Doctoral Thesis

Title: **Structural and functional analysis of cytoplasmic region of synapse organizer Neurexin-1 that mediates intracellular signals for synapse formation**

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Introduction

Chemical synapses are highly specialized inter-neuronal junctions that consist of pre- and postsynaptic parts where signals are released and received, respectively. Cell adhesion molecules are known to be required for recognition and connection between pre- and postsynapses. Neurexins are a family of synaptic cell adhesion molecules that bridge pre- and postsynapses and considered to be involved not only in synapse formation, but also maturation of synapses [1]. Neurexins are originally isolated as receptors for α-Latrotoxin, a widow spider venom, which attacks presynaptic terminals and induces massive neurotransmitter release resulting in dysfunction of synapses [2, 3].

Mammals possess three neurexin genes (Nrx1, Nrx2, and Nrx3), each of which has two independent promoters that produce longer α- and shorter β-form [4, 5]. α-Neurexin proteins contain six LNS (laminin, neurexin, sex-hormone globulin) domains and three EGF (epidermal growth factor) -like repeats in their extracellular regions. α-Neurexins share the last LNS-domain to cytoplasmic region with β-Neurexins. Cytoplasmic tails of Neurexins are short, with ~ 50 amino acids, and they contain PDZ binding sequences at carboxyl terminals, where they bind a synaptic scaffolding protein, CASK [6].

All three neurexin genes are subjected to alternative splicing at five sites, all in their extracellular coding regions [5]. Among these, the effect of splice site 4 (ss4) has been best characterized. ss4 is located within the last LNS domain and the insertion affects interaction with Neuroligins or LRRTM2, postsynaptic ligands expressed predominantly in the cerebral cortex and the hippocampus [7-9]. On the other hand, the insertion of this exon confers the binding affinity to Cbln1, the soluble protein secreted from granule cells in the cerebellum [10, 11].

Neuroligins are the first identified postsynaptic ligands for Neurexins [12]. They are also single transmembrane proteins with relatively long extracellular regions and

short cytoplasmic tails. Extracellular region of Neuroligins contains acetylcholinesterase-like domain at which they bind to the last LNS domain of Neurexins in the presence of calcium [13-15]. Neurexin-Neuroligin interaction has been demonstrated to induce synapse formation by artificial synapse formation assay [16]. Cultured neurons form synapses only onto surface of neurons, but not on non-neuronal cells. However, when non-neuronal cells, such as COS or HEK293 cells, were transfected with Neuroligins and co-cultured with neurons, neurons form presynaptic structures onto Neuroligin expressing non-neuronal cells [16, 17]. An addition of recombinant extracellular domain of Neurexin in this culture blocks the formation of presynaptic structures onto non-neuronal cells, suggesting that Neuroligins induce presynaptic formation via the interaction with Neurexins [16].

LRRTM2 are also enriched in postsynaptic terminals in pyramidal neurons in the hippocampus and the cerebral cortex and induce presynaptic differentiation through interaction with Neurexins [18, 19]. Cbln1 links between Neurexin at parallel fiber terminals and GluRδ2 at dendritic spines of the Purkinje neurons in the cerebellum and induce the synapse formation [10]. These findings suggest that Neurexins may function as receptors for presynapse formation and maturation. But the mechanisms by which Neurexins transmit those signals remain unknown.

In my PhD work, I studied Neurexin functions at intracellular signaling. For this, I first studied subcellular localization and function of Neurexins in synapses using electron microscopy and electrophysiology, respectively. Then I analyzed the structure of cytoplasmic tail of Neurexin-1. I also studied lipid binding with Neurexins because this may be critical for the signals beneath plasma membrane. Based on the results, I searched the mechanism for signal transduction required for synapse formation by focusing on phosphorylation and molecular interaction.

Materials and methods

Clones and constructs

(Lentiviral vectors) L315 and L315-Nrx-TKD were obtained from Dr. Thomas C. Südhof at Stanford University School of Medicine [20]. L315 contains two shRNA cloning sites under the human H1 promoters, two shRNA cloning sites under the mouse U6 promoters, and an EGFP coding sequence under the Ubiquitin-C promoter. L315-Nrx-TKD contains shRNAs for mouse neurexin-1, mouse neurexin-2 and mouse neurexin-3 genes cloned into L315 backbone vector. FSyGW-2xHA-Nrx1β: N-terminal two HA tagged mouse Neurexin-1β coding sequence with shRNA resistant mutations was cloned into FSyGW lentiviral vector under the mouse synapsin 1

promoter. FSyGW-2xHA-Nrx1β SD6-8: FSyGW-2xHA-Nrx1β was mutated to substitute aspartic acids for the last three serines of Neurexin-1. FSyGW-2xHA-Nrx1β SA6-8: FSyGW-2xHA-Nrx1β was mutated to substitute alanins for the last three serines of Neurexin-1. Vectors for lentiviral production, pRRE, pVSVG and pREV, were obtained from Dr. Südhof.

(Eukaryotic expression vectors) pCSMT-ratCaMKIIa T286D was obtained from Dr. Yasunori Hayashi at RIKEN Brain Science Institute (BSI) [21]. pCMV-NL1-EGFP, in which EGFP coding sequence was inserted in the cytoplasmic region of rat Neuroligin-1 coding sequence, was obtained from Dr. Südhof. pCAGGS-Flag-NRXN1β: Flag-epitope sequences were inserted between signal sequence and N-terminal of mouse neurexin-1β gene and cloned into pCAGGS vector. pCAGGS-Flag-NRXN1β SA6-8: pCAGGS-Flag-NRXN1β was mutated to substitute aspartic acids for the last three serines of Neurexin-1.

(Prokaryotic expression vectors) pGEX-KG, for expression of glutathione-S-transferase (GST) protein or for construction of GST-fusion protein vectors, was obtained from Dr. Südhof. pGEX-NxIc WT: cytoplasmic region of rat Neurexin-1 coding sequence was inserted into pGEX-KG in frame to express GST-Neurexin-1C protein. pGEX-NxIc SD6-8: pGEX-NxIc was mutated to substitute aspartic acids for the last three serines of Neurexin-1. pGEX-NxIc SD1-8: pGEX-NxIc was mutated to substitute aspartic acids for eight serines of cytoplasmic tail of Neurexin-1. pGEX-CASK: DNA fragment encoding PDZ, SH3, and guanylate kinase domain of rat CASK was cloned into pGEX-KG in frame to express GST-CASK protein [22].

Generation and specificity test for Pan-Neurexin polyclonal antibodies

Synthetic peptides corresponding to the last nine amino acid sequence of mouse Neurexins were dissolved in 100 μl of PBS, followed by mixing and conjugating with 100 μl of complete Freund's adjuvant (Sigma, F-5881). Then the mixture was injected into one rabbit subcutaneously and one guinea pig intraperitoneally. The animals were boosted every 14 days with the same peptides in 100 μl of PBS mixed with 100 μl of incomplete Freund's adjuvant (Sigma, F-5506). The antisera were taken on the seventh day after the last boost. The specificity test for the antisera was performed by western blotting. For this, Neurexin proteins from mouse brain lysate were concentrated by GST-CASK affinity pull-down and loaded on the SDS-polyacrylamide gel. Mouse brain lysate and GST, without fusion protein, pull-down product were loaded in parallel on the same gel for positive and negative controls, respectively. 1μg/ml of the antibodies were used for western blotting.

Pre-embedding immuno electron microscopy

Adult mice were perfusion-fixed with 4% w/v paraformaldehyde (PFA), 0.05% v/v glutaradldehyde (25%), and 15% v/v saturated solution of picric acid in phosphate buffer (PB) (0.1 mM, pH 7.4) for 10 minutes. Brains were then immediately removed from the skull, washed in 0.1 m PB and sliced coronally in 70-μm-thick sections on a vibratome (VT1000S, Leica Microsystems, Vienna, Austria). Sections were stored in 0.1 mM PB containing 0.05% sodium azide at 6℃ until use. For immunostaining, free-floating sections were freeze–thawed twice after cryoprotection in 20% sucrose in 0.1 mM PB to allow antibody penetration, and then incubated in 20% normal goat serum (NGS) in Tris-buffered saline (TBS) for 2 hours at 21℃–23℃ (room temperature, RT). After blocking, sections were exposed to primary antibodies for ~72 hours at 6℃. The immunoreaction was visualized by means of a nanogold–silver-enhanced reaction. Sections were incubated overnight with Fab′fragment secondary antibodies conjugated to nanogold particles (1.4 nm), then extensively washed in milliQ water followed by silver enhancement of the gold particles using the HQ kit (Nanoprobes) for ~10–12 minutes. After extensive washes in milliQ water and 0.1 mM PB, the sections were treated with 2% OsO4 in 0.1 mM PB for 40 minutes at room temperature. Contrast was enhanced with 1% uranyl acetate in 50% ethanol for 30 minutes at room temperature. Sections were dehydrated by graded ethanol (50%, 70%, 90%, 100%) and propylene oxide at room temperature, and then quickly transferred into weighing boats containing epoxy resin (Durcupan ACM-Fluka, Sigma) where they were kept overnight at room temperature. The following day, the sections were transferred onto siliconized slides, coverslipped with ACLAR® film coverslips (Ted Pella, Inc., Redding, CA, USA), and incubated for 3 days at 60℃. Blocks of the cerebellar cortex were cut under a stereomicroscope and re-embedded in epoxy resin. Ultrathin sections (70 nm) were cut using a diamond knife (Diatome, Switzerland) on an ultramicrotome (Ultracut, Leica), collected on single-slot copper grids coated with pioloform (Agar Scientific Ltd) and analysed in a transmission electron microscope (CM120, Philips, Eindhoven, the Netherlands) equipped with a Morada CCD camera (Soft Imaging Systems, Münster, Germany).

Neuron culture

Primary dissociated neuron cultures were prepared from P0-P2 ICR mice. The hippocampi from P0-P2 mice were dissected, minced, and treated with trypsin solution containing 1% trypsin, 0.1% DNase I in $CaCl_2$/$MgCl_2$-free PBS for 5 minutes at 37℃. The trypsinized hippocampi were washed with a Neurobasal-A medium (life technologies) supplemented with 5% fetal bovine serum (FBS) (life

technologies) to inactivate and dilute trypsin. They were then dissociated by passing through a fire-polished Pasteur pipette in $CaCl_2$-free PBS containing 0.05% DNase I, 0.03% trypsin inhibitor, and 2 mM $MgCl2_2$. The cells were placed on polyethilenimine (PEI) coated 12 mm diameter round coverslips (Matsunami) in 24 well plates (BD Falcon) at the density of 1.5×10^5 cells/well. The cells were cultured in Neurobasal-A supplemented with 2% B-27 supplement (life technologies), 5% FBS, 100 U/ml penicillin (life technologies), 100 μg/ml streptomycin (life technologies) and 0.5 mM L-glutamine (life technologies). After 24 hours, the culture medium was replaced with the same medium, but without FBS. The medium was changed twice a week by replacing 30% of the old medium with a fresh one.

