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Supplemental Information

Transsynaptic Signaling by Activity-Dependent Cleavage of Neuroligin-1

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SUPPLEMENTAL FIGURE LEGENDS

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Figure S1

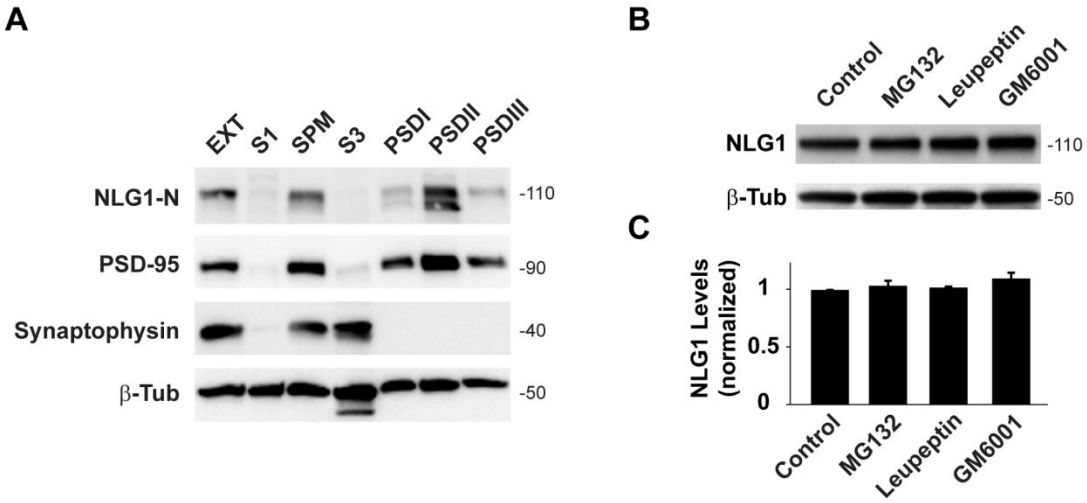

Figure S1. Biochemical Fractionation of Cultured Cortical Neurons. **Related to Figure 1**.

(A) Immunoblots of biochemical fractions from DIV21 cortical neuron cultures for the indicated proteins. EXT, whole cell extract; S1, supernatant; SPM, synaptic plasma

membranes; S3, synaptic vesicle fraction; PSDI, Triton-washed SPM pellet; PSDII, Triton-washed PSDI pellet; PSDIII, Sarcosyl-washed PSDI pellet. Note that 4-fold less protein by mass was loaded in PSD fraction lanes. See Experimental Procedures for details. Molecular mass markers in kDa are shown.

(B) Immunoblot analysis of NLG1 in whole cell extracts from DIV21 cortical neuronal cultures following 2 h incubation in Neurobasal medium (Control) or medium supplemented with MG132 (10 μM), leupeptin (200 μM), or GM6001 (10 μM).

(C) Data represent means ± SEM of total NLG1 levels under the indicated conditions. n = 3.

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Figure S2

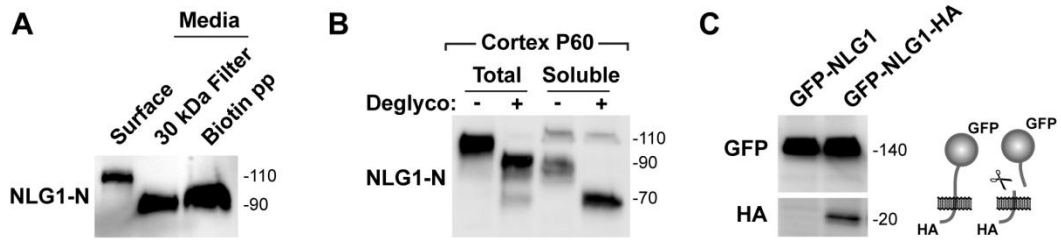

Figure S2. Biochemical Detection and Analysis of Neuroligin-1 Cleavage Fragments. **Related to Figure 2.**

(A) Mass cutoff filtration (30 kDa) of media collected from cultured cortical neurons (DIV21) reveals the presence of ~90 kDa NLG1-NTFs similar to those isolated by surface biotinylation and strepavidin pull-down (Biotin pp). The slight increase in molecular mass of the streptavidin-isolated NLG1-NTF relative to filter-isolated NLG1-NTF is likely due to biotinylation. For (A)-(C), molecular mass markers in kDa are shown on the right.

(B) Deglycosylation of N- and O-linked glycans (Deglyco) in whole homogenate (Total) and soluble fractions from P60 WT mice cortex results in a ~20 kDa apparent mass shift of NLG1-NTFs. Note that NLG-NTFs are less abundant at P60 (compare to Figures 2E and 2G).

