Zhang et al., Inventory of Supplemental Information

Supplemental Figures 1-8

Figure S1. Analysis of mIPSC and mRNA levels in hippocampal neurons infected with lentivirus expressing neurexin-2β; related to Figure 1.

Figure S2. Analysis of mIPSC and synapse number in hippocampal neurons transfected with a vector expressing neurexin-2β; related to Figure 2.

Figure S3. Analysis of IPSCs in hippocampal neurons transfected with Nrx-2β expression and control vectors, and treated for 48 hrs with pharmacological agents that either activate or silence the neurons, to test the effect of synaptic activity on the IPSC-inhibitory action of neurexin-2β; related to Figure 3.

Figure S4. Presents control experiments demonstrating that the NL2 KO does not produce a suppression of IPSCs in cultured neurons (A), that a pan-neurexin knockdown restricted to a postsynaptic neuron does not alter the synaptic strength of inhibitory synapses (B-F), and that truncated neurexin-2β ending with a KDEL sequence still binds to neuroligin-1 effectively but becomes retained in the ER (G-I); related to Figure 4.

Figure S5. EPSCs and IPSCs measurements of cultured hippocampal neurons treated with recombinant Ig-neurexin-1β (IgNrx-1β) or control fusion protein (IgC), and protein quantitations of such neurons; and related to Figure 5.

Figure S6. Extensive additional control affinity chromatography experiments using immobilized Ig- or GST-fusion proteins, related to Figure 6.

Figure S7. Affinity chromatography experiments of the binding of endogenous brain $GABA_A\alpha1$ and NL1 to immobilized Ig-neurexin-1β or IgC control fusion protein, related to Figure 7.

Figure S8. Lack of changes in apparent GABA-affinity from HEK293 cells stably expressing $GABA_A$ receptors transfected with either control or Neurexin-2β, related to Figure 8.

Supplemental Tables S1-S4

Table S1, related to Figure 1-5. Passive membrane parameters (membrane capacitance (Cm), membrane resistance (Rm) and access resistance (Rs) in cultured neurons

Table S2, related to Figure 6. Numerical summary of results of affinity chromatography experiments

Table S3, related to Figures 1-5, 8 and Figures S1-S5 and S8. Numerical summary of values obtained in the electrophysiological experiments, as depicted in the main and supplementary figures, to allow independent assessment of raw data by others.

Table S4, related to all Figures. Summary of constructs/expressed proteins used in this study.

Supplemental Experimental Procedures

Detailed description of all experimental procedures to allow others to follow our experimental protocols

Supplemental References

References unique to the Supplementary Materials.

Supplemental Information for Zhang et al., "Neurexins Physically and Functionally Interact with $GABA_A\alpha1$-receptors"

Supplemental Figures

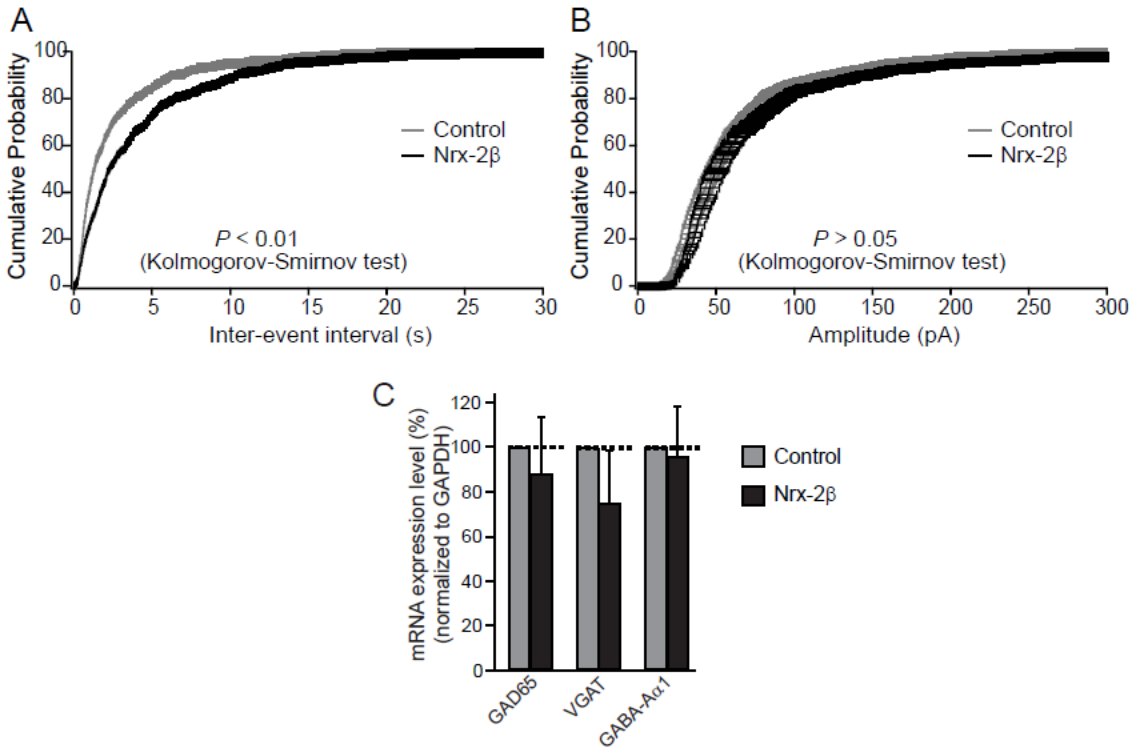

Figure S1

Analysis of hippocampal neurons infected with lentiviruses expressing EGFP (control) or neurexin-2β: mIPSC parameters analyzed using cumulative probability plots (A and B), and mRNA levels determined using quantitative PCR (C) (related to Figure 1)

A and **B.** Cumulative graphs of the mIPSC inter-event interval (A) and amplitude (B) in hippocampal cultured neurons infected with lentiviruses expressing neurexin-2β (Nrx-2β) or EGFP (control). Statistical comparisons were made by Kolmogorov-Smirnov test. Note that there is no significant difference in the distribution of mIPSCs amplitude (control: n=29/3; Nrx-2β: n=27/3).

