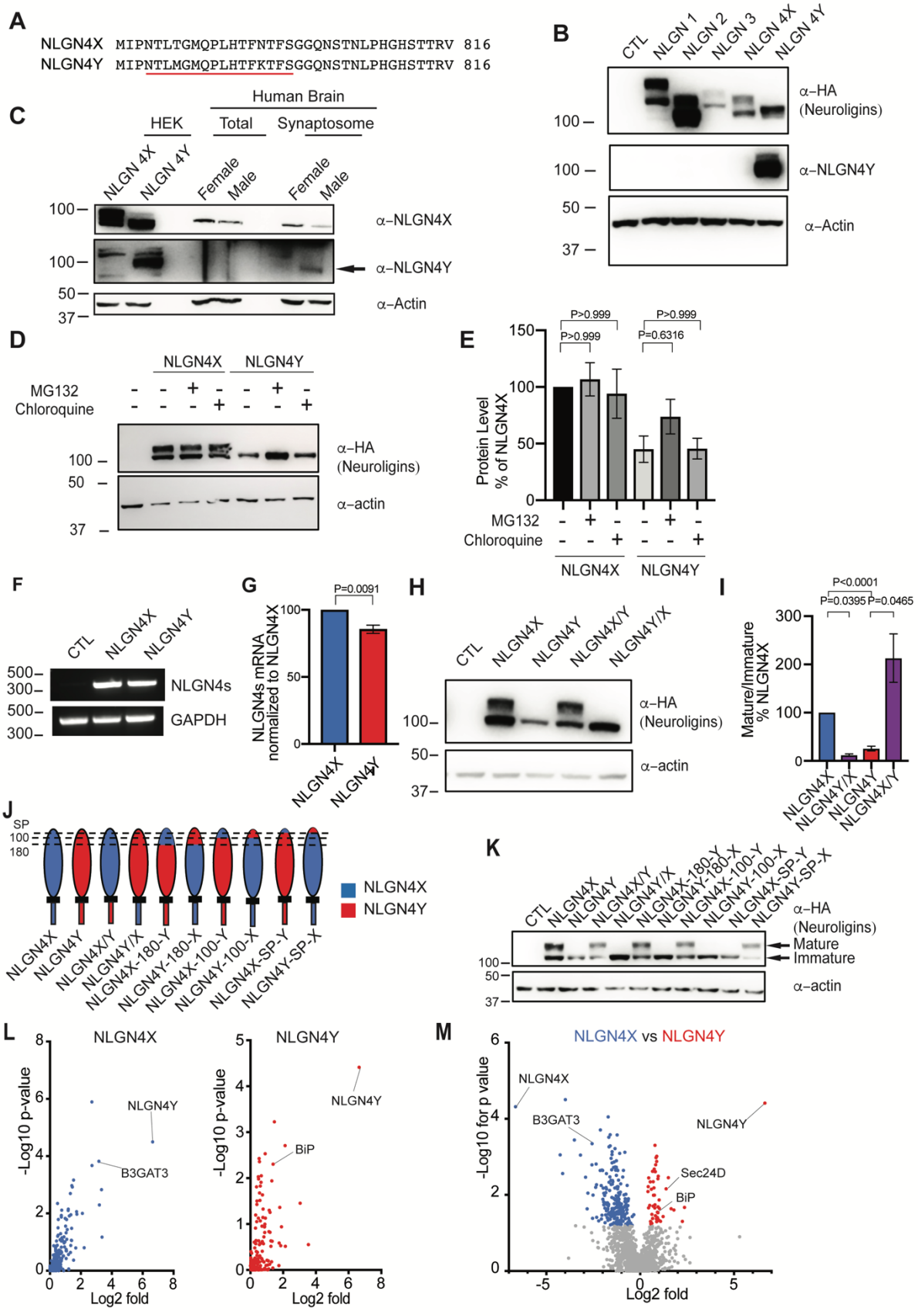
A
NLGN4X MIPNTLTGMQPLHTFNTFSGGQNSTNLPHGHSTTRV 816
NLGN4Y MIPNTLMGMQPLHTFKTFSGGQNSTNLPHGHSTTRV 816
B
CTL
NLGN 1
NLGN 2
NLGN 3
NLGN 4X
NLGN 4Y
100
50
37
α–HA (Neuroligins)
α–NLGN4Y
α–Actin
C
Human Brain
HEK
Total
Synaptosome
NLGN 4X
NLGN 4Y
Female
Male
Female
Male
100
100
50
37
α–NLGN4X
α–NLGN4Y
α–Actin
D
NLGN4X
NLGN4Y
MG132
Chloroquine
100
50
37
α–HA (Neuroligins)
α–actin
E
P>0.999
P>0.999
P>0.999
P=0.6316
150
100
50
0
Protein Level % of NLGN4X
MG132
Chloroquine
NLGN4X
NLGN4Y
F
CTL
NLGN4X
NLGN4Y
500
300
500
300
NLGN4s
GAPDH
G
P=0.0091
100
50
0
NLGN4s mRNA normalized to NLGN4X
NLGN4X
NLGN4Y
H
CTL
NLGN4X
NLGN4Y
NLGN4X/Y
NLGN4Y/X
100
50
37
α–HA (Neuroligins)
α–actin
I
P<0.0001
P=0.0395
P=0.0465
300
200
100
0
Mature/Immature % NLGN4X
NLGN4X
NLGN4Y/X
NLGN4Y
NLGN4X/Y
J
SP
100
180
NLGN4X
NLGN4Y
NLGN4X/Y
NLGN4Y/X
NLGN4X-180-Y
NLGN4Y-180-X
NLGN4X-100-Y
NLGN4Y-100-X
NLGN4X-SP-Y
NLGN4Y-SP-X
K
CTL
NLGN4X
NLGN4Y
NLGN4X/Y
NLGN4Y/X
NLGN4X-180-Y
NLGN4Y-180-X
NLGN4X-100-Y
NLGN4Y-100-X
NLGN4X-SP-Y
NLGN4Y-SP-X
100
50
37
α–HA (Neuroligins)
Mature
Immature
α–actin
L
NLGN4X
NLGN4Y
-Log10 p-value
Log2 fold
NLGN4Y
B3GAT3
BiP
M
NLGN4X vs NLGN4Y
-Log10 for p value
Log2 fold
NLGN4X
B3GAT3
NLGN4Y
Sec24D
BiP