(C) Lysates from COS7 cells expressing GFP-NLG1 or GFP-NLG1-HA were analyzed by immunoblot using anti-GFP and anti-HA antibodies. Anti-HA immunoblot revealed a fragment of approximately 20 kDa corresponding to a C-terminal fragment.

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Figure S3

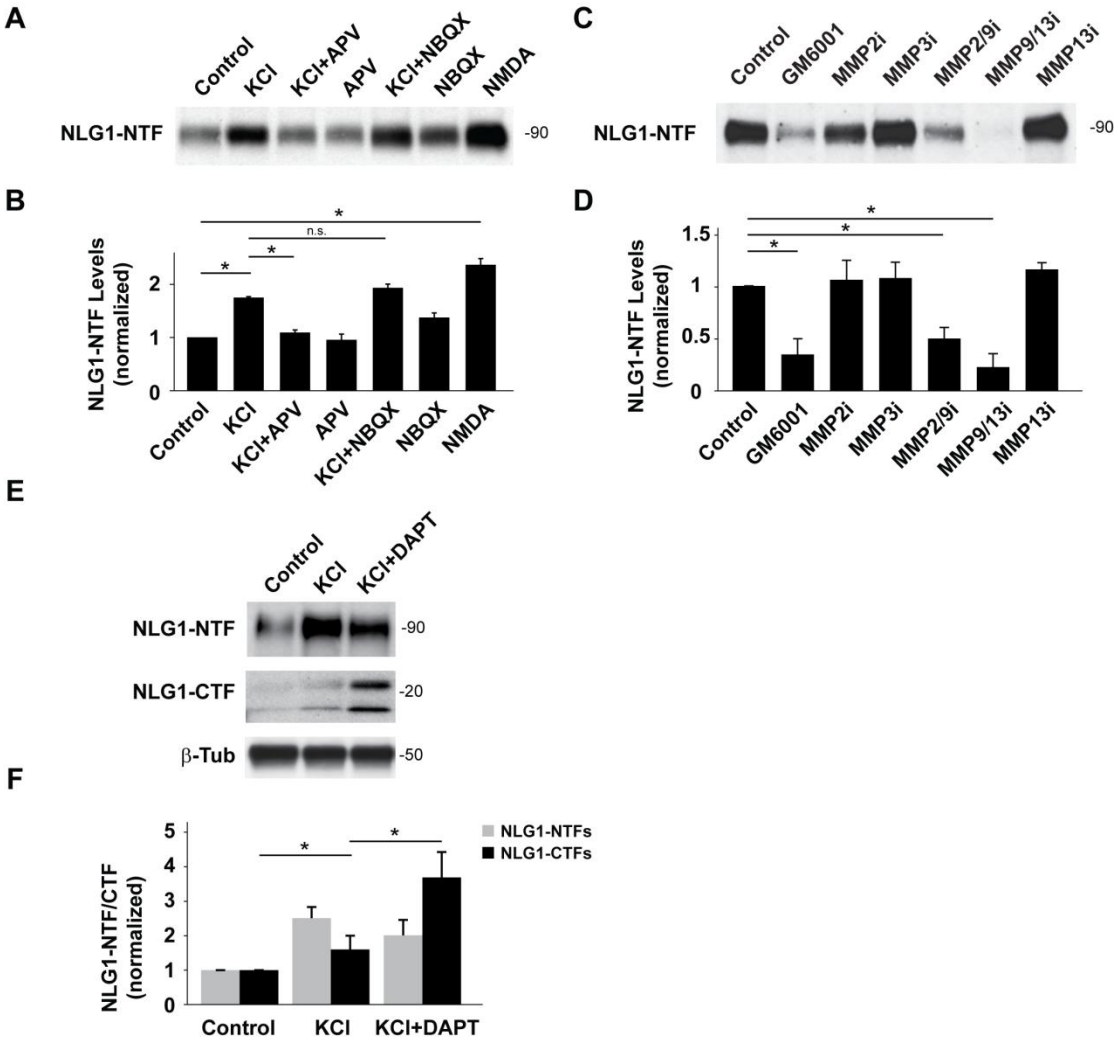

Figure S3. Characterization of the Signaling Pathways Regulating Activity-Dependent Cleavage of NLG-1. **Related to Figure 3.**

(A) Biotinylation-based isolation and detection of NLG1-NTFs from DIV21 cortical neurons incubated in Neurobasal media (Control), or Neurobasal media supplemented with 30 mM KCl (KCl) with APV (50 µM) or NBQX (20 µM), or stimulated with 50 µM

NMDA for 5 min (NMDA). Note that unlike APV, NBQX does not block the KCl-mediated increase in NLG1-NTFs. Direct stimulation with NMDA elicits robust NLG1 cleavage.