C. Measurements of the mRNA levels for GAD65, VGAT, and $GABA_A\alpha1$-receptor using quantitative rt-PCR in neurons treated as described in the legend to Figure 1 (n=3 independent cultures). Summary graphs show means ± SEMs; statistical comparisons were made by Student's t-test, and no significant differences were detected.

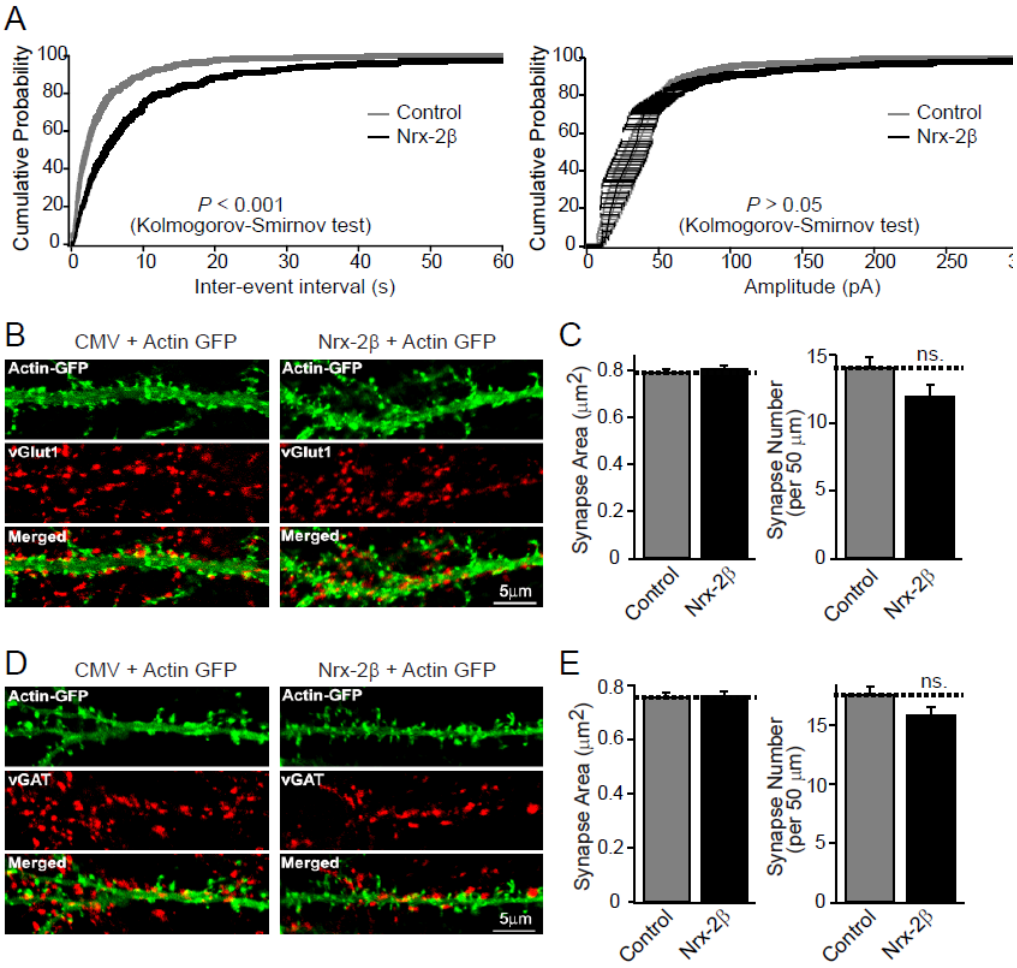

Figure S2

Analysis of mIPSCs (A) and of synapse numbers (C-E) in transfected hippocampal neurons expressing neurexin-2β (related to Figure 2)

A. Cumulative probability plots of the mIPSC inter-event interval (left) and amplitude (right) in hippocampal cultured neurons transfected with a CFP expression plasmid and either an empty vector (control), or a vector encoding neurexin-2β (Nrx-2β). Statistical comparisons were made by Kolmogorov-Smirnov test (control: n=26/3; Nrx-2β: n=24/3).

B. Representative fluorescence images of neurons transfected with vectors expressing an actin-EGFP fusion protein alone (control), or together with neurexin-2β (Nrx-2β). Images show the EGFP fluorescence (green), vGLUT1 immunofluorescence labeling (red), and the merged view. Calibration bar applies to all panels.

C. Quantitations of the size (left) and density (right) of vGLUT1-positive synapses on dendrites of transfected neurons (control: n = 62/4 neurons/cultures; Nrx-2β: n = 62/4).

D. Same as B, except that the neurons were stained for VGAT instead of vGLUT1.

E. Quantitations of the size (left) and density (right) of vGAT-positive synapses on dendrites of transfected neurons (control: n = 64/4 neurons/cultures; Nrx-2β: n = 64/4).

For C and D, data shown are means ± SEMs. Statistical significance was determined by Student's t test (n.s., non-significant).