Figure S1. Related to Figure 1 and 2. NLGN4Y is expressed in male brain and selectively binds to BiP

A. Sequence alignment for the intracellular domain of NLGN4X and NLGN4Y. The NLGN4Y antibody epitope is underlined in red. **B.** Immunoblot of transfected HA-tagged NLGN 1, 2, 3, 4X, and 4Y. **C.** Immunoblot of male and female brain synaptosomal preps. **D.** NLGN4X and NLGN4Y expressed in HEK293T cells were treated with MG132 or chloroquine for 24 hours to examine protein degradation. Cells were lysed and protein level was determined by immunoblotting with HA-Ab. **E.** Protein level (means ± S.E.M.) normalized to NLGN4X. P values were calculated by one-way ANOVA with Bonferroni's comparison test. (n=4). **F.** NLGN4X and NLGN4Y expression (319 bp) in transfected HEK293T cells. **G.** Expression level (means ± S.E.M.) normalized to NLGN4X. P value was calculated by t-test. **H.** Immunoblot of HEK293T cells expressing NLGN4X, NLGN4Y, NLGN4X/Y, NLGN4Y/X. **I.** Ratio of mature/immature level (means ± S.E.M.) normalized to NLGN4X or NLGN4Y. P values were calculated by ANOVA with Bonferroni's comparison test. (n=8). **J.** Schematic for domain swapping of NLGN4X and NLGN4Y. **K.** Immunoblot comparing chimeras of NLGN4X and NLGN4Y expressed in HEK293T cells. NLGN4X-100-4Y is sufficient to promote NLGN4Y maturation. **L.** Volcano plots of enrichment of protein interactors for NLGN4X or NLGN4Y referenced to HA beads. **M.** Volcano plot of protein interactor enrichment for NLGN4X vs NLGN4Y

