Use of MAP2 in co-cultures
- exclude dendrites crossing the co-cultured HEK cells i.e. confirm absence of MAP2 in CFP positive regions
- reference point for the beginning of the z-stack
- ensure that CFP positive cells are within reasonable distance to a MAP2 positive, healthy looking dendrite

Mixed-culture assays for analyzing neuronal synapse formation

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The assembly of synapses in the vertebrate central nervous system requires bidirectional signaling across the synaptic cleft that directs the differentiation of pre- and postsynaptic membrane domains. Biochemical and genetic studies have identified several adhesion and signaling molecules that localize to synapses and might participate in organizing synaptic structures. Understanding how individual proteins contribute to synaptic organization is complicated by the fact that there are significant numbers of separate signals that cooperate in this process. This protocol describes an assay system that permits examination of synaptogenic activities of individual cell-surface proteins in isolation. Besides the time needed for preparation and growth of primary neuronal cultures (6–14 days), the execution and analysis of the assay is rapid, requiring approximately 2 days. Using this assay, recent studies revealed that single synaptic adhesion complexes can direct a remarkable degree of synaptic differentiation and provided new insights into the cell biological mechanisms of synaptogenesis.

INTRODUCTION

The formation of synaptic junctions in the central nervous system is thought to require *trans*-synaptic interactions mediated by adhesion molecules[1–4]. Although many synaptic adhesion complexes have been identified, determining their specific activity at synapses has been a challenge. The bidirectional nature of synaptic signaling and the presence of a multitude of *trans*-synaptic signals that might act in parallel make it complicated to separate direct effects from indirect effects on synapse assembly. These problems can be circumvented for soluble growth factors, where purified proteins can be added directly to cultured neurons and signaling responses examined[5,6]. However, this is not feasible for adhesion molecules that require membrane anchoring and lateral interactions for normal function.

The assay described here uses a mixed-culture system where primary neurons are combined with non-neuronal cells transfected with a cDNA encoding the synaptic adhesion molecule of interest (**Fig. 1**). Thereby, a single neuronal protein is presented in a non-neuronal plasma membrane, greatly reducing the complexity of the *trans*-synaptic signals present at the neuronal cell surface. When brought into contact with primary neuronal cells *in vitro*, responses to the presented molecule can be measured using biochemical, histochemical, imaging and electrophysiological approaches. In essence, this approach has been used in many previous studies to examine effects on neuronal growth, axon guidance, as well as synaptic differentiation. The more recent realization that single pre- or postsynaptic factors can induce a substantial degree of synaptic differentiation highlighted the use of this assay strategy for analyzing specific aspects of synapse assembly and function.

Using mixed-culture assays, several postsynaptic proteins that are sufficient to direct the assembly of functional presynaptic specializations have been identified. For example, the postsynaptic adhesion molecule neuroligin-1 is sufficient to induce in axons the formation of sites with presynaptic ultrastructure, active zone components and clusters of synaptic vesicles that undergo depolarization-dependent exocytosis[7]. Similar activities have been demonstrated for the immunoglobulin domain protein SynCAM 1 and Netrin-G ligand, a leucine-rich repeat protein[8,9]. Conversely, neurexin-1, a presynaptic receptor for neuroligin-1, has been shown to trigger the assembly of postsynaptic specializations containing neuroligin-1, the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor, as well as cytoplasmic scaffolding proteins of glutamatergic synapses[10]. The induction of presynaptic vesicle clustering and recruitment of postsynaptic NMDA receptors to synapses are phenocopied in experiments where neuroligin-1 is overexpressed in cultured hippocampal neurons[11–13]. However, only the mixed-culture assays demonstrate that the pre- and postsynaptic effects are directly induced by the neuroligin–neurexin complex, rather than being an indirect consequence of recruitment of other neuronal signaling proteins to synaptic membranes. Using a similar mixed-culture assay, Narp, a lectin secreted by neurons, has been demonstrated to drive the aggregation of alpha–amino–3–hydroxy–5–methyl–4–isoxazolepropionic acid (AMPA)-type glutamate receptors in dendrites[14]. In summary, these studies exemplify