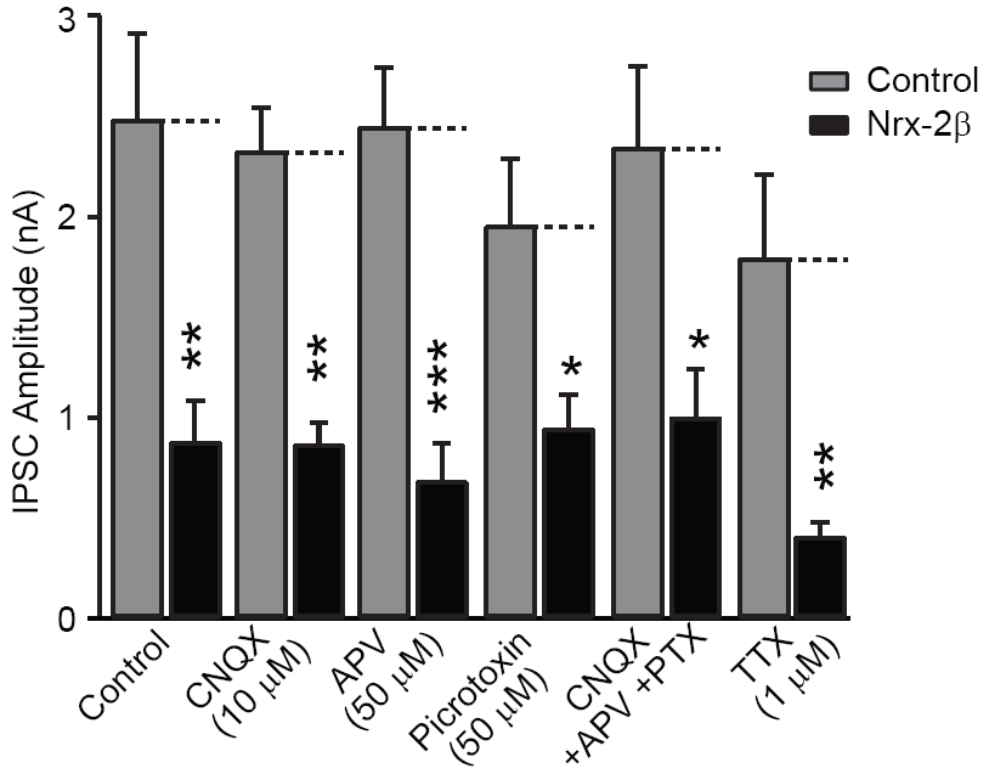

Figure S3

Neuronal activity does not alter the decrease in IPSCs induced by overexpression of neurexin-2β (related to Figure 3)

Summary graphs of the amplitudes of evoked IPSCs from neurons transfected with CFP expression plasmid and either an empty vector (control), or a vector encoding neurexin-2β (Nrx-2β). Cultured hippocampal neurons were transfected at DIV10, and treated with the indicated drugs from DIV10 to DIV14, except for the tetrodotoxin (TTX) group which was treated from DIV12 to DIV14 (untreated group: control, n = 9/3; Nrx-2β: n = 10/3; CNQX group: control, n = 14/3; Nrx-2β: n = 14/3; APV group: control, n = 21/3; Nrx-2β: n = 24/3; Picrotoxin group: control, n = 12/3; Nrx-2β: n = 14/3; CNQX + APV + PTX group: control, n = 18/3; Nrx-2β: n = 15/3; TTX group: control, n = 12/3; Nrx-2β: n = 15/3). Abbreviations used: CNQX, 6-cyano-7-nitro-quinoxaline-2,3-dione; APV, 5-amino-phosphono valerate; PTX, picrotoxin; TTX, tetrodotoxin. All data shown are means ± SEMs. Statistical significance was determined by Student's t test (compared to control; *=p<0.05; **=p<0.01; ***=p<0.001)..

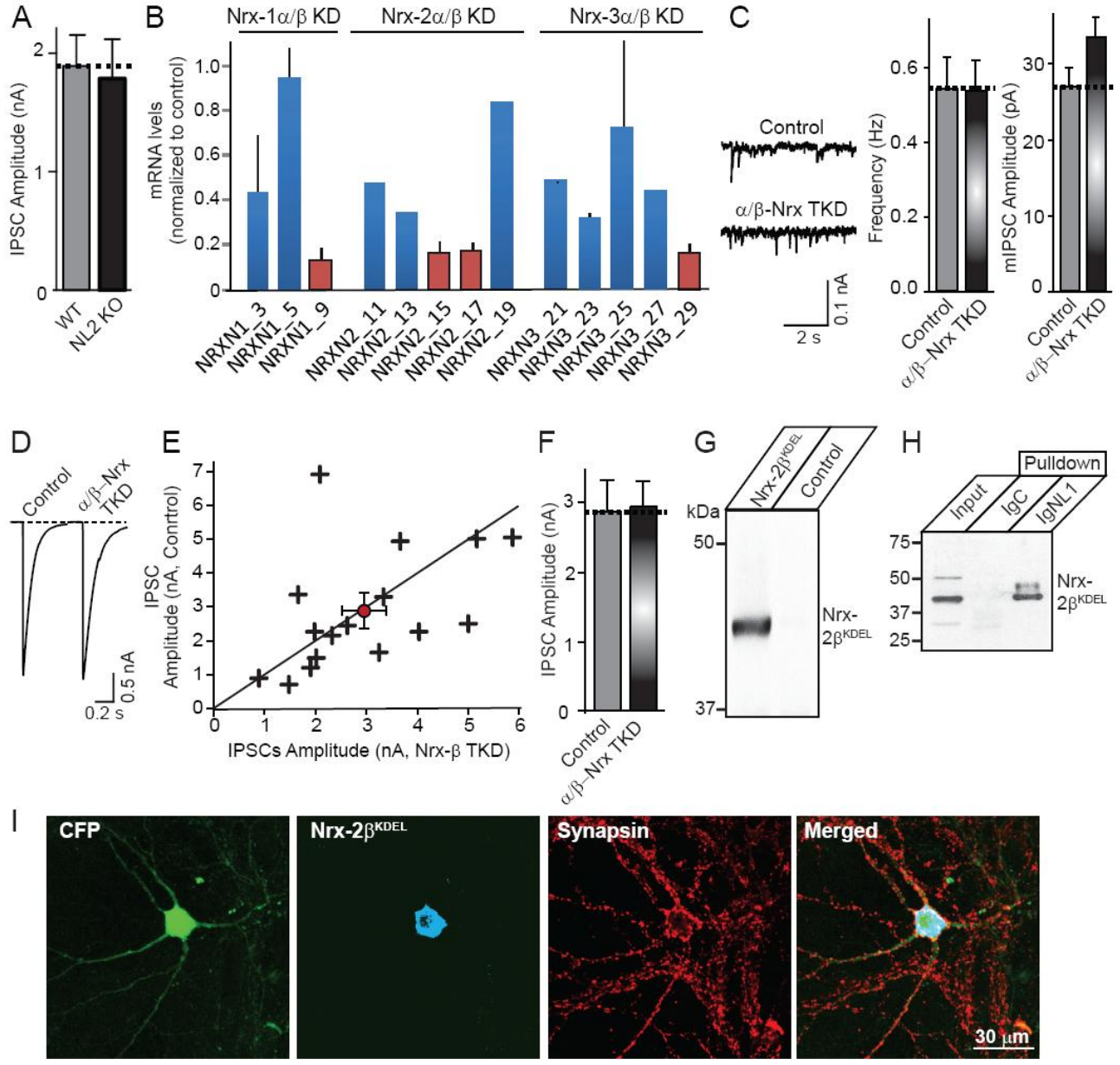

Figure S4

Control experiments ensuring that the inhibitory effect of neurexin-2β on inhibitory synaptic strength does not depend on neuroligin-2, but does require transport of neurexin-2β to the cell-surface without an intracellular action (related to Figure 3)

