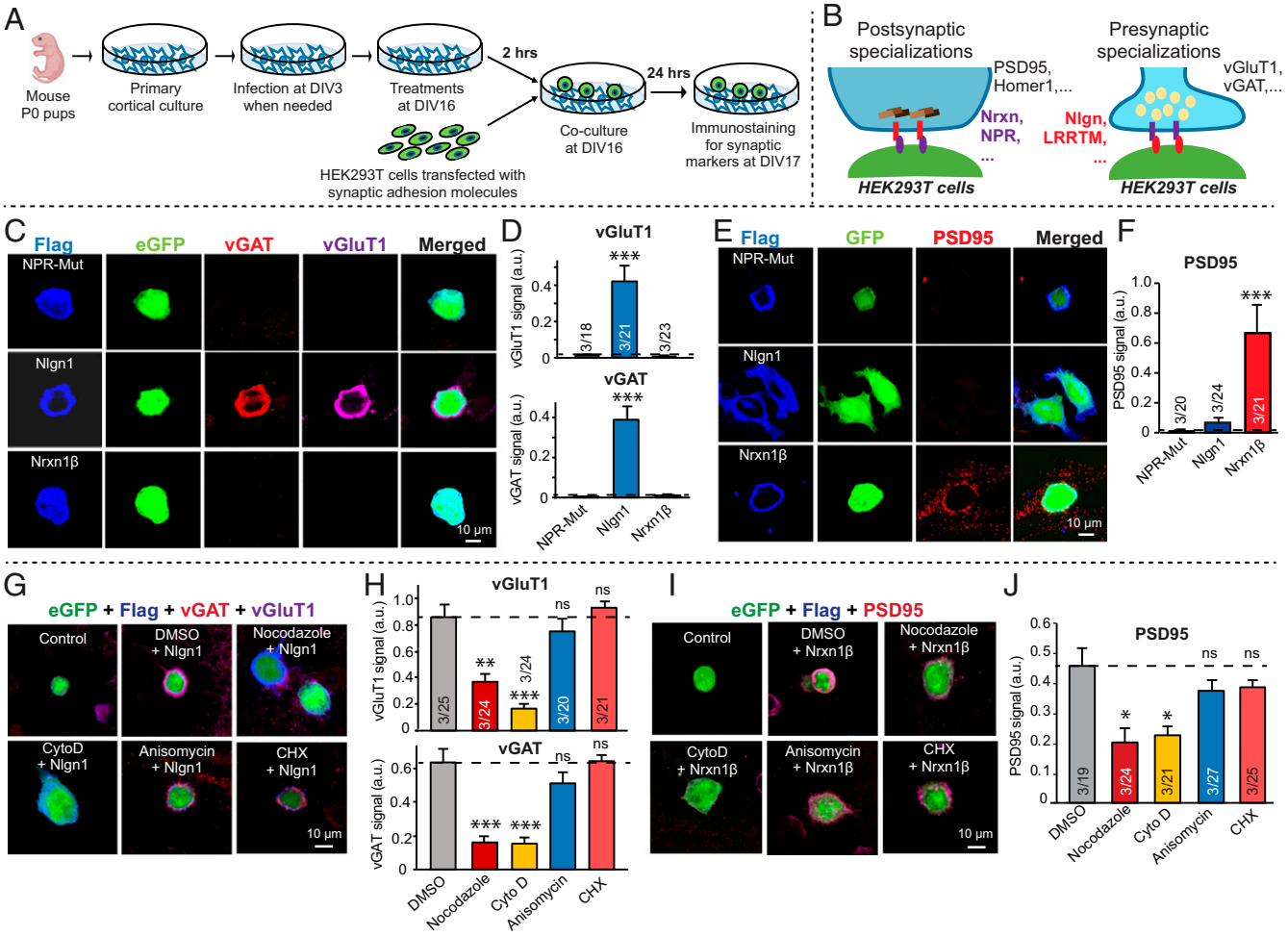

Fig. 1. Inhibition of microtubule and microfilament assembly, but not of protein synthesis, blocks induction of presynaptic specializations by neuroligin-1 (Nlgn1) or of postsynaptic specializations by neurexin-1β (Nrxn1β) during heterologous synapse formation assays. (*A* and *B*) Experimental design of heterologous synapse formation assays in which pre- or postsynaptic adhesion molecules expressed in HEK293T cells selectively induce post- or presynaptic specializations, respectively, in cocultured neurons (*A*), and schematic of the resulting heterologous synapses (*B*). (*C–F*) Nlgn1 specifically induces pre- but not postsynaptic specializations in heterologous synapse formation assays, whereas Nrxn1β induces post- but not presynaptic specializations. In contrast, an inactive mutant adhesion molecule (NPR-Mut, for neuronal pentraxin receptor mutant) does neither (*C* and *E*, representative images; *D* and *F*, quantifications of pre- and postsynaptic specializations, respectively). Presynaptic specializations were labeled by immunostaining for vGluT1 (purple; specific for excitatory synapses) and vGAT (red; specific for inhibitory synapses). Transfected cells were identified by imaging coexpressed eGFP, and adhesion molecules were visualized by labeling for the FLAG epitope included in the Nlgn1, Nrxn1β, and NPR-Mut constructs. (*G–J*) Inhibitors of microtubule (nocodazole, 1 μg/mL) or microfilament assembly (cytochalasin D; 2 μM) block formation of pre- (*G* and *H*) and postsynaptic specializations (*I* and *J*) in heterologous synapse formation assays, whereas inhibitors of protein synthesis (anisomycin, 25 μg/mL; cycloheximide, 50 μM) have no effect (*G* and *I*, representative images; *H* and *J*, quantifications of pre- and postsynaptic specializations, respectively). Mouse cortical neurons were treated with the indicated chemicals for 2 h before and continuing during the 24-h coculture with HEK293T cells expressing Nlgn1 (*G* and *H*) or Nrxn1β (*I* and *J*). Controls expressing only eGFP were not quantified since no heterologous synapse formation was detectable. Representative images show merged views; for individual staining patterns, see *SI Appendix*, Fig. S1. All numerical data are means ± SEM. Numbers in bars list the number of independent experiments/cells analyzed. Statistical significance was examined by one-way ANOVA comparing individual test conditions to control (ns = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

from newborn mice at DIV16 for 24 h (Fig. 1*A*). Then pre- or postsynaptic specializations are visualized on the surface of the HEK293T cells by immunocytochemistry for pre- or postsynaptic markers, such as vGluT1 and vGAT for presynaptic or PSD95 and Homer1 for postsynaptic specializations (Fig. 1 *B*–*F*). Heterologous synapse formation exhibits specificity in that presynaptic adhesion molecules, such as neurexins or the neuronal pentraxin receptor (NPR), induce only postsynaptic specializations, whereas postsynaptic adhesion molecules, such as neuroligins or LRRTMs, induce only presynaptic specializations (Fig. 1 *B*–*F* and *SI Appendix*, Fig. S1 *A* and *B*).

