J. Biochem. 2017;162(4):295–302 doi:10.1093/jb/mvx030

JB THE JOURNAL OF BIOCHEMISTRY

In situ screening for postsynaptic cell adhesion molecules during synapse formation

Received April 06, 2017; accepted April 13, 2017; published online April 25, 2017

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Neuronal synapse formation is regulated by pre- and postsynaptic cell adhesion molecules. Presynaptic neurexins (NRXNs) and receptor protein tyrosine phosphatases (RPTPs; PTP*δ*, PTPσ and LAR in mammals) can induce postsynaptic differentiation through the interaction with various postsynaptic cell adhesion molecules. Here, we developed a novel *in situ* screening method to identify postsynaptic membranous proteins involved in synaptogenesis. Magnetic beads coated with the extracellular domains of NRXN1β(−S4) and PTP*δ*-A6 variants preferentially induced excitatory postsynaptic differentiation on the beads' surface when co-cultured with cortical neurons. After inducing postsynaptic sites on these beads, protein complexes including NRXN1β(−S4)/PTP*δ*-A6 and their ligands on the neuronal membrane were chemically cross-linked and purified using a magnetic separator. Liquid chromatography-tandem mass spectrometry analysis of the complexes revealed two types of postsynaptic ligands for NRXN1β(−S4) and PTP*δ*-A6, one has an activity to induce presynaptic differentiation in a *trans* manner, whereas the other has no such activity. These results suggest that synapse formation is regulated by the interplay between presynaptic NRXN/PTP*δ* and their postsynaptic ligands with functionally different impacts on pre- and postsynaptic differentiation. Thus, our *in situ* screening method for identifying synapse-organizing complexes will help to understand the molecular basis for elaborate neuronal networks.

Keywords: *in situ* screening; mass spectrometry; protein–protein interaction; synapse formation; synaptic cell adhesion molecule.

Abbreviations: Cbln, cerebellin precursor protein; DIV, days *in vitro*; DTSSP, 3,3'-dithiobis(sulfosuccinimidylpropionate); ECD, extracellular domain; Ig, immunoglobulin-like; IL1RAPL1, interleukin-1 receptor accessory protein-like 1; LNS, laminin-neurexin-sex hormone-binding globular; NLGN, neuroligin; NMDA, *N*-methyl-D-aspartate; NRXN, neurexin; PDZ, PSD-95/Dlg/ZO-1; PTP, protein tyrosine phosphatase; S4, splice site 4; SALM, synaptic adhesion-like molecule; Slitrk, Slit and Trk-like; SorCS2, Sortilin-related receptor CNS expressed 2.

Neuronal synapse formation, one of the key steps in the development of neural networks, is triggered by the *trans*-synaptic interaction between pre- and postsynaptic cell adhesion molecules called synaptic organizers including presynaptic neurexins (NRXN1-3) and type IIa receptor protein tyrosine phosphatases (RPTPs), and postsynaptic neuroligins (NLGN1-4), glutamate receptor GluRδ2/GluD2, interleukin-1 receptor accessory protein-like 1 (IL1RAPL1) and Slit and Trk-like (Slitrk1-6) *1–4*). *Trans*-synaptic interactions between pre- and postsynaptic synapse organizers induce bidirectional signals into both pre- and postsynaptic neurons to trigger synapse formation. Coordinated assembly of large numbers of synaptic receptor complexes is thought to regulate synapse formation and maturation during development in the brain (*1–12*). During this century, many efforts have been made to identify receptor complexes. The affinity chromatography technique and expression cloning, which uses the physical interactions, have widely been used to identify receptor complexes. However, physically identified receptor complexes do not necessarily reflect the spatiotemporal patterns and/or functions in the brain.

Presynaptic NRXNs and type IIa RPTPs play an important role in synapse formation and maturation