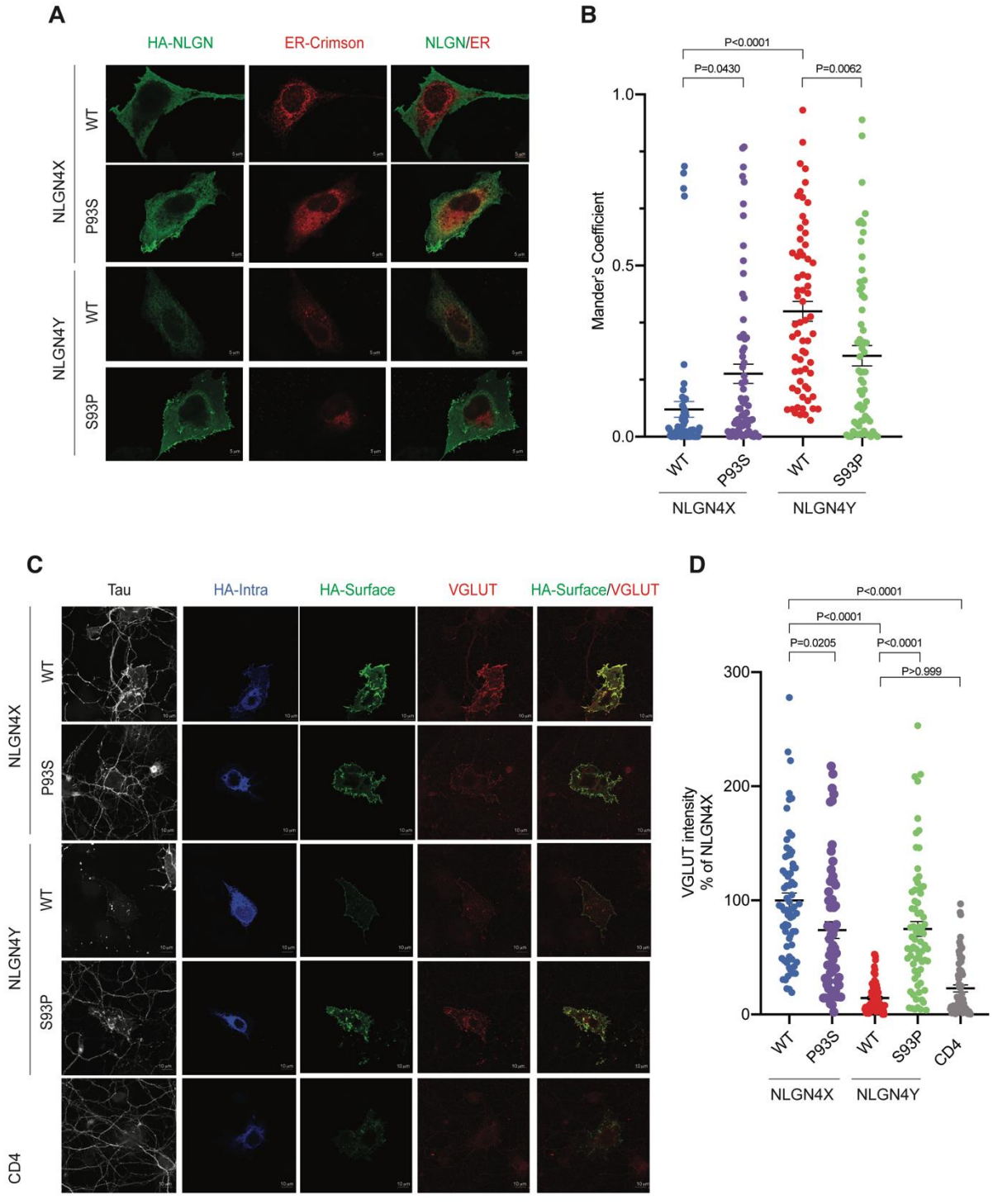
A
HA-NLGN
ER-Crimson
NLGN/ER
NLGN4X
WT
P93S
NLGN4Y
WT
S93P
B
P<0.0001
P=0.0430
P=0.0062
Mander's Coefficient
1.0
0.5
0.0
WT
P93S
WT
S93P
NLGN4X
NLGN4Y
C
Tau
HA-Intra
HA-Surface
VGLUT
HA-Surface/VGLUT
NLGN4X
WT
P93S
NLGN4Y
WT
S93P
CD4
D
P<0.0001
P<0.0001
P=0.0205
P<0.0001
P>0.999
VGLUT intensity
% of NLGN4X
300
200
100
0
WT
P93S
WT
S93P
CD4
NLGN4X
NLGN4Y