We first asked whether basic cell-biological features of neurons, such as gene transcription, protein synthesis, or the actin- and microtubule-based cytoskeleton, are required for heterologous synapse formation. Application of drugs inhibiting the actin- or microtubule-based cytoskeleton blocked most heterologous synapse formation (Fig. 1 *G*–*J* and *SI Appendix*, Fig. S1). In contrast, drugs inhibiting protein translation had no effect. These data show that consistent with its relatively fast time course, heterologous synapse formation involves assembly of presynthesized components into synaptic specializations via a mechanism involving the cytoskeleton.

Multiple Kinases Are Required for the Induction of Both Pre- and Postsynaptic Specializations during Heterologous Synapse Formation. To explore potential intracellular signaling pathways that regulate synapse formation, we tested whether the nonspecific kinase inhibitors staurosporine and genistein (*SI Appendix*, Table S1) interfere with heterologous synapse formation. Both significantly decreased the development of presynaptic specializations during heterologous synapse formation, as measured by the accumulation of vGluT1 and vGAT signals on the surface of the HEK293T cells expressing Nlgn1 (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S2*A*). This result prompted us to examine specific major kinase pathways, such as the MAP kinase, PKA, PI3 kinase, and PKC pathways.

The JNK inhibitor SP6000125, the PKA inhibitors H89 and PKI (16), and the DLK inhibitor GNE-3511 (17) significantly inhibited Nlgn1-induced presynaptic specializations (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S2*A*). Other kinase inhibitors, however, including inhibitors of PKC, MLCK, JAK2, p56, ERK MAP kinases, and MEK, did not show a significant effect on Nlgn1-induced