through various postsynaptic cell adhesion molecules including NLGNs, GluD2-cerebellin precursor protein (Cbln) 1 complex and leucine-rich repeat transmembrane neuronal proteins for NRXNs, and IL1RAPL1, Slitrks, synaptic adhesion-like molecules (SALMs) for type IIa RPTPs (*1–4*, *10–12*). In mammals, NRXNs are encoded by three genes. Two alternative promoters in each gene yield a long α-NRXN and short β-NRXN (*13*). Type IIa RPTPs consist of three family genes products: PTPδ, PTPσ and LAR (*4*, *10*, *11*). The extracellular domain (ECD) of β-NRXN consists of a short β-NRXN-specific sequence, a single laminin–neurexin–sex hormone-binding globular (LNS) domain and the *O*-linked glycosylated stretch with two splice sites (*14*, *15*). The first splice site, splice site 4 (S4), is located in the LNS domain. The second splice site is between the *O*-linked glycosylated stretch and the transmembrane region (*14–16*). The ECD of type IIa RPTPs commonly consists of three immunoglobulin-like (Ig) domains and 4–8 fibronectin type-III domains with two splice sites within Ig domains (*17*). The first splice site, site A, is located within the second Ig domain, and the second one, site B, is located at the junction between the second and the third Ig domains. PTPδ exists in at least eight splice variants lacking or containing short-peptide inserts, a three-residue peptide (A3), a six-residue peptide (A6) and their tandem combination (A9) at site A and a four-residue peptide at site B (*17*). Previously, we identified synapse-organizing complexes selectively formed between splice variant of NRXNs carrying the splice insert 4 and GluD2-Cbln1 or GluD1-Cblns, and between splice variants of PTPδ and IL1RAPL1 or interleukin-1 receptor accessory protein (*17–21*). So far, several splice variants selective postsynaptic ligands for NRXNs and PTPδ have been reported (*4*, *10*, *12*, *22*). Thus, the alternative splicing in the ECDs of both NRXNs and type IIa RPTPs seem to enable the binding to multiple postsynaptic ligands. Nevertheless, only limited *in situ* information for the interaction of these molecules was available.

Previously, we have developed an *in situ* screening method for finding glutamate receptor GluD2 binding partner, and identified presynaptic NRXN-secreted Cbln1 complex as a GluD2 ligand (*18*). Here, we attempt to apply this method to identify postsynaptic membranous proteins involved in the regulation of synapse formation. Postsynaptic specializations of cortical neurons were induced on the surface of magnetic beads coated with the ECDs of NRXN1β(-S4) and PTPδ-A6 variants. Then NRXN1β(-S4)/PTPδ-A6 on the beads' surface and their postsynaptic ligands on the dendritic membrane were chemically cross-linked, and purified. Liquid chromatography-tandem mass spectrometry of the purified cross-linked protein complexes successfully revealed several postsynaptic ligands for NRXN1β(-S4) and PTPδ-A6; some, but not all, of these showed synaptogenic activity. Thus, multiple ligands with different properties seem to be involved in the coordination of synaptogenesis.

Materials and Methods

Screening of proteins interacting with ECDs of NRXN1β(–S4) and PTPδ-A6

Primary cerebral neurons were prepared as previously described (*17*) and placed on cell culture dish coated with poly-L-lysine and laminin at the density of 1.0×10^7 cells/10-mm dish. Magnetic beads coated with Fc fusion proteins for NRXN1β(–S4)-ECD or PTPδ-A6-ECD were prepared as described previously (*23*). The coated magnetic beads were added to the cultured cortical neurons at days *in vitro* (DIV) 13 at the density of 1.2×10^7 beads/10-mm dish. After 24 h, cultures were washed once with D-PBS and cross-linked with 1 mM 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce). Cross-linked proteins on the beads were collected and extensively washed using a magnetic separator as previously described (*18*). The purified proteins were treated with dithiothreitol to cleave DTSSP, separated by SDS-PAGE. All animal procedures were approved by the Animal Care and the Use Committee of Graduate School of Medicine, the University of Tokyo (Approval #1721T062), Shinshu University (Approval #280017) and Toyama University (Approval #A2016MED-32).

Mass spectrometry

The excised protein bands from a silver-stained gel were reduced with dithiothreitol and alkylated with iodoacetamide. Band slices were then digested with trypsin (12 μg/ml) at 37°C overnight and desalted with ZipTip C18 (Millipore). The obtained peptides were separated via nano-flow liquid chromatography (nanoLC) (Paradigm MS4, AMR) using a reverse-phase C18 column. The LC eluent was coupled to a nano ion spray source attached to an LCQ Fleet mass spectrometer (Thermo Fisher Scientific). For protein identification and semi-quantification, we used the score of the SEQUEST algorithm from BioWorks software (Thermo Fisher Scientific) (*24*, *25*).