A. Mean amplitudes of IPSCs recorded from hippocampal neurons cultured from littermate wild-type (WT) and neuroligin-2 KO mice (NL2 KO) (control, n = 7/2; NL2 KO: n = 7/2). Note that in culture, neuroligin-2 KO neurons do not exhibit a major phenotype themselves. **B.** Measurements of the mRNA levels for neurexin-1, -2 and -3 in cortical neurons after expression of shRNAs targeting the corresponding neurexin. Neurons were infected with lentiviruses expressing the indicated shRNAs at DIV4, and the levels of the corresponding mRNAs were measured by quantitative rt-PCR at DIV13. Note that the NRX1_3 shRNA

corresponds to the commercial shRNA (Open Biosystems) used in a previous paper (de Wit et al., 2009).

C. Sample traces of mIPSCs recorded from neurons co-transfected with a CFP expression plasmid and three vectors encoding shRNA against neurexin-1, -2 and -3 (NRXN1_9, NRXN2_15 and NRXN3_29), and neighboring non-transfected neurons as controls (left), and summary graphs of the frequency (middle) and amplitudes (right) of mIPSCs. mIPSCs were recorded in 1 μM TTX and 10 μM CNQX (n=15 pairs/3 cultures).

D. and **E**. Representative traces (D) and two-dimensional plot (E) of IPSC amplitudes from pairs of neighboring neurons, one of which was co-transfected with three vectors encoding CFP and shRNAs against neurexin-1, -2 and -3. The red circle shows that means ± SEMs (n=16 pairs/4 cultures).

F. Bar diagram of the means ± SEMs from the data shown in E.

G. Representative immunoblot of proteins from HEK293 cells that were transfected with control vector, or a vector expressing myc-tagged neurexin-2β in which a KDEL sequence followed by a stop codon was inserted into the protein at the O-linked sugar region (Nrx-2β^{KDEL}; C-terminal sequence: RESSSTEQKLISEEDLKDEL-stop; analyzed by immuno-blotting for the myc-tag).

H. Pulldown of Nrx-2β^{KDEL} expressed in transfected HEK293 cells by immobilized IgNL1. Ig-fusion proteins of a short N-terminal control sequence (IgC) and the extracellular sequences of neuroligin-1 (NL1) were immobilized, and used as an affinity matrix. Bound proteins were analyzed by immunoblotting for the myc-tag.

I. Representative fluorescence images of neurons transfected with a vector expressing ER-trapped neurexin-2β (Nrx-2β^{KDEL}, myc-tagged) together with CFP. Images show the CFP (green), myc (blue), synapsin (red) immunofluorescence labeling and the merged view. Scale bars apply to all pictures. The data represent the full images for Figure 3.

All data shown are means ± SEMs. Statistical significance was determined by Student's t test (no significant differences were detected in any comparison)

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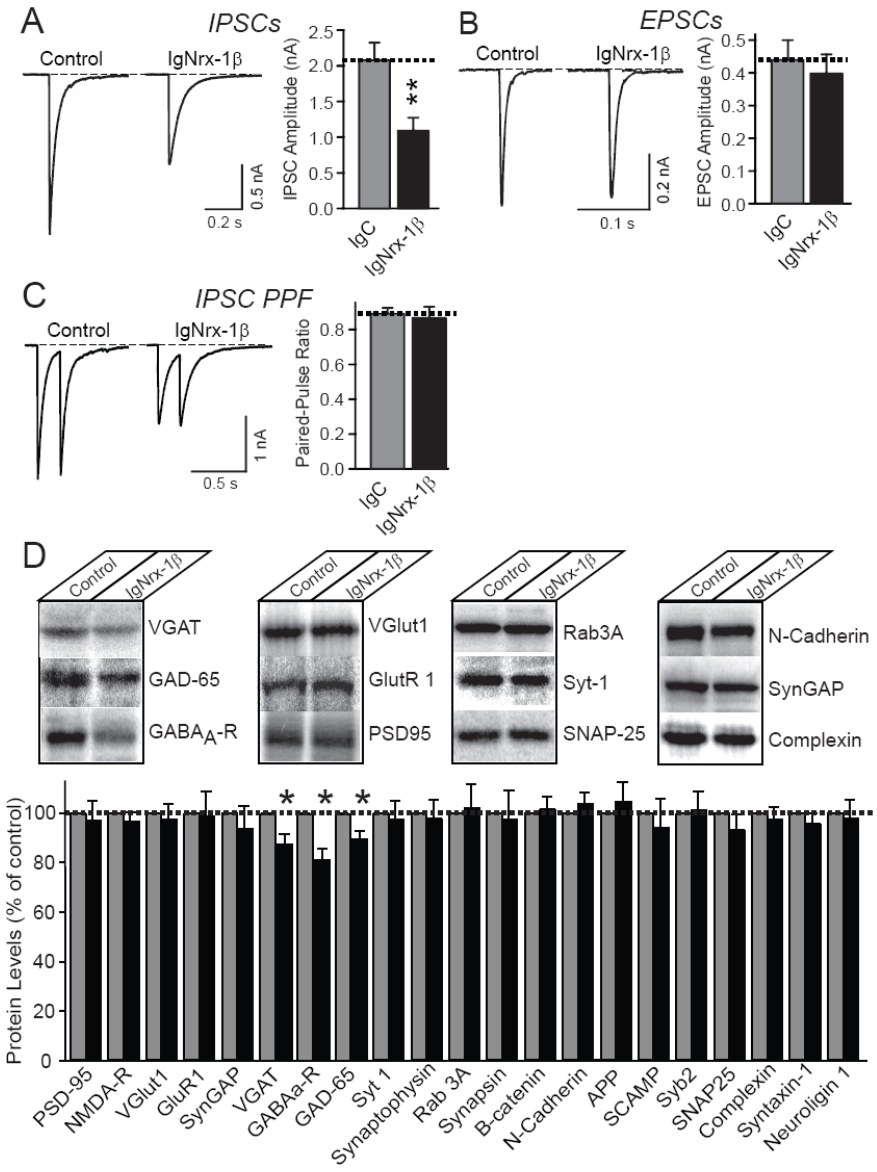