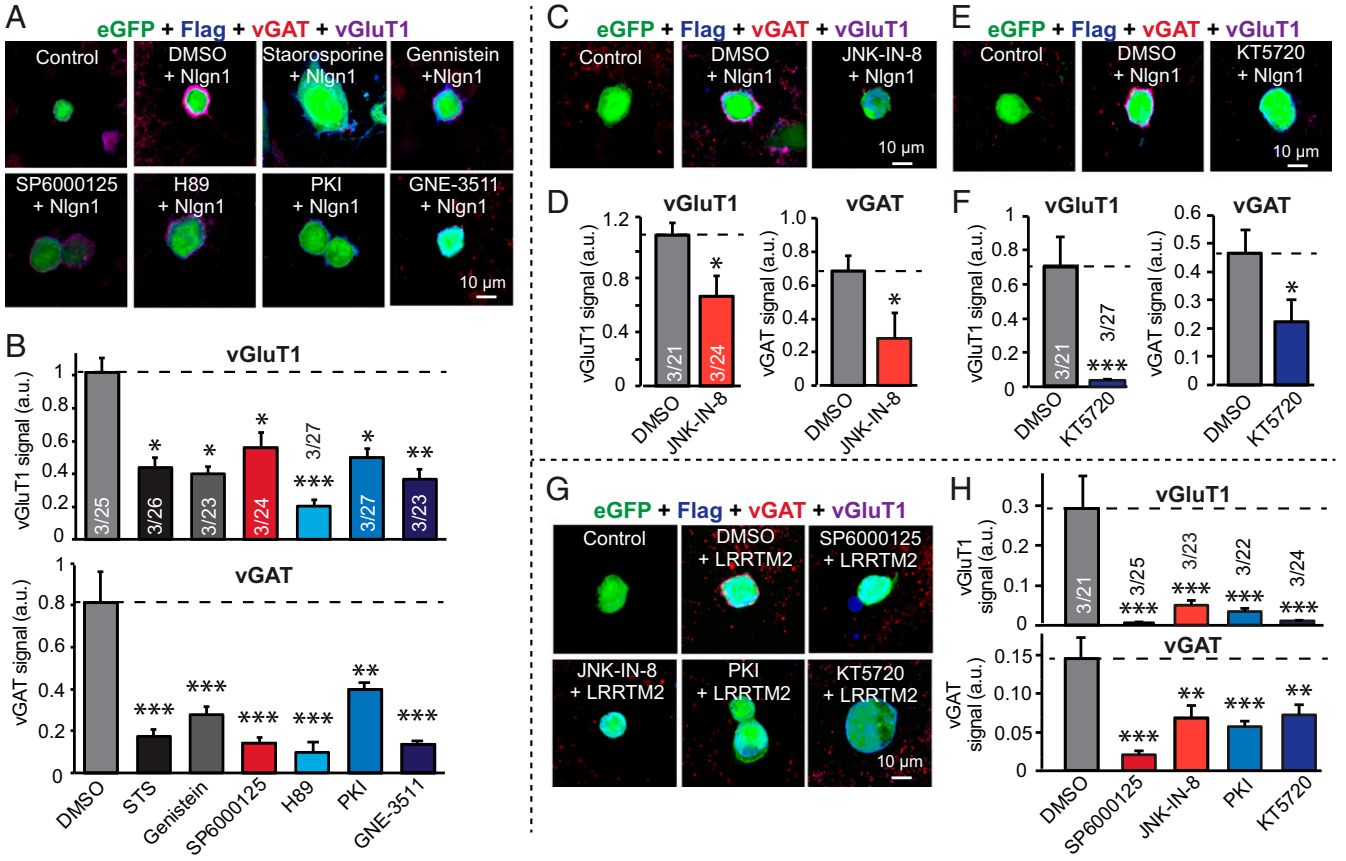

Fig. 2. Protein kinase inhibitors targeting the DLK→JNK or the PKA pathway impair formation of presynaptic specializations induced by Nlgn1 or LRRTM2 during heterologous synapse formation. In all experiments, cortical mouse neurons were treated with the indicated chemicals starting 2 h before and continuing during the 24-h coculture with HEK293T cells expressing Nlgn1 or LRRTM2. Representative images show merged views; for individual staining patterns, see *SI Appendix*, Fig. S2. (*A* and *B*) Two broad-spectrum protein kinase inhibitors (staurosporine A [STS], 0.1 μM; genistein, 50 μg/mL), a DLK inhibitor (GNE-3511 1 μM), a JNK inhibitor (SP6000125, 25 μM), and two PKA inhibitors (H89, 20 μM; PKI, 0.1 μM) impair Nlgn1-induced formation of presynaptic specializations during heterologous synapse formation assays (*A*, representative merged images; *B*, quantifications). (*C* and *D*) An independent JNK inhibitor (JNK-IN-8, 0.1 μM) also impairs Nlgn1-induced formation of postsynaptic specializations (*C*, representative merged images; *D*, quantifications). (*E* and *F*) An independent PKA inhibitor (KT5720, 0.1 μM) similarly impairs Nlgn1-induced formation of postsynaptic specializations induced by Nlgn1 (*E*, representative merged images; *F*, quantifications). (*G* and *H*) Two JNK inhibitors (SP6000125, 25 μM; JNK-IN-8, 0.1 μM) and two PKA inhibitors (KT5720, 0.1 μM; PKI, 0.1 μM) impair LRRTM2-induced formation of presynaptic specializations during heterologous synapse formation assays (*G*, representative merged images; *H*, quantifications). All numerical data are means ± SEM. Numbers in bars list the number of independent experiments/cells analyzed. Statistical significance was examined by one-way ANOVA comparing individual test conditions to control (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

presynaptic specializations (*SI Appendix*, Fig. S4*A*). In addition, inhibitors of proteasomes that often interfere with signaling pathways were also without significant effect on heterologous synapse formation (*SI Appendix*, Fig. S4*A*).

Since JNKs function downstream of DLK in a linear signaling pathway (18), these results suggest that the DLK→JNK and PKA signaling pathways are required for Nlgn1-induced heterologous synapse formation. We verified the potential roles of the JNK and PKA pathways in Nlgn1-induced presynaptic specializations by testing other JNK and PKA inhibitors. Again, we observed that the other JNK inhibitor JNK-IN-8 and PKA inhibitor KT5720 significantly blocked Nlgn1-induced presynaptic specialization (Fig. 2 *C*–*F* and *SI Appendix*, Fig. S2 *B* and *D*). Moreover, we observed a robust signal for JNK protein in Nlgn1-induced presynaptic specializations during heterologous synapse formation; this signal was decreased when heterologous synapse formation was inhibited with the JNK antagonist SP6000125 (*SI Appendix*, Fig. S2*C*).

Next, we investigated neurexin-induced postsynaptic specializations, using neurexin-1β (Nrxn1β) as the inducing agent. We found that the JNK inhibitor SP6000125 and JNK-IN-8, the PKA inhibitors PKI and KT5720, and the DLK inhibitor GNE-3511 also significantly suppressed the induction of postsynaptic specializations by Nrxn1β in heterologous synapse formation assays, as probed via the accumulation of PSD95 or of Homer1 signals on the surface of the HEK293T cells (Fig. 3 and *SI Appendix*, Fig. S3 *A*, *C*, and *D*). We detected during heterologous synapse formation a strong immunocytochemical signal for JNK protein in Nrxn1β-induced postsynaptic specializations; this signal was also decreased when heterologous synapse formation was inhibited with the JNK antagonist SP6000125 (*SI Appendix*, Fig. S3*B*). In contrast to JNK and PKA inhibitors, inhibitors for PKC, JAK2, p38, MEK, and proteasomes again did not block Nrxn1β-induced postsynaptic specializations (*SI Appendix*, Fig. S4*B*). Thus, both JNK and PKA signaling play important roles in the formation of both pre- and postsynaptic specializations.

Interestingly, the JNK inhibitor JNK-IN-8 only partly decreased the Nlgn1-induced formation of presynaptic specializations, but severely impaired the Nrxn1β-induced formation of postsynaptic specializations. In contrast, the PKA inhibitor KT5720 blocked formation of excitatory but not inhibitory presynaptic specializations completely (Figs. 2 and 3). The inhibition of synapse formation in the heterologous assay by inhibitors of the DLK→JNK or the PKA pathways was independent of the adhesion molecule used for synapse induction since presynaptic NPR or postsynaptic LRRTM had the same effect (Figs. 2 *G* and *H* and 3 *G* and *H*).

PI3 Kinase Inhibitors Specifically Inhibit Induction of Post- but Not of Presynaptic Specializations. In addition to inhibitors of PKA and DLK→JNK signaling pathways that impaired induction of both pre- and postsynaptic specializations, we observed that inhibitors