Production and preparation of lentiviruses

HEK293T cells were transfected with the control vector (L315), Neurexin triple knockdown vector (L315-Nrx-TKD), or Neurexin rescue vectors (FSyGW-2xHA-Nrx1β, FSyGW-2xHA-Nrx1β SD6-8, or FSyGW-2xHA-Nrx1β SA6-8) together with pPRE, pVSVG and pREV by using PEI or calcium phosphate method. After 24 hours, the transfected cells were incubated with fresh Dulbecco's Modified Eagle Medium (DMEM) containing 10% FCS for 48-72 hours. The culture media containing virus particles were filtrated with 0.45 μM filter and aliquoted in 5 mL microtubes. They were centrifuged at 10,000 rpm overnight at 4 ^{0}C to concentrate the viral particles. The supernatants were discarded and virus particles were suspended in PBS. Control (L315) and Neurexin triple knockdown (L315-Nrx-TKD) viruses were added to the neuron culture at DIV4. Rescue viruses were added to the neuron culture at DIV7.

Heterologous synapse formation assay, immunocytochemistry and image acquisition and analysis

For heterologous synapse formation assay, HEK293T cells were transfected with plasmids expressing EGFP-neuroligin-1 (EGFP-NL1) or EGFP only using polyethylenimine (PEI) and cultured for 48 hours. Then the cells were trypsinized and plated onto DIV14–DIV16 mouse hippocampal neuron cultures and co-cultured for 48 hours. For immmunostaining, co-cultured neurons and HEK293T cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 15 minutes. After removing and washing the PFA, cells were incubated with blocking solution (0.1% Triton X-100 and 5% normal goat serum in PBS) at room temperature for 30 minutes followed by incubation with rabbit anti-synapsin I antibody (1:1,000) in the blocking solution at room temperature for 1 hour. After removing and washing the antibody, they were incubated with fluorescent secondary antibody at room temperature for 1

hour. Immunostained samples were mounted on the slide glasses and the fluorescent signals were detected by confocal microscopy. The ratio between synapsin I signals from neurons on the HEK293T cells and the GFP signals from transfected HEK293T cells (i.e., EGFP-NL1 or EGFP) were analyzed by Image J software (NIH) to evaluate synaptogenic activity induced by Neuroligin-1.

Induction of phosphorylation

HEK293T cells were transfected with pCAGGS-Flag-NRXN1β or pCAGGS-Flag-NRXN1β SA6-8 mutant, alone, or together with constitutively active CaMKII (pCSMT-ratCaMKIIa T286D). 24 hours after transfection, transfected HEK293T cells were treated with 2 μM 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma), 30 μM forskolin (Sigma), alone, or together with 5 μM cyclosporine A (Sigma) and 20 nM calyculin A (Sigma) at 37℃ for 5 min. After drug treatment, cells were washed with TBS and lysed in RIPA buffer containing 50 mM Tris-HCl (pH, 7.5), 150 mM NaCl, 1% Trition-X, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM sodium fluoride, and phosphatase and protease inhibitor cocktails (Nacalai Tesque). For phosphatase treatment, cell lysates were incubated with λ-phosphatase (New England Biolabs) at 37ºC for 3 hours. Cultured mouse neurons for TPA treatment were prepared from P0-P2 mouse pups as described above. The cells were infected with lenti viruses for 2xHA-Nrx1β at DIV4. At DIV14, cells were incubated with 200 nM TPA, 5 μM cyclosporine A, and 20 nM calyculin A at 37 ºC for 1 hour. Cell lysates were prepared as described above.

Detection of phosphorylation

Cell lysates were separated by SDS-PAGE and Phos-tag SDS-PAGE [23]. Phos-tag SDS-PAGE was performed with 7.5% polyacrylamide gels containing 50 μM and 100 μM phos-tag acrylamide (Wako Pure Chemical), essentially according to the manufacturer's instructions. After electroblotted onto polyvinylidene difluoride membrane (GE Healthcare), the membranes were incubated with 5% skim milk or StartingBlock Blocking buffer (Pierce), followed by incubation with horseradish peroxidase (HRP)-conjugated anti-FLAG tag (anti-DDDDK; MBL), anti-HA tag (SIGMA), anti-phospho-ERK (Cellular Signaling), anti-pan ERK (Cellular Signaling), anti-CREB (Cellular Signaling), or anti-phospho CREB antibody (Cellular Signaling). HRP-conjugated anti-rabbit and anti-mouse antibodies (GE Healthcare) were used for secondary antibody. Immunoreactive proteins were detected using ECL prime (GE Healthcare) western blotting detection system with ImageQuant LAS 4000mini (GE Healthcare).

Measurement of frequency and amplitude of mini EPSCs

Whole-cell patch-clamp recording was performed at ambient temperature using a HEKA EPC10 double USB (HEKA Elektronik). Cultured mouse hippocampal neurons were bathed with an external solution containing (in mM) 100 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 1 NaH_2PO_4 and 10 D-glucose (pH7.4). The patch electrodes were filled with an internal solution containing (in mM): 90 K-gluconate, 10 CsCl, 5 NaCl, 10 HEPES, 10 EGTA, 4 Mg-ATP and 0.3 Na-GTP (pH 7.2). The mEPSCs were recorded at the holding potential of -70 mV, in the presence of 1 μM tetrodotoxin to block action potentials, 50 μM D-AP5 (D-2-amino-5-phosphonopentanoate) to block NMDA receptors and 50 μM picrotoxin to block GABA (γ-aminobytyric acid) receptors. Data were digitized at 10 kHz and filtered at 2 kHz for acquisition using HEKA EPC10 double USB operated by PatchMaster software (HEKA Elektronik). Data were analyzed with Mini Analysis 6 software (Synaptosoft). The threshold for detecting mEPSC events was set at 5 pA.

Circular dichroism spectroscopy

Far-UV CD spectra of purified hexa histidine (His) -tagged Neurexin-1 cytoplasmic tail protein were collected on a Jasco J-720 circular dichroism spectropolarimeter over the range of 200–250 nm in 0.1-cm path-length rectangular cell. Proteins were dialyzed against PBS and then diluted to a concentration of 0.5 μg/ml in the same buffer. Spectra were recorded at 20℃ (5.0 nm bandwidth) at a rate of 10 nm/min, and 1 second response.

Lipid binding test

lipid membrane strips (echelon, Membrane lipid Strips, Product number: P-6001 and P-6002) were incubated in the blocking solution (PBS + 3% BSA + 0.01% v/v Tween-20) for 1 hour at room temperature, followed by addition of probe proteins (GST-PIP2 Grip for positive control, GST-Nrx1 C-tail wild-type, GST-Nrx1 C-tail SD1-8 mutant, or GST-Nrx1 C-tail SD6-8 mutant proteins) to final concentration of 1 μg/ml and incubated overnight at 4 ℃. After washing 3 times with PBST (PBS + 0.1% v/v Tween-20) for 10 minutes at room temperature, they were incubated with anti-GST mouse monoclonal antibody (Nacalai Tesque, INC, Code 04435-84) in the blocking solution at 1:2,000 dilution for 1 hour at room temperature. After washing with PBST 3 times for 10 minutes at room temperature, they were incubated with anti-mouse 800 secondary antibody (Li-COR) in the blocking solution at 10,000 dilution for 1 hour at room temperature. After washing 3 times with PBST for 10 minutes each, signals were detected by Odyssey Scanner (Li-COR).

Results

Neurexin proteins are specifically localized at presynaptic terminal.

Neurexins have been hypothesized to be localized at presynaptic terminals because of the following reasons; 1. They were identified as α-Latrotoxin receptors [2, 24]. 2. Major extracellular binding partners including Neuroligins, LRRTMs, GluRδ2 are postsynaptic. But this remained unproved due to lack of antibodies sufficient to detect their subcellular localization. To perform immuno electron microscopy, I first generated Neurexin antibodies under the support of Comprehensive Brain Science Network and raised anti-rabbit and anti-guinea pig polyclonal antibodies against peptide corresponding to the last nine amino acid sequence of mouse Neurexins that were conserved among all isoforms. Both anti-rabbit and anti-guinea pig Neurexin antibodies detected weak signals at 180 kD and 100 kD corresponding to α- and β-Neurexins, respectively from mouse brain lysate in western blotting (Figure 1A). Both signals were enhanced when endogenous Neurexins were concentrated by GST-CASK affinity pull-down (Figure 1A). Using these antibodies, I tested subcellular localization of Neurexins in parallel fiber-Purkinje synapses by preembedding immuno electron microscopy under the support of Dr. Ryuichi Shigemoto. In this experiment, I found that Neurexin signals were exclusively localized at active zone area of presynaptic terminals (Figure 1B).

Neurexins are required for synaptic activity.

Amino acid sequence of rodent Neurexin-1, -2, 3 proteins are highly conserved and their expression patterns are widely overlapped in the brain [4, 25]. This indicates Neurexins may act redundantly each other. Up to now, studies in triple knockout of all three Neurexins have not been published. To study the synaptic effect of Neurexins, I knocked down all three Neurexins in dissociated mouse hippocampal neuron cultures using shRNAs (Nrx-TKD) introduced by lentiviruses (Figure 2A). I studied synaptic function in Neurexin triple knockdown neurons using patch clamp electrophysiology under the support of Dr. Moyuru Hayashi. In this experiment, I found that the frequency of miniature excitatory postsynaptic currents (mEPSCs) was significantly decreased in the Neurexin triple knockdown neurons compared to control (Figure 2B). This reduction was rescued by overexpression of wild-type Neurexin-1β (Figure 2B). But there was no significant change in the amplitude of mEPSCs (Figure 2B). These results indicate that Neurexin effects are dominant on presynapse.

Cytoplasmic tail of Neurexin-1 is intrinsically disordered.

