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Supplementary Information for

PSD-95 Binding Dynamically Regulates NLGN1 Trafficking and Function

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SI Materials and Methods

Plasmids and antibodies.

pCAG-HA-NLGN1 WT (or S839A or S839E)-IRES-mCherry, pCAG-NLGN miRs-GFP, pCMV-myc-rat PKA T198E, and PSD-95-myc (or GFP) plasmids were used for biochemical and imaging experiments. pCAG-HA-NLGN1 and pCAG-NLGN miRs-GFP plasmids were characterized in a previous study (1). The rat PKA catalytic subunit was subcloned into the pcDNA3.1/myc-His vector (Invitrogen). All point mutations were generated by PCR-based mutagenesis (QuickChange Site-Directed Instruction Manual). To generate the phosphorylation state-specific antibody (pS839-Ab), the synthetic phosphopeptide Ac-CHPHPHSH(pS)TTR-amide corresponding to amino acids 832-842 of NLGN1 was immunized to rabbits by New England Peptide. Sera were collected and affinity purified with an antigen phosphopeptide. The commercial antibodies used in this study were mouse anti-NLGN1 (Synaptic Systems, clone 4C12), rabbit anti-GST (Bethyl Laboratories), mouse anti-PSD-95 (Neuromab, clone K28/43), guinea pig anti-VGLUT1 (Millipore, AB5905), mouse anti-myc (Cell Signaling, clone 9B11), rabbit anti-PKA (Cell Signaling, 4782), rabbit anti-CREB (Cell Signaling, 48H2), rabbit anti-pCREB (Cell Signaling, 87G3), rabbit anti-HA (Abcam, ab9110), rat anti-HA (Roche), rabbit anti-β tubulin (Sigma, T2200), mouse anti-β actin (ABM, G043), and mouse anti-transferrin receptor (Thermo Fisher Scientific, #13-6800).

Lambda protein phosphatase assay.

The protein phosphatase assay was performed following the manufacturer's instructions. Briefly, we cotransfected HA-NLGN1 WT and the constitutively active PKA catalytic subunit in HEK293 cells. HA-NLGN1 was immunoprecipitated using anti-HA agarose resin (Thermo Fisher Scientific, #26181) and the beads were washed with PBS. The beads were incubated with or without lambda protein phosphatase (NEB, P0753S) in the phosphatase buffer for 30 min at 37°C. The phosphatase reaction was stopped by adding 2 x SDS sample loading buffer.

and subsequently washed with TBS buffer containing 1 mM EDTA and 0.1% Triton X-100.

Neuronal cultures.

For biochemical analyses and immunocytochemistry experiments, we used primary cultured cortical and hippocampal neurons from embryonic day 18 (E18) Sprague Dawley rats of either sex. Briefly, embryonic hippocampal or cortical tissues were dissociated at 37°C for 30 min by 0.05% trypsin in 10 mM HBSS containing 1.37 mg/ml DNase I. Neurons were plated and maintained in serum-free Neurobasal Medium supplemented with 2% (vol/vol) B-27 and 2 mM L-Glutamine. We adhered to the guidelines of the National Institutes of Health's Animal Care and Use Committee regarding the care and use of animals for this study.

Transfection and immunoblot.

HEK293 cells were transfected with Lipofectamine^{2000} and 2 days after transfection the cells were lysed in a TBS buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100. Lysates were centrifuged at 16,000*g* for 15 min at 4°C and supernatant were used for the analysis. For coimmunoprecipitation, lysates were incubated with an appropriate antibody at 4°C overnight and protein-A-Sepharose beads (GE Healthcare) at 4°C for 1 hour. Cultured cortical neurons were transfected at DIV 5 using calcium phosphate method as described previously (2). The neurons were further lysed and fractionated for desired experiments. All chemiluminescence blots were captured with a ChemiDoc™ Imaging System (Bio-Rad).

Pharmacological treatments on cultured neurons.