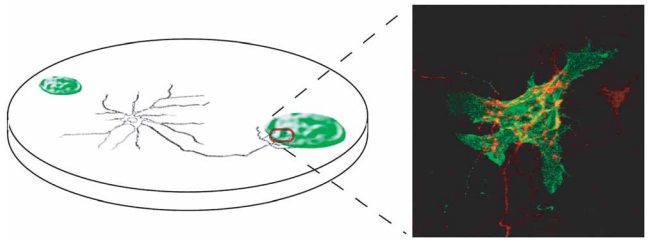

Figure 1 | Co-culture design. Left panel: Non-neuronal cells (green) expressing the surface protein to be analyzed are seeded over dissociated neurons (black) and co-cultured for 1–2 days before analysis. Sites of cell contact with neuronal axons (red box) are analyzed for the recruitment of presynaptic markers (see option 15A(ii)) and presence of recycling synaptic vesicles (see options 15B and 15C). Sites of contact with dendrites (not shown) can be examined for recruitment of postsynaptic markers (see option 15A(iii)). For simplicity, only one neuron is shown. Right panel: The inset depicts a representative immunofluorescence image of an HEK 293 cell expressing HA-tagged Neuroligin-1 in mixed culture (green, HA; red, synapsin as presynaptic marker).

BOX 1 | CHOICE OF HETEROLOGOUS CELLS AND EXPRESSION APPROACH

Choosing the right cell line. The HEK 293 cell line used in this protocol has been employed successfully by multiple laboratories. However, other cell types have also been utilized in this assay, notably primary astroglia, COS7 cells and PC12 cells. Astroglia provide a better growth substrate for the neuronal cultures, allowing to grow neurons at very low density, but astroglia are harder to transfect than HEK 293 cells. COS7 cells have a larger surface area than HEK 293 cells, but are not well suited for electrophysiological analysis, due to their flat and irregular morphology, which complicates voltage clamp control. PC12 cells endogenously express several neuronal proteins. This can be an advantage, for example, with respect to some neuron-specific carbohydrate modifications that would be lacking COS7 and HEK 293 cells. However, other neuronal proteins present in PC12 cells may interact with the transfected candidate protein or with neuronal receptors and thereby complicate the interpretation of results. In all cell line choices, it needs to be taken into account that many of the membrane proteins analyzed in mixed culture studies are glycosylated, which can affect their extracellular interactions and function. Therefore, it should be tested whether the post-translational modifications of proteins in heterologous cells are comparable to those in neurons.

Most cell lines other than HEK 293 need to be trypsinized before seeding in the mixed culture assay. Trypsinization raises two concerns: first, trypsin carryover into neuronal cultures can compromise neuronal cell health and second, trypsinization may cause proteolysis of the heterologously expressed protein and rapid re-expression of intact protein at cell surface needs to be confirmed.

Considerations for heterologous protein expression. For analysis, heterologous cells expressing the surface protein of interest have to be visualized. This can be achieved either through detection of an epitope tag on the cell-surface protein in fixed cells or by coexpression of a fluorescent protein from a bi-cistronic vectors. Surface expression of the proteins to be analyzed in non-neuronal cells is tested by confocal microscopy and confirmed in surface biotinylation experiments. If multiple surface proteins are compared side by side, their surface expression levels need to be equivalent. This can be ensured by directly tagging these proteins with the same epitope (e.g., HA tag or cyan fluorescent protein (CFP)). In all studies, a known synapse-inducing molecule, such as neuroligin-1 or SynCAM 1, and an adhesive but non-inductive molecule, such as N-cadherin, should be used as positive and negative controls, respectively. As additional negative control, HEK 293 expressing a mutant membrane protein lacking the extracellular domain or a soluble marker such as CFP can be used.

how bidirectional signaling at neuron–neuron contacts can be broken down into separate one-directional signals originating from a non-neuronal cell that organizes hemisynapses in a primary neuron.

In this protocol, we describe established procedures for performing and analyzing mixed-culture assays with dissociated hippocampal neurons and human embryonic kidney 293 (HEK 293) cells. In addition, we will discuss some important methodological considerations pertinent to developing related assays, for example, the choice of non-neuronal cell type, expression vector design, neuronal cell preparation, maturity of neuronal cultures and reagents for analysis (discussed in **Boxes 1** and **2**, and **Table 1**).