Figure S5

EPSC and IPSC recordings and protein quantitations of hippocampal neurons in culture treated with recombinant Ig-neurexin-1β (IgNrx-1β) or control fusion protein (IgC) (related to Figure 5)

A. Representative traces (left) and mean amplitudes (right) of IPSCs recorded from neurons incubated with IgNrx-1β (1 μM) or IgC (1 μM) for 96 h at 37 °C (IgC: n = 25/3; IgNrx-1β: n = 26/3).

B. Representative traces (left) and mean amplitudes (right) of EPSCs recorded from neurons incubated with IgNrx-1β (1 μM) or IgC (1 μM) for 96 h at 37°C (IgC: n = 15/3; IgNrx-1β: n = 15/3).

C. Representative traces (left) and paired-pulse ratio (right) of IPSCs evoked by two closely spaced action potentials (200 ms interval) recorded from neurons incubated with IgC or IgNrx-1β (IgC: n = 12/3; IgNrx-1β: n = 12/3).

D. Representative immunoblots (top) and summary graphs (bottom) of protein levels from protein samples collected from hippocampal cultured neurons treated with IgC (1 μM) or IgNrx-1β (1 μM) for 96 h. Protein levels were measured by quantitative immuno-blotting with ^{125}I-labeled secondary antibodies and phosphoImager detection (n = 4 independent cultures).

All data shown are means ± SEMs. Statistical significance was determined by Student's t test (comparison of IgNrx-1β to IgC; *=p<0.05; **=p<0.01; other comparisons reveal no statistically significant differences).

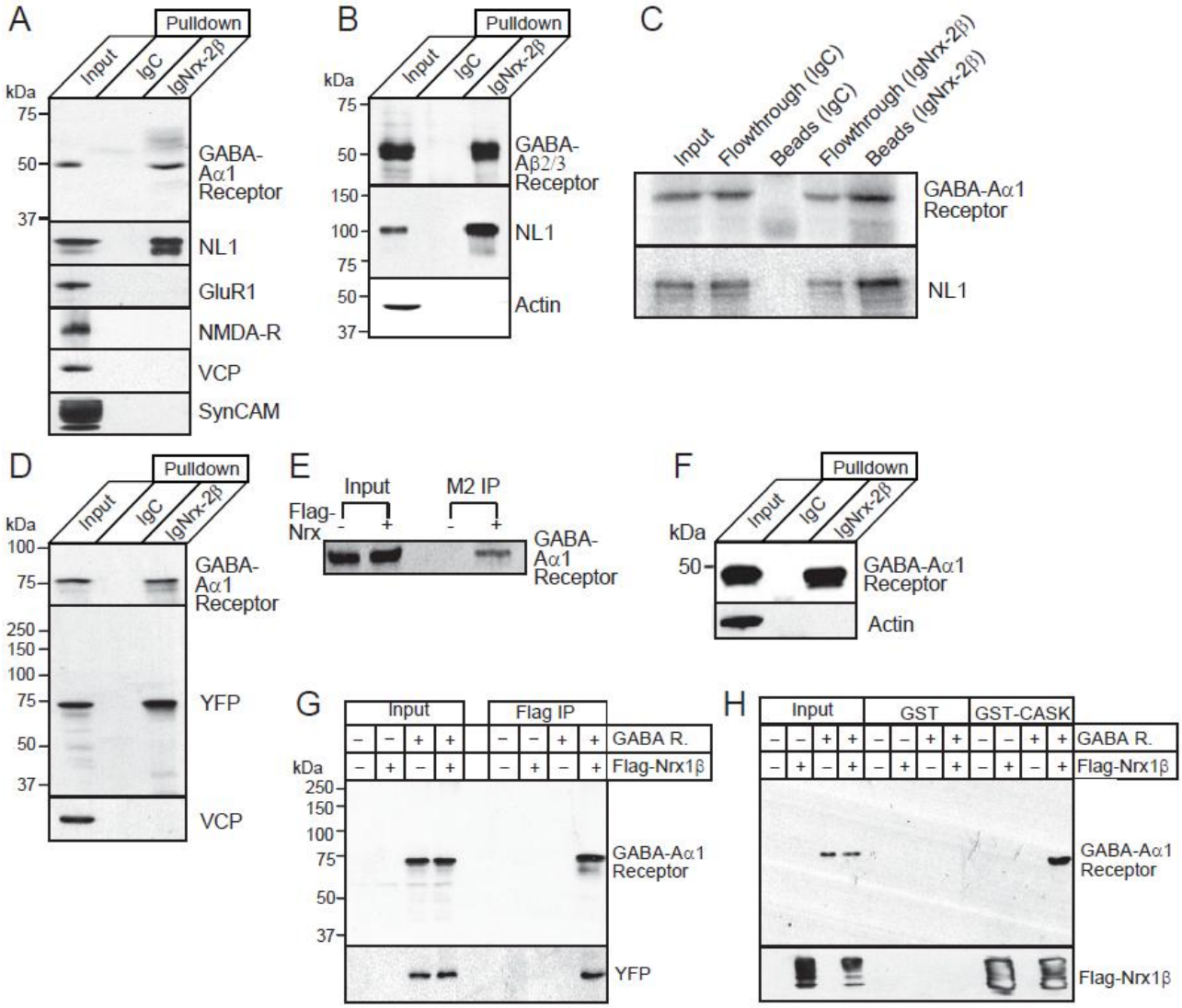

Figure S6

Miscellaneous affinity chromatography experiments using immobilized Ig- or GST-fusion proteins (related to Figure 6)

A and **B**. Selective binding of $GABA_A\alpha1$-receptor (A) and $GABA_A\beta2/3$-receptors (B) from rat brain homogenates to immobilized Ig-fusion protein of neurexin-2β (IgNrx-2β), but not to control Ig-fusion protein (IgC). Rat brain proteins solubilized in Triton X-100 were bound to immobilized Ig-fusion proteins, and bound proteins were analyzed by immunoblotting with antibodies to neuroligin-1 (NL1), glutamate receptor 1 (GluR1), NMDA-receptor (NMDA-R), vasolin-containing protein (VCP), and SynCAM.