Cultured neurons were treated with chemical reagents as descibed for the biochemical experiments. Neuronal cultures were treated with 100 μM forskolin (Sigma, F3917) for 10 min at 37°C to activate PKA. Neurons were treated with 20 mM KCl for 1 h at 37°C to activate neurons and 20

μM GABA or 20 μM glutamate were applied for 10 min at 37°C to stimulate neurotransmitter receptors.

GST fusion protein production.

Cytoplasmic regions of NLGNs were subcloned into the pGEX-4T plasmid and transformed to BL21 bacterial cells. Bacterial cultures were grown at 37°C to an absorbance at 600 nm of 1.1–1.2 of the culture media. 50 μM isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to the cultures and incubated at 16°C overnight to induce fusion protein expression. The bacterial pellets were then lysed in a Tris-buffered saline (TBS) buffer containing protease inhibitors (Roche), 100 μg/ml lysozyme, 15 mM dithiothreitol (DTT), 10 mM ethylenediaminetetraacetic acid (EDTA), and 1.5% sarkosyl. The sonicated lysate was neutralized with Triton X-100 to a final concentration of 4%. The lysates were incubated with glutathione–Sepharose 4B (GE Healthcare) for 1 hour at 4°C

Mass spectrometry.

In-gel samples were digested with chymotrypsin at 25°C for 18 hours. Peptides were extracted then desalted before being injected into a nano-LC/MS/MS system where an Ultimate 3000 HPLC (Thermo-Dionex) was coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific) via an Easy-Spray ion source (Thermo Scientific). Peptides were separated on a ES802 Easy-Spray column (75-μm inner diameter, 25 cm length, 3 μm C18 beads; Thermo Scientific) with a 25-min linear gradient of 2–27% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 98% acetonitrile, 0.1% formic acid). The HPLC flow rate was 300 nl/min.

Thermo Scientific Orbitrap Elite mass spectrometer was operated in positive data-dependent LC-MS/MS mode. The resolution of the survey scan was set at 60k at m/z 400. The m/z range for MS scans was 300–1600. For MS/MS data acquisition, the decision-tree mode was activated, the

minimum signal intensity required to trigger MS/MS scan was 1e4, the top ten most abundant ions were selected for product ion analysis, the isolation width was 1.9 m/z, and the dynamic exclusion window was 9 sec.

Xcalibur RAW files were converted to peak list files in mgf format using Mascot Distiller. Database search was performed using Mascot Daemon (2.4.0) against NCBInr_Human database. Spectra of phosphopeptides were manually checked.

SI Figure legends

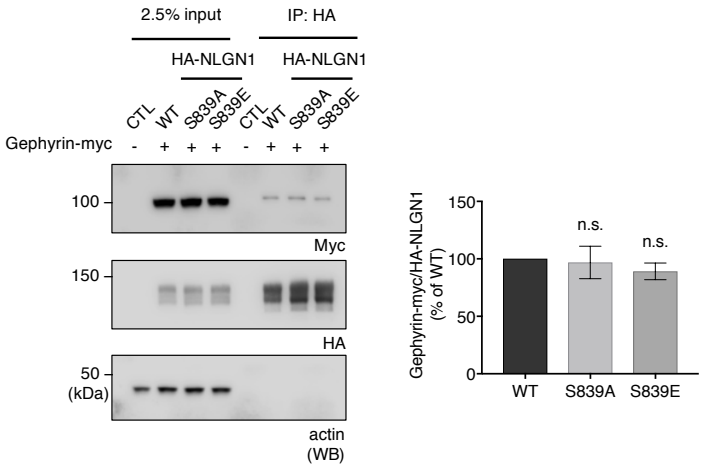

Fig. S1. NLGN1 S839 phosphorylation does not affect gephyrin binding.

HA-NLGN1 (WT, S839A or S839E) and Gephyrin-myc were transfected in HEK293 cells as indicated in the figures. Gephyrin-myc that co-precipitated with NLGN1 was analyzed by immunoblotting. The coimmunoprecipitated ratio of Gephyrin-myc/immunoprecipitated NLGN1 was analyzed. Graph indicates mean ± SEM (n=5). The statistical significance between the mean of WT and the mean of each condition was calculated using one-way ANOVA with Dunnett's multiple comparison test.

