**Supplemental Information**

# Molecular dissection of Neuroligin2 and Slitrk3 reveals an essential framework for GABAergic synapse development

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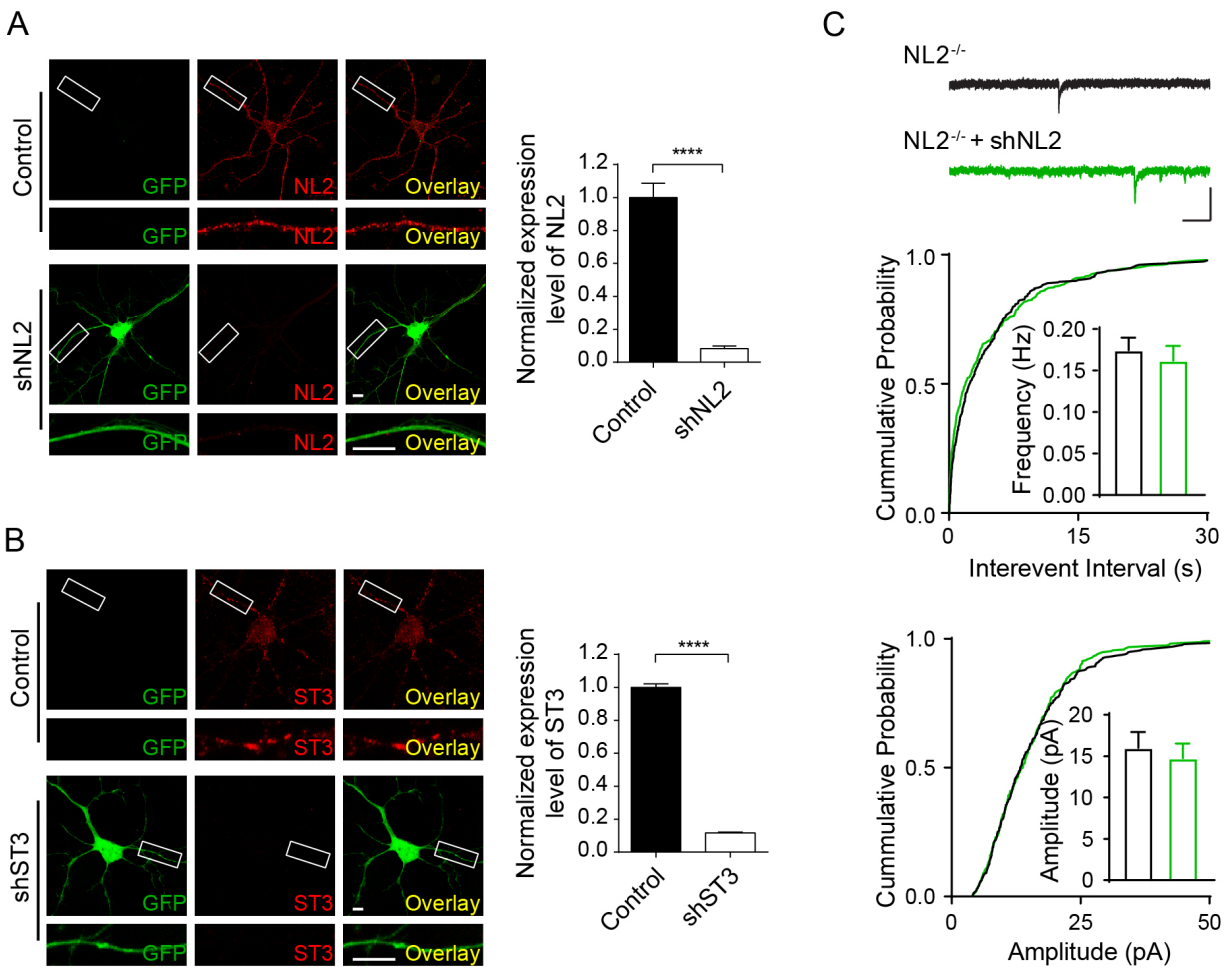



**Figure S1. Characterization of shRNAs in cultured hippocampal neurons and NL2 KO mice (related to Figures 1-2)**

(A) Confocal images and quantification analysis showed the knockdown effect of NL2 shRNA on endogenous NL2 in cultured hippocampal neurons. Neurons were transfected with shRNA against NL2 (also expressing GFP) at DIV2 and immunostained at DIV8. Controls were nearby non-transfected neurons. Control, 1.0 ± 0.09, n = 13; shNL2, 0.08 ± 0.02, n = 13, unpaired *t*-test, $p < 0.0001$ compared to Control. Scale bar, 10 μm.

(B) Confocal images and quantification analysis showed knockdown effect of ST3 shRNA on endogenous ST3 in cultured hippocampal neurons. Neurons were transfected with shRNA against ST3 (also expressing GFP) at DIV2 and immunostained at DIV8. Controls were nearby

non-transfected neurons. Control, 1.0 ± 0.02, n = 14; shST3, 0.12 ± 0.02, n = 14, unpaired *t*-test, $p < 0.0001$ compared to Control. Scale bar, 10 μm.