(B) Data represent means ± SEMs of NLG1-NTFs produced under the indicated conditions normalized to control. $n = 3$, * $p < 0.05$.

(C) Isolation and detection of ~90 kDa NLG1-NTFs following biotinylation of DIV21 cortical neurons incubated under control conditions or in the presence of MMP inhibitors. Note that the broad spectrum MMP inhibitor GM6001 and the specific MMP2/9i and MMP9/13i inhibitors reduce NLG1-NTF release under basal conditions.

(D) Data represent means ± SEMs of NLG1-NTFs produced under the indicated conditions normalized to control. $n = 3$, *$p < 0.05$.

(E) Immunoblot analysis of NLG1-NTF and NLG1-CTF production in DIV21 cortical neurons incubated under control conditions, the presence of 30 mM KCl (KCl), or in the presence of KCl plus the γ-secretase inhibitor DAPT (20 μM).

(F) Data represent means ± SEMs of NLG1-NTFs (black bars) and NLG1-CTFs (gray bars) produced under the indicated conditions normalized to control. $n = 3$, *$p < 0.05$.

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Figure S4

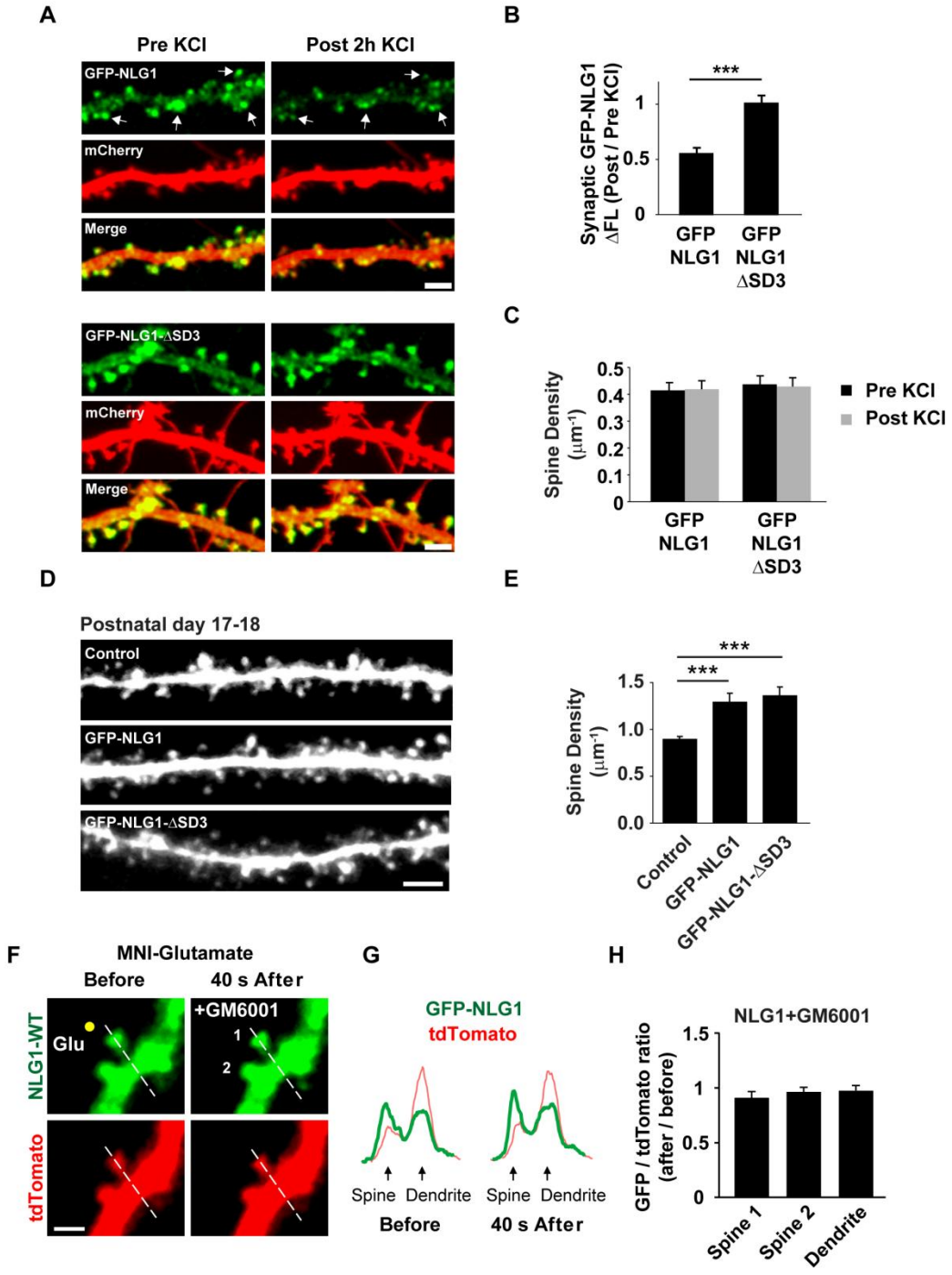

Figure S4. NLG1-ΔSD3 is Resistant to Activity-Dependent Cleavage and Retains NLG1 Synaptogenic Activity. **Related to Figure 4.**

(A) Hippocampal neurons (DIV21) expressing GFP-NLG1 or GFP-NLG1-ΔSD3 plus mCherry for 3-5 d were imaged before (Pre) and 2 h after (Post) 30 mM KCl incubation. Note that synaptic GFP-NLG1 fluorescence is decreased after KCl treatment whereas GFP-NLG1-ΔSD3 fluorescence remains constant. Scale bars, 3 μm.