Figure S2. Related to Figure 2 and 3. NLGN4Y localizes to ER thus inhibits it to induce synapses

A. Cos-7 cells coexpressing NLGN4X (WT, P93S) or NLGN4Y (WT, S93P) with E2-Crimson, an ER marker. NLGN4X (WT, P93S) and NLGN4Y (WT, S93P) were labeled with anti-HA and Alexa 488-conjugated secondary (green). E2-Crimson fluorescent was excited using far-red laser (red) to label ER. DAPI was used to visualize the nucleus (blue). **B.** Manders Coefficient of NLGN4X (WT, P93S) or NLGN4Y (WT, S93P) with ER. P value was calculated by ANOVA with Bonferroni's (n=3). **C.** Cos-7 cells expressing NLGN4X (WT, P93S), NLGN4Y (WT, S93P), or CD4 were cocultured with rat hippocampal neurons. NLGN4X (WT, P93S), NLGN4Y (WT, S93P) were labeled with rat anti-HA and Alexa 555 -conjugated secondary (green) for surface expression, and with rabbit anti-HA and Alexa 488-conjugated secondary (blue) for intracellular expression. Endogenous tau and VGLUT were labeled with their respective antibody with Alexa 405-conjugated secondary for tau (white) and Alexa 647-conjugated secondary for VGLUT (red). **D.** VGLUT intensity levels (means ± S.E.M.) were normalized to NLGN4X. P values were calculated by ANOVA with Bonferroni's comparison test.