(C) mIPSC recordings showed that NL2 knockdown did not significantly change GABAergic synaptic transmission in CA1 pyramidal neurons in acute hippocampal slices prepared from P8-9 NL2 KO mice that were *in utero* electroporated with NL2 shRNA plasmids at E14.5 (Frequency (Hz): $NL2^{-/-}$, 0.17 ± 0.02, n = 6; $NL2^{-/-}$+shNL2, 0.16 ± 0.02, n = 6, $p = 0.61$; Amplitude (pA): $NL2^{-/-}$, 16.0 ± 1.9, n = 6; $NL2^{-/-}$+shNL2, 14.7 ± 1.8, n = 6, $p = 0.64$; *t*-test; Kolmogorov–Smirnov test was used for cumulative distributions, $p > 0.05$ for both conditions). Scale bar,20 pA and 1 s.

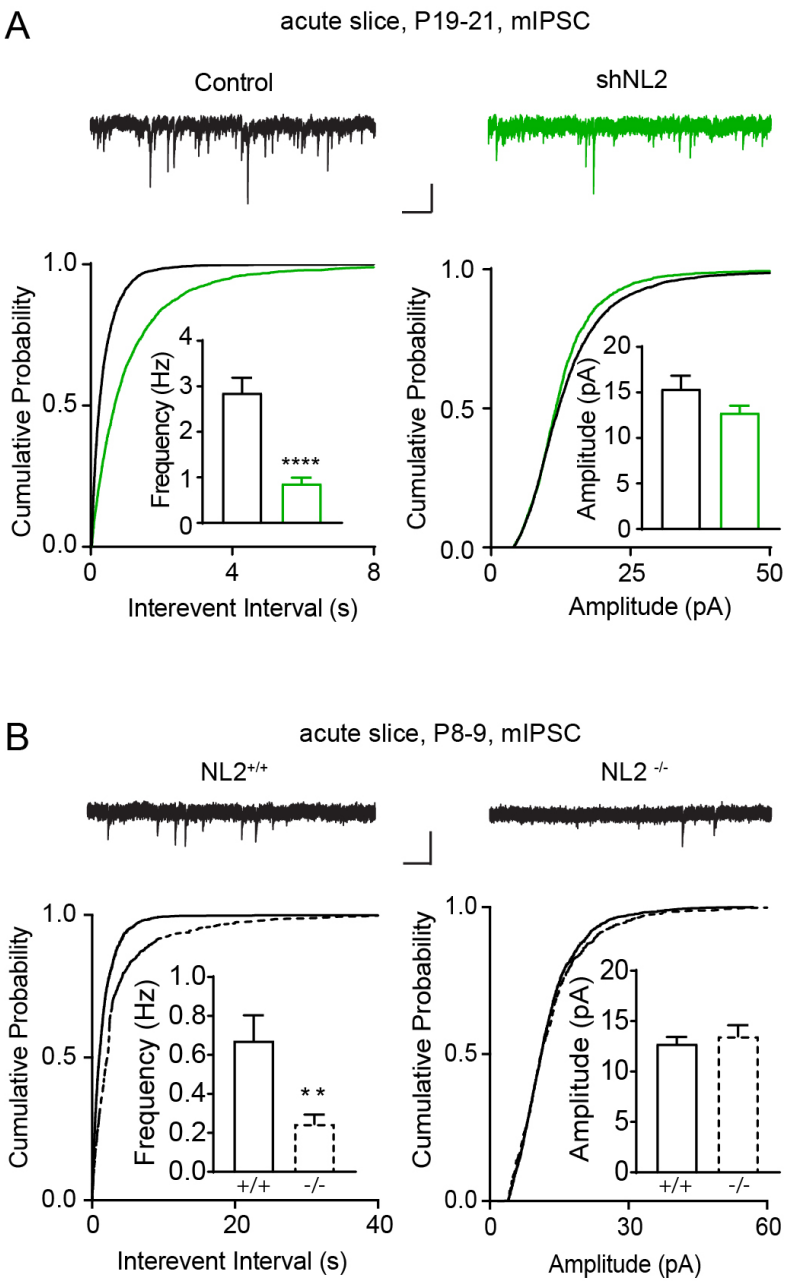



**Figure S2. Characterization of GABAergic synaptic transmission in hippocampal neurons expressing NL2 shRNAs and in NL2 KO mice (related to Figure 1)**

(A) mIPSC recordings showed that there was a ~70% reduction of mIPSC frequency in GFP-positive NL2 knockdown CA1 pyramidal neurons in acute hippocampal slices from P19-21 WT mice that were in utero electroporated with NL2 shRNA plasmids at E14.5 (Frequency (Hz): Control, 2.86 ± 0.33, n = 11; shNL2, 0.86 ± 0.13, n = 10, $p < 0.0001$; Amplitude (pA): Control,

15.4 ± 1.5, n = 11; shNL2, 12.7 ± 0.8, n = 10, $p = 0.14$, *t*-test; Kolmogorov–Smirnov test was used for cumulative distributions, $p < 0.0001$ for both conditions). Scale bar,10 pA and 1 s.