To elucidate the molecular mechanism by which Neurexins transmit signals for

synapse formation and function at presynaptic terminals, I first focused on the structure of cytoplasmic tails of Neurexins. Cytoplasmic tails of Neurexins are likely to play an important role in Neurexin function. Spanish group generated a mouse line that expresses Neurexin-1β lacking cytoplasmic tail and found synaptic defects in those mice [26]. The frequency of mEPSCs and mIPSCs in the transgenic mice were significantly decreased [26]. They also observed autism like-behaviors, such as increased self-grooming and impaired social interaction and preference for social odors in these mice [26].

To study the structure of Neurexin-1, I used a bioinformatic software named "PSIPRED" [27]. This program predicted that cytoplasmic tail of Neurexin-1 was intrinsically disordered (Figure 3A). Next, I confirmed this using circular dichroism spectroscopy in six histidine tagged cytoplasmic tail of rat Neurexin-1 (Figure 3B). Intrinsically Disordered Proteins (IDPs) lack of stable secondary or tertiary structures when the protein exists as an isolated polypeptide chain under physiological conditions *in vitro* [28]. But once they interact with their target proteins, IDPs tend to change their conformation immediately to become structured proteins [28, 29]. There are two notable characteristics of IDPs. First, IDPs tend to exhibit molecular interaction simply based on the property of amino acids constituting the region [28, 29]. Second, phosphorylation changes the conformation or affects the interaction property of the region [30].

Positively charged cytoplasmic tail of Neurexin-1 bind to negatively charged phospholipids that constitute plasma membrane.

Cytoplasmic tail of Neurexin-1 is enriched by basic amino acids (histidine[H], lysine[K], and arginine[R]) (Figure 4A). In addition, this region contains eight serines which can be potential phosphorylation sites (Figure 4A). I speculated that this region may interact with negatively charged molecules by electrostatic manner. Especially the cytoplasmic tail of Neurexin-1 is short, only with around 55 amino acids, and it resides just beneath the plasma membrane. Thus, the negatively charged phospholipids constituting plasma membrane are the first candidates for interaction with the region. I examined these interaction using lipid membrane strips on which fifteen lipids were dotted and incubated with GST-Neurexin-1 C-tail fusion protein as a probe. In this experiment, I obtained positive signals from lipids including PIP: Phosphatidylinositol (4) Phosphate, PIP2: Phosphatidylinositol (4,5) Bisphosphate, PIP3: Phosphatidylinositol (3,4,5) TrisPhosphate, PA: Phosphatidic Acid;CL: Cardiolipin) (Figure 4B). I assume these interactions are due to electrostatic affinity between positive charge of cytoplasmic tail of Neurexin-1 and

the negative group of phospholipids.

Serine phosphorylation in cytoplasmic tail of Neurexin-1 abolishes phospholipid binding.

Next question is whether or not serine phosphorylation in cytoplasmic tail of Neurexin-1 affects the interaction with phospholipids. Phosphorylation confers negative charge to proteins resulting in neutralization of the positively charged cytoplasmic tail of Neurexin-1 (Figure 4C). To address this question, I made two different GST-fused Neurexin-1 C-tail proteins with phosphor-mimic mutations. The first construct substitutes aspartic acids for all eight serines (SD1-8). The second one substitutes aspartic acids for only last three serines (SD6-8), of which phosphorylation has previously been confirmed by mass spectrometry (Figure 4D) [31]. Interestingly, both phospho- mimic Neurexin-1 completely abolished the phospholipid binding, suggesting that phosphorylation of the last three serines is sufficient to abolish the interaction (Figure 4E). Now, I decided to focus on the phosphorylation of the last three serines.

PKC, but not PKA or CaMKII, phosphorylates last three serines of Neurexin-1 in both HEK293T cells and cultured neurons.

It is known that synaptic scaffold protein CASK phosphorylates last three serines in cytoplasmic tail of Neurexin-1. In the hippocampal cultures, Neurexin phosphorylation is reduced 40% in the CASK knockout mice [31]. Next question is which kinases phosphorylate remaining 60% of Neurexin? To address this question, I transfected HEK293T cells with Flag-Neurexin-1 construct and induced kinase activities using chemicals (TPA for PKC, forskolin for PKA) or an active form CaMKII (CaMKII T286D) construct together with phosphatase inhibitors (Calyculin A and Cyclosporin), and monitored phosphorylation of transfected Neurexin-1 by mobility shift on Phos-tag gel. In this experiment, I found PKC, but not PKA or CaMKII, phosphorylated Neurexin-1 in transfected HEK293T cells (Figure 5A and 5B). Alanine substitution for last three serines abolished the mobility shift after TPA treatment suggesting that these serines are the target of PKC (Figure 5C). A similar result was obtained in cultured mouse neurons in which 2xHA-Neurexin-1 was introduced by lentiviral infection (Figure 5D).

Phosphorylation of the last three serines in Neurexin-1 decreases synaptogenic activity in co-culture system.

In order to study the physiological consequence of this phospholylation in cytoplasmic tail of Neurexin-1, I employed heterologous synapse formation assay that

can evaluate synapse formation between HEK293T cells and co-cultured hippocampal neurons (Figure 6A). In this system, HEK293T cells were transfected with EGFP tagged-neuroligin-1(EGFP-NL1) and co-cultured neurons were infected with lentiviruses that express neurexin shRNAs or neurexin rescue genes (Figure 6B). Co-cultured neurons with control infection formed synapses on Neuroligin-1 expressing HEK293T cells (Figure 6B: L315 control/None, Figure 6C). The level of the synapse formation was dramatically decreased when neurexins were knocked down in co-cultured neurons (Figure 6B: NrxnTKD, None, Figure 6C). This reduction was rescued or reversed when neurexin knocked down neurons were superinfected with wild-type neurexin-1β expressing viruses (Figure 6B: NrxnTKD/2HA-Nrxn1β WT, Figure 6C). But this rescue effect was not observed when superinfected with SD6-8 mutant neurexin-1β (Figure 6B: NrxnTKD/2HA-Nrxn1β SD6-8, Figure 6C). Superinfection with SA6-8 neurexin-1β rescued synapse formation up to control level (Figure 6B: NrxnTKD, 2HA-Nrxn1β SA6-8, Figure 6C). These result suggest that phosphorylation of the last three serines in Neurexin-1 decreases synaptogenic activity

Neurexin-1 cytoplasmic tail binds to phosphoinositides involved in the protein trafficking.

What is the consequence of the interaction between phospholipid and the cytoplasmic tail of Neurexin-1? It is known: (1) Phosphatidy-inositolphosphates can rapidly switch between different forms through phosphorylation of the inositol ring (at carbon positions 3, 4 and 5) by kinases, and through its dephosphorylation by phosphatases. (2) Different phosphoinositides are involved in different stages of exocytosis, endocytosis, phagocytosis, protein targeting and trafficking. I performed lipid binding assay using another lipid strip that contains more variants of phosphatidyl inositols. In this experiment, I found that Neurexin-1 interacts most of PIP, PIP2, and PIP3 (Figure 7).

Neurexin-1 cytoplasmic tail interacts with AP-2, an adaptor protein involved in clathrin-mediated endocytosis.

I performed pull-down experiments with GST-Neurexin-1 cytoplasmic tail and found it interacted with AP-2, an adaptor protein involved in clathrin-mediated endocytosis (Figure 8). The phosphorylation of the last three serines of cytoplasmic tail of Neurexin-1 did not affect this interaction, but phosphorylation of all eight serines abolished the interaction, suggesting that phoshporylation of one or more serines within 1-5 may regulate the interaction with AP-2.

Discussion

In my PhD work, I found: (1) Neurexins were specifically localized at presynaptic terminals. (2) Neurexins were required for presynaptic functions. (3) The cytoplasmic tail of Neurexin-1 was intrinsically disordered. (4) The cytoplasmic tail of Neurexin-1 interacted with phosphatidyl inositols and the interaction was abolished by phosphorylation of the last three serines. (5) The last three serines were phosphorylated by PKC. (6) Signals for synapse formation were negatively regulated by phosphorylation of the last three serines of Neurexin-1. (7) Neurexin-1 cytoplasmic tail bound to phosphoinositides involved in subcellular protein trafficking. (8) Neurexin-1 cytoplasmic tail interacted with AP-2, an adaptor protein involved in clathrin-mediated endocytosis. The phosphorylation of the last three serines of cytoplasmic tail of Neurexin-1 did not affect the interaction between Neurexin-1 and AP-2, but phosphorylation of all eight serines abolished the interaction.

Neurexins have been considered to be major presynaptic organizers. Although several evidences suggested that they are presynaptic, the specificity had remained elusive. Indeed, there is a report that overexpressed Neurexin-1β is localized at both pre- and postsynapses and the postsynaptic Neurexin-1β disturbs the function of Neuroligin by cis-interaction [32]. This is probably due to the artifact of the overexpression. My immuno electronmicroscopy experiment did not detect endogenous Neurexins in postsynapse. The antibody was raised against the last nine amino acids of Neurexins and this sequence is conserved in all Neurexin isoforms. Thus, I could conclude Neurexins are very presynaptic.

The structure of Neurexins is extremely complicated. Three genes have two independent promoters that produce α- and β-Neurexins, resulting in the production of six canonical isoforms [5]. Furthermore, several different combinations of exons are inserted into their five alternative splice sites. As a result, up to ~4,000 Neurexin isoforms are expressed in mammals [5]. Thus far, α- specific or single Neurexin knockout mice have been studied [33-35], but due to the complicated structure, knockout mice of all isoform of Neurexins have not been generated. I successfully knocked down all isoforms of Neurexins in cultured neurons using shRNAs targeted α- and β- common coding region of all three neurexin genes. In the whole cell patch clamp electrophysiology, I found that the frequency of mEPSCs was significantly decreased, whereas the amplitude was unchanged. This suggests that Neurexin function is specific to presynapse, which is consistent with my immuno EM result.