Although this assay represents a reductionist approach, it is extremely powerful for identifying new synaptogenic activities of adhesion molecules and can be applied to characterize almost any

BOX 2 | AN EYE ON NEURONS AND SYNAPSES

Developmental stage of culture, neuronal cell types, and culture substrates. The protocol describes analysis of synapse formation by dissociated hippocampal neurons starting at days 6–7 *in vitro*. During this early phase of synapse formation in culture, endogenous synapse-forming signals are still scarce, facilitating detection of presynaptic structures induced by exogenous signals. Induction of postsynaptic structures is more readily observed in more mature cultures (days 12–16 *in vitro*) when expression of postsynaptic glutamate receptors and scaffolding proteins increases.

Instead of dissociated hippocampal neurons, this protocol has been successfully adapted for dissociated cerebellar granule cells, cortical neurons, spinal cord neurons and explant cultures with tissue from pontine nuclei. The Matrigel substrate used in this protocol can be replaced by poly-D-lysine, laminin or any substrate that facilitates best neuronal cell health without causing a large degree of non-synaptic clustering of synaptic proteins.

Monitoring the quality of cultured neurons. The quality of the neuronal preparations needs to be regularly monitored. Criteria are proper neuronal differentiation *in vitro*, normal morphology of neuronal processes, punctate distribution of pre- and postsynaptic markers, a high extent of their proper apposition, and a low number of microglia. A good additional indicator of culture health is low non-synaptic background fluorescence after FM dye labeling (Step 15C(v)). In addition, spontaneous responses of neurons in the neuronal culture preparations can be recorded. This determines the extent of network activity, which is an excellent additional criterion to assess and compare the quality of the dissociated neuron preparations. Regular tests of these parameters need to be complemented by visual inspection of mixed cultures before analysis. Density and differentiation of the dissociated neurons, as well as proper seeding density and mono-dispersion of the non-neuronal cells need to be confirmed before proceeding to analysis.

Induced versus endogenous synaptic specializations. Besides the hemi-synapses induced by the heterologous cells, most dissociated neuronal cultures will form a substantial number of synaptic neuron–neuron contacts. Therefore, it has to be confirmed which pre- or postsynaptic marker accumulations are induced by physical contact with the non-neuronal cells, and which represent neuron-neuron contacts. If induction of presynaptic specializations is analyzed, parallel staining for postsynaptic and dendritic markers should be performed to exclude any presynaptic staining that shows overlap with postsynaptic markers. Conversely, if postsynaptic induction is analyzed, immunostaining for presynaptic or axonal markers should be conducted to ensure that only postsynaptic specializations are analyzed that lack the presynaptic or axonal markers. An alternative approach to circumvent these problems is the analysis of explant cultures where dendritc processes are restricted to the tissue explant and non-neuronal cells can be applied to a field of axons emerging from the explant.

TABLE 1 | Antibodies for immunostaining.