(B) mIPSC recordings showed a significant reduction of mIPSC frequency in CA1 pyramidal neurons in acute hippocampal slices from P8-9 $NL2^{-/-}$ mice (Frequency (Hz): $NL2^{+/+}$, 0.67 ± 0.13, n = 12; $NL2^{-/-}$, 0.24 ± 0.05, n = 11, $p < 0.01$; Amplitude (pA): $NL2^{+/+}$, 12.7 ± 0.7, n = 12; $NL2^{-/-}$, 13.5 ± 1.1, n = 11, $p = 0.57$, *t*-test; Kolmogorov–Smirnov test was used for cumulative distributions, $p < 0.0001$ for mIPSC frequency). Scale bar, 20 pA and 1 s.

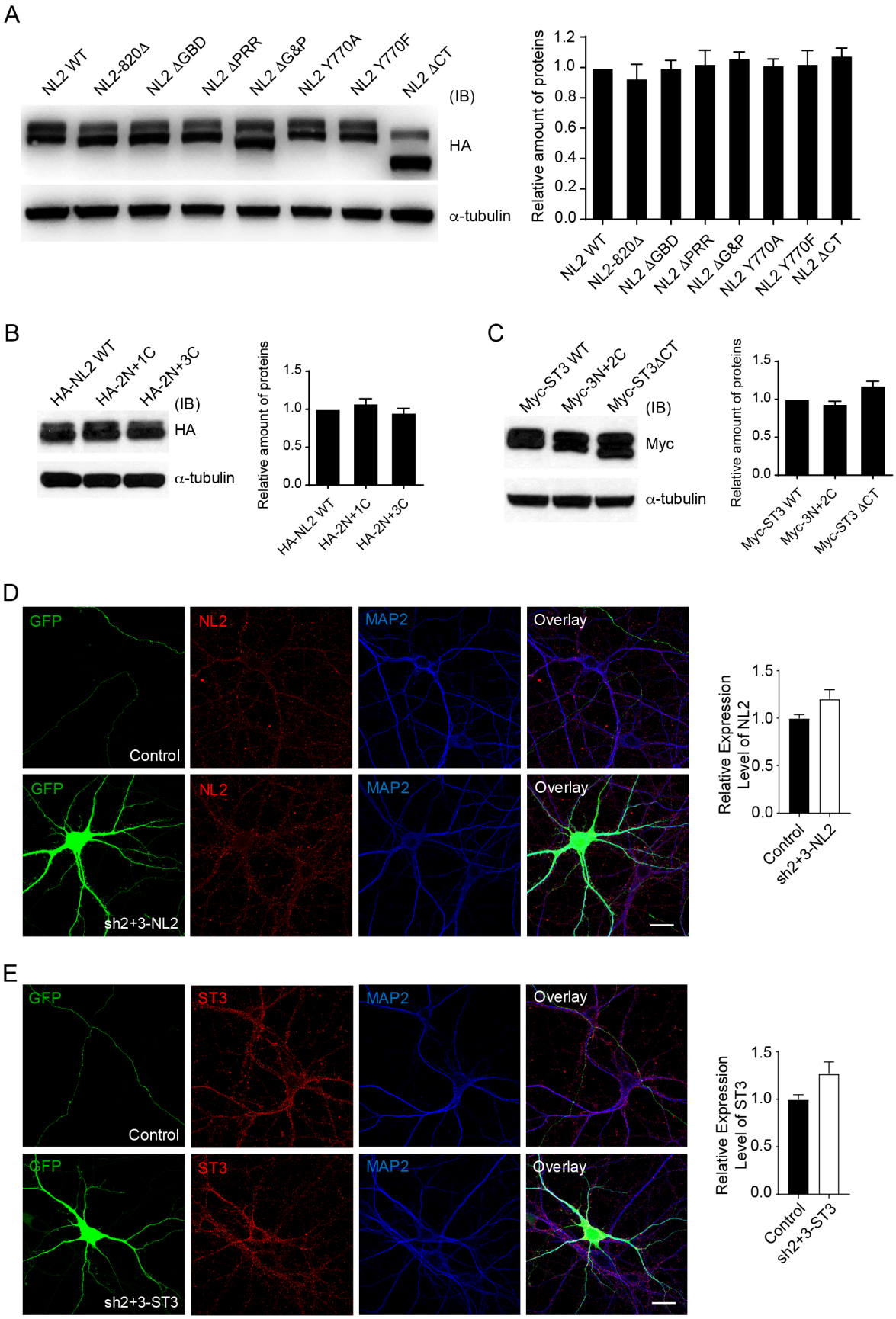

A
NL2 WT
NL2-820Δ
NL2 ΔGBD
NL2 ΔPRR
NL2 ΔG&P
NL2 Y770A
NL2 Y770F
NL2 ΔCT
(IB)
HA
α-tubulin
Relative amount of proteins
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B
HA-NL2 WT
HA-2N+1C
HA-2N+3C
(IB)
HA
α-tubulin
Relative amount of proteins
1.5
1.0
0.5
0.0
C
Myc-ST3 WT
Myc-3N+2C
Myc-ST3ΔCT
(IB)
Myc
α-tubulin
Relative amount of proteins
Myc-ST3 ΔCT
D
GFP
NL2
MAP2
Overlay
Control
sh2+3-NL2
Relative Expression Level of NL2
Control
sh2+3-NL2
E
ST3
sh2+3-ST3
Relative Expression Level of ST3
Control
sh2+3-ST3

**Figure S3. Characterization of expression of NL2, ST3 or their mutants in neurons and HEK cells (related to Figure 2)**

(A) Representative immunoblots and the summary graph showed no significant difference of protein levels between HA-tagged NL2 WT and NL2 C-tail mutants expressed in HEK293T cells (n = 3, $p > 0.05$, One-way ANOVA test).