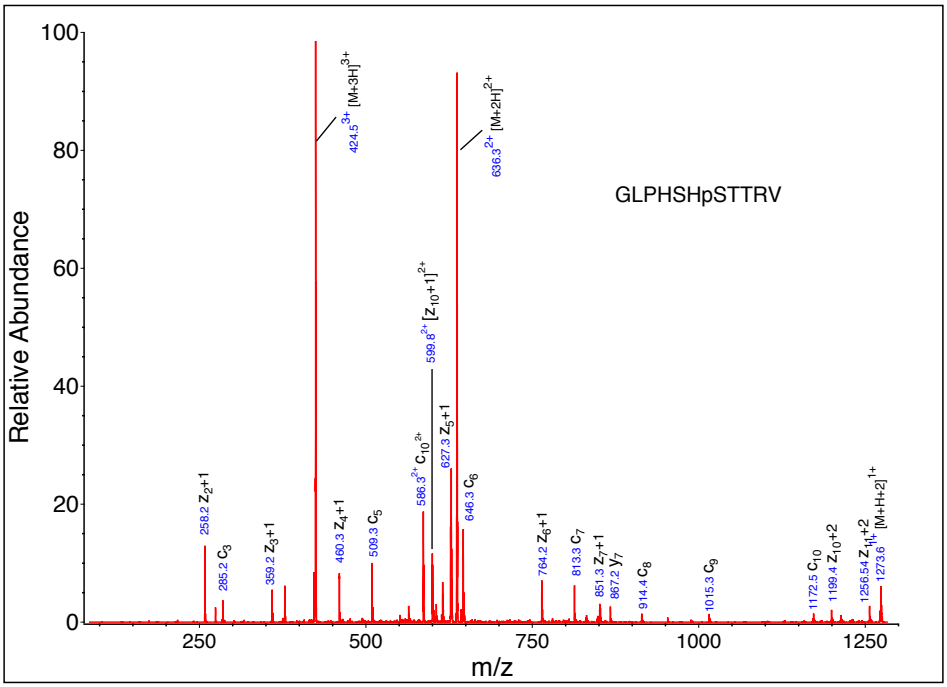

Fig. S2. PKA phosphorylates NLGN3 S824 *in vitro*.

MS/MS spectrum of the phosphorylated NLGN3 peptide found in GST-NLGN3 fusion proteins incubated with ATP and purified PKA. Samples were digested with trypsin and analyzed using the LC/MS/MS method. PKA phosphorylates NLGN3 on S824 *in vitro*.