Antigen	Detection	Vendor	Species, antibody subtype	Dilution in immunocytochemistry
Synapsin 1	Synaptic vesicles	Chemicon[7]	Rabbit	1:500
Syntaxin 1	Synaptic vesicles	Synaptic Systems[8]	Mouse IgG1	1:500
Synaptophysin 1	Synaptic vesicles	Synaptic Systems[7,8]	Mouse IgG1	1:200
Synaptotagmin 1	Synaptic vesicles	Synaptic Systems Cl 604.1[19]	Mouse IgG3	1:100
SV2	Synaptic vesicles	Developmental Studies Hybridoma Bank[6]	Mouse IgG1	1:400
vGlut1	Synaptic vesicles of glutamatergic presynaptic terminals	Chemicon[10]	Guinea-pig	1:5,000
		Synaptic Systems[13]	Rabbit	1:1,000
vGAT	Synaptic vesicles of GABAergic presynaptic terminals	Synaptic Systems[13]	Rabbit	1:1,000
GAD65	GABAergic presynaptic terminals	Chemicon[20]	Rabbit	1:300
tau	Axons	Chemicon MAB3420[21]	Mouse	1:2,000
GAP-43	Axons[22]	Zymed[23]	Mouse IgG2a	1:300
PSD-95	Postsynaptic sites of glutamatergic synapses	Affinity Bioreagents[10]	Mouse IgG2a	1:500
NMDA receptor subunit NR1	Postsynaptic sites of glutamatergic synapses	Synaptic Systems[13]	Mouse IgG2b	1:200
		PharMingen[10]	Mouse IgG2a	1:1,000
Glutamate receptor subunit GluR1	Postsynaptic sites of glutamatergic synapses	Upstate Biotechnology[10,13]	Rabbit	1:5,000
Gephyrin	Postsynaptic sites of GABAergic synapses	Cederlane[24]	Mouse IgG1	1: 500
		Alexis[10]	Mouse IgG1	1:500
$GABA_A$ receptor subunit $\gamma 2$	Postsynaptic sites of GABAergic synapses	Alomone[10]	Rabbit	1:200
MAP2	Dendrites	Chemicon[10]	Mouse IgG1	1:500
		Chemicon[25]	Chicken	1:2,000

synaptic protein. More recent applications extended mixed-culture systems to the reconstitution of postsynaptic multiprotein complexes by co-transfection of several factors in the non-neuronal cell and the reconstitution of synaptic transmission[8,15,16]. One of the caveats of the assay is that some proteins require neuron-specific post-translational modifications. Moreover, it cannot be excluded that endogenous proteins of the non-neuronal cell might modify the function of the neuronal protein of choice. Clearly, the assay primarily provides a first test that then needs to be expanded by additional studies. However, the simplicity of the experimental manipulations make this approach suitable for screening purposes with expression libraries or chemical libraries to identify new synapse-organizing factors and molecules or drugs that modify the synaptogenic activity of a specific protein.

MATERIALS

REAGENTS

- Rat hippocampal neurons **! CAUTION** All experiments involving live animals must conform to Institutional and Federal regulations.
- HEK 293 or similar cells (see **Box 1** for further details)

EQUIPMENT

- Laminar flow hood
- Tissue culture hood
- Cell culture incubator (37 °C, 100% humidity, 5% CO_2)
- Hemocytometer
- 6- and 24-well tissue culture plates
- Sterile cover glasses (thickness 0, Assistent Brand)
- Inverted epifluorescence microscope equipped with an intensified CCD camera (for FM imaging) or a confocal microscope with standard set of lasers and suitable image analysis software such as Metafluor (Universal Imaging) or IPLab (Scanalytics)
- Perfusion chamber for FM imaging (e.g., RC-26, Warner Scientific)
- Standard whole-cell patch clamp setup for electrophysiological analysis

REAGENT SETUP

Dissection solution HEPES pH 7.2–7.5 (10 mM; Invitrogen, 15630-080), sodium pyruvate (1 mM; Invitrogen, 11360-070), prepared in Hank's balanced salt solution (Invitrogen, 14170-112); stable for 1 month at 4 °C; store light protected.

Enzyme solution Papain (0.4 U ml^{-1}; Worthington, LS003127), calcium (1.5 mM), EDTA (0.75 mM), L-cysteine (0.2 mg ml^{-1}), prepared in dissection solution; incubate at 37 °C for 20 min before use; solution is to be prepared fresh each time and filtered through a 0.2 µm syringe filter.

Plating medium Glutamax (6 mM; Invitrogen, 35050-061), sodium pyruvate (1 mM; Invitrogen, 11360-070), glucose (0.6% (w/v); Sigma, G5146), FBS (10% (v/v); Invitrogen, 16140-07; qualified, heat-inactivated), prepared in MEM (without glutamate; Invitrogen, 12360-038).

Neuronal medium B27 supplement (1:50; Invitrogen, 17504-044), Glutamax (6 mM; Invitrogen, 35050-061), prepared in neurobasal medium (Invitrogen, 21103-049).

Neuronal medium plus 2-AraC Neuronal medium adjusted to 2 µM arabinocytidine hydrochloride (Ara-C; Sigma, C6645).