(B-C) Representative immunoblots and the summary graphs showed no significant difference of protein levels between HA-tagged NL2 WT and NL2 swap mutants (B), or between Myc-tagged ST3 WT and ST3 swap mutants (C) expressed in HEK293T cells (n = 3, $p > 0.05$, One-way ANOVA test).

(D-E) Confocal images and quantification analysis showed that the expression levels of recombinant NL2 or ST3 together with sh2+3 double knockdown vector were similar to endogenous NL2 or ST3 expression in cultured hippocampal neurons, respectively (normalized expression levels: D, Control, 1.0 ± 0.04, n = 15; sh2+3-NL2, 1.2 ± 0.10, n = 15, unpaired *t*-test, $p = 0.0555$; E, Control, 1.0 ± 0.05, n = 12; 1.27 ± 0.12, n = 12, unpaired *t*-test, $p = 0.052$). Scale bar, 10 μm.

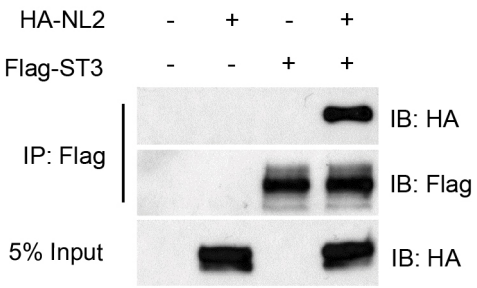
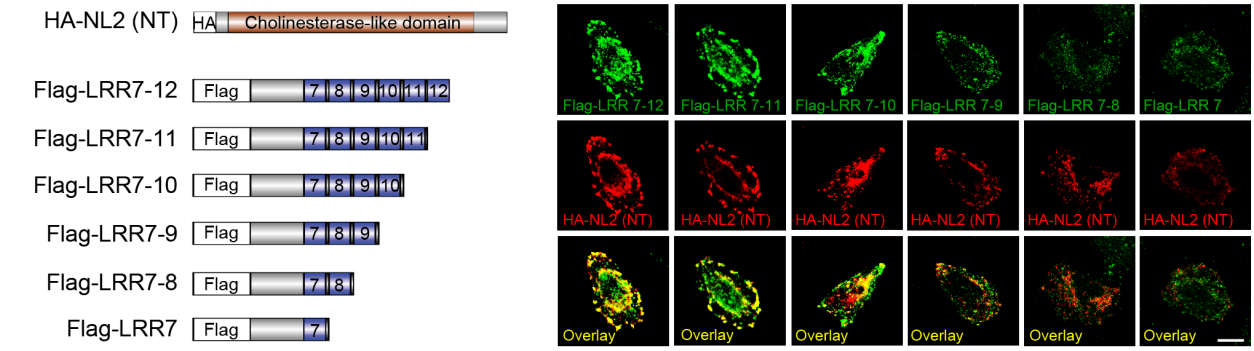


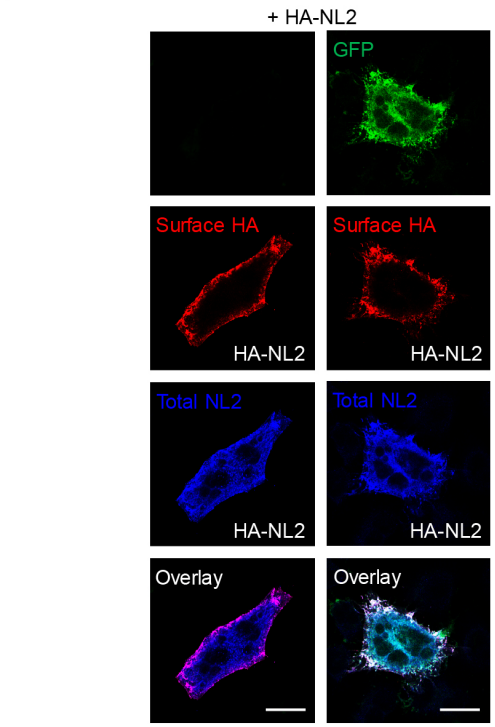

**Figure S4. Co-IP of NL2 and ST3 in HEK cells (related to Figure 4)**

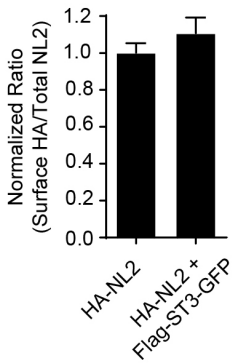
A co-IP assay in HEK293T cells showed that HA-NL2 was co-immunoprecipitated with Flag-ST3. Lysates from cells transfected with HA-NL2 or Flag-ST3 alone, HA-NL2 together with Flag-ST3 or an empty vector as a control, were immunoprecipitated with an anti-Flag antibody and probed with indicated antibodies.