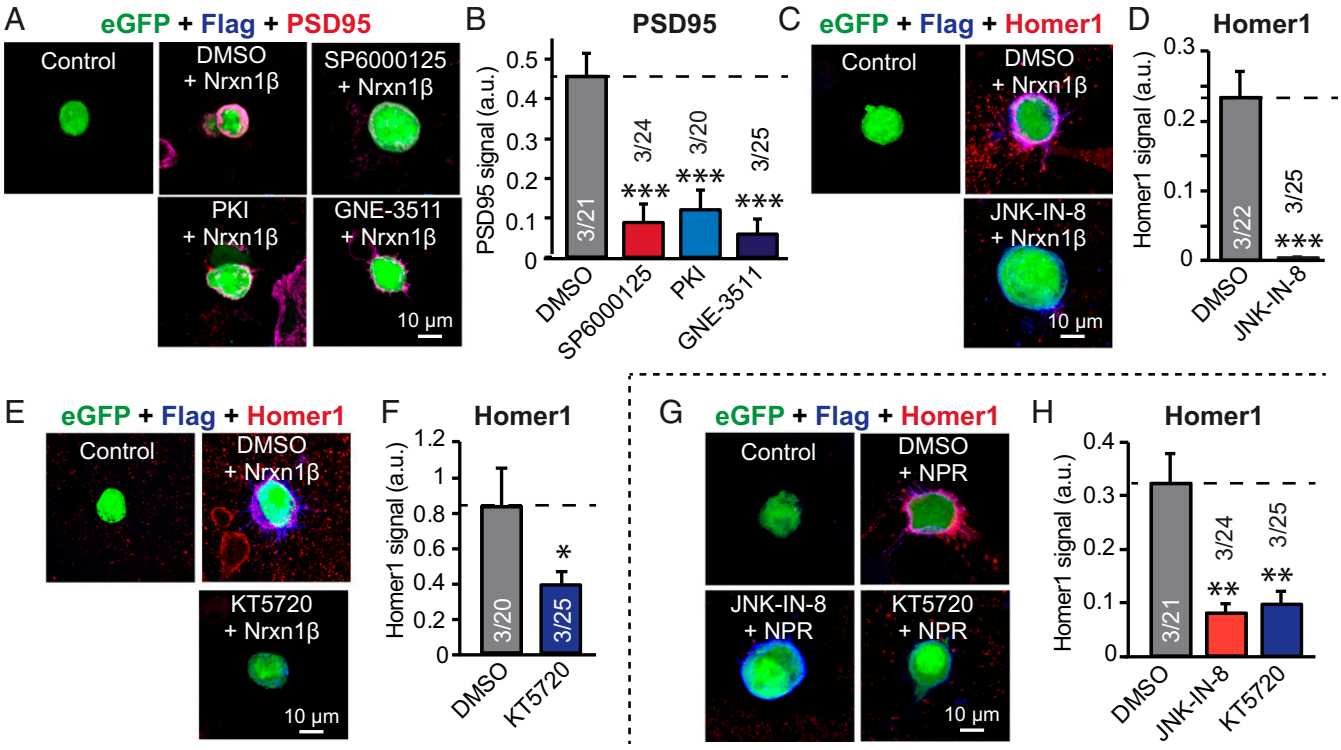

Fig. 3. Protein kinase inhibitors targeting the DLK→JNK or the PKA pathway also suppress formation of postsynaptic specializations induced by Nrxn1β or by the NPR during heterologous synapse formation assays. Cortical mouse neurons were treated with the indicated compounds for 2 h before and continuing during the 24-h coculture with HEK293T cells expressing Nrxn1β or NPR. Postsynaptic specializations were visualized by staining for PSD95 (*A* and *B*) or Homer1 (*C*–*H*), while transfected HEK293T cells were identified by coexpressed eGFP and by labeling for the FLAG epitope present on adhesion molecules. Representative images show merged views; for individual staining patterns, see *SI Appendix*, Fig. S3. (*A* and *B*) Inhibitors of DLK (GNE-3511 1 μM), JNK (SP6000125, 25 μM), or PKA (PKI, 0.1 μM) suppress formation of postsynaptic specializations induced by Nrxn1β in heterologous synapse formation assays (*A*, representative merged images; *B*, quantifications). (*C* and *D*) A second JNK inhibitor (JNK-IN-8, 0.1 μM) also impairs heterologous postsynaptic synapse formation induced by Nrxn1β (*C*, representative merged images; *D*, quantifications). (*E* and *F*) A second PKA inhibitor (KT5720, 0.1 μM) also impairs heterologous postsynaptic synapse formation induced by Nrxn1β (*E*, representative merged images; *F*, quantifications). (*G* and *H*) A JNK inhibitor (JNK-IN-8, 0.1 μM) and a PKA inhibitor (JNK-IN-8, 0.1 μM) similarly suppress heterologous postsynaptic synapse formation induced by NPR (*G*, representative merged images; *H*, quantifications). All numerical data are means ± SEM. Numbers in bars list the number of independent experiments/cells analyzed. Statistical significance was examined by one-way ANOVA comparing individual test conditions to control (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

of phosphatidylinositol-3 kinases (PI3 kinases) or of AKT protein kinase that is activated by the product of PI3 kinases, phosphatidylinositol-3-phosphate (PI3P), impaired induction of postsynaptic specializations by presynaptic adhesion molecules (Fig. 4 and *SI Appendix*, Fig. S5). Strikingly, the PI3 kinase and AKT inhibitors had no effect on the induction of presynaptic specializations by postsynaptic adhesion molecules. This observation was confirmed with multiple pharmacological agents, including the AKT inhibitors GSK2110183 and ARQ092 (19, 20) and the PI3 kinase inhibitors BEZ235 and GDC-0941 (21, 22), suggesting that the PI3 kinase pathway is specifically required for induction of post- but not presynaptic specializations (Fig. 4 and *SI Appendix*, Fig. S5). Immunocytochemistry showed that AKT localizes to postsynaptic specializations induced by HEK293T cells expressing Nrxn1β and that the AKT signal is decreased when formation of Nrxn1β-induced postsynaptic specializations is inhibited by the AKT antagonist GDC-0941 (*SI Appendix*, Fig. S5*G*), suggesting recruitment of AKT to nascent postsynaptic sites. These results further indicated the importance of the PI3 kinase pathway in the postsynaptic specialization.

PTEN Targeted to Postsynaptic Locations Specifically Inhibits Induction of Postsynaptic Specializations. Identification of signaling pathways by pharmacological inhibition is potentially limited owing to a lack of drug specificity. To independently test whether postsynaptic activation of AKT is essential for the formation of functional synapses, we targeted a PTEN to postsynaptic sites. PTEN is an enzyme that hydrolyzes PI3P to PI3, thereby preventing activation of AKT (23). Specifically, we fused PTEN to Homer1, using Homer1 alone as a negative control. The PTEN-Homer1 fusion protein (PTEN-Hr1) severely depressed Nrxn1β-induced formation of postsynaptic specializations but had no effect on Nlgn1-induced formation of presynaptic specializations (Fig. 4 *G* and *H* and *SI Appendix*, Fig. S5 *H* and *I*). Thus, PI3 kinases regulate formation of post- but not presynaptic specializations in the heterologous synapse formation assay.

In a final set of experiments, we asked whether the role of the PI3 kinase pathway extends to synapse formation between neurons. Since all of the signaling pathways implicated in synapse formation in our current experiments operate at many different subcellular locations in a neuron, we cannot test this question by simply incubating neurons with a pharmacological inhibitor. The PTEN-Hr1 tool, however, provides us with an approach to pursue this question. Thus, we infected cultured cortical neurons at DIV3 with lentiviruses expressing Homer1 alone or PTEN-tagged Homer1 (PTEN-Hr1) and measured the density of synapsin-, vGluT1-, or vGAT-positive synaptic puncta on dendrites at DIV16 (Fig. 5 *A–D*). In addition, we examined the soma size and dendrite length (*SI Appendix*, Fig. S5 *J* and *K*). We found that overexpression of the homer1-tagged PTEN significantly reduced the puncta size and density of vGluT1, but not those of synapsin or vGAT (Fig. 5 *A–D*). There was a small but significant decrease in the density of vGluT1-positive puncta that, however, was modest compared to the effect of the inhibition of the PI3 kinase pathway on heterologous synapse formation.