While Neurexins bind several postsynaptic ligands, Neurexins seem to be unique presynaptic receptors, at least, for Neuroligins. In my co-culture synapse formation assay, Neuroligin-1 on HEK293T cells failed to induce presynaptic structure when all three Neurexins were knocked down in co-cultured neurons. Synaptogenic activity of Neuroligins has been intensively studied in this co-culture assay. It has been demonstrated that presynaptic apparatus, such as synaptic vesicles, are accumulated at synaptic terminals formed on Neuroligin expressing fibloblasts [16, 17, 36]. These results suggest that Neurexin could induce signals for presynaptic differentiation triggered by binding to Neuroligins. But the interacellular signaling mediated by Neurexins remained elusive. I first examined the structure of cytoplasmic tails of Neurexins and found that they are intrinsically disordered. Based on the property of this type of proteins, I examined and found that at least C-tail of Neurexin-1 bound phospholipids and the affinity was deleted by serine phosphorylation of Neurexin-1. My result in co-culture assay indicates that this phosphorylation affects synapse formation. But the mechanism is still unsolved. One clue to the mechanism is the studies in Transmembrane AMPA regulatory protein (TARP), an AMPA receptor binding protein that transports AMPA receptors to postsynaptic site. Like Neurexins, C-tail of TARP is also enriched with basic amino acids surrounded by nine serine phosphorylation sites. This region also binds to PI(4,5)P2 and the serine phosphorylation remove the affinity to PI(4,5)P2 [37]. Unlike Neurexins, these serines are phosphorylated in the steady state level. When they are dephosphorylated, TARP starts coupling with PI(4,5)P2 and AP-2 and consequently it is endocytosed in the clathirine mediated manner [38, 39]. Phosphorylation of Neurexin may also be involved in the endocytosis of the protein. But if Neurexin undergoes same mechanism as TARP, phosphorylation should increase the surface localization of Neurexins. The present results are somewhat controversial to this, because phospho mimic mutation (SD6-8) decreased the synaptogenic activity in co-culture assay. Further studies are needed to solve this problem.

Recent genome wide screenings implicate Neurexins to be involved in neuropsychiatric disorders including Autism Spectrum Disorders (ASDs) [40, 41]. This is likely due to the dysfunction of synapse because Neuroligins also have been implicated to be involved in ASDs [42, 43]. Continuous study of intracellular signaling mediated by Neurexins will provide insight into not only basic understanding of synaptogenesis, but also the pathophysiology of ASDs.

Acknowledgements

I would like to thank Professors Katsuhiko Tabuchi at Shinshu University, Makoto

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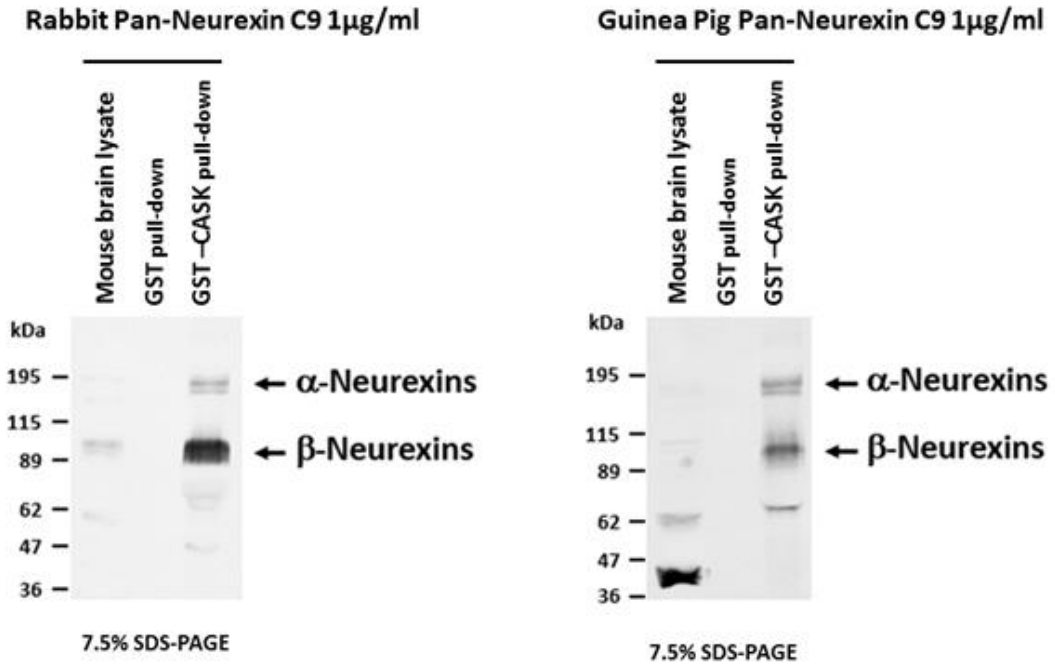
A



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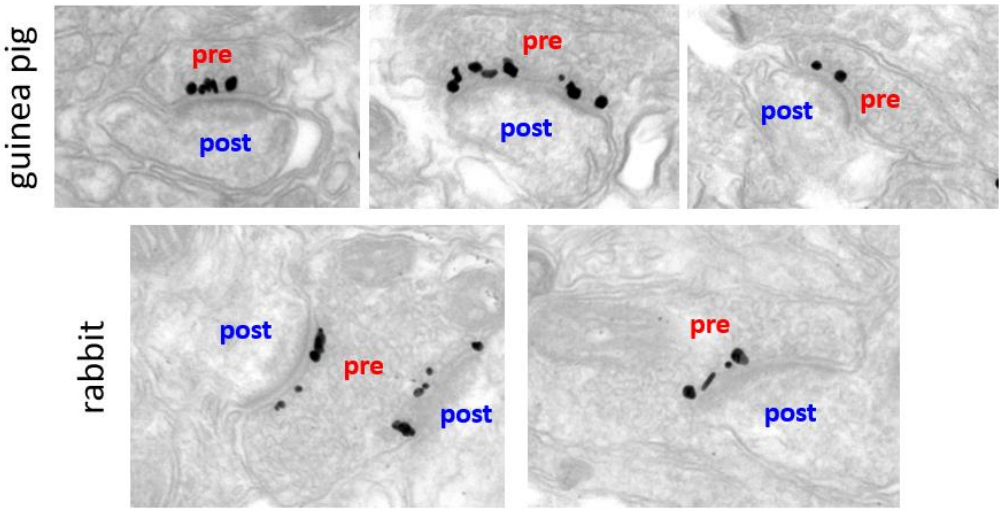


Figure 1: Neurexin proteins are specifically localized at presynaptic terminal. (A) Specificity test for rabbit (left) and guinea pig (right) Pan-Neurexin C9 antibodies. Both antibodies detected weak signals from mouse brain lysate corresponding to α- (~180 kDa) and β- (~100 kDa) Neurexins (left lanes). These signals became stronger when endogenous Neurexins were concentrated by GST-CASK affinity pull-down (right lanes). (B) Pre-embedding immuno electron microscopy in parallel fiber-Purkinje synapses using Pan-Neurexin C9 antibodies. Immunoreactivity to Neurexins was detected specifically at presynaptic terminals in both guinea pig (upper panels) and rabbit (lower panels) Neurexin antibodies.

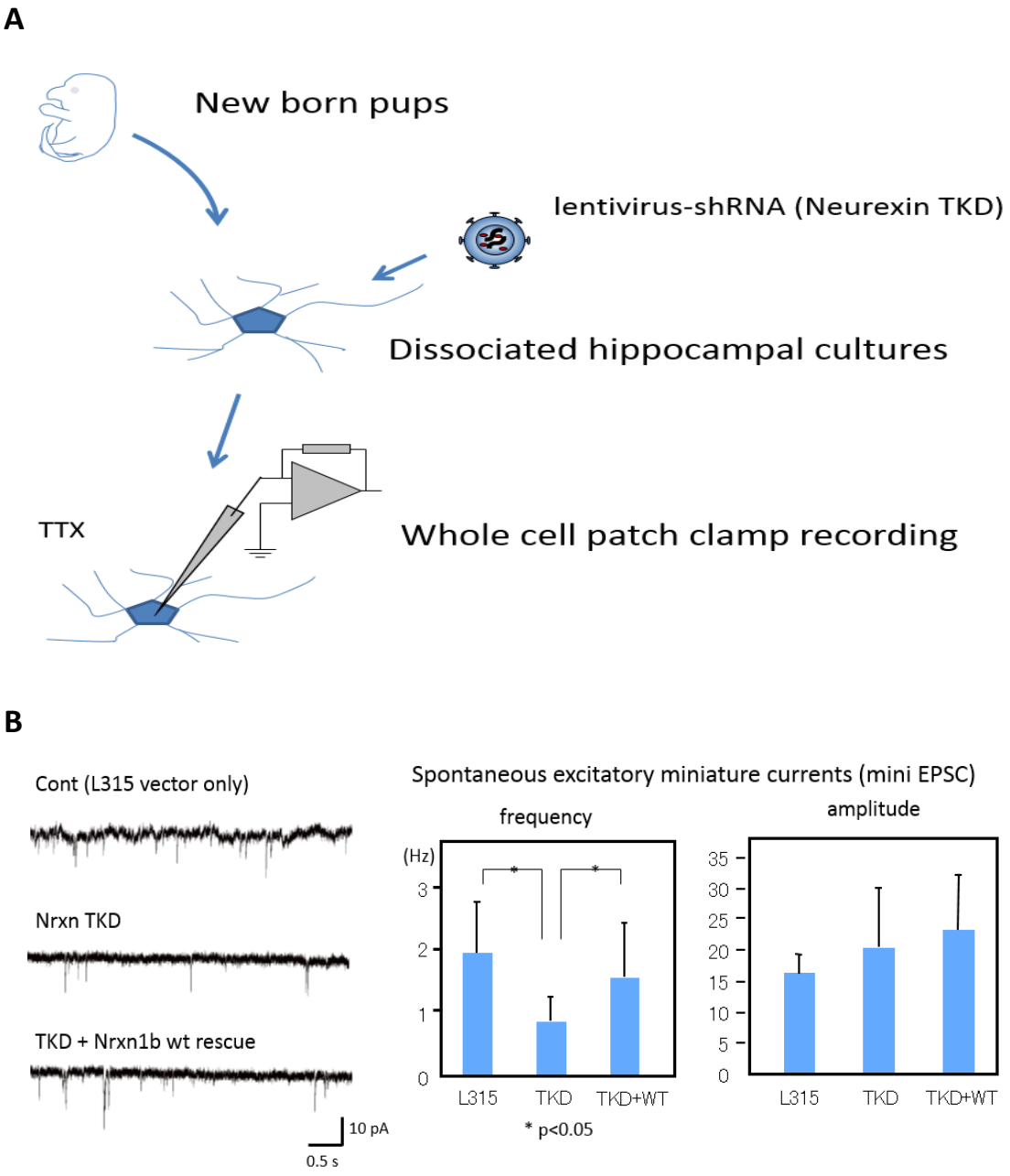
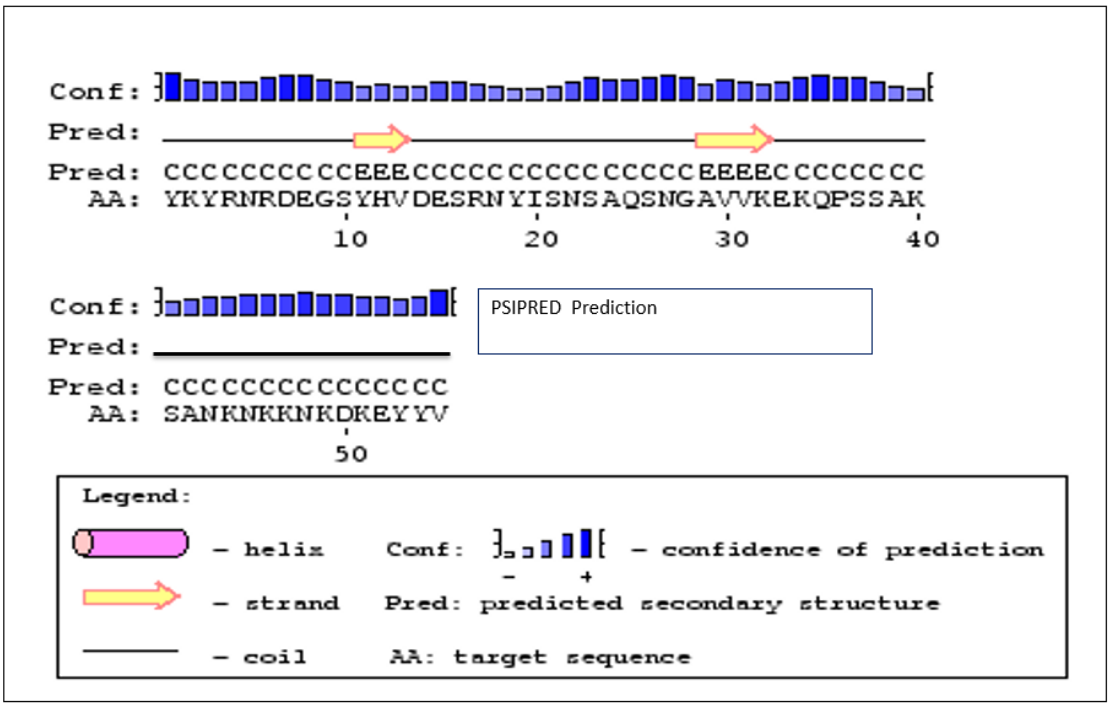