A

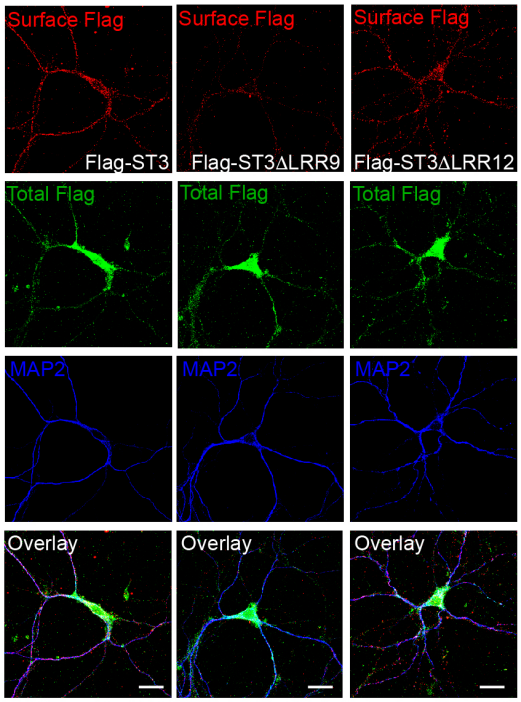

HA-NL2 (NT)
HA
Cholinesterase-like domain
Flag-LRR7-12
Flag
7
8
9
10
11
12
Flag-LRR7-11
Flag-LRR7-10
Flag-LRR7-9
Flag-LRR7-8
Flag-LRR7
Flag-LRR 7-12
Flag-LRR 7-11
Flag-LRR 7-10
Flag-LRR 7-9
Flag-LRR 7-8
Flag-LRR 7
HA-NL2 (NT)
Overlay

B


Flag-ST3-GFP
-
+
+ HA-NL2
GFP
Surface HA
HA-NL2
Total NL2
Overlay


Normalized Ratio
(Surface HA/Total NL2)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HA-NL2
HA-NL2 +
Flag-ST3-GFP

C


Surface Flag
Flag-ST3
Flag-ST3ΔLRR9
Flag-ST3ΔLRR12
Total Flag
MAP2
Overlay

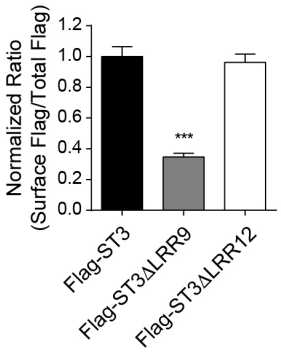

Normalized Ratio
(Surface Flag/Total Flag)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
Flag-ST3
Flag-ST3ΔLRR9
Flag-ST3ΔLRR12

**Figure S5. Immunostaining of ST3 or ST3 mutants in heterologous cells and in neurons (related to Figures 4-6)**

(A) Confocal images showed that the ST3 mutants that contained the LRR9 domain, but not those lacking the LRR9 domain, co-localized with NL2 N-terminus in HeLa cells. Scale bar, 20 μm.

(B) Confocal images (top) and summary bar graph (bottom) showed that ST3 did not change NL2 trafficking to the plasma membrane in HeLa cells. Cells were transfected with full-length HA-NL2 alone or together with Flag-ST3-GFP. Surface HA was immunolabeled under non-permeabilized condition, total NL2 was labeled by anti-NL2 antibody after permeabilization and ST3 expression was indicated by GFP fluorescence (GFP was fused to the C-terminus of ST3) (HA-NL2, 1.0 ± 0.05, n = 15; HA-NL2+Flag-ST3-GFP, 1.11 ± 0.09, n = 15, unpaired *t*-test, $p = 0.30$). Scale bar, 10 μm.

(C) Confocal images (top) and quantification graph (bottom) showed that deletion of the LRR9 domain significantly decreased surface expression of Flag-ST3 in cultured neurons. In contrast, Flag-ST3ΔLRR12 showed similar surface expression as compared to WT Flag-ST3 (Flag-ST3, 1.0 ± 0.06, n = 10; Flag-ST3ΔLRR9, 0.35 ± 0.02, n = 10; Flag-ST3ΔLRR12, 0.96 ± 0.05, n = 11, one-way ANOVA, $p < 0.001$ compared to Flag-ST3). Scale bar, 10 μm.