We next investigated the possibility that PTEN-Hr1 may have altered synaptic transmission, a plausible possibility given that deletion of many adhesion molecules, such as neuroligins, that are active in the heterologous synapse formation assay nevertheless does not produce a synapse loss in cultured neurons, but instead causes a change in synaptic transmission (24–26). Overexpression of PTEN-Hr1 caused a large decrease (~60%) in the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs), but not of miniature inhibitory postsynaptic currents (mIPSCs), with the analysis of the means between control and experimental groups, suggesting that it also impairs organization of excitatory synapses in synapses formed between neurons (Fig. 5 *E–J*).

Discussion

Significant progress has been made in the identification and characterization of adhesion molecules that may mediate synapse formation (reviewed in refs. 1–3). How synaptic adhesion molecules organize pre- and postsynaptic specializations, however, remains largely unknown. It seems likely that adhesion molecules activate transsynaptic signal transduction cascades in promoting synapse formation, but what signals are involved has not yet been explored. Here, we took advantage of the heterologous synapse formation assay (4) to ask whether any of the canonical signal transduction pathways that govern cellular functions are required for the organization of synapses. In examining this question, we used both pharmacological inhibitors that affect an entire neuron and a genetically encoded tool that disrupts signaling specifically at postsynaptic localizations in a neuron. Our results suggest that at least two canonical protein kinase pathways, the PKA and the DLK→JNK pathway, are required for the formation of pre- as well as postsynaptic specializations during heterologous synapse formation, whereas a third canonical protein kinase pathway, the PI3 kinase→AKT pathway, is selectively required for assembly of post- but not presynaptic specializations. Since these signaling pathways interconnect and engage in cross-talk with each other at multiple levels, they may converge at certain points to mediate the synaptogenic processes initiated by various adhesion molecules. We validated these results using multiple synaptic adhesion molecules during heterologous synapse formation to ensure that our observations do not selectively apply to only one set of adhesion interactions and used a panoply of pharmacological agents in addition to the genetically encoded signaling disrupters to confirm specificity. Moreover, we observed recruitment of JNK kinase to nascent pre- and postsynaptic sites and the recruitment of AKT to nascent postsynaptic sites in the heterologous synapse formation paradigm. Notably, other canonical protein kinase pathways tested, such as the PKC and the ERK MAP kinase pathways, were not required for heterologous synapse formation. Viewed together, our results suggest that synapse formation as visualized by the generation of pre- or postsynaptic specialization during heterologous synapse formation assays involves multiple canonical signaling pathways that are activated by synaptic adhesion molecules.

The heterologous synapse formation assay produces functional hemisynapses at least on the presynaptic side (6). These hemisynapses that are generated in the assay are remarkably similar to synapses observed in vivo, both in terms of their ultrastructure and in terms of their protein composition (5–9). Nevertheless, the heterologous synapse formation assay has notable limitations. The fact that nearly all synaptic adhesion molecules induce pre- or postsynaptic specializations in this assay, even though most of them are not required for synapse formation in vivo but are involved in organizing specific properties of synapses (1), suggests that any transsynaptic signal activated by a synaptic adhesion molecule can trigger a synapse organization reaction. As a result, the requirement for PKA, JNK, and AKT signaling in heterologous synapse formation should not be interpreted as evidence for a necessary role of these kinases in the initial formation of synapses, but rather as an indication that these kinases are involved in one of the many processes that likely generate synapses and endow synapses with specific properties. In support of this conclusion, we found that blocking postsynaptic AKT signaling in neurons using the PTEN-Homer1 fusion protein only modestly decreased vGlut1 puncta density, but suppressed spontaneous excitatory synaptic events much more effectively (Fig. 5). This result suggests that in a "real" synapse, AKT signaling is primarily required for organizing the components mediating synaptic transmission and not necessarily for establishing a synaptic junction.