C. Images of quantitative immunoblotting for the comparative analysis of binding of endogenous $GABA_A\alpha1$ and NL1 in brain homogenates to immobilized Ig-neurexin-2β or IgC control fusion protein (see Fig. 6B). Total rat brain proteins were solubilized in Triton X-100, and bound to IgNrx-2β or IgC in the presence of 2.5 mM Ca^{2+}. The flowthrough was collected, and bound proteins were eluted with SDS-sample buffer. Fractions were

analyzed by immunoblotting for $GABA_A\alpha1$-receptor and for neuroligin-1 (NL1) with ^{125}I-labeled secondary antibodies and PhosphoImager detection.

D. Selective binding of YFP-tagged $GABA_A\alpha1$ receptor expressed in transfected HEK293 cells to immobilized IgNrx-2β.

E. Co-immunoprecipitation with Flag-antibodies of GABA-Aα1 receptor with Flag-tagged neurexin-1β from HEK293 cells stably expressing $GABA_A$ receptors (α1β2γ2, CRL-2029 from ATCC) transfected with either control or Flag-tagged neurexin-1β.

F. Selective binding of $GABA_A\alpha1$-receptor from HEK293 cells stably expressing functional surface $GABA_A$ receptors (CRL-2029 from ATCC) to immobilized Ig-fusion protein of neurexin-2β (IgNrx-2β), but not to control Ig-fusion protein (IgC).

G. Co-immunoprecipitation of $GABA_A\alpha1$-receptor with neurexin-1β. HEK293 cells were transfected with a control plasmid and/or plasmids encoding YFP-tagged $GABA_A\alpha1$-receptor (GABA R.) and/or Flag-tagged neurexin-1β (Flag-Nrx-1β) as indicated, with a constant amount of total transfected DNA. Neurexin-1β was immunoprecipitated from lysates of transfected cells with Flag antibodies, and precipitated proteins were analyzed by immunoblotting with antibodies to $GABA_A\alpha1$-receptor or YFP.

H. Binding of $GABA_A\alpha1$-receptor/neurexin-1β complexes expressed in HEK293 cells transfected as described for A. Cell lysates were affinity-chromatographed on immobilized GST or GST-CASK as described in Fig. 6C, and analyzed by immunoblotting.

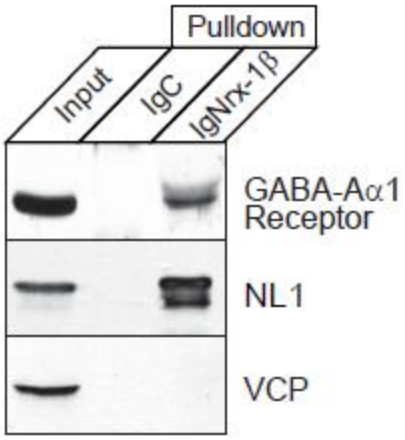

Figure S7

Affinity chromatography experiments of the binding of endogenous $GABA_A\alpha1$ and NL1 in brain homogenates to immobilized Ig-neurexin-1β or IgC control fusion protein (related to Figure 7)

Selective binding of $GABA_A\alpha1$-receptor from rat brain homogenates to immobilized Ig-fusion protein of neurexin-1β (IgNrx-1β), but not to control Ig-fusion protein (IgC). Rat brain proteins solubilized in Triton X-100 were bound to immobilized Ig-fusion proteins, and bound proteins were analyzed by immunoblotting with antibodies to $GABA_A\alpha1$-receptor, neuroligin-1 (NL1) and vasolin-containing protein (VCP). This control experiment ensures that the neurexin-1β used for the measurements of binding affinities in Fig. 7 fully bind sto the $GABA_A\alpha1$-receptor.

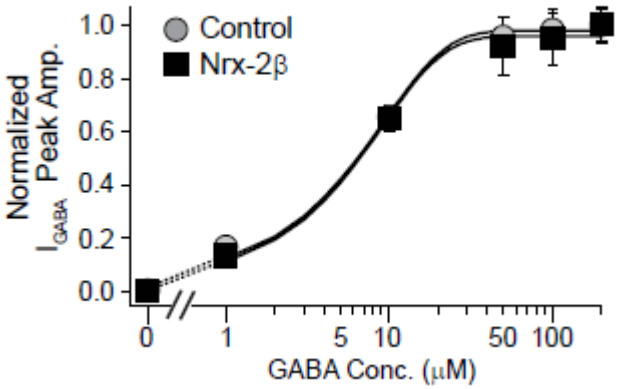

Figure S8

Lack of changes in apparent GABA-affinity from HEK293 cells stably expressing $GABA_A$ receptors transfected with either control or Neurexin-2β (related to Figure 8)

Normalized GABA-induced responses are plotted with GABA concentrations and fitted with Hill relation (control: n = 19/4; Nrx-2β: n = 17/4). Data are same as in Fig. 8B.

Supplemental Tables

Table S1 (related to Figures 1-5)

Passive membrane parameters (membrane capacitance (Cm), membrane resistance (Rm) and access resistance (Rs) in cultured neurons

	Recombinant protein treatment		Lentiviral Infection		Calcium-phosphate Transfection				
	IgC	IgNrx-1β	Control	Nrx-2β	Control	Nrx-1β	Nrx-2β	Nrx-3β	Nrx-1α
Cm (pF)	40 ± 2 (38/3)	37 ±2 (40/3)	42 ± 2 (86/10)	38 ±2 (86/10)	43 ± 1 (72/4)	39 ± 3 (54/4)	36 ±2 (86/4)	40 ± 4 (50/4)	44 ± 2 (23/2)
Rm (MΩ)	362 ± 30	380 ± 37	389 ± 31	320 ± 17	340 ± 24	341 ± 48	366 ± 37	335 ± 40	332 ± 40
Rs (MΩ)	10 ± 1	9 ± 1	12 ± 1	13 ± 1	11 ± 1	12 ± 1	12 ± 1	13 ± 1	12 ± 1

Data represent the summary from all electrophysiological experiments. Numerical values are given as means ± SEMs. The numbers in the bracket indicate the number of cells analyzed/of independent batches of cultures (applies to all parameters for a given construct).