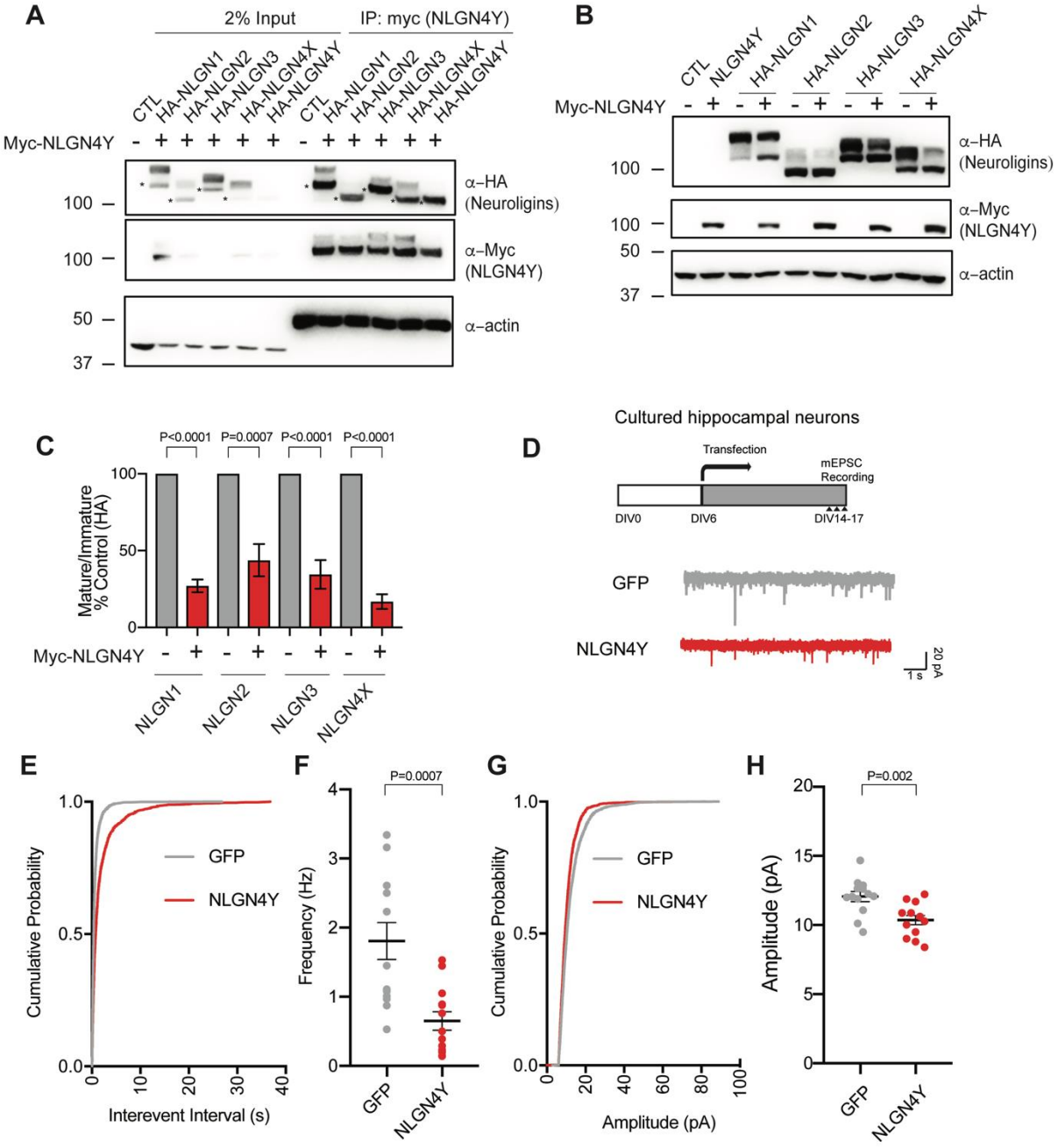
A
2% Input
IP: myc (NLGN4Y)
CTL
HA-NLGN1
HA-NLGN2
HA-NLGN3
HA-NLGN4X
HA-NLGN4Y
Myc-NLGN4Y
100
100
50
37
α–HA (Neuroligins)
α–Myc (NLGN4Y)
α–actin
B
CTL
NLGN4Y
HA-NLGN1
HA-NLGN2
HA-NLGN3
HA-NLGN4X
Myc-NLGN4Y
100
100
50
37
α–HA (Neuroligins)
α–Myc (NLGN4Y)
α–actin
C
P<0.0001
P=0.0007
P<0.0001
P<0.0001
Mature/Immature % Control (HA)
100
50
0
Myc-NLGN4Y
NLGN1
NLGN2
NLGN3
NLGN4X
D
Cultured hippocampal neurons
Transfection
mEPSC Recording
DIV0
DIV6
DIV14-17
GFP
NLGN4Y
20 pA
1 s
E
Cumulative Probability
1.0
0.5
0.0
GFP
NLGN4Y
0
10
20
30
40
Interevent Interval (s)
F
P=0.0007
Frequency (Hz)
4
3
2
1
0
GFP
NLGN4Y
G
Cumulative Probability
1.0
0.5
0.0
GFP
NLGN4Y
0
20
40
60
80
100
Amplitude (pA)
H
P=0.002
Amplitude (pA)
20
15
10
5
0
GFP
NLGN4Y