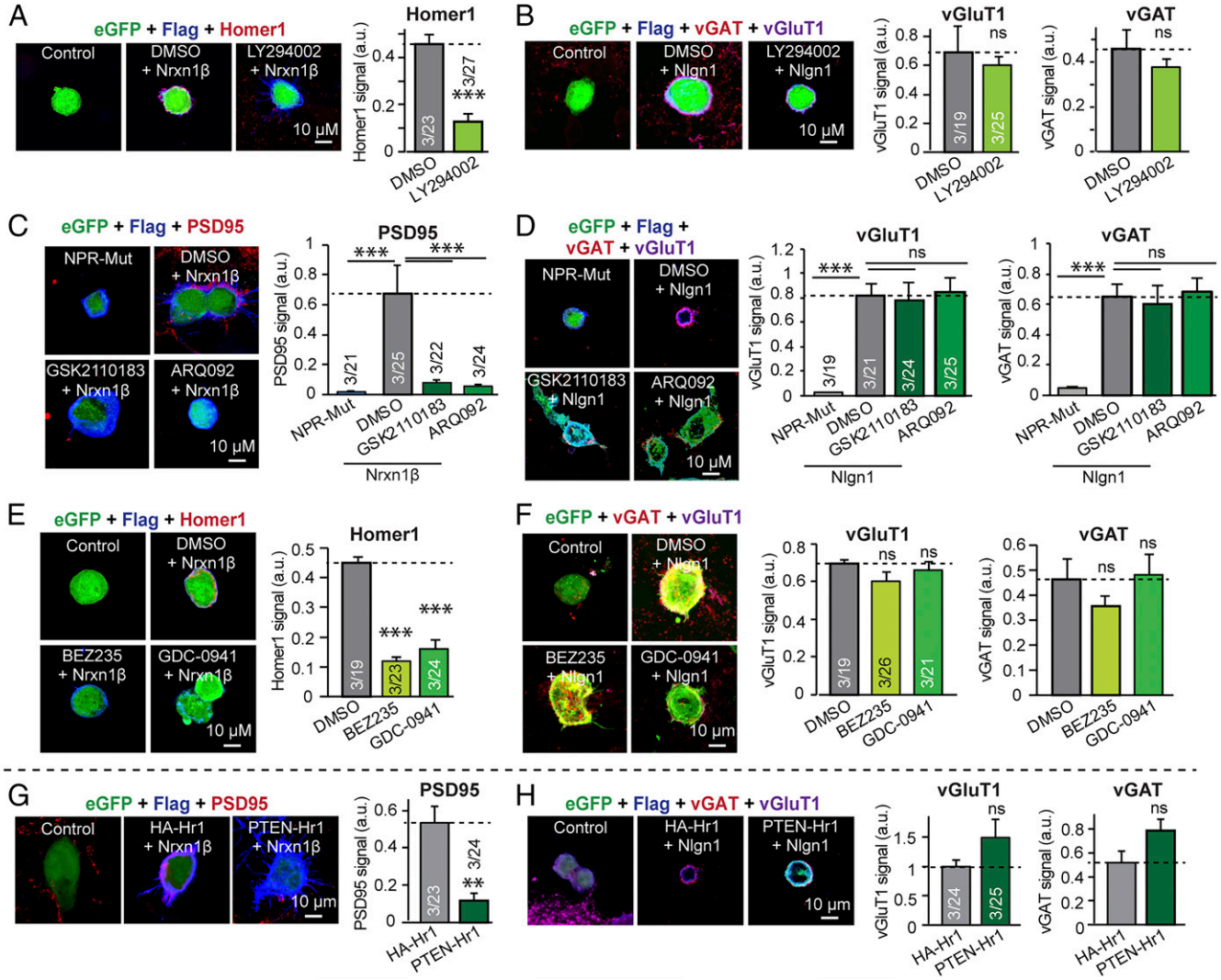

Fig. 4. Inhibition of the PI3 kinase → AKT pathway severely impairs formation of Nrxn1β-induced postsynaptic specializations, but not of Nlgn1-induced presynaptic specializations. Pre- and postsynaptic specializations that are formed on transfected HEK293T cells (green owing to coexpressed eGFP, with synaptic FLAG-tagged Nrxn1β or Nlgn1 shown in blue) were visualized by staining for the indicated markers. All representative images show merged views; for individual staining patterns, see *SI Appendix*, Fig. S5. (*A*) The PI3 kinase inhibitor LY294002 (50 μM) severely impaired Nrxn1β-induced postsynaptic specializations as visualized by Homer1 staining (*Left*, representative merged images; *Right*, quantifications). In this and the experiments shown in *B*–*F*, cortical mouse neurons were treated with the indicated chemicals at DIV16 for 2 h before and for 24 h during coculture with HEK293T cells expressing Nlgn1, Nrxn1β, or NPR-Mut (as a further negative control). (*B*) LY294002 had no effect on Nlgn1-induced presynaptic specializations imaged by staining for vGluT1 and vGAT. (*C*) Inhibitors of PI3P-activated AKT kinase (GSK2110183, 10 nM; ARQ092, 50 nM) suppressed Nrxn1β-induced postsynaptic specializations visualized by staining for PSD95. NPR-Mut was included as an additional negative control. (*D*) GSK2110183 and ARQ092 had no effect on Nlgn1-induced presynaptic specializations examined by staining for vGluT1 and vGAT. (*E*) The PI3 kinase inhibitors BEZ235 (0.1 μM) and GDC-0941 (0.1 μM) decreased Nrxn1β-induced postsynaptic specialization. (*F*) The PI3 kinase inhibitors BEZ235 and GDC-0941 did not affect Nlgn1-induced presynaptic specialization. (*G*) Targeting PTEN that hydrolyzes PI3P and thereby blocks AKT activation to postsynaptic sites by fusing PTEN to Homer1 (PTEN-Hr1) greatly impairs formation of Nrxn1β-induced postsynaptic specialization during heterologous synapse formation (*Left*, representative images; *Right*, quantifications). In this panel and in *H*, neurons were infected at DIV3 with lentiviruses expressing a PTEN-HA-Homer1 fusion protein (PTEN-Hr1) or HA-Homer1 (HA-Hr1, as a control), cocultured with transfected HEK293T cells at DIV16, and analyzed at DIV17. (*H*) Pten-Homer1 did not affect Nlgn1-induced presynaptic specializations visualized using vGluT1 and vGAT staining. All numerical data are means ± SEM. Numbers in bars list the number of independent experiments/cells analyzed. Statistical significance was examined by one-way ANOVA comparing individual test conditions to control (ns = $P > 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

How might the various protein kinases act in organizing pre- and/or postsynaptic specializations? As canonical intracellular signaling pathways, PKA-, JNK-, and AKT-mediated signals obviously contribute to many cellular processes. Since our data show that the microtubule- and actin-based cytoskeleton is critical for heterologous synapse formation (Fig. 1), it is possible that these signaling pathways contribute to synapse formation by regulating the cytoskeleton. Moreover, the DLK→JNK pathway was previously shown to function in synapse formation in classical studies in *Caenorhabditis elegans* and *Drosophila* (27–30). In these organisms, JNK acts by phosphorylating RPM1 (a.k.a. highwire), a large scaffolding protein that functions as a ubiquitin E3-ligase (31, 32). However, it is unclear whether RPM1 functions in synapse formation via its E3-ligase activity or another molecular process (33, 34). Indeed, we found that inhibitors of proteasomes do not interfere with heterologous synapse formation (*SI Appendix*, Fig. S4), indicating that heterologous synapse formation is not mediated by an