Table S2 (related to Figure 6)

Numerical summary of results of affinity chromatography experiments

Parameter	**Transfect with**	**Infect. with**	**Value (%, mean)**	**Value (sem.)**	**n***	**p-value** (p<0.05)	**Stat. test**	**Fig.**
Flow-through (GABA-Aα1)			88	2	8			6B
Flow-through (NL1)			67	1	8			6B
Bound (GABA-Aα1)			14	2	8			6B
Bound (NL1)			29	2	8			6B
Flow-through (GABA-Aα1)			93	4	6			6D
Flow through (NL1)			97	4	6			6D
Bound (GABA-Aα1)			5.3	1.2	6			6D
Bound (NL1)			4.6	0.2	6			6D

Data are from figure 6. Bound proteins were analyzed by immunoblotting for GABAAα1-receptor and neuroligin-1 (NL1) with ^{125}I-labeled secondary antibodies and phosphoimager detection for quantitation. The percentage of bound protein and unbound protein (Flow through) was calculated by dividing the amount of bound protein and unbound protein (GABA-Aα1 or NL1) by the total amount protein (GABA-Aα1 or NL1) in the input that was used for pulldowns. *n = number of independent affinity chromatography experiments.

Table S3 (related to Figures 1-5, 8 and Figures S1-S5 and S8)

Numerical summary of values obtained in the electrophysiological experiments, as depicted in the main and supplementary figures

Parameter	**Transfect with**	**Infect. with**	**Value (mean)**	**Value (sem.)**	**n***	**p-value** (p<0.05)	**Stat. test**	**Fig.**
mEPSC freq. (Hz)		control	0.42	0.06	25/3			1A
mEPSC freq. (Hz)		Nrx-2β	0.50	0.09	26/3	p>0.05	t-test	1A
mEPSC amp. (pA)		control	26.85	1.11	25/3			1A
mEPSC amp. (pA)		Nrx-2β	27.27	0.78	26/3	p>0.05	t-test	1A
mIPSC freq. (Hz)		control	0.63	0.08	29/3			1B
mIPSC freq. (Hz)		Nrx-2β	0.30	0.04	27/3	p<0.001	t-test	1B
mIPSC amp. (pA)		control	53.07	4.12	29/3			1B
mIPSC amp. (pA)		Nrx-2β	51.95	5.83	27/3	p>0.05	t-test	1B
eEPSC amp. (pA)		control	459	44	54/8			1C
eEPSC amp. (pA)		Nrx-2β	398	40	59/8	p>0.05	t-test	1C
eEPSC charge (pc)		control	3.25	0.53	54/8			1C
eEPSC charge (pc)		Nrx-2β	2.84	0.49	59/8	p>0.05	t-test	1C
eIPSC amp. (pA)		control	2764	155	12/3			1D
eIPSC amp. (pA)		Nrx-2β	1210	130	13/3	p<0.001	t-test	1D
eIPSC amp. (pA)		Ct-Nrx-1β	2916	255	15/3	p>0.05	t-test	1D
eIPSC charge (nc)		control	0.28	0.04	12/3			1D
eIPSC charge (nc)		Nrx-2β	0.17	0.02	13/3	p<0.01		1D
eIPSC charge (nc)		Ct-Nrx-1β	0.31	0.04	15/3	p>0.05		1D
eEPSC amp. (pA)		control	384	50	11/2			1E
eEPSC amp. (pA)		Nrx-2β (-ss4)	338	102	11/2	p>0.05	t-test	1E
eEPSC amp. (pA)		Nrx-2β (+ss4)	341	32	11/2	p>0.05	t-test	1E
eIPSC amp. (pA)		control	2926	357	50/8			1F
eIPSC amp. (pA)		Nrx-2β (-ss4)	1496	214	51/8	p<0.01	t-test	1F
eIPSC amp. (pA)		Nrx-2β (+ss4)	1237	230	46/8	p<0.001	t-test	1F
Synapse area (μm^2)	control		0.91	0.1	29/3			1H
Synapse area (μm^2)	Nrx-2β		1.0	0.1	40/3	p>0.05	t-test	1H