(B) Data represents means ± SEM of GFP fluorescence intensity changes for each GFP-NLG1 or GFP-NLG1-ΔSD3 puncta before and 2 h after KCl incubation. Final fluorescence intensity values were normalized to values before treatment. GFP-NLG1, n = 150 spines, 5 neurons; GFP-NLG1-ΔSD3, n = 240 spines, 5 neurons; ***p <0.0005.

(C) Data represents means ± SEM of dendritic spine density in neurons expressing GFP-NLG1 or GFP-NLG1-ΔSD3 before and after 2 h KCl incubation. GFP-NLG1, n = 811 spines, 5 neurons; GFP-NLG1-ΔSD3, n = 648 spines, 5 neurons.

(D) Representative images from dendrites of cortical neurons in acute slices of P17-P18 mice electroporated *in utero* at E15.5 with tdTomato alone or together with GFP-NLG1 or GFP-NLG1-ΔSD3. Scale bar, 5 μm.

(E) Data represents means ± SEM of spine density for each condition depicted in (D). Note that GFP-NLG1 and GFP-NLG1-ΔSD3 induce a similar increase in spine density.

(F) Time-lapse images of hippocampal neurons expressing GFP-NLG1 and tdTomato before and after glutamate uncaging in the presence of the broad spectrum MMP inhibitor GM6001 (10 μM). The yellow dot represents the uncaging spot. The number "1" represents the dendritic spine stimulated with glutamate and "2" represents a neighboring spine within 10 μm of spine 1. The dashed white line represents the contour used for fluorescence analysis. Scale bar, 2 μm.

(G) Fluorescence intensity profiles of GFP (green) and tdTomato (red) along the white dashed lines depicted in (F).

(H) Data represent means ± SEMs of the ratio of GFP/tdTomato fluorescence intensity after glutamate uncaging in dendritic spines and adjacent dendritic shaft regions in the conditions represented in (F). n = 11.