Matrigel solution Prepared in neuronal medium with added Matrigel (1:50; Becton Dickinson).

Modified Tyrode solution (310 mOsm): HEPES pH 7.2–7.5 (20 mM; Invitrogen, 15630-080), NaCl (150 mM), KCl (4 mM), $MgCl_2$ (2 mM), glucose (10 mM).

Tyrode high K solution (310 mOsm): HEPES pH 7.2–7.5 (20 mM; Invitrogen, 15630-080), NaCl (64 mM), KCl (90 mM), $MgCl_2$ (2 mM), glucose (10 mM),

$CaCl_2$ (2 mM); before use, add AP-5 (50 μM) and CNQX (10 μM); solution containing AP-5 and CNQX is stable for 2 weeks at 4 °C; store light protected.
Loading solution Tyrode high K solution with added $CaCl_2$ (to a final concentration of 4 mM); before use, add AP-5 (50 μM) and CNQX (10 μM).
Dye FM 1-43 (Invitrogen; λ absorption/emission 502/625 nm; stock 2.0 mM in H_2O; store at −20 °C)[17].
Dye FM 4-64 (Invitrogen; λ absorption/emission 560/767 nm; stock 2.0 mM in H_2O; store at −20 °C)[17].

PROCEDURE

Coverslip preparation

1| Add ~100 μl Matrigel solution per cover glass ∅ 12 mm and incubate for >1 h. Aspirate the solution to leave a thin, wet film on the glass.

Dissociated hippocampal culture preparation

2| Dissociate rat hippocampal neurons on the day of birth (P0) or first postnatal day (P1) to prepare mixed neuron/glia cultures. Dissect hippocampi in a laminar flow hood, collect in dissection solution on ice and allow to settle on ice.

3| In a tissue culture hood, wash these hippocampal preparations 3× with ice-cold dissection solution and incubate with 4 ml enzyme solution for 30–45 min at 37 °C on a rocking platform.

4| Aspirate the enzyme solution gently and wash the hippocampal preparations 3× with 10 ml plating medium.

5| Dissociate neurons very gently by pipetting in 2 ml plating medium with a flame-polished Pasteur pipette. Add plating medium to a final volume of 5 ml and allow debris to settle for several minutes.
▲ CRITICAL STEP Gentle cell trituration is very critical for neuronal survival.

6| Transfer the supernatant into a Falcon tube and count cells using a hemocytometer.

7| Plate the dissociated neurons at ~10×10^3 to 30×10^3 cells cm^{-2} on Matrigel-covered cover glasses in tissue culture plates and place in the cell culture incubator.

8| After cells have settled for 7–8 h, flood the wells with neuronal medium that has been warmed and CO_2-equilibrated in the cell culture incubator, and place the plates back in the cell culture incubator.

9| Refresh the neuronal medium every 3–4 days. This procedure follows a published protocol[18].
▲ CRITICAL STEP Medium change is performed by replacing only half of the old medium with fresh medium. Changing the medium completely will result in neuronal cell death.

HEK 293 cell transfection

10| At days 5–6 *in vitro* of the neuronal culture, transfect non-neuronal cells to express the membrane protein to be tested for its role in synapse induction. One well of a six-well tissue culture plate yields a sufficient number of transfected non-neuronal cells for a typical co-culture experiment. In this protocol, we will describe the use of HEK 293 cells, but other cell types have also been used successfully (see **Box 1**).

Preparation of mixed HEK 293 cell–hippocampal neuron culture

11| One day later, at days 6–7 *in vitro* of the neuronal culture, seed transfected HEK 293 cells atop the dissociated hippocampal neurons as follows.

12| Wash HEK 293 cells with warm neuronal medium and collect by gently pipetting up/down in 2 ml warm neuronal medium 2-AraC per well of a six-well tissue culture plate. Cell clumps need to be completely dispersed in this procedure. Do not perform trypsinization (see **Box 1** for comments on the usage of trypsin). HEK 293 cells have to be fully dissociated before plating to allow individual analysis, as clumps of heterologous cells cannot be examined for synapse induction on their surface.
? TROUBLESHOOTING

13| Count the fully suspended HEK 293 cells using a hemocytometer. Expect to collect a total of 200×10^3 to 300×10^3 HEK 293 cells from one confluent well in a six-well tissue culture plate. HEK 293 cells should be seeded at a density of 30×10^3 per ∅ 12 mm cover glass with the mixed neuron–glia cultures.