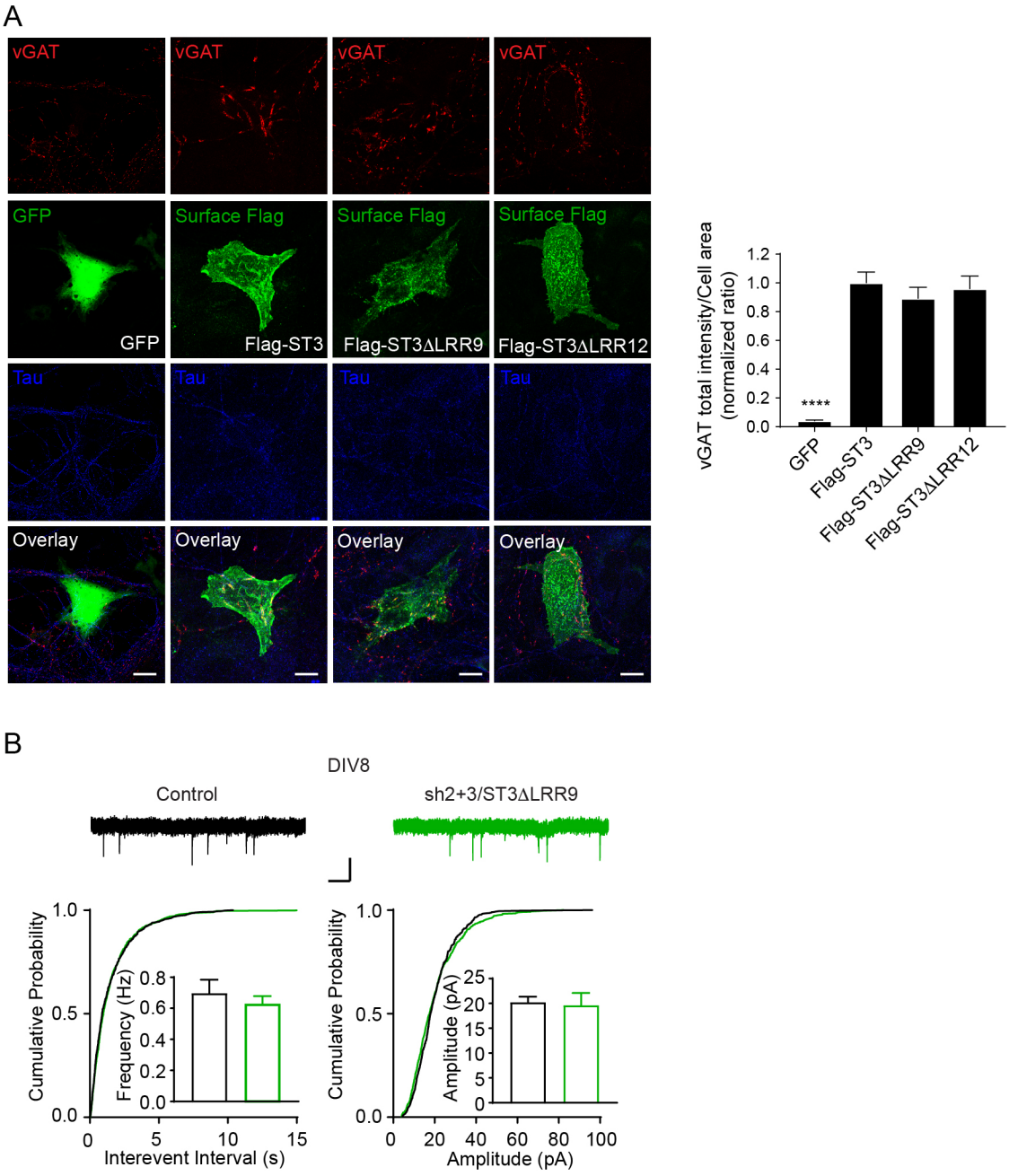



**Figure S6. ST3 ΔLRR9 mutant has similar synaptogenic activity compared with WT ST3 (related to Figure 6)**

(A) Confocal images and quantification bar graph of neuron-fibroblast co-culture showed that deletion of LRR9 or LRR12 domain in ST3 did not significantly change its ability to induce GABAergic presynaptic differentiation (GFP, 0.04 ± 0.01, n = 12; Flag-ST3, 1.0 ± 0.08, n = 10;

Flag-ST3ΔLRR9, 0.89 ± 0.08, n = 11; Flag-ST3ΔLRR12, 0.96 ± 0.09, n = 10, One-way ANOVA, $p < 0.0001$ compared to Flag-ST3). Scale bar, 10 μm.

(B) mIPSC recordings showed that expression of the ST3 mutant lacking LRR9 domain could rescue mIPSC deficits in NL2/ST3 double knockdown neurons in DIV8 developing neurons (Frequency (Hz): Control, 0.70 ± 0.09, n = 9; sh2+3/ST3ΔLRR9, 0.63 ± 0.05, n = 11, $p = 0.49$; Amplitude (pA): Control, 20.2 ± 1.1, n = 9; sh2+3/ST3ΔLRR9, 19.6 ± 2.5, n = 11, $p = 0.83$, unpaired *t*-test; Kolmogorov–Smirnov test was used for cumulative distributions, $p < 0.05$ for mIPSC amplitude). Scale bar, 20 pA and 1 s.