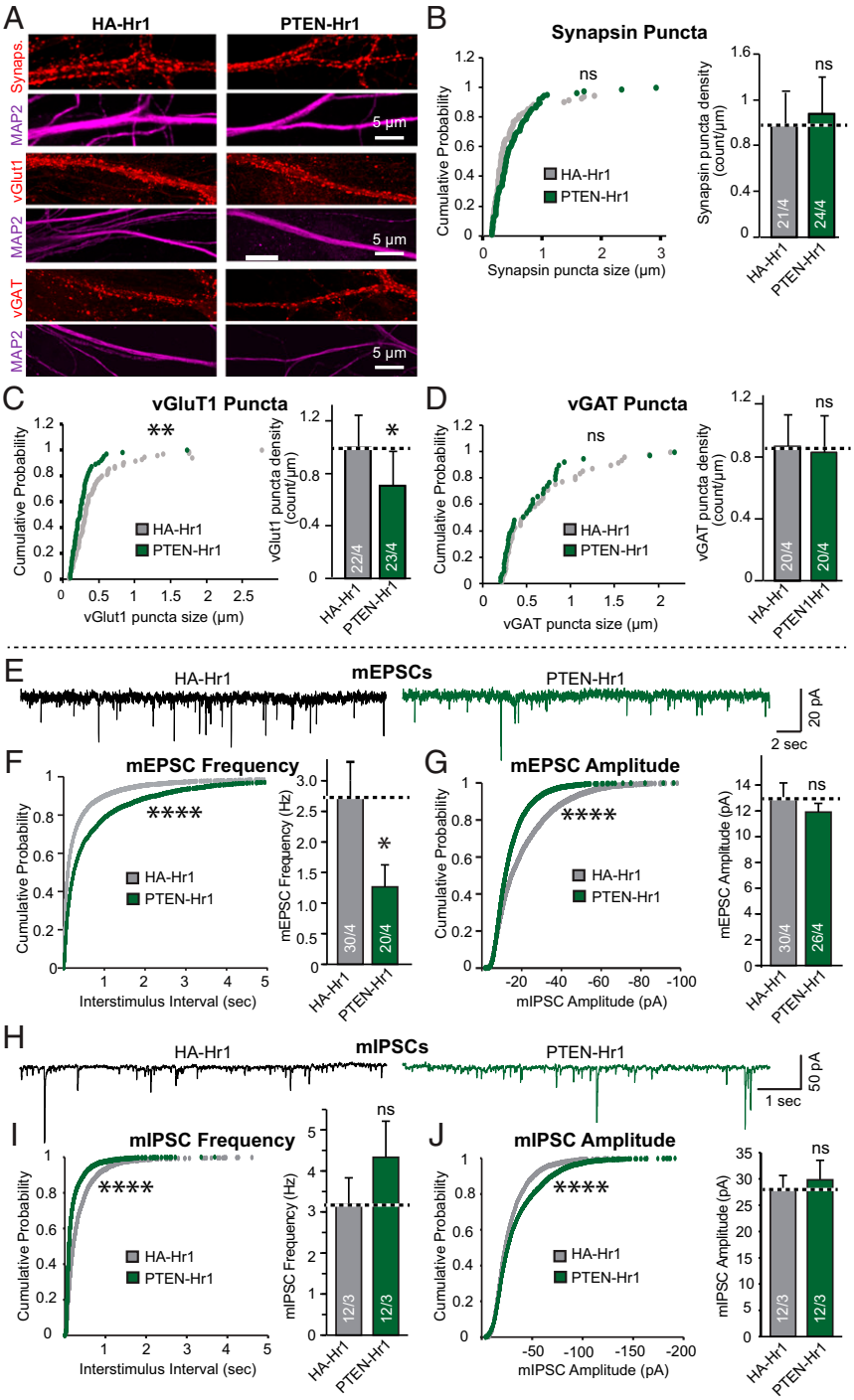

Fig. 5. Lowering postsynaptic PI3P levels by expression of a PTEN-Homer1 fusion protein selectively suppresses excitatory synaptic transmission in cultured cortical mouse neurons. Cortical neurons cultured from newborn mice were infected at DIV3 with lentiviruses expressing eGFP alone or together with PTEN-Hr1 and analyzed at DIV16. (*A–D*) Inhibition of local AKT signaling in postsynaptic specializations by expression of PTEN-Hr1 produced only minor changes in the density of excitatory or inhibitory synapses (*A*, representative images of neuronal dendrites immunolabeled for MAP2 [purple] and synapsins, vGluT1, or vGAT [all red] as indicated; *B–D*, cumulative distribution plots and summary graphs of the puncta density per µm dendrite as determined by synapsin [*B*], vGluT1 [C], or vGAT staining [*D*]; for other parameters, see *SI Appendix*, Fig. S5). (*E–J*) Postsynaptic PTEN diminished the mEPSC frequency (*E–G*) but not the mIPSC frequency (*H–J*). Representative mEPSC (*E*) and mIPSC (*H*) traces, quantifications of mEPSC (*F*) and mIPSC (*I*) frequencies, and quantifications of mEPSC (*G*) and mIPSC (*J*) amplitudes are shown. All numerical data are means ± SEM. Numbers in bars list the number of independent experiments/coverslips analyzed. Statistical significance was examined by one-way ANOVA (bar graphs) or the Kolmogorov–Smirnov test (cumulative distributions), with ns = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, **** = $P < 0.0001$.

ubiquitin-dependent protein degradation pathway. In addition, JNK has been implicated in the postsynaptic clustering of PSD95 (35).

PI3P signaling is primarily known for its role in regulating mitosis, cell growth, and cellular differentiation (36), but not in organizing synapses. PI3 kinases were implicated in controlling

synaptic plasticity by a protein synthesis-dependent process (37, 38). However, heterologous synapse formation is independent of protein synthesis (Fig. 1), and the number of kinases that have been implicated in synaptic plasticity include not only AKT and PKA, but also PKC (39) and ERK-type MAP kinases (40) that do not appear to participate in heterologous synapse formation (*SI Appendix*, Fig. S4). Thus, heterologous synapse formation appears to be mediated by a narrower range of signaling pathways than synaptic plasticity, raising the possibility that there are a few canonical synapse organization pathways as opposed to multifarious synaptic plasticity pathways. The biggest question really is whether the conclusions made here apply to real synapses—after all, the adhesion molecules used to induce heterologous synapses are not actually essential for formation of "real" synapses.

Taken together, our results provide an initial characterization of the signaling pathways involved in the assembly of synapses, thus offering an entry point for future mechanistic studies on how synapses are formed. We identified the canonical JNK, PKA, and AKT protein kinase pathways as essential components of the machinery that enables assembly of pre- and postsynaptic specializations during heterologous synapse formation. Remarkably, JNK and PKA signaling were required on both the pre- and postsynaptic sides, whereas AKT signaling was only required on the postsynaptic side.

Materials and Methods

Chemicals and pharmacological agents, plasmids, and antibodies are described in *SI Appendix, SI Materials and Methods*. Also see *SI Appendix, SI Materials and Methods* for details for primary cultures from mouse cortex, heterologous synapse formation assays, immunocytochemistry of neurons, sparse transfections of neurons, production of lentiviruses, and electrophysiology experiments.

Data Availability. All study data are included in the article and *SI Appendix*.