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Figure S5

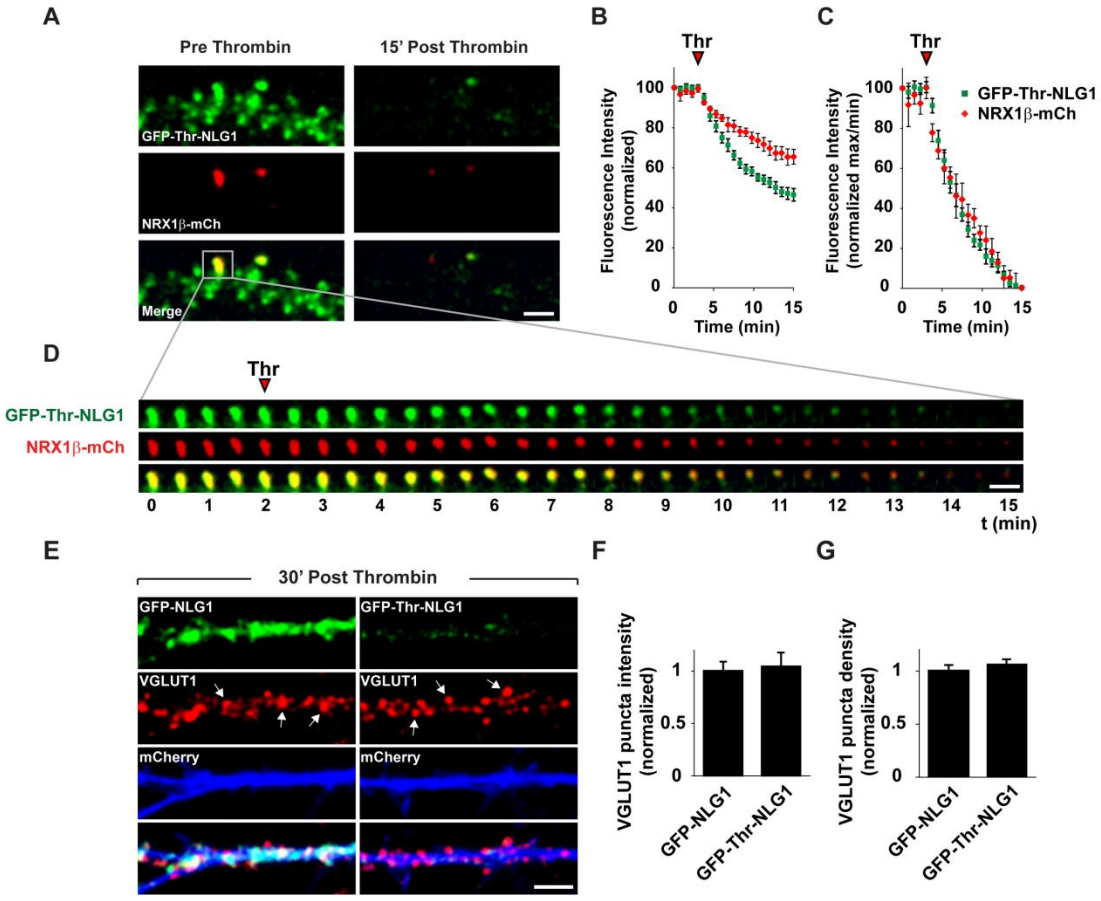

Figure S5. Neuroligin-1 Cleavage Causes Simultaneous Loss of Presynaptic Neurexin-1β and Does Not Alter Presynaptic VGLUT1. **Related to Figure 5.**

(A-D) DIV17-18 hippocampal cultures were sequentially transfected with GFP-Thr-NLG1 (green) and NRX1β-mCh (red) for 3-4 d to label distinct postsynaptic and presynaptic neurons, respectively. Neurons were incubated with thrombin and imaged live by confocal microscopy every 30 s. Note the thrombin-induced loss of GFP-Thr-NLG1 and NRX1β-mCh. (A) Images before (Pre) and 15 min after (Post) thrombin. Scale bar, 5 µm. (B) Quantification of time lapse experiments as depicted in (A). Plotted is fluorescence intensity before and after thrombin application at synapses containing presynaptic NRX1β-mCh along with postsynaptic GFP-Thr-NLG1. The red arrowhead shows the time of thrombin (Thr) application. n = 88 synaptic pairs. (C) Same as (B) but curves were scaled to both maximum and minimum values in order to compare decay

kinetics. Note that NRX1β-mCh and GFP-Thr-NLG1 fluorescence loss occurs in tandem. (D) Time-lapse of the highlighted region depicted in (A). The red arrowhead shows the time of thrombin (Thr) application. Note that thrombin-induced cleavage of GFP-Thr-NLG1 is followed by concurrent loss of NRX1β-mCh. Scale bar, 5 µm.
(E) Hippocampal neurons (DIV21) expressing mCherry and either GFP-NLG1 or GFP-Thr-NLG1 were incubated with 5 U/ml thrombin for 30 min, fixed, and immunolabeled for endogenous VGLUT1. Arrows indicate VGLUT1-positive presynaptic terminals. Note that VGLUT1 staining is unaffected by the thrombin-induced loss of GFP-Thr-NLG1. Scale bar, 5 µm.
(F) Data represent means ± SEM of VGLUT1 puncta intensity apposing GFP-Thr-NLG1 or GFP-NLG1 after thrombin treatment normalized to the GFP-NLG1 group. GFP-NLG1, n = 2316 puncta, 9 neurons; GFP-Thr-NLG1, n = 2408 puncta, 8 neurons.
(G) Data represent means ± SEM of VGLUT1 puncta density after thrombin normalized to the GFP-NLG1 group. n values as in (F).