14| In a cell culture incubator, maintain the mixed cultures in Neuronal Medium 2-AraC to prevent HEK 293 and glia cell overgrowth.
▲ CRITICAL STEP Consistent high quality of the neuronal preparations at the time of seeding and throughout the mixed culture incubation is critical and needs to be controlled (see **Box 2**).

- wash the HEK cells twice with neuronal medium
- resuspend in neuronal medium, count cell density, adjust with neuronal medium to 300,000 cells/ml
- add 15,000 cells (50 ul) to each coverslip with neurons (300 ul medium)

Analysis

15| Perform analysis 24–48 h after seeding of the HEK 293 cells and co-culture with dissociated neurons. To determine induction of pre- and postsynaptic specializations atop the heterologous cells in co-culture by immunostaining, follow option A. To examine induction of functional presynaptic terminals by analysis of synaptic vesicle recycling, follow options B and C. To functionally analyze presynaptic terminal induction and neurotransmission in reconstituted synapses, follow option D.

(A) Synapse induction analysis by immunostaining for synaptic markers

(i) Quantitative immunostaining analysis can be performed for co-cultured HEK 293 cells expressing the surface protein of interest that are in direct physical proximity to axons and dendrites. Proximity is determined by phase-contrast microscopy or immunostaining for axonal and dendritic markers. Expect to observe induction of presynaptic (option A(ii)) or postsynaptic (option A(iii)) specializations mainly along the cell boundary and also atop these co-cultured HEK 293 cells. Sources, application and references for the antibodies described below are stated in **Table 1**.

■ **PAUSE POINT** Mixed cultures can be fixed and stored at 4 °C before processing for immunocytochemistry.

(ii) To investigate induction of specializations containing presynaptic markers atop HEK 293 cells in co-culture, markers that can be successfully used include synapsin, syntaxin, synaptophysin and SV2. Antibodies used to specifically detect markers for glutamatergic presynaptic terminals are directed against the vesicular glutamate transporters vGlut1 and vGlut2. To study induction of GABAergic presynaptic specializations, antibodies detecting the GABA-synthesizing enzyme GAD65 or the vesicular GABA transporter vGAT are used. Antibodies against tau or GAP-43 serve to visualize axons.

(iii) All postsynaptic markers used in immunostaining analysis of co-cultures directly distinguish induction of glutamatergic versus GABAergic specializations. To analyze glutamatergic postsynaptic induction, antibodies used are directed against postsynaptic scaffolding proteins of the PSD-95 family (PSD-95/PSD-93/SAP-97/SAP-102) and the NMDA- and the AMPA-type glutamate receptors. To examine induction of GABAergic postynaptic specializations, immunostainings are performed for gephyrin and GABA receptor subunits. Antibodies against MAP2 are used to visualize dendrites.

(iv) The induction of synaptic marker clustering can be analyzed in four ways. First, determine the fractional area occupied by pre- or postsynaptic marker staining per surface area of transfected HEK 293 cells. Second, count the number of individual pre- or postsynaptic puncta per surface area of co-cultured HEK 293 cells (this method is useful only if individual puncta are detected that do not fuse into larger immunopositive areas). Third, determine the fractional area occupancy or the number of synaptic puncta per length of axon or dendrite crossing the co-cultured HEK 293 cell. Fourth, measure the intensity of synaptic puncta fluorescence signals atop co-cultured HEK 293 cells (this readout is particularly helpful when comparing synapse formation activities between proteins or mutants of one protein). In general, applying a combination of two or three of these methods for quantitative analysis is recommended. In all cases, threshold signals from images of the transfected cells are compared with signals obtained for negative control cells (see **Box 1**). Thresholds should be chosen such that all recognizable punctuate structures are included in the analysis. Alternatively, structures can be selected that show a specific increase in staining intensity over surrounding puncta (e.g., fivefold higher than synaptic markers in isolated axons). All image analyses should be conducted blinded with respect to the transfected expression construct.