Construction of expression vectors

The entire coding sequence of Sortilin-related receptor CNS expressed 2 (SorCS2) was amplified by RT-PCR using mRNA prepared from ICR mouse cortex and cloned into pcDNA3.1D/V5-His-TOPO (Invitrogen) to yield pcDNA3.1-SorCS2. The entire coding sequences of GluN1 and GluN2B lacking signal sequences were amplified by PCR using pBKSA ζ1 (*26*) and pBKSA ϵ2 (*27*) and cloned into the pDisplay containing an amino-terminal Igκ signal peptide followed by HA tag (Clontech) and pFLAG-CMV1 (SIGMA) containing an amino-terminal preprotrypsin signal sequence followed by FLAG tag to yield pHA-GluN1 and pFLAG-GluN2B, respectively. The entire coding sequences of mouse IL1RAPL1, Slitrk4, SALM2 and SALM4 lacking signal sequences were cloned into pFLAG-CMV1 to yield pFLAG-IL1RAPL1, pFLAG-Slitrk4, pFLAG-SALM2 and pFLAG-SALM4, respectively. The DNA fragments encoding ECDs of NRXN1β(–S4) and PTPδ-A6 without signal sequences were cloned into pEB6-Igk-Fc (*23*) to yield pEB6-NRXN1β(–S4)-ECD-Fc and pEB6-PTPδ-A6-Fc, respectively. The NRXN1β(–S4) cDNA used for the expression vector construction includes the splice insert 5.

Preparation of soluble recombinant proteins

Soluble recombinant proteins were prepared as described previously (*23*). In brief, pEB6-Igk-Fc, pEB6-NRXN1β(–S4)-ECD-Fc or pEB-Multi-PTPδ-A6-Fc was transfected into the Freestyle 293 cells (Invitrogen). Soluble recombinant Fc fusion proteins in the medium were purified by Protein A Sepharose Fast Flow (GE Healthcare), eluted with 3 M $MgCl_2$, and dialyzed against Hanks' Balanced Salt Solution.

Cell cultures and co-culture assay

Primary cortical neuron cultures were prepared from neonatal mice at postnatal day 0 as described previously (*17*). Co-culture assay was performed as described previously (*17*). In brief, HEK293T cells transfected with respective expression vector were co-cultured with cortical neurons at DIV7. After 24 h, cells were fixed with 4% paraformaldehyde and incubated with mouse anti-Bassoon antibody (Stressgen) and rabbit anti-FLAG (SIGMA) or sheep anti-SorCS2 antibody (R&D Systems), followed by incubation with species

specific Alexa Fluor 488- and 555-conjugated antibodies. Magnetic beads coated with NRXN1β(-S4) and PTPδ-A6 were prepared as described above. The coated beads were applied to the cultured cortical neurons at DIV13. After 24 h incubation, cells were fixed with 4% paraformaldehyde and incubated with rabbit anti-Shank2 (*28*) and mouse anti-gephyrin (Synaptic Systems) antibodies, followed by incubation with species specific Alexa Fluor 488- and 555-conjugated antibodies.

Image acquisition and quantification

Image acquisition and quantification were performed as described previously (*17*). In brief, the intensities of immunostaining signals for presynaptic or postsynaptic proteins were measured as the mean fluorescent intensity within circles of 30- and 7-μm diameters enclosing transfected HEK293T cells and coated-beads, respectively. Statistical significance was evaluated by Student's *t* test or one-way ANOVA followed by post hoc Tukey's test. Statistical significance was assumed when $P<0.05$.

Results

Screening of postsynaptic proteins interacting with ECDs of NRXN1β(−S4) and PTPδ-A6

We first examined the activity of NRXN1β(−S4) and PTPδ-A6 to induce postsynaptic differentiation, because both splice variants are highly expressed in cortical neurons (*29*) (Yoshida et al., unpublished observation). Cultured cortical neurons were incubated with magnetic beads coated with an equal amount of the respective ECDs of NRXN1β(−S4) and PTPδ-A6 (Fig. 1). After incubation for 24 h, the cultures were immunostained with antibodies against excitatory and inhibitory postsynaptic scaffold proteins, Shank2 and gephyrin, respectively. We detected numerous punctate signals for Shank2 on the surface of both NRXN1β(−S4)-ECD- and PTPδ-A6-ECD -coated beads (Fig. 1A). The staining signals on the surface of these beads were significantly stronger than those on the control Fc-coated beads ($P<0.01$; Fig. 1B). On the other hand, faint accumulation of gephyrin signals was detected on the surface of the NRXN1β(−S4)-ECD -coated beads (Fig. 1A). There was a significant difference in the staining signals for gephyrin between NRXN1β(−S4)-ECD- and control Fc-coated beads (Fig. 1B). These results suggest that the ECDs of NRXN1β(−S4) and PTPδ-A6 on the beads' surface preferentially induced the excitatory postsynaptic differentiation of co-cultured cortical neurons in a *trans* manner through the binding partners on the dendritic membrane.