Figure 2: Neurexins are required for presynaptic activity. (A) Schematic diagram of electrophysiology experiment in dissociated mouse hippocampal neuron cultures in which all three neurexins were knocked down by lentivirus mediated shRNA introduction. Spontaneous miniature excitatory postsynaptic currnts (mEPSCs) in the presence of tetorodotoxin (TTX) were analyzed in this experiment. (B) Frequency of miniature excitatory postsynaptic currents were signifiantly decreased in Neurexin triple knockdown (TKD) neurons compared to control (L315). This effect was rescued by co-infection with wild-type Neurexin-1β expressing viruse (TKD + WT). Significant change in amplitude was undetectable in Neurexin triple konockdown neurons (TKD).

A



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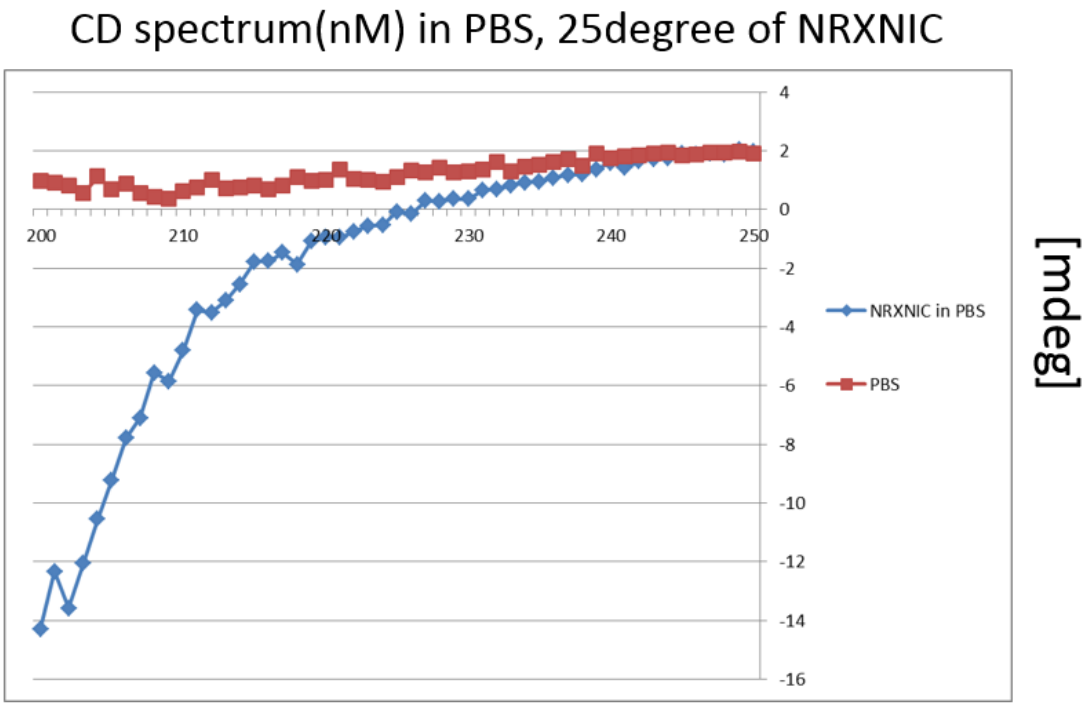
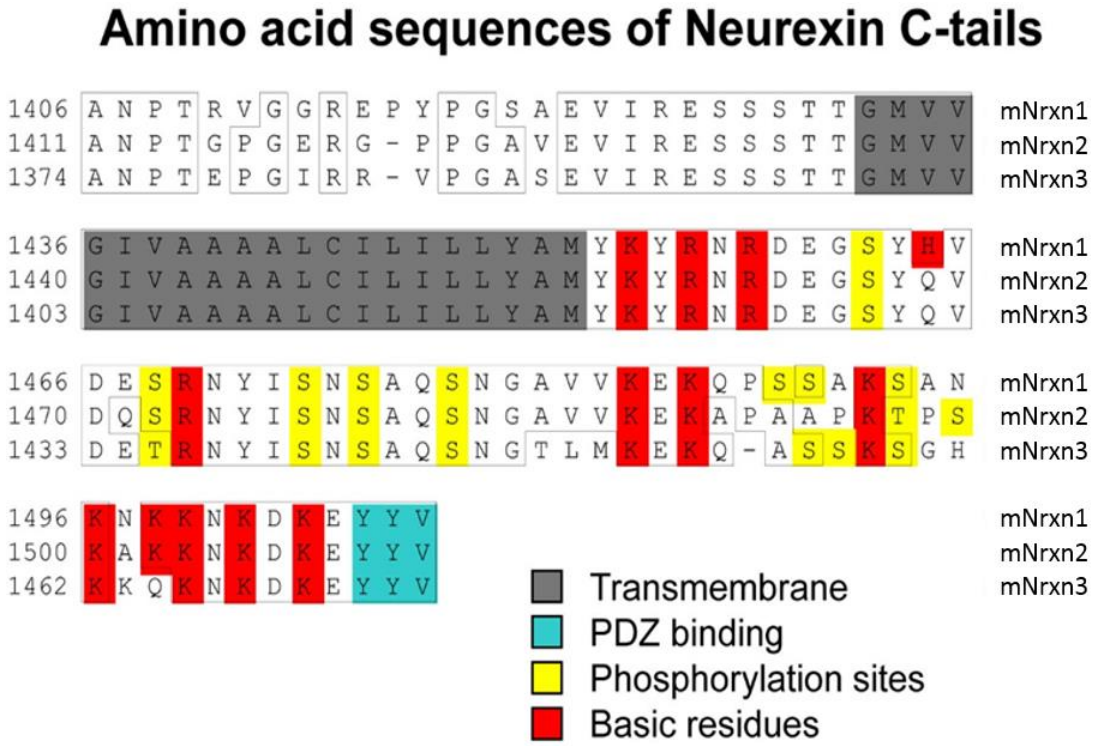


Figure 3: The cytoplasmic tail of Neurexin-1 is intrinsically disordered. (A) The bioinformatics software named "PSIPRED" predicted that cytoplasmic tail of rat Neurexin-1 (Neurexin-1C) was intrinsically disordered. (B) The trait of intrinsically disordered structure was confirmed by using circular dichroism (CD) spectroscopy in His-tag rat Neurexin-1C protein.