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Figure S6

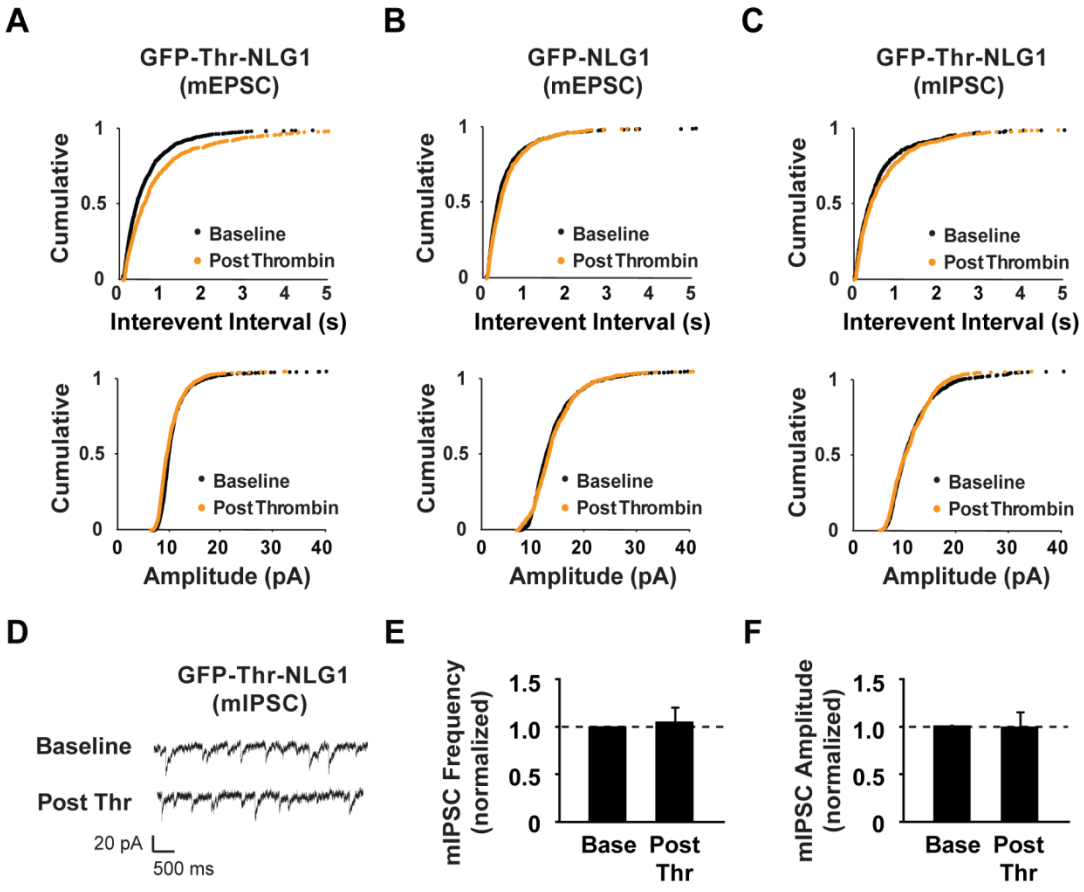

Figure S6. NLG1 Cleavage Regulates mEPSCs but Not mIPSCs. **Related to Figure 6.**
(A) Cumulative distribution of interevent intervals (top) and amplitudes (bottom) of mEPSCs recorded before (Baseline) and 30 min after (Post) thrombin application from cells expressing GFP-Thr-NLG1. Note the increase in interevent interval after thrombin application.

(B) Same as (A) but cells expressed GFP-NLG1.

(C) Cumulative distribution of interevent intervals (top) and amplitudes (bottom) of mIPSCs recorded before (Baseline) and after (Post) thrombin application from cells expressing GFP-Thr-NLG1. Note that thrombin affects neither mIPSC frequency nor amplitude.

(D) Representative mIPSCs before (Baseline) and after (Post) thrombin treatment in neurons expressing GFP-Thr-NLG1.
(E-F) Means ± SEM of normalized mIPSC frequency (E) and amplitude (F) after thrombin incubation for 30 min. n = 6.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. Acute Cleavage of GFP-Thr-NLG1 Following Thrombin Application. **Related to Figure 5.**
Shown is a hippocampal neuron (DIV21) expressing GFP-Thr-NLG1 and mCherry imaged live by confocal microscopy before and during thrombin incubation (5 U/ml, 30 min). Thrombin application is indicated by the white circle. Lower panels represent the dendritic region inside the white box. Note the stability of dendritic spines after thrombin application despite dramatic loss of GFP fluorescence due to GFP-Thr-NLG1 cleavage. Time is indicated in min:sec. Scale bars, 20 μm and 5 μm in upper and lower panels, respectively.