Figure S3. Related to Figure 3. Heterodimerization with NLGN4Y decreases NLGN1, 2, 3, 4X maturation and synaptic strength

A. HA-NLGN1, 2, 3, 4X, or 4Y were coexpressed with myc-NLGN4Y and then immunoprecipitated using Myc antibody. Immunoblot of HA-NLGN1, 2, 3, 4X, or 4Y from the immunoprecipitates. The immature bands are enriched in the immunoprecipitated fraction. **B.** Immunoblot of HA-NLGN1, 2, 3, or 4X coexpressed with Myc-NLGN4Y. **C.** Mature/immature HA-NLGNs level (means ± S.E.M.) normalized to NLGN1, 2, 3, or 4X. P values were calculated using an unpaired *t* test. (n=6). **D.** Experimental design for recording, and representative mEPSC traces recorded in cultured hippocampal neurons expressing GFP or GFP with NLGN4Y. **E and F.** mEPSC frequency cumulative probability and mean. P values were calculated by t-test (n=3). **G and H.** mEPSC amplitude cumulative probability and mean. P values were calculated by t-test. (n=3)

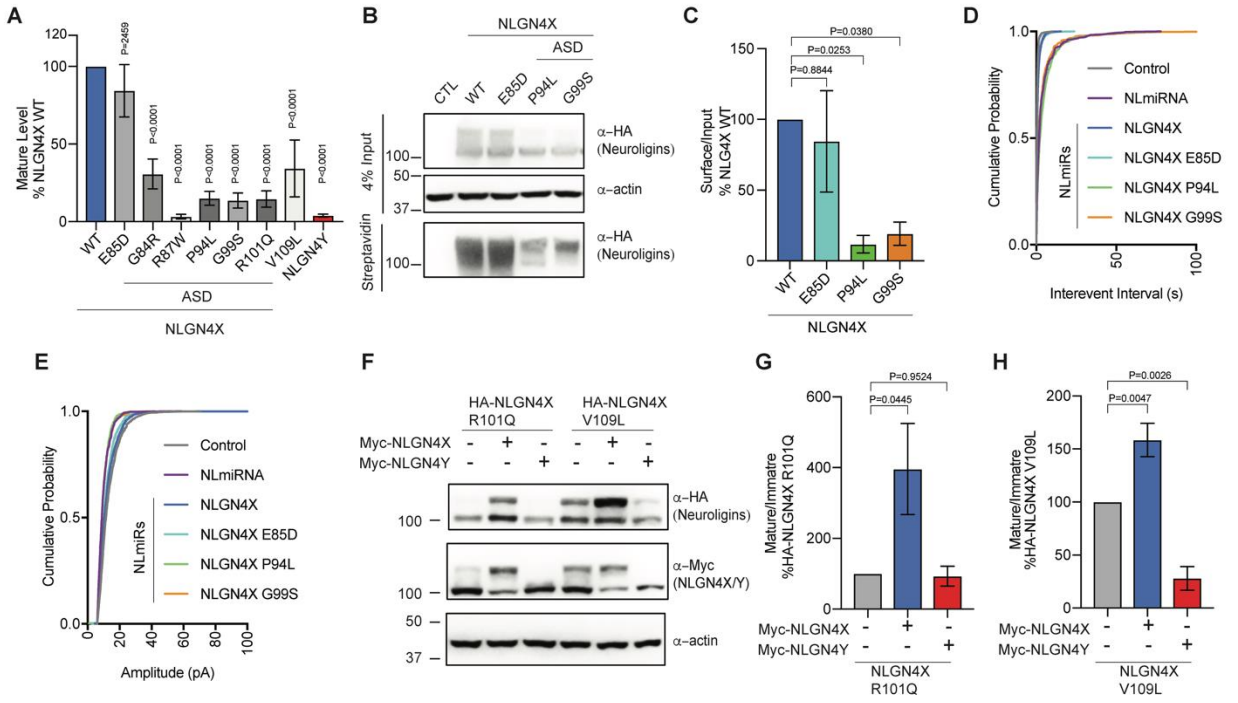

Figure S4. Related to Figure 4. Decreased surface expression and synaptic transmission in NLGN4X ASD-associated mutations

A. Mature band levels (means ± S.E.M.) normalized to NLGN4X WT. P values were calculated by one-way ANOVA Bonferroni's comparison test. (n=4) **B.** Surface expression of NLGN4X (WT, E85D, P94L, or G99S) analyzed by immunoblotting isolated surface biotinylated proteins in transfected HEK293T cells. **C.** Surface expression level (means ± S.E.M.) normalized to NLGN4X WT. P values were calculated using one-way ANOVA Bonferroni's comparison test. (n=3). **D and E.** Cumulative probability of spontaneous mEPSC frequency and amplitude. P values were calculated by one-way ANOVA Bonferroni's comparison test. (n=3). **F.