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1. T. C. Südhof, Towards an understanding of synapse formation. *Neuron* **100**, 276–293 (2018).
2. J. R. Sanes, M. Yamagata, Many paths to synaptic specificity. *Annu. Rev. Cell Dev. Biol.* **25**, 161–195 (2009).
3. Z. Nusser, Creating diverse synapses from the same molecules. *Curr. Opin. Neurobiol.* **51**, 8–15 (2018).
4. T. Biederer, P. Scheiffele, Mixed-culture assays for analyzing neuronal synapse formation. *Nat. Protoc.* **2**, 670–676 (2007).
5. P. Scheiffele, J. Fan, J. Choih, R. Fetter, T. Serafini, Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* **101**, 657–669 (2000).
6. T. Biederer *et al*., SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* **297**, 1525–1531 (2002).
7. A. A. Chubykin *et al*., Dissection of synapse induction by neuroligins: Effect of a neuroligin mutation associated with autism. *J. Biol. Chem.* **280**, 22365–22374 (2005).
8. E. R. Graf, X. Zhang, S. X. Jin, M. W. Linhoff, A. M. Craig, Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* **119**, 1013–1026 (2004).
9. C. I. Nam, L. Chen, Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 6137–6142 (2005).
10. R. Sando, X. Jiang, T. C. Südhof, Latrophilin GPCRs direct synapse specificity by coincident binding of FLRTs and teneurins. *Science* **363**, eaav7969 (2019).
11. J. Li *et al*., Structural basis for teneurin function in circuit-wiring: A toxin motif at the synapse. *Cell* **173**, 735–748.e15 (2018).
12. S. J. Lee *et al*., Presynaptic neuronal pentraxin receptor organizes excitatory and inhibitory synapses. *J. Neurosci.* **37**, 1062–1080 (2017).
13. K. A. Han *et al*., PTPσ drives excitatory presynaptic assembly via various extracellular and intracellular mechanisms. *J. Neurosci.* **38**, 6700–6721 (2018).
14. W. Mah *et al*., Selected SALM (synaptic adhesion-like molecule) family proteins regulate synapse formation. *J. Neurosci.* **30**, 5559–5568 (2010).
15. H. Takahashi *et al*., Postsynaptic TrkC and presynaptic PTPσ function as a bidirectional excitatory synaptic organizing complex. *Neuron* **69**, 287–303 (2011).
16. N. A. Otmakhova, N. Otmakhov, L. H. Mortenson, J. E. Lisman, Inhibition of the cAMP pathway decreases early long-term potentiation at CA1 hippocampal synapses. *J. Neurosci.* **20**, 4446–4451 (2000).
17. S. Patel *et al*., Discovery of dual leucine zipper kinase (DLK, MAP3K12) inhibitors with activity in neurodegeneration models. *J. Med. Chem.* **58**, 401–418 (2015).
18. G. Fan, S. E. Merritt, M. Kortenjann, P. E. Shaw, L. B. Holzman, Dual leucine zipper-bearing kinase (DLK) activates p46SAPK and p38mapk but not ERK2. *J. Biol. Chem.* **271**, 24788–24793 (1996).
19. J. M. Hetman, S. H. Soderling, N. A. Glavas, J. A. Beavo, Cloning and characterization of PDE7B, a cAMP-specific phosphodiesterase. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 472–476 (2000).
20. C. Ranieri *et al*., In vitro efficacy of ARQ 092, an allosteric AKT inhibitor, on primary fibroblast cells derived from patients with PIK3CA-related overgrowth spectrum (PROS). *Neurogenetics* **19**, 77–91 (2018).
21. D. Kong, T. Yamori, Advances in development of phosphatidylinositol 3-kinase inhibitors. *Curr. Med. Chem.* **16**, 2839–2854 (2009).
22. D. Sarker *et al*., First-in-human phase I study of pictilisib (GDC-0941), a potent pan-class I phosphatidylinositol-3-kinase (PI3K) inhibitor, in patients with advanced solid tumors. *Clin. Cancer Res.* **21**, 77–86 (2015).
23. C. A. Worby, J. E. Dixon, PTEN. *Annu. Rev. Biochem.* **83**, 641–669 (2014).
24. F. Varoqueaux *et al*., Neuroligins determine synapse maturation and function. *Neuron* **51**, 741–754 (2006).
25. A. A. Chubykin *et al*., Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. *Neuron* **54**, 919–931 (2007).
26. S. Chanda, W. D. Hale, B. Zhang, M. Wernig, T. C. Südhof, Unique versus redundant functions of neuroligin genes in shaping excitatory and inhibitory synapse properties. *J. Neurosci.* **37**, 6816–6836 (2017).
27. C. A. Collins, Y. P. Wairkar, S. L. Johnson, A. DiAntonio, Highwire restrains synaptic growth by attenuating a MAP kinase signal. *Neuron* **51**, 57–69 (2006).
28. D. T. Byrd *et al*., UNC-16, a JNK-signaling scaffold protein, regulates vesicle transport in *C. elegans*. *Neuron* **32**, 787–800 (2001).
29. K. Nakata *et al*., Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. *Cell* **120**, 407–420 (2005).
30. O. Crawley *et al*., A MIG-15/JNK-1 MAP kinase cascade opposes RPM-1 signaling in synapse formation and learning. *PLoS Genet.* **13**, e1007095 (2017).
31. M. Zhen, X. Huang, B. Bamber, Y. Jin, Regulation of presynaptic terminal organization by *C. elegans* RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. *Neuron* **26**, 331–343 (2000).
32. H. I. Wan *et al*., Highwire regulates synaptic growth in *Drosophila*. *Neuron* **26**, 313–329 (2000).
33. A. Russo *et al*., The E3 ligase Highwire promotes synaptic transmission by targeting the NAD-synthesizing enzyme dNmnat. *EMBO Rep.* **20**, e46975 (2019).
34. B. Grill, R. K. Murphey, M. A. Borgen, The PHR proteins: Intracellular signaling hubs in neuronal development and axon degeneration. *Neural Dev.* **11**, 8 (2016).
35. G. G. Farías *et al*., Wnt-5a/JNK signaling promotes the clustering of PSD-95 in hippocampal neurons. *J. Biol. Chem.* **284**, 15857–15866 (2009).
36. D. A. Fruman *et al*., The PI3K pathway in human disease. *Cell* **170**, 605–635 (2017).
37. H. Y. Man *et al*., Activation of PI3-kinase is required for AMPA receptor insertion during LTP of mEPSCs in cultured hippocampal neurons. *Neuron* **38**, 611–624 (2003).
38. M. Cammalleri *et al*., Time-restricted role for dendritic activation of the mTOR-p70S6K pathway in the induction of late-phase long-term potentiation in the CA1. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 14368–14373 (2003).
39. T. C. Sacktor, J. W. Hell, The genetics of PKMζ and memory maintenance. *Sci. Signal.* **10**, eaao2327 (2017).
40. M. G. Giovannini, The role of the extracellular signal-regulated kinase pathway in memory encoding. *Rev. Neurosci.* **17**, 619–634 (2006).