Movie S2. Acute NLG1 Cleavage Destabilizes Presynaptic NRX1β. **Related to Figure 5.**
Hippocampal cultures were sequentially transfected with GFP-Thr-NLG1 or GFP-NLG1 followed by NRX1β-mCh to label distinct postsynaptic and presynaptic neurons, respectively. Synaptic pairs from labeled neurons were imaged live by confocal microscopy before and during thrombin incubation (5 U/ml, 30 min). Arrows indicate loss of presynaptic NRX1β-mCh apposed to postsynaptic GFP-Thr-NLG1 puncta following thrombin application. Arrowheads show newly generated mobile NRX1β-mCh puncta along the axon. Note the stability of NRX1β-mCh apposed to non-thrombin cleavable GFP-NLG1 puncta in the right panels. Time is indicated in min:sec. Thrombin application is indicated by the white circle. Scale bars, 2 μm.

EXTENDED EXPERIMENTAL PROCEDURES

DNA Constructs

Thrombin inducible GFP-Thr-NLG1 was generated by replacing the endogenous sequence TTTKVP from GFP-NLG1(ΔA) with the thrombin recognition sequence LVPRGS. This was achieved by sequential PCR using primers 5'-ctagtacctagaggatcctcaacagacatcactctcag-3' and 5'-ggatcctctaggtactagcgaggtatactgagaaatgtca-3', and primers 5'-ggatcctctaggtactagctgagaaatgtcattgag-3'and 5'-gttggagctggtacctcatc-3'. The two self-annealing PCR products were templates in a reaction with primer 5'-gttggagctggtacctcatc-3' containing a KpnI site and 5'-gtcactcgagctataccctggttgttgaatg-3'. The resulting PCR product was subcloned into pcDNA3-GFP-NLG1 using KpnI and XhoI. The NLG1-ΔSDfull mutant was generated using a similar strategy by substitution of endogenous 636-695 residues with a GAAAAA linker using primers 5'-ggcgccgcagcagcagcactaagtgtcacaatcgcagtg-3' and 5'-tgctgctgctgcggcgccattatgcagatgaggtacc-3'; NLG1-ΔSD1 (636-660) 5'-ggcgccgcagcagcagcaaggaaaaattccactccag-3' and 5'-tgctgctgctgcggcgccattatgcagatgaggtacc-3; NLG1-ΔSD2 (654-677) 5'-ggcgccgcagcagcagcacccaaacaacaaccaagtc-3' and 5'-tgctgctgctgcggcgccgtctgttgatggcactttag-3'; NLG1-ΔSD3 (672-695) 5'-ggcgccgcagcagcagcactaagtgtcacaatcgcagtg-3'and 5'-tgctgctgctgcggcgccgggaaaggctgatgtgactgg-3'. For GFP-NLG1-HA the HA peptide YPYDVPDYA was inserted at the end of GFP-NLG1(ΔA) using the primer 5'-gcatctcgagctaagcgtaatctggaacatcgtatgggtataccctggttgttgaatgtg-3'.

Antibodies

Commercial antibodies used include N-terminal NLG1 antibody clone 4C12 (mouse, Synaptic Systems), C-terminal NLG1 polyclonal antibody (rabbit, Synaptic Systems), VGLUT1 (mouse, Synaptic Systems), PSD-95 (mouse, Chemicon), Arc (C-7, rabbit,

Santa Cruz), Na^+,K^+-ATPase (rabbit, Cell Signaling), α-Actinin clone EA-53 (Sigma), Tuj1 (mouse, Covance), GFP (mouse, Millipore), HA.11 (mouse, Covance). Rabbit anti-panNLG antibody was a kind gift from Peter Scheiffele (Biozentrum).

Biochemical Analysis of Brain, Whole Cell Extracts, and PSD Fractions

For immunoblot analysis of PSD fractions, 5 µg of PSD fractions and 20 µg of remaining fractions were used. Brain tissue was homogenized in 4 mM HEPES, 0.32 M sucrose, pH 7.4 with protease and phosphatase inhibitors (Roche). Crude membrane fractionation was performed by centrifugation at 150,000 x g for 30 min. Deglycosylation of O- and N- glycans was performed using an enzymatic deglycosylation kit (Calbiochem, see supplemental methods for details). Whole cell extracts of dissociated cultures were solubilized in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.4). Protein concentration was determined by Dc protein assay (Bio-Rad) and proteins of interest were detected and quantified by immunoblot (ECl Plus, Amersham) on LAS-3000 gel reader (Fujifilm) using Multigauge 3.0 software. Immunoblots depicted in Figures 3I and 3J were quantified on a Li-Cor Odyssey infrared imaging system.