To find the *trans*-synaptic postsynaptic binding partners of NRXN1β(−S4) and PTPδ-A6 in the cortical neurons, postsynaptic differentiation was induced with magnetic beads conjugated with the ECD of NRXN1β(−S4) and that of PTPδ-A6, respectively (Fig. 1C). After co-culturing for 24 h, the surface proteins of the dendrites of cortical neurons were cross-linked to the ECDs of NRXN1β(−S4) and PTPδ-A6 on the magnetic beads with non-membrane-permeable chemical cross-linker DTSSP, respectively, as described previously (*18*). Then, the cross-linked proteins were identified by liquid chromatography-tandem mass

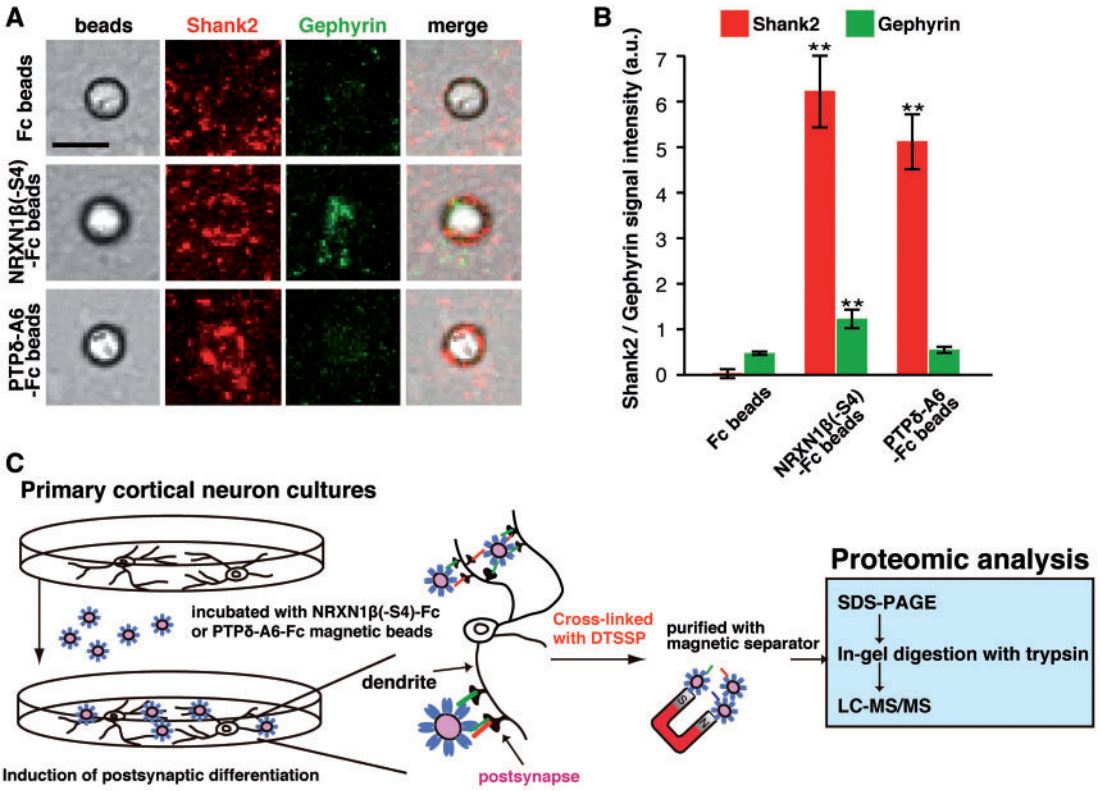

Fig. 1 ***In situ*** **screening for NRXN1β(−S4) and PTPδ-A6 ligands during postsynaptic differentiation.** (**A**) Induction of postsynaptic differentiation by magnetic beads coated with ECDs of NRXN1β(−S4) and PTPδ-A6 fused to Fc. Fc beads served as a control. Excitatory and inhibitory postsynaptic terminals induced by the beads were visualized by immunostaining for Shank2 and gephyrin, respectively. (**B**) Intensity of staining signals for Shank2 and gephyrin on the beads (n = 16–22) was quantified. (**C**) Schema for *in situ* screening of postsynaptic binding partners for NRXN1β(−S4) and PTPδ-A6. Scale bar represents 5 μm. All values represent mean ± SEM. $^{**}P<0.01$; Tukey's test.