▲ **CRITICAL STEP** It needs to be confirmed that the analyzed synaptic specializations are not formed between neurons, but in physical contact with the analyzed non-neuronal cells expressing the cell-surface protein of interest (see **Box 2**).

(B) Analysis of synaptic vesicle recycling in induced presynaptic terminals through antibody uptake

(i) Recycling synaptic vesicles can be visualized by internalization of an antibody directed against the lumenal domain of the synaptic vesicle protein synaptotagmin-1. Incubate cultures for 5 min with prewarmed, isotonic Tyrode high K solution containing mouse anti-synaptotagmin-1 lumenal domain antibodies (**Table 1**) to depolarize the neurons in this hyperkalemic solution and induce synaptic vesicle recycling and antibody uptake.

(ii) Use control mouse IgG (Sigma) at the same concentration.

(iii) After incubation, wash cultures three times with prewarmed modified Tyrode solution, fix and process for immunocytochemistry using secondary antibodies.

(C) Analysis of synaptic vesicle recycling in induced presynaptic terminals through FM dye imaging

(i) Recycling of synaptic vesicles can be analyzed through live-cell imaging with FM styryl dyes[17,18]. FM imaging has demonstrated that presynaptic terminals induced in the co-culture system can have synaptic vesicle recycling properties similar to synapses formed between neurons[8,11]. Perform all staining and washing steps in 10 μM CNQX and 50 μM AP-5 to prevent recurrent activity in neuronal cultures. Based on the imaging requirements, either FM 1-43 or the red-shifted FM 4-64 can be used to achieve optical separation. A fluorescence microscope with a heated stage is preferable.

(ii) At the imaging setup, mount two empty syringes with stopcocks and tubes for perfusion and suction. Warm modified Tyrode solution and Tyrode high K solution containing CNQX and AP-5 to 37 °C before the experiment and fill into the syringes. Thaw the aliquoted FM dye (stored at −20 °C) and store on ice under light protection. Repeated freeze–thaw steps are not advised, but can be performed as long as dye precipitation is excluded.

(iii) The volumes given below assume that the perfusion chamber holds 170 µl. Fill the perfusion chamber with 150 µl of modified Tyrode solution and place a cover glass carrying the co-culture into the perfusion chamber. Briefly superfuse the cover glass with modified Tyrode solution from one of the mounted syringes by gravity flow (1–2 ml min^{-1}) and then stop the flow.

(iv) Mix 200 µl Tyrode high K solution with added $CaCl_2$ (to a final concentration of 4 mM) freshly with 4.0 µl FM dye (stock 2.0 mM; 40 µM). Add 170 µl of this mix to the perfusion chamber to obtain a 1:1 dilution with the modified Tyrode solution present in the chamber. Incubate the co-culture for 90 s to ensure depolarization of the neurons in this hyperkalemic solution and induce synaptic vesicle recycling with maximal dye uptake into presynaptic terminals. Alternatively, dye uptake can be performed through field stimulation for 90 s at frequencies of 10–40 Hz in modified Tyrode solution (with added $CaCl_2$, 2 mM).

(v) Superfuse the chamber with modified Tyrode solution for 10 min to wash off surface-accessible dye. Ensure successful washing at the end of the superfusion by loss of FM dye fluorescence from plasma membranes.

? TROUBLESHOOTING

(vi) Perform detection in a field of interest containing a non-neuronal cell expressing the surface protein to be tested in proximity to neurons. Stimulate FM dye release with four pulses of Tyrode high K solution from the second mounted syringe (60–90 s each); separate the pulses by 60 s wash intervals with modified Tyrode solution. Quantify dye loss from individual loaded puncta at 1 Hz. As internal positive controls, choose several FM dye-loaded puncta at synaptic contact sites between neurons in each frame. Acquisition time of each frame can be expected to last 20–100 ms, depending on the CCD camera used for detection.

(vii) Calculate the loss of dye fluorescence $\Delta F/F$ for each punctum over time by data analysis. This determines to which extent the FM dye loaded into the presynaptic vesicles in Step 15C(iv) was released in Step 15C(vi). Presynaptic terminals can be expected to release $>80\%$ of the loaded FM dye.