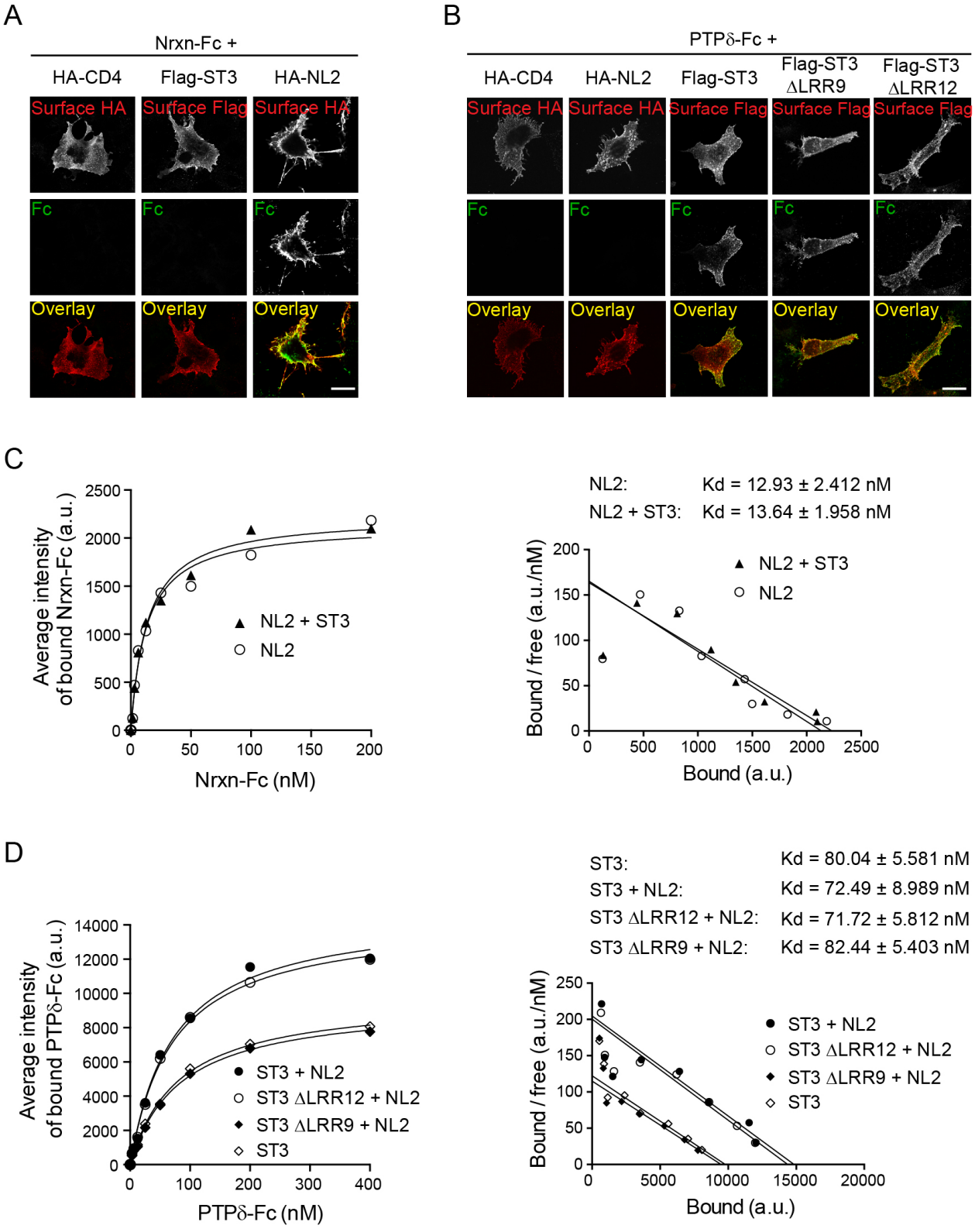

A
Nrxn-Fc +
HA-CD4
Flag-ST3
HA-NL2
Surface HA
Surface Flag
Surface HA
Fc
Fc
Fc
Overlay
Overlay
Overlay
B
PTPδ-Fc +
HA-CD4
HA-NL2
Flag-ST3
Flag-ST3 ΔLRR9
Flag-ST3 ΔLRR12
Surface HA
Surface HA
Surface Flag
Surface Flag
Surface Flag
Fc
Fc
Fc
Fc
Fc
Overlay
Overlay
Overlay
Overlay
Overlay
C
Average intensity of bound Nrxn-Fc (a.u.)
Nrxn-Fc (nM)
NL2 + ST3
NL2
NL2: Kd = 12.93 ± 2.412 nM
NL2 + ST3: Kd = 13.64 ± 1.958 nM
Bound / free (a.u./nM)
Bound (a.u.)
NL2 + ST3
NL2
D
Average intensity of bound PTPδ-Fc (a.u.)
PTPδ-Fc (nM)
ST3 + NL2
ST3 ΔLRR12 + NL2
ST3 ΔLRR9 + NL2
ST3
ST3: Kd = 80.04 ± 5.581 nM
ST3 + NL2: Kd = 72.49 ± 8.989 nM
ST3 ΔLRR12 + NL2: Kd = 71.72 ± 5.812 nM
ST3 ΔLRR9 + NL2: Kd = 82.44 ± 5.403 nM
Bound / free (a.u./nM)
Bound (a.u.)
ST3 + NL2
ST3 ΔLRR12 + NL2
ST3 ΔLRR9 + NL2
ST3

**Figure S7. Binding affinity of NL2, ST3, ST3 mutants or the NL2-ST3 complex to presynaptic binding partners (related to Figure 6)**

(A) Confocal images showed that purified Neurexin (Nrxn)-Fc (Fc fusion to C-terminus of Nrxn1βAS4(+) extracellular domain) protein specifically bound to surface-expressing HA-NL2, but not HA-CD4 or Flag-ST3, in COS7 cells. Scale bar, 20 μm.