Deglycosylation of Brain Fractions

Deglycosylation of O- and N- glycans was performed using an enzymatic deglycosylation kit (Calbiochem) containing N-glycosidase F, endo-α-N-acetylgalactosaminidase, α2-3,6,8,9-neuraminidase, β1,4-galactosidase and β-N-acetylglucosaminidase according to the manufacturer's protocol.

Biotinylation-Based Internalization Assay

High-density (700,000 cells / 60 mm plate) DIV21 cortical neuron cultures were incubated with 100 µg/ml of the lysosomal protease inhibitor leupeptin beginning 1 h prior to biotinylation with 1 mg/ml sulfo-NHS-SS-biotin. Leupeptin was present throughout all steps and incubations except the 4ºC biotinylation reaction. Neurons were then incubated at either 4ºC to block membrane trafficking or 37ºC for various times to

allow endocytosis to occur. The remaining surface biotin was cleaved by reducing its disulfide linkage with glutathione cleavage buffer (50 mM glutathione in 75 mM NaCl and 10 mM EDTA containing 1% BSA and 0.075 N NaOH) two times for 15 min each at 4ºC. Whole cell extracts were prepared, and biotinylated proteins were precipitated essentially as described. Biotinylated receptors were detected by immunoblot (ECL Plus, Amersham), and quantification was performed on an LAS-3000 gel reader (Fujifilm), using Multigauge 3.0 software. Percent internalization was determined by measuring the band intensity after 37ºC incubation, subtracting the nonspecific band intensity obtained after 4ºC incubation (always <5%), and comparing to the total surface receptor calibration curve.

Confocal Imaging and FM4-64 Unloading Experiments

Confocal images of fixed samples and live cells were obtained using a Perkin Elmer Ultraview spinning disc confocal microscope with either a 40x 1.3 N.A. objective or a 60x 1.4 N.A. objective. For immunocytochemistry, DIV21 hippocampal neurons were fixed in 4% paraformaldehyde/4% sucrose in PBS for 20 min, permeabilized with 0.2% Triton X-100 for 15 min, and incubated with indicated antibodies at 1:250 (panNLG) or 1:1000 (PSD-95 and VGLUT1) dilution overnight at 4ºC. For pan-NLG labeling, cells were incubated in -20ºC methanol for 10 min after paraformaldehyde fixation. For live imaging, cells were imaged in E4 media (10 mM HEPES, 120 mM NaCl, 3 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$) at 37ºC. Maximum projections of individual stacks were analyzed using Metamorph software (Universal Imaging Corporation). Integrated fluorescence intensity of synaptic puncta was measured using circular regions of fixed diameter (15 pixels). Following background subtraction, integrated intensity values were collected and pooled per neuron and normalized to control conditions. For total NLG1-4, full dendritic arbors were traced from maximum projections of confocal z-stacks (0.75 µm), average intensity values determined after background subtraction, and values pooled and averaged per neuron. For time lapse movies, analysis was done at every time point and regions of interest were adjusted at each frame. Results shown correspond to the average of all neurons per condition. Statistical significance was determined by unpaired t-test and error bars represent standard error of the means.

For FM4-64 unloading experiments, DIV16-17 neurons were transfected with GFP-NLG1 or GFP-NLG1-ΔSD3 and analyzed at DIV21. Bicuculline (50 µM) and 4-aminopyridine (4AP, 25 µM) were added to neuronal cultures 2 d prior to the experiment. Images were acquired in E4 media. FM4-64 (10 µM) uptake was performed using 90 mM KCl loading media (10 mM HEPES, 33 mM NaCl, 90 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 20 µM NBQX, 50 µM APV) for 1 min. Cells were then washed three times in washing buffer for 30 s (E4 containing 0.5 mM $CaCl_2$ and 10 mM $MgCl_2$), twice for 15 s in washing buffer containing 1 mM ADVASEP-7 (Sigma), and three more times in washing buffer. FM4-64 signal was acquired with a 567 nm excitation laser and a 647 nm emission filter. GFP fluorescence was acquired at the end of the time lapse to minimize photobleaching. Unloading solution containing 60 mM KCl (10 mM HEPES, 63 mM NaCl, 60 mM KCl, 10 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 20 µM NBQX, 50 µM APV) was added with a precision of <2 s. For FM4-64 puncta, integrated intensity was quantified at each frame using circular regions of fixed diameter (15 pixels) and background intensity levels were subtracted. Fluorescence intensity values for each bouton were normalized to value at time zero and the resulting curve fitted to a single exponential function using Prism software (GraphPad software). Time constants (τ) of FM4-64 fluorescence decay at boutons corresponding to each condition were pooled and analyzed by a Kolmogorov-Smirnov test. Statistical significance was determined using unpaired t-tests.