A



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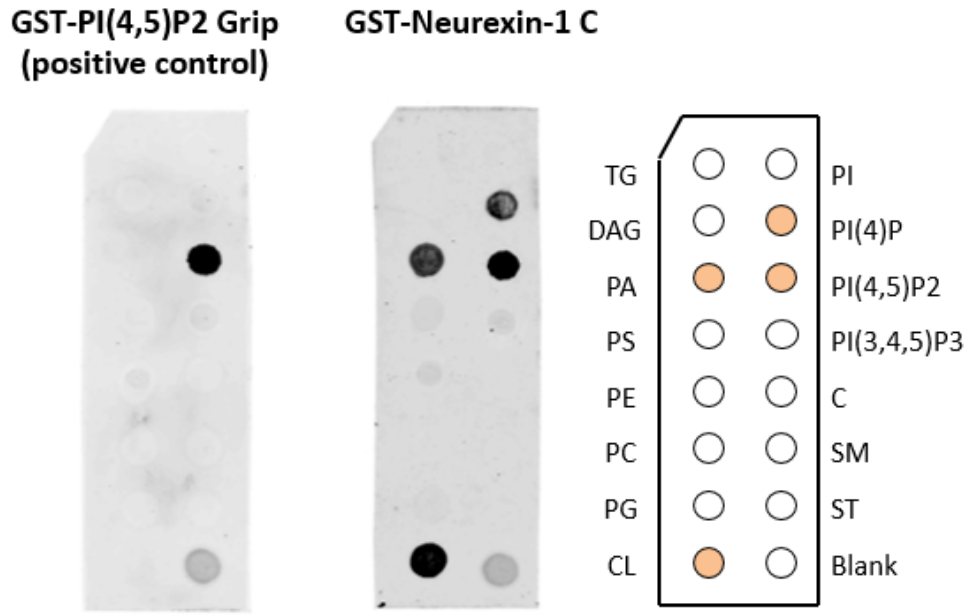
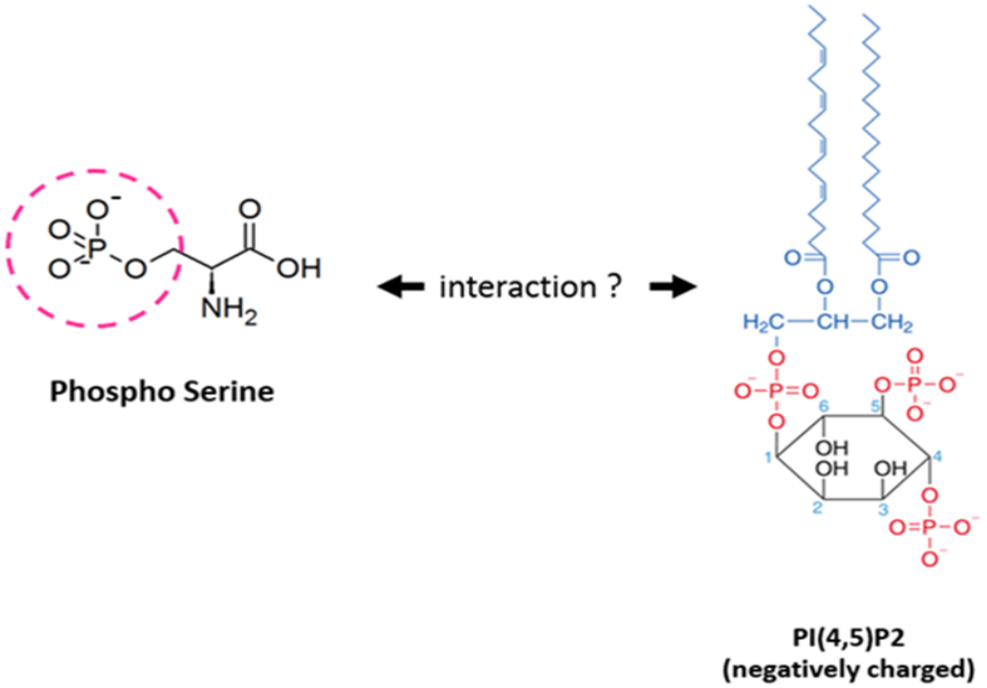


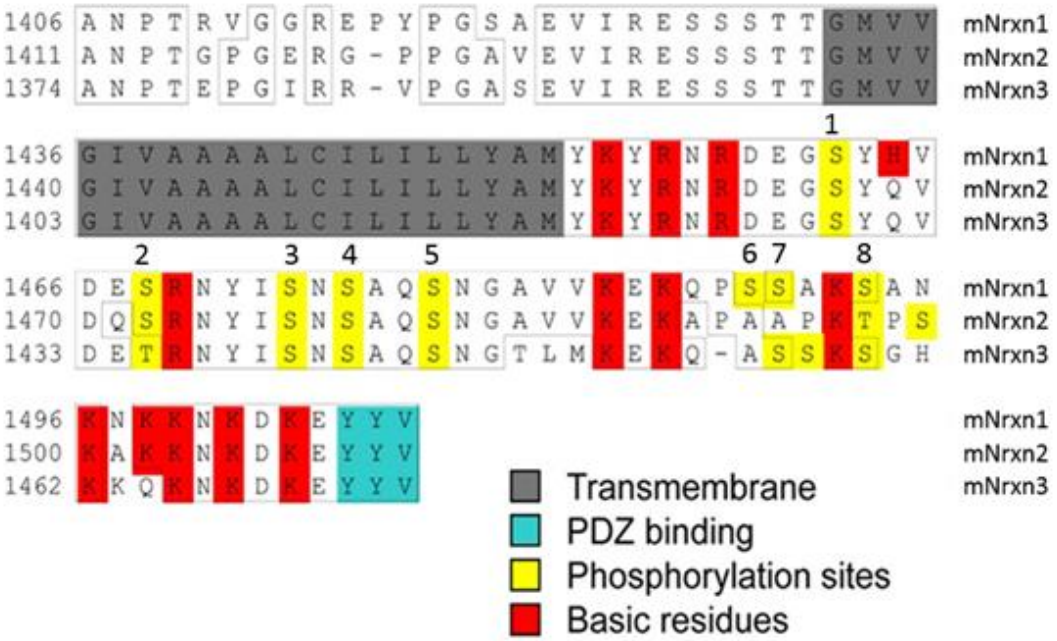
TG: Triglyceride, PI: Phosphatidylinositol, PIP: Phosphatidylinositol (4) Phosphate, PIP2: Phosphatidylinositol (4,5) Bisphosphate, PIP3: Phosphatidylinositol (3,4,5) TrisPhosphate, PS: Phosphatidylserine, PA: Phosphatidic Acid, PE: Phosphatidylethanolamine, DAG: Diacylglycerol, C: Cholesterol, PC: Phosphatidylcholine, SM: Sphingomyelin, PG: Phosphatidylglycerol, ST: 3-sulfogalactosylceramide (Sulfatide)
CL: Cardiolipin

C



D

Phospho-mimic mutations of Neurexin C-tail



* SD1-8: all serines (number 1-8) in Neurexin-1C are mutated to aspartic acids.
** SD6-8: the last three serines (number 6-8) in Neurexin-1C are mutated to aspartic acids.

E

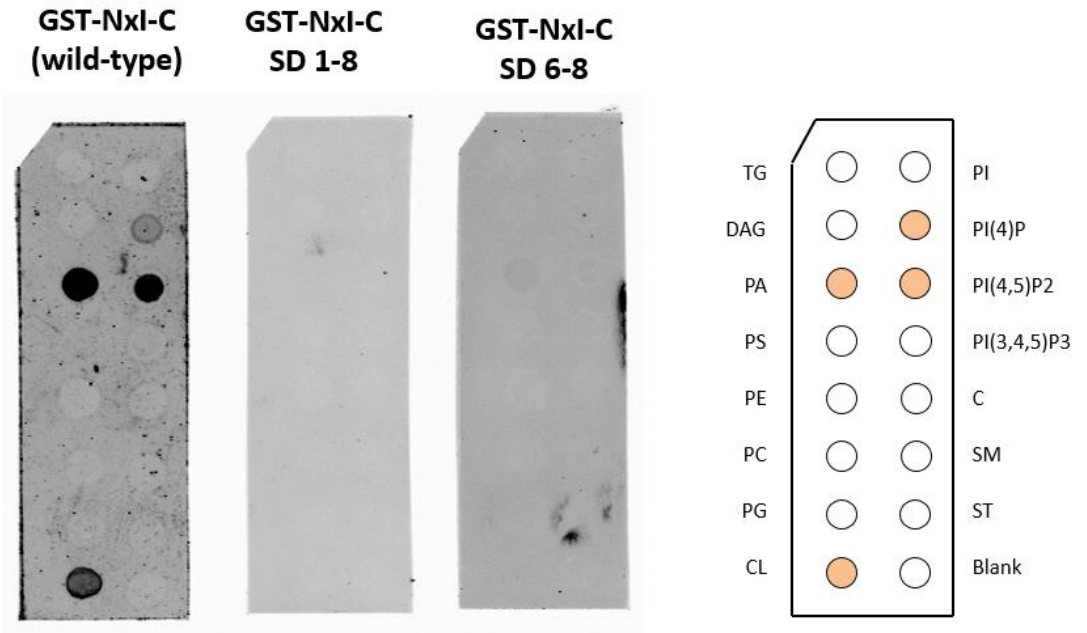
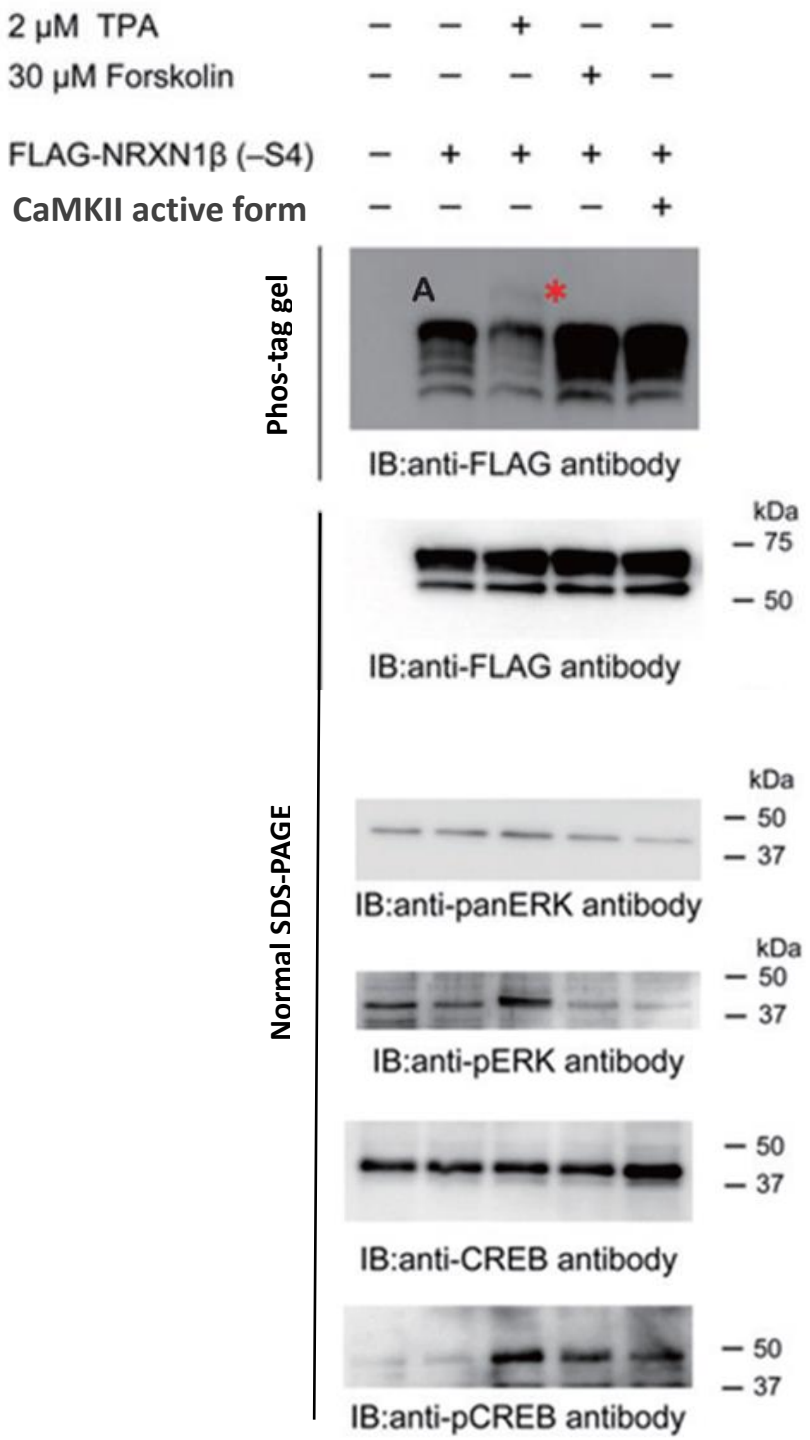