(B) Confocal images showed that purified PTPδ-Fc (Fc fusion to C-terminus of PTPδ extracellular domain) protein specifically bound to surface expressing Flag-ST3, but not HA-CD4 or HA-NL2, in COS7 cells. PTPδ-Fc also bound similarly to Flag-ST3ΔLRR9 or Flag-ST3ΔLRR12. Scale bar, 20 μm.

(C) Scatchard analysis of dissociation constant (Kd) showed that NL2 co-expression with ST3 did not change the binding affinity of Nrxn-Fc to NL2. Binding affinity of Nrxn-Fc to HA-NL2 without ST3 or with ST3 was characterized by an estimated dissociation constant (Kd) of 12.93 and 13.64 nM, respectively.

(D) Scatchard analysis for Kd showed that co-expression with NL2 did not change the binding affinity of ST3 to PTPδ-Fc. In addition, WT ST3, ST3ΔLRR9 or ST3ΔLRR12 in the presence of NL2 had similar binding affinity to PTPδ-Fc.

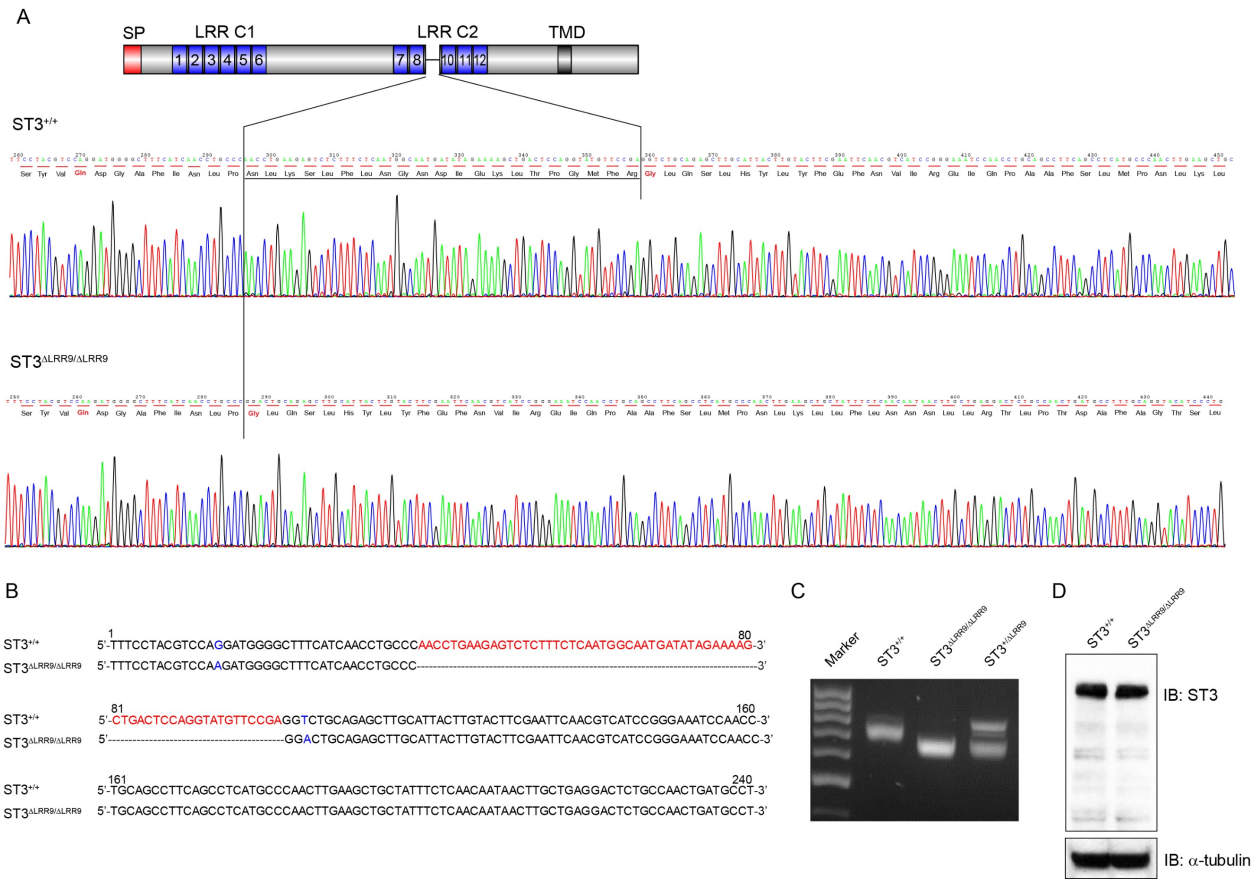



**Figure S8. Characterization of ST3$^{\Delta LRR9/\Delta LRR9}$ mice (related to Figure 7)**

(A-B) Schematic diagram illustrated the domain structure of ST3 lacking LRR9 motif (A). DNA sequencing and nucleotides alignment showed successful deletion of the LRR9 domain in ST3$^{\Delta LRR9/\Delta LRR9}$ mice (A-B).

(C) Genotyping of ST3$^{+/+}$, ST3$^{+/\Delta LRR9}$, and ST3$^{\Delta LRR9/\Delta LRR9}$ mice.

(D) Western blot analysis of hippocampal lysates showed that genetic deletion of ST3 LRR9 domain in ST3$^{\Delta LRR9/\Delta LRR9}$ mice did not alter the expression levels of ST3.