Table I. A list of membrane proteins identified from NRXN1β(-S4)-Fc and PTPδ-A6-Fc coated beads

Bait	Protein[a]	Probability[b]	Score[c]	Hits[d]
NRXN1β(-S4)-Fc	NLGN1	3.70E-07	10.15	1
	GluN2B	3.99E-07	22.15	3
	NLGN3	9.66E-07	38.16	5
	SorCS2	2.74E-06	30.15	3
	GluN1	5.43E-05	26.13	4
PTPδ-A6-Fc	GluN2B	9.83E-08	52.21	7
	IL1RAPL1	4.66E-05	8.07	1
	Slitrk4	2.21E-06	16.17	2
	SALM2	7.90E-06	10.18	1
	SALM4	1.11E-05	20.19	2

Protein[a], proteins with probability value (<1.0E−04) are listed; Probability[b], probability (protein) of finding a match as good as or better than the observed match by chance; Score[c], SEQUEST scores; Hits[d], number of unique parent peptides found.

spectrometry. NLGN1, NLGN3, and SorCS2, and *N*-methyl-D-aspartate (NMDA)-type glutamate receptor subunits GluN1, and GluN2B proteins were identified from the NRXN1β(−S4)-ECD -coated magnetic beads. IL1RAPL1, GluN2B, Slitrk4, SALM2 and SALM4 proteins were identified from the PTPδ-A6-ECD-coated magnetic beads (Table I).

The NRXN1β(−S4)-ECD and the PTPδ-A6-ECD bind to identified membrane proteins

We next examined the interaction of NRXN1β(−S4)-ECD with NLGN1, NLGN3, SorCS2, GluN1 and GluN2B through a cell surface binding assay. Each protein was expressed in HEK293T cells and incubated with the NRXN1β(−S4)-ECD fused to Fc followed by staining with antibody against Fc. Because functional NMDA receptor forms a heterotetramer between two GluN1 and two GluN2 subunits (*27*), HEK293T cells were co-expressed with both GluN1 and GluN2B for the binding assay. We detected strong staining signals for the Fc fusion protein on the surface of NLGN1, NLGN3 or SorCS2 expressing HEK293T cells but not on GluN1/GluN2B expressing cells (Fig. 2A). The staining signals on the HEK293T cells expressing NLGN1, NLGN3, or SorCS2 were significantly stronger than those cells incubated with the control Fc ($P<0.001$, $P<0.001$ and $P<0.01$, respectively; Fig. 2B).

Then, we examined the interaction of PTPδ-A6 with IL1RAPL1, Slitrk4, SALM2, SALM4 and GluN1/GluN2B. The transfected HEK293T cells expressing IL1RAPL1, Slitrk4, SALM2, SALM4 or GluN1/GluN2B were incubated with the PTPδ-A6-ECD fused to Fc and then stained as described above. We detected strong staining signals for the Fc fusion protein on the surface of IL1RAPL1, Slitrk4, SALM2 or SALM4 expressing HEK293T cells but not on GluN1/GluN2B expressing cells (Fig. 3A). The staining signals on the HEK293T cells expressing IL1RAPL1, Slitrk4, SALM2 or SALM4 were significantly stronger than those cells incubated with the control Fc ($P<0.001$, respectively; Fig. 3B). Thus, we successfully identified the *trans*-synaptic postsynaptic binding partners of NRXN1β(−S4) and PTPδ-A6. To the best of our knowledge, the direct interactions between NRXN1β(−S4) and SorCS2 and those between PTPδ-A6 and SALM2 or SALM4 have never been reported.