Figure 4: Positively charged cytoplasmic tail of Neurexin-1 binds to negatively charged phospholipids. (A) Amino acid sequence alignment of cytoplasmic tail of mouse Neurexin-1, -2, and -3. These regions are enriched by basic amino acids (histidine[H], lysine[K], and arginine[R]), as well as serines that are potential phosphorylation sites. (B) GST-Neurexin-1 c-tail fusion protein showed interaction with phospholipids, including PIP: Phosphatidylinositol (4) Phosphate, PIP2: Phosphatidylinositol (4, 5) Bisphosphate, PIP3: Phosphatidylinositol (3, 4, 5) Tris Phosphate, PA: Phosphatidic; CL: Cardiolipin in dot blot experiment. (C) Phosphorylation of serine confers negative charged to the proteins. (D) Two different phospho-mimic mutants of GST-Neurexin-1C protein, SD1-8 and SD6-8 were produced. SD1-8 substitutes aspartic acids for all eight serines. SD6-8 substitutes aspartic acids for only the last three serines. Numbers (1-8) in the sequence alignment indicate serines for mutation. (E) Both SD1-8 and SD6-8 mutations abolished the interaction with phospholipids, suggesting that the phosphorylation of the last three serines is enough to affect the interaction.

A


2 μM TPA
30 μM Forskolin
FLAG-NRXN1β (–S4)
CaMKII active form
Phos-tag gel
A
IB:anti-FLAG antibody
Normal SDS-PAGE
kDa
75
50
IB:anti-FLAG antibody
kDa
50
37
IB:anti-panERK antibody
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50
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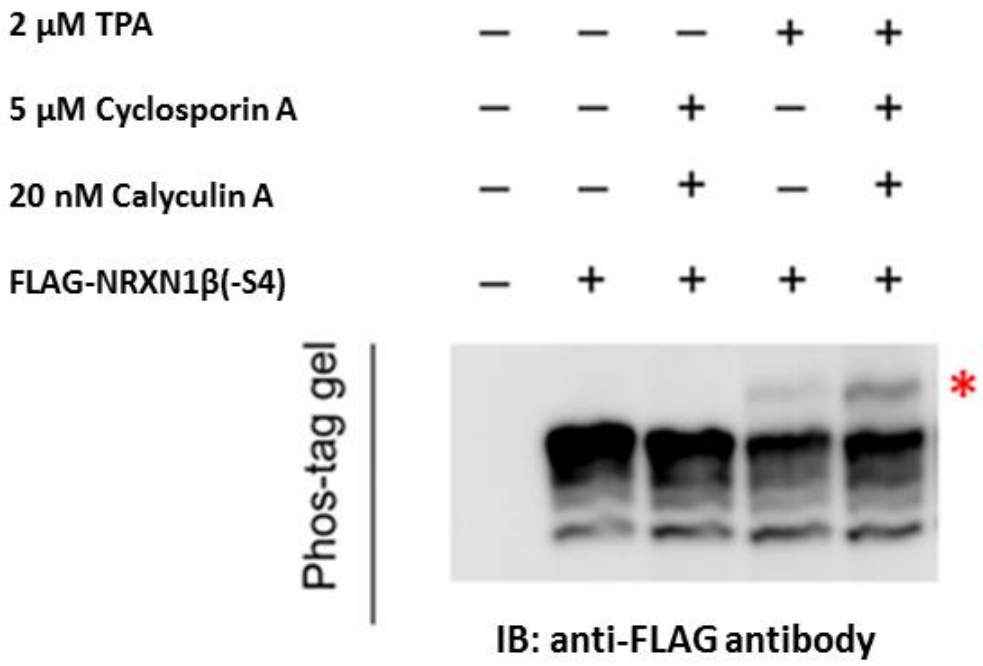
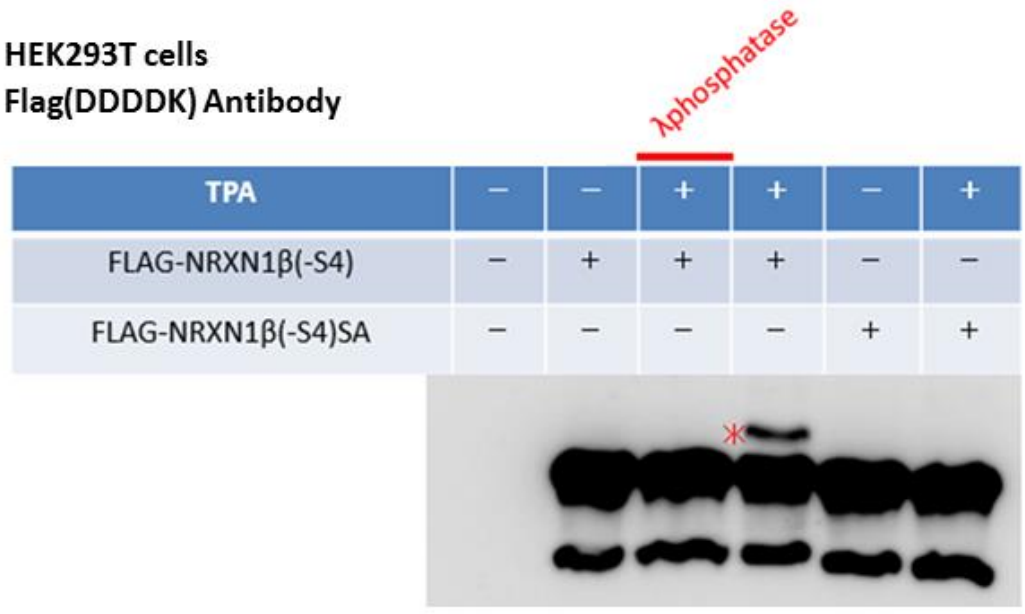
IB:anti-pERK antibody
50
37
IB:anti-CREB antibody
50
37
IB:anti-pCREB antibody

B


2 μM TPA
5 μM Cyclosporin A
20 nM Calyculin A
FLAG-NRXN1β(-S4)
– – – + +
– – + – +
– – + – +
– + + + +
Phos-tag gel
*
IB: anti-FLAG antibody

C


HEK293T cells
Flag(DDDDK) Antibody
λphosphatase
TPA – – + + – +
FLAG-NRXN1β(-S4) – + + + – –
FLAG-NRXN1β(-S4)SA – – – – + +
*

D

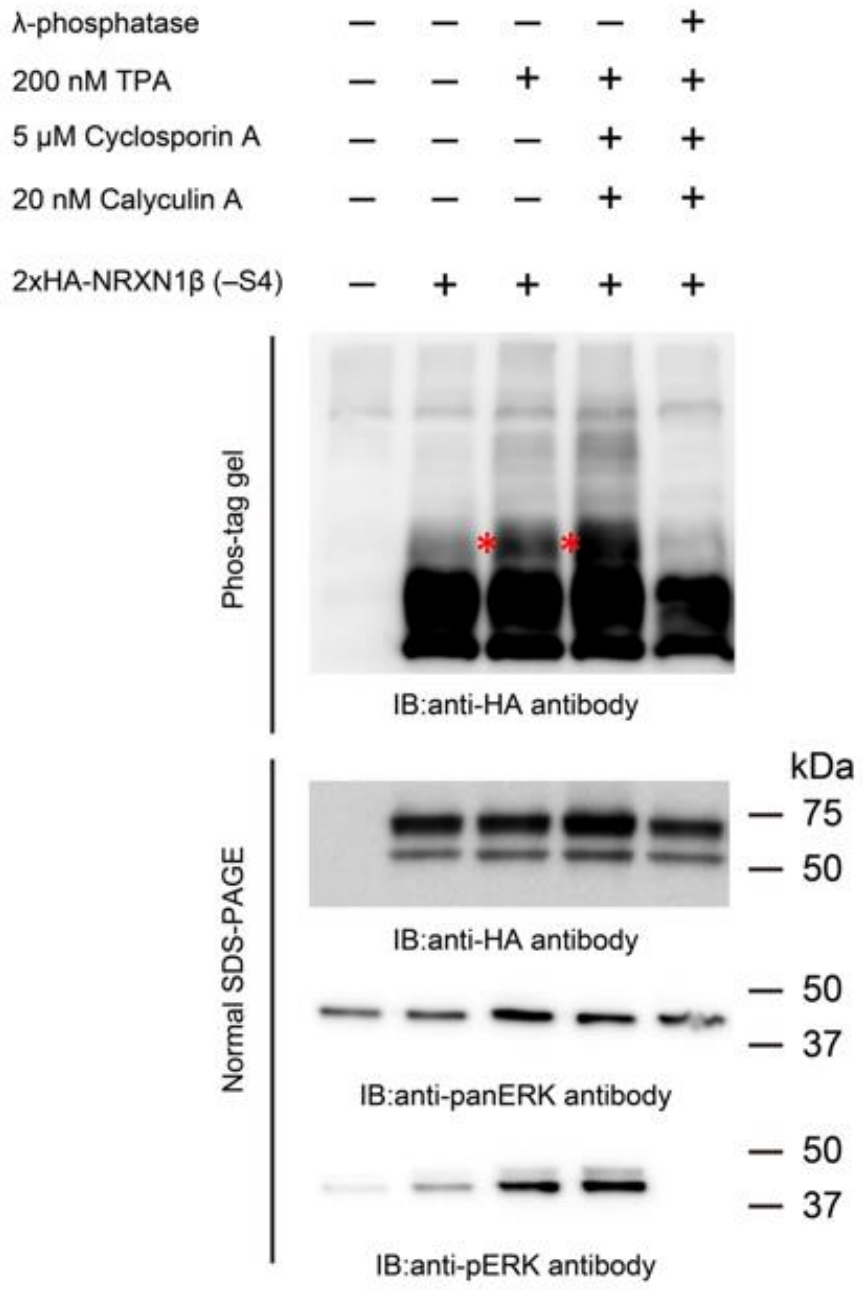


Figure 5: PKC, but not PKA and CaMKII, phosphorylates the last three serins of Neurexin-1 both in HEK293T cells and cultured mouse neurons. (A) Induction of PKC signals by TPA in HEK293T cells showed mobility shift of Neurexin-1 protein (asterisk) on phos-tag gel indicating the phosphorylation. Forskolin and CaMKII did not show mobility shif on the phos-tag gel. (B) Addition of phosphatase inhibitors (Cyclosporin A and Calyculin A) enhanced the phosphorylation (asterisk). (C) Alanine substitution for last three serines abolished mobility shift after TPA treatment. (D) Induction of PKC activation phosphorylated Neurexin-1 in cultured mouse neurons.