(D) Electrophysiological analysis of neurotransmission reconstituted between induced presynaptic terminals and co-cultured non-neuronal cells

(i) With this read-out, the functionality of induced presynaptic terminals is directly determined by measurement of neurotransmitter release onto the surface of the non-neuronal cells that are coexpressing the protein of interest and a neurotransmitter receptor.

(ii) HEK 293 cells are well suited for these studies as they can be analyzed in patch-clamp recordings. Co-transfect HEK 293 cells a day before seeding with vectors encoding the surface protein to be analyzed for synapse-inducing activity and a neurotransmitter receptor subunit that forms a functional homo-oligomeric receptor such as the glutamate receptor subunit GluR2[8]. The NMDA receptor has also been used[15]. Confirm successful transfection in living cells by expressing the surface protein to be tested and the neurotransmitter receptor subunit as constructs that are individually tagged with either of two suitable fluorescent proteins. Negative control conditions include HEK 293 cells expressing either the surface protein to be analyzed for synapse-inducing activity or the neurotransmitter receptor.

(iii) Perform electrical recordings of action potential-evoked synaptic transmission in HEK 293 cells 24–72 h after seeding with an internal pipette solution containing 10 mM HEPES (pH 7.4), 140 mM CsCl, 0.6 mM EGTA and 2 mM Mg-ATP (pH 7.35). The extracellular solution should contain 10 mM HEPES (pH 7.4), 150 mM NaCl, 4 mM KCl,, 4 mM $MgCl_2$, 10 mM glucose and 4 mM $CaCl_2$ (310 mOsm at 21 °C). Add 10 µM cyclothiazide to the culture medium to prevent desensitization of glutamate receptors. HEK 293 cells have input resistances between 0.5 and 2 GΩ. Test GluR2 dependency of events in HEK 293 cells with the GluR2 glutamate receptor antagonist CNQX (10 µM).

(iv) Determine amplitude distribution and rise time distribution of depolarization responses obtained in this reconstituted system by data analysis. Compare results to events from synapses formed between neurons in cultures of the same age. It can be expected that synaptic amplitudes are significantly larger in neurons owing to the proper assembly of their postsynaptic components, but that rise times of the responses are comparable. Frequencies of depolarization responses will depend on the number of presynaptic specializations induced on HEK 293 cells expressing the cell-surface protein of interest and on the extent of spontaneous network activity of co-cultured neurons.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Solution
12	Cells are not fully dissociated	Suspension of HEK 293 can be improved by pipetting the cells with a siliconized Pasteur pipette that has been flame polished and reduced to about $^2/_3$ to $^1/_2$ of the regular diameter
15C(v)	High FM dye background	High FM dye background can result from the unhealthy condition of the dissociated neurons, which will need to be improved. Alternatively, it occurs of FM dye stocks are prepared in solvents other than H_2O

ANTICIPATED RESULTS

Newly analyzed synaptic proteins may have one-directional signaling capabilities, for example, induce the assembly of pre- but not post-synaptic structures, or may act bidirectionally, as observed for the neuroligin–neurexin complex. It is expected that synapse-inducing factors will differ in their ability to recruit specific neurotransmitter receptors, such as NMDA type, AMPA type or GABA receptors. In contrast, other newly analyzed synaptic adhesion molecules would be expected to lack potent synapse-organizing functions and might only slightly increase synaptic marker accumulation, by their ability to increase contact between neuronal and non-neuronal cells. Therefore, it is essential to directly compare newly analyzed proteins with known factors with potent synapse-inducing activities such as neuroligins and SynCAM.

Data obtained in this assay have implications for both developmental and molecular neurobiology. The assay can be used not only for identification of novel synaptogenic molecules, but also for quantitative comparison of activities and analysis of regulatory mechanisms (but see discussion of limitations in **Boxes 1** and **2**). Results obtained in such studies will then provide the basis for more detailed analysis of the synaptic proteins using complementary approaches, dissecting the cell biological mechanism of their action as well as their contribution to neuronal development *in vivo*.

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