** Immunoblot of HA-NLGN4X and NLGN4Y coexpressed with Myc-NLGN4X (R101Q or V109L). **G.** Ratio of mature/immature levels (means ± S.E.M.) normalized to NLGN4X R101Q. **H.** Mature/immature level (means ± S.E.M.) normalized to NLGN4X V109L. P values were calculated by one-way ANOVA Bonferroni's comparison test. (n=3)

Variants	Inheritance Pattern	Sex	Primary Phenotype	Additional Comments/Reference
G84R	Maternal	M	ASD	Asymptomatic mothers. (Xu *et al.*, 2014)
R87W	De novo	M	ASD	(Zhang *et al.*, 2009)
P94L	N/A	N/A	n/a	GeneDX submitted on ClinVar with unknown significant.
G99S	Maternal	F	ASD	Mother also has learning disability. A brother also has learning disability. (Yan *et al.*, 2005)
		M	ASD	Mother also has learning disability. Sibling of above. (Yan *et al.*, 2005)
R101Q	Maternal	M	ASD	New variant from this manuscript
V109L	Maternal	M	ID	New variant from this manuscript
Q162K	De novo	F	ASD	(Xu *et al.*, 2014)
L211X	N/A	N/A	Anxiety, ADHD, Cerebral palsy	(Yuen *et al.*, 2017)
Q274X	Maternal	M	ADHD	(Yuen *et al.*, 2017)
A283T	Maternal	M	ASD	(Xu *et al.*, 2014)
Q329X	Maternal	M	ASD	(Yu *et al.*, 2013)
K378R	Maternal	M	ASD	(Pampanos *et al.*, 2009)
		M	ASD	(Yan *et al.*, 2005)
396X frameshift 1186t	Maternal	2XM	Asperger Syndrome/ASD	(Jamain *et al.*, 2003)
V403M	Maternal	M	ASD	have both affected and unaffected siblings. (Xu *et al.*, 2014)
429X (nt1253del(AG)	Maternal	13XM	ASD/ID	(Laumonnier *et al.*, 2004)
V454_A457X	De novo	M	ID	(Mayo *et al.*, 2016)
V522M	De novo	N/A	TD	(Wang *et al.*, 2018)
R704C	Maternal	M	ASD	unaffected sister (+/-)
R766Q	Maternal	M	ASD	(Yan *et al.*, 2005)

Table S1. Related to Figure 4. NLGN4X-associated variants. Variants found in NLGN4X in published literature are listed with inheritance pattern.