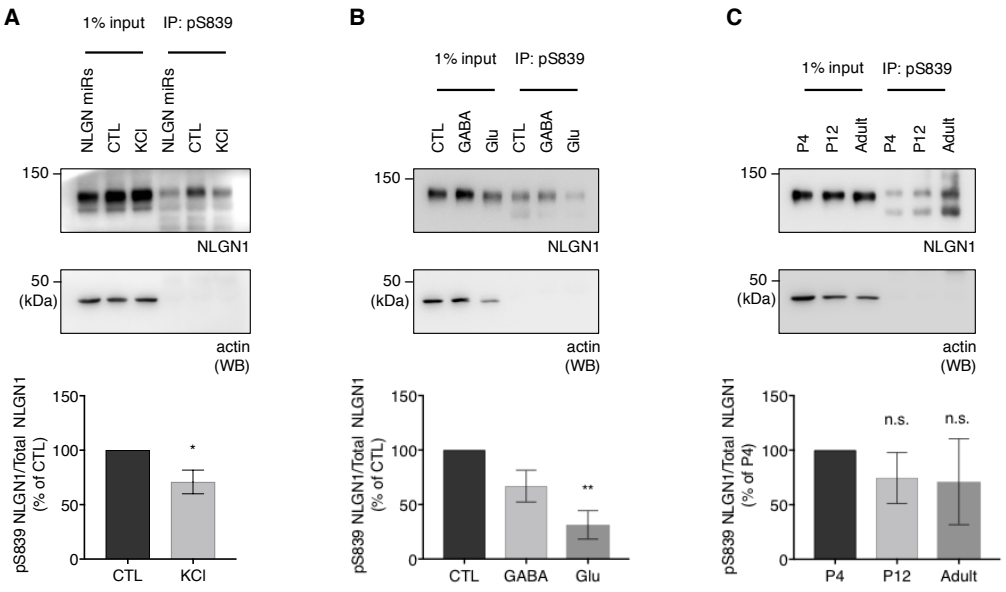

Fig. S3. Changes in NLGN1 phosphorylation upon neuronal activation, neurotransmitter stimulation and at different developmental stages.

Cultured rat cortical neurons were treated with 20 mM KCl for 1 hour to potentiate neuronal activity or treated with 20 μM GABA or 20 μM glutamate for 10 min for neurotransmitter receptor stimulation. NLGN microRNAs (miRs) lentivirus were transduced in cultured neurons to test the specificity of the pS839 immunoprecipitation. (A) Neuronal activation by KCl treatment significantly decreases NLGN1 S839 phosphorylation. *p = 0.0138 using unpaired *t*-test (n=6). (B) Glutamate also reduces pS839 NLGN1, although the neurons became unhealthy by glutamate toxicity (3, 4). The statistical significance between every condition was calculated using one-way ANOVA with Tukey's multiple comparison test. **p = 0.0092 (n=3). To investigate the changes of NLGN1 S839 phosphorylation in development, crude synaptosomal fractions (P2) from mouse brains of different developmental stages (P4, P12, and adult) were prepared. Phosphorylated NLGN1 was immunoprecipitated from the P2 fraction using the pS839 antibody. (C) The phosphorylation changes were not significantly observed. The statistical significance between every condition was calculated using one-way ANOVA with Tukey's multiple comparison test (n=6). All graphs indicate mean ± SEM.

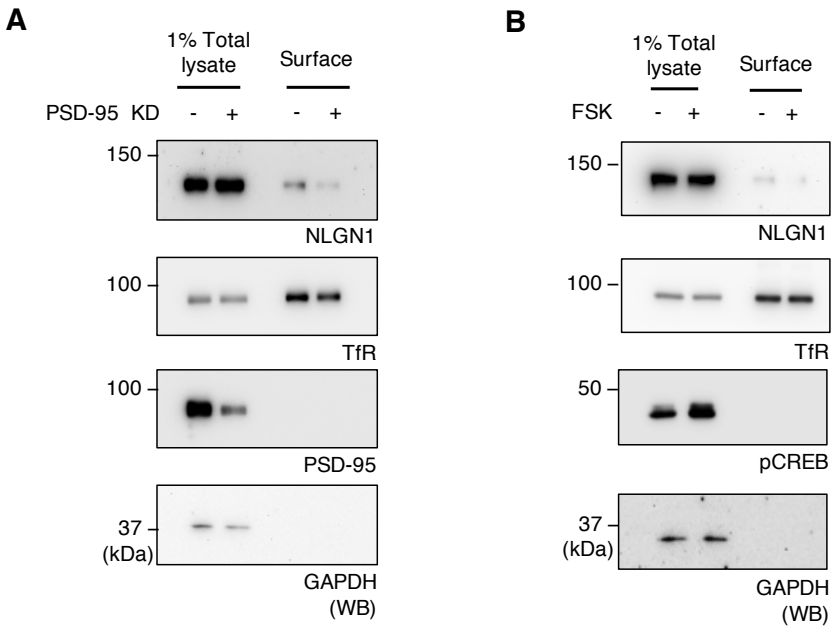

Fig. S4. PSD-95 knockdown or forskolin treatment do not disrupt neuronal plasma membrane.

Cultured cortical neurons were transduced with shPSD-95 lentivirus or treated forskolin as described previously. (A, B) The surface biotinylation assays were performed and transferrin receptor (TfR) levels were analyzed as a negative control by immunoblotting.

References

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