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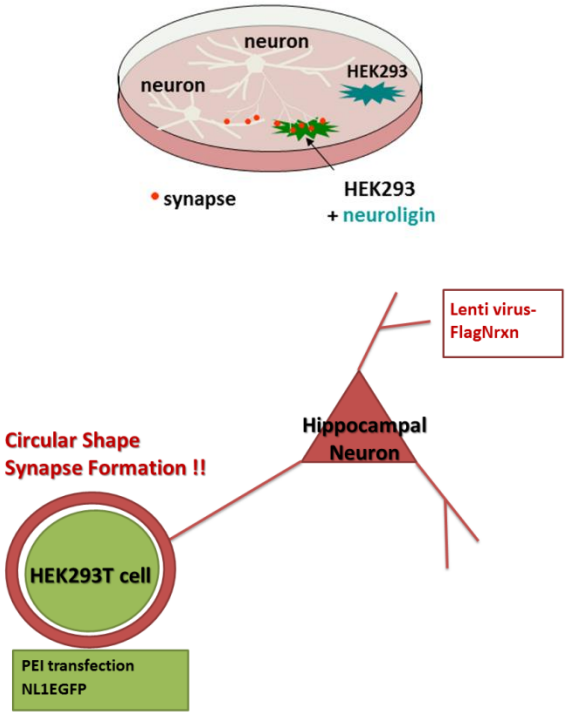
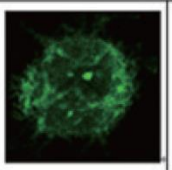
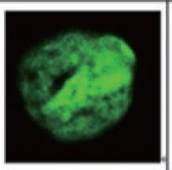
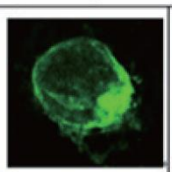
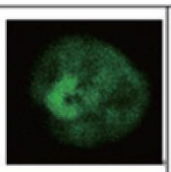
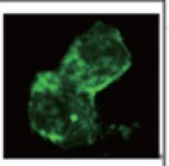
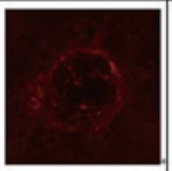
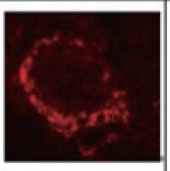
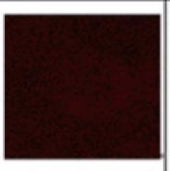
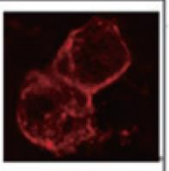
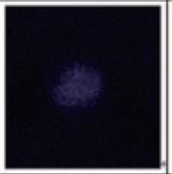
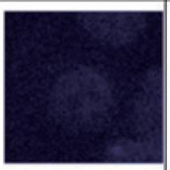
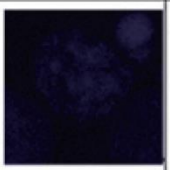
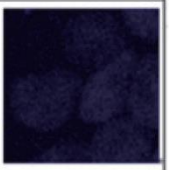
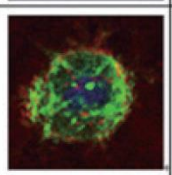
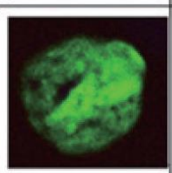
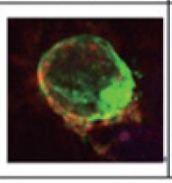
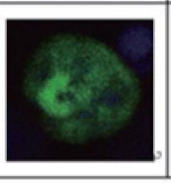
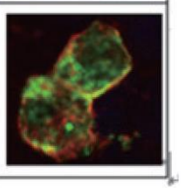


B

	L315 control	NrxnTKD			
Rescue	None	None	2HA-Nrxn1β WT	2HA-Nrxn1β SD6-8	2HA-Nrxn1β SA6-8
	HEK293T cells / EGFP-NL1				
EGFP					
Synapsin					
DAPI					
Merge					

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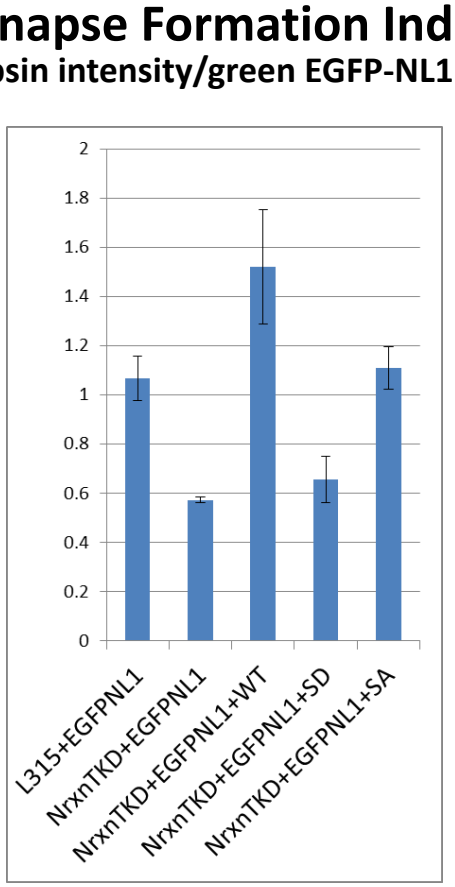

Figure 6: Synapse formation is decreased by SD6-8, but not SA6-8, mutation in Neurexin-1 in co-culture system. (A) Schematic diagram of neuron-HEK293T co-culture assay that can monitor synaptogenic activity for cell adhesion molecules. Artificial synapses between cultured mouse neurons and HEK293T cells were detected by anti-synapsin I antibody. (B) HEK293T cells were transfected with EGFP fused rat Neuroligin-1 (EGFP-NL1) and co-cultured with mouse neurons infected with control lentivirus (left lane) or lentivirus expressing neurexin-1, -2, -3 shRNAs (Nrxn TKD) as well as rescue construct expressing lentiviruses, as indicated. In this system, co-cultured neurons without knock down virus form synapses on Neurolign-1 expressing HEK293T cells (left lane). The synapse formation was significantly decreased when neurexins were knocked down in co-culture neurons (Nrxn TKD/None). When the neurons were superinfected with wild-type neurexin-1β expressing viruses, increased level of synapse formation was detected on HEK293T cells (Nrxn TKD/2HA-Nrxn1β WT). When neurons were superinfected with SD6-8 mutant neurexin-1β, the level of synapse formation was not restored as wild-type. When neurons were superinfected with SA6-8 mutant neurexin-1β, the level of synapse formation was restored up to wild-type level. (C) Statistical analysis of synapse formation in (B).

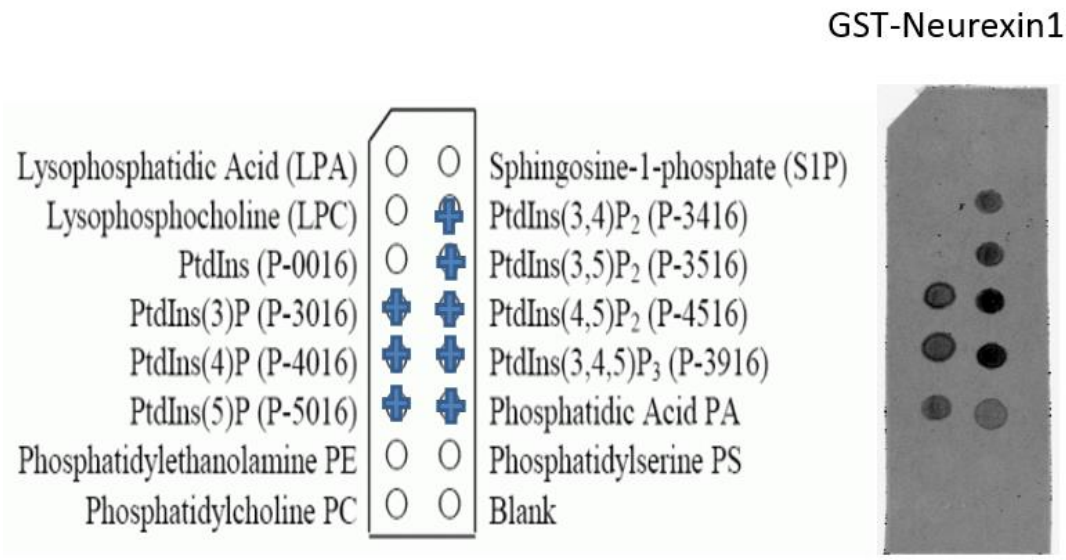

Figure 7: Neurexin-1 cytoplasmic tail binds to phosphoinositides involved in protein trafficking. (A) Dot blot experiment using another lipid strip probed with GST-Neurexin-1 c-tail fusion protein. Neurexin-1 showed interaction with PI3P, PI4P, PI5P, PI(3,4)P2, PI(3,5)P2, PI(4,5)P2, and PI(3, 4, 5)P3.

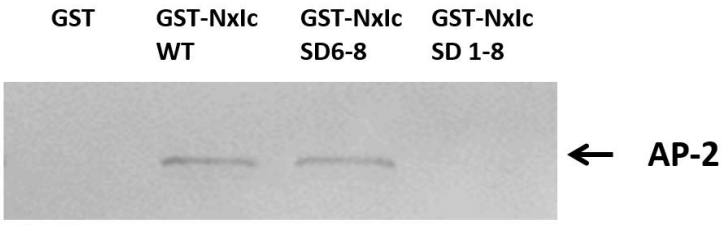

Figure 8: Neurexin-1 cytoplasmic tail interacts with AP-2, an adaptor protein involved in clathrin-mediated endocytosis. (A) Cytoplasmic tail of Neurexin-1 interacted with AP-2. This interaction was independent of phosphorylation of the last three serines of Neurexin-1. But phosphorylation of all eight serines abolished the interaction.

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