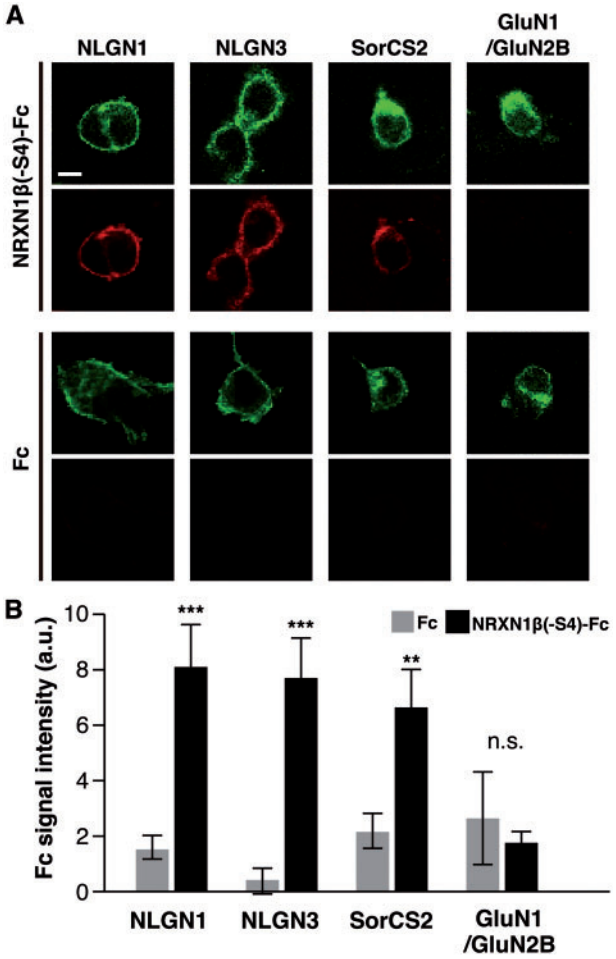

Fig. 2 Interactions between NRXN1β(−S4) and proteins identified by the screening. (**A**) Binding of ECD of NRXN1β(−S4) fused to Fc (NRXN1β(−S4)-Fc) or Fc to HEK293T cells expressing NLGN1, NLGN3, SorCS2 or GluN1/GluN2B. Expressed proteins (upper panels) and cell surface-bound Fc or Fc fusion proteins (lower panels) were immunostained. Scale bar represents 10 μm. (**B**) Ratios of staining signals for Fc or Fc fusion proteins and expressed proteins in (A) (n = 10 HEK293T cells each). All values represent mean ± SEM. $^{**}P<0.01$ and $^{***}P<0.001$, respectively; *t*-test.

NLGN1, NLGN3, IL1RAPL1 and Slitrk4 showed presynapse-inducing activity, whereas SorCS2, SALM2 and SALM4 showed no such activity

Axo-dendritic contacts bidirectionally induce the pre- and postsynaptic differentiation at the contact sites (*30*), and the *trans*-synaptic interactions between NRXNs and NLGNs and between PTPδ and IL1RAPL1 are well known to bidirectionally induce synaptic differentiation (*17*, *31*–*33*). Thus, by using HEK293T cell-neuron co-cultures, we examined whether the identified postsynaptic ligands for NRXN1β(−S4) and PTPδ-A6 show presynapse-inducing activity or not. The HEK293T cells expressing NLGN1, NLGN3, SorCS2, IL1RAPL1, Slitrk4, SALM2 or SALM4 were co-cultured with cultured cortical neurons. After co-culturing for 24 h, the cells

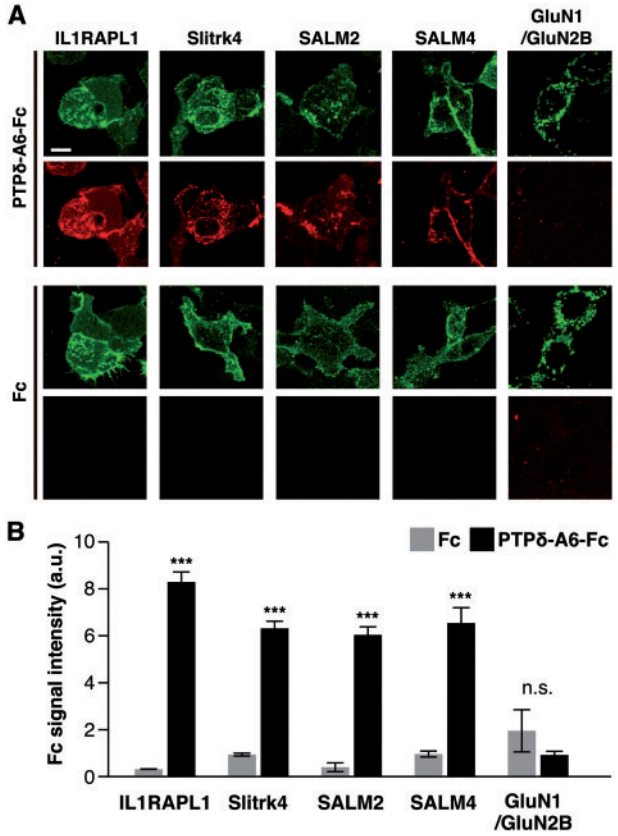

Fig. 3 Interactions between PTPδ-A6 and proteins identified by the screening. (**A**) Binding of ECD of PTPδ-A6 fused to Fc (PTPδ-A6-Fc) or Fc to HEK293T cells expressing IL1RAPL1, Slitrk4, SALM2, SALM4 or GluN1/GluN2B. Expressed proteins (upper panels) and cell surface-bound Fc or Fc fusion proteins (lower panels) were immunostained. Scale bar represents 10 μm. (**B**) Ratios of staining signals for Fc or Fc fusion proteins and expressed proteins in (A) (n = 10 HEK293T cells each). All values represent mean ± SEM. ***$P<0.001$; t-test.

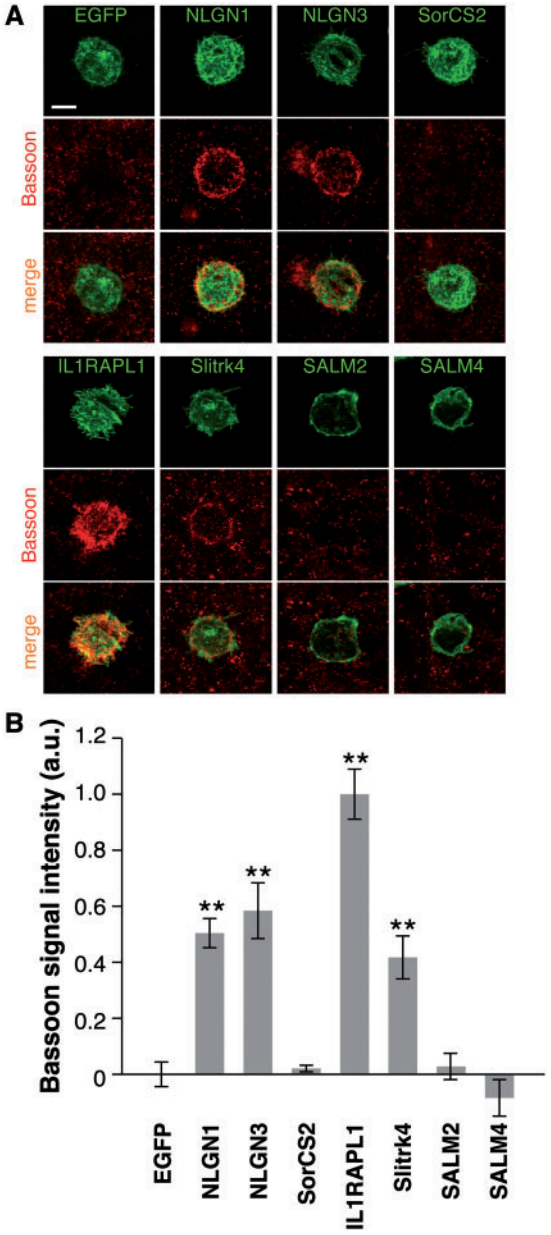

Fig. 4 Presynapse-inducing activities of NRXN1β(–S4) and PTPδ-A6 ligands. (**A**) Induction of presynaptic differentiation of cultured cerebral cortical neurons by HEK293T cells expressing each NRXN1β(–S4) and PTPδ-A6 ligand. HEK293T cells and presynaptic terminals induced around HEK293T cells were visualized by immunostaining for each NRXN1β(–S4) and PTPδ-A6 ligand (top panels) and for presynaptic active zone marker Bassoon (middle panels). (**B**) Intensity of staining signals for Bassoon around HEK293T cells (n = 15–21) was quantified. Scale bar represents 10 μm. All values represent mean ± SEM. **$P < 0.01$; Tukey's test.

were fixed and stained with antibody against the presynaptic active zone protein Bassoon. We detected the accumulation of Bassoon signals on the surface of NLGN1, NLGN3, IL1RAPL1 or Slitrk4 expressing HEK293T cells (Fig. 4A). The staining signals for Bassoon on the transfected HEK293T cells were significantly stronger than those on HEK293T cells expressing EGFP ($P<0.01$; Fig. 4B). In contrast, the staining signals for Bassoon on HEK293T cells expressing SorCS2, SALM2 or SALM4 were comparable to those on HEK293T cells expressing EGFP (Fig. 4B), indicating that these ligands have no presynapse-inducing activity.

Discussion

Synapse formation is highly spatiotemporally organized by multiple and diverse synaptic cell adhesion molecules. Much evidence has been presented for cell-type-, region-specific or biased expression of synaptic cell adhesion molecules in the brain (*12*). Thus, certain cell-type- or region-specific *trans*-synaptic cell adhesion complexes must be elucidated for understanding of wiring specificity and synaptic diversity. Herein, we developed a novel screening method to identify postsynaptic membranous proteins involved *in situ* in synaptogenesis.

In this study, by using artificially induced hemi-synapses between recombinant synapse organizer-coated beads and cultured cortical neurons, we identified NLGN1, NLGN3 and SorCS2 as postsynaptic ligands of presynaptic NRXNβ(–S4) as well as IL1RAPL1, Slitrk4, SALM2 and SALM4 as postsynaptic ligands of presynaptic PTPδ-A6. Postsynaptic NLGN1 and NLGN3 are reported as NRXN ligands, and

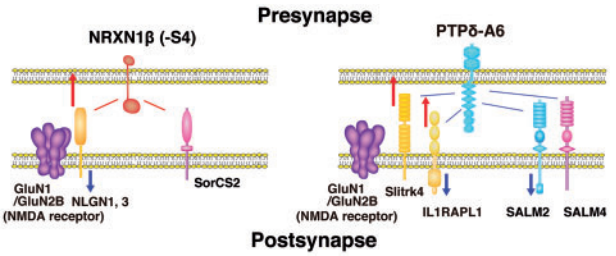

Fig. 5 A proposed model for NRXN1β(−S4)- and PTPδ-A6-induced excitatory synapse formation. Postsynaptic NRXN1β(−S4) (left) and PTPδ-A6 (right) ligands identified in this study are classified into two groups with regard to presynapse-inducing activity. These ligands also differ in the impact on the regulation of postsynaptic differentiation. Presynapse- and postsynapse-inducing activities of each postsynaptic ligand are indicated by upward and downward arrows, respectively, based on previous reports (see Discussion section).

IL1RAPL1 and Slitrk4, as PTPδ ligands (*1*, *11*, *17*, *34*). On the other hand, to the best of our knowledge, no direct interactions between NRXN1β(−S4) and SorCS2, and between PTPδ-A6 and SALM2 or SALM4 have been reported.

We showed that there are two types of postsynaptic ligands for NRXN1β(−S4) and PTPδ-A6, one has an activity to induce presynaptic differentiation in a *trans* manner (NLGN1, NLGN3, IL1RAPL1 and Slitrk4), whereas the other has no such activity (SorCS2, SALM2 and SALM4; Fig. 5). These results are consistent with previous observations (*31*, *35–38*). Synapse organizer complexes such as NRXNs-NLGNs can bidirectionally induce pre- and postsynaptic differentiation at the contact sites (*31–33*). Some synapse organizer complexes may unidirectionally regulate synapse formation. Because SALM2 possesses the carboxyl-terminal PSD-95/Dlg/ZO-1 (PDZ)-binding motif that interacts with the excitatory postsynaptic scaffold protein postsynaptic density protein-95 (*39*, *40*), it may unidirectionally induce postsynaptic differentiation upon making contact with PTPδ-A6. On the other hand, SALM4 lacks the PDZ-binding motif and is reported to suppress excitatory synaptogenesis (*41*). The cytoplasmic region of SorCS2 lacks the PDZ-binding motif (*42*). SorCS2 is a member of the vacuolar protein sorting 10 domain-containing receptor proteins family that includes sortilin, SorLA, SorCS1, SorCS2 and SorCS3 (*43*), and it plays important roles in the control of neuronal viability and functions through interactions with neurotrophic factors and their receptors (*44–46*). Recently, it was reported that SorCS1 and NRXN1β preferentially interact in *a cis* manner and regulates the trafficking of NRXN (*36*). The functional meaning of the interaction between NRXN1β(−S4) and SorCS2 remained unclear. Further investigation will be required to understand the functions of this interaction. Interestingly, GluN1 and/or GluN2B protein were identified from both the NRXN1β(−S4)-ECD- and PTPδ-A6-ECD -coated magnetic beads (Table I), indicating that NMDA-type receptors are physically close at these synapse organizers. A previous study showed that NLGN1 interacts with GluN1 through its extracellular cholinesterase domain (*47*). Therefore, GluN1 and GluN2B may indirectly interact with NRXN1β(−S4) through NLGN1. On the other hand, the mechanism by which PTPδ-A6 recruits NMDA-type glutamate receptors into the synapse-organizing complex remains elusive.

Collectively, our results suggest that synapse formation is regulated by the complex interplay between presynaptic NRXN/PTPδ and their postsynaptic ligands with functionally different impacts on pre- and postsynaptic differentiation. Our *in situ* screening method to identify synapse-organizing complexes can be useful for understanding the molecular basis for elaborate neuronal networks in the brain.

Acknowledgements

We are grateful to Dr. Masayoshi Mishina for generous support and helpful discussions. We also thank Dr. Masahiko Watanabe for antibody against Shank2, and Dr. Hisashi Mori for helpful comments on the manuscript.

Funding

This study was supported in part by JST CREST Grant Number JPMJCR1XM5, Japan (to T.U.): JST PRESTO Grant Number 4145, Japan (to T.Y.).

Conflict of Interest

None declared.

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