Parameter	**Transfect with**	**Infect. with**	**Value (mean)**	**Value (sem.)**	**n***	**p-value** (p<0.05)	**Stat. test**	**Fig.**
Synapse number (per 50 µm)	control		14.1	0.7	29/3			1H
Synapse number (per 50 µm)	Nrx-2β		13.2	0.6	40/3	p>0.05	t-test	1H
mIPSC freq. (Hz)	control		0.22	0.04	26/3			2A
mIPSC freq. (Hz)	Nrx-2β		0.06	0.02	24/3	p<0.01	t-test	2A
mIPSC amp. (pA)	control		46.31	7.36	26/3			2A
mIPSC amp. (pA)	Nrx-2β		48.54	11.49	24/3	p>0.05	t-test	2A
eIPSC amp. (pA, 1 mM $[Ca^{2+}]_e$)	control		489.7	80.47	17/3			2D
eIPSC amp. (pA, 2 mM $[Ca^{2+}]_e$)	control		1779	208.5	17/3			2D
eIPSC amp. (pA, 5 mM $[Ca^{2+}]_e$)	control		2498	251.1	20/3			2D
eIPSC amp. (pA, 1 mM $[Ca^{2+}]_e$)	Nrx-2β		317.7	134.1	17/3	p>0.05	t-test	2D
eIPSC amp. (pA, 2 mM $[Ca^{2+}]_e$)	Nrx-2β		561.1	157.1	18/3	p<0.001	t-test	2D
eIPSC amp. (pA, 5 mM $[Ca^{2+}]_e$)	Nrx-2β		896.3	246.9	23/3	p<0.001	t-test	2D
eIPSC amp. (pA)	control		1576.1	354.8	11/3			2B
eIPSC amp. (pA)	Neigh. To control		1381.3	305.8	12/3	p>0.05	t-test	2B
eIPSC amp. (pA)	Neigh. To Nrx-2β		1316.4	173.1	11/3			2B
eIPSC amp. (pA)	Nrx-2β		440.9	67.0	12/3	p<0.001	t-test	2B
eIPSC ratio	control		0.932	0.041	11/3			2B
eIPSC ratio	Nrx-2β		0.328	0.038	12/3	p<0.001	t-test	2B
eEPSC amp. (pA)	control		434.7	82.48	28/4			2E
eEPSC amp. (pA)	Nrx-2β		391.3	60.71	32/4	p>0.05	t-test	2E
eEPSC charge. (pC)	control		3.90	0.73	28/4			2E
eEPSC charge. (pC)	Nrx-2β		3.34	0.63	32/4	p>0.05	t-test	2E
Sucrose (IPSC, nC)	control		10.45	1.20	36/8			2F
Sucrose (IPSC, nc)	Nrx-2β		6.51	0.99	42/8	p<0.05	t-test	2F
NL1 Fluro. Inten. (A.U)	control		100	3.34135	55/4			2G
NL1 Fluro. Inten. (A.U)	Nrx-2β		104.533	4.31267	62/4	p>0.05	t-test	2G
Syt1 Fluro. Inten. (A.U)	control		100	1.62489	55/4			2G
Syt1 Fluro. Inten. (A.U)	Nrx-2β		104.148	2.59603	62/4	p>0.05	t-test	2G
GABAα1 Fluro. Inten. (A.U)	control		100	3.24765	52/4			2G

Parameter	**Transfect with**	**Ig-Nrx**	**Value (mean)**	**Value (sem.)**	**n***	**p-value** (p<0.05)	**Stat. test**	**Fig.**
GABAα1 Fluro. Inten. (A.U)	Nrx-2β		81.1016	2.55337	69/4	p<0.001	t-test	2G
GAD65 Fluro. Inten. (A.U)	control		100	1.2493	52/4			2G
GAD65 Fluro. Inten. (A.U)	Nrx-2β		91.3215	2.18088	69/4	p<0.01	t-test	2G
eEPSC amp. (pA)	control		523.9	100	30/4			3A
eEPSC amp. (pA)	Nrx-1β		419.5	70.61	31/4	p>0.05	t-test	3A
eEPSC amp. (pA)	Nrx-2β		332.2	65.45	28/4	p>0.05	t-test	3A
eEPSC amp. (pA)	Nrx-3β		472.9	84.45	29/4	p>0.05	t-test	3A
eIPSC amp. (pA)	control		2093	213.7	42/4			3A
eIPSC amp. (pA)	Nrx-1β		900.1	167.8	23/3	p<0.001	t-test	3A
eIPSC amp. (pA)	Nrx-2β		555.6	120.7	48/4	p<0.001	t-test	3A
eIPSC amp. (pA)	Nrx-3β		913.3	156.2	21/3	p<0.001	t-test	3A
eIPSC amp. (pA)	control		1724	523.9	32/5			3B
eIPSC amp. (pA)	Nrx-2β		472.8	419.5	52/5	p<0.001	t-test	3B
eIPSC amp. (pA)	Nrx-2β$^{\Delta Ct}$ without SS4		437.6	332.2	32/5	p<0.001	t-test	3B
eIPSC amp. (pA)	Nrx-2β$^{\Delta Ct}$ with SS4		437.6	472.9	24/4	p<0.001	t-test	3B
eIPSC amp. (pA)	Ct-Nrx-1β		2032	523.9	42/3	p<0.001	t-test	3B
eIPSC amp. (pA)	Control (24h)		1304	121.1	15/3			3C
eIPSC amp. (pA)	Nrx-2β (24h)		1341	172.45	15/3	p>0.05	t-test	3C
eIPSC amp. (pA)	Control (48h)		1999	153.1	15/3			3C
eIPSC amp. (pA)	Nrx-2β (48h)		1336	131.85	15/3	p<0.05	t-test	3C
eIPSC amp. (pA)	Control (72h)		2199	207.2	15/3			3C
eIPSC amp. (pA)	Nrx-2β (72h)		1129	133.35	15/3	p<0.01	t-test	3C
eIPSC amp. (pA)	Control (96h)		2351	174.95	15/3			3C
eIPSC amp. (pA)	Nrx-2β (96h)		915.7	124.85	15/3	p<0.001	t-test	3C
mIPSC freq. (Hz)	Control		0.5453	0.08065	15/3			S4C
mIPSC freq. (Hz)	Nrx TKD		0.5375	0.07897	15/3	p>0.05	t-test	S4C
eIPSC amp. (pA)	Control		2883	441.8	16/3			S4D
eIPSC amp. (pA)	Nrx TKD		2954	364	16/3	p>0.05	t-test	S4D
eIPSC amp. (pA)	control		1832	218	42/5			4A
eIPSC amp. (pA)	Nrx-1β		1231	203	55/5			4A
eIPSC amp. (pA)	NL-2		3124	338	9/1			4A
eIPSC amp. (pA)	Nrx-1β + NL-2		2083	347	25/4			4A
eIPSC amp. (pA)	Nrx-1β + NL-1		1251	297	26/4			4A
eIPSC amp. (pA)	control		2354	168	48/5			4B

Parameter	**Transfect with**	**Ig-Nrx**	**Value (mean)**	**Value (sem.)**	**n***	**p-value** (p<0.05)	**Stat. test**	**Fig.**
eIPSC amp. (pA)	Nrx-1α		1491	92	38/5			4B
eIPSC amp. (pA)	Nrx-1α + NL-2		2378	331	16/3			4B
eIPSC amp. (pA)	Nrx-1α + NL-1		1537	251	19/3			4B
eIPSC amp. (pA)	control		1883	137	20/3			4C
eIPSC amp. (pA)	Nrx-2β		1047	135	19/3	p<0.001	t-test	4C
eIPSC amp. (pA)	control		1979	107	9/3			4D
eIPSC amp. (pA)	Nrx-2β		1224	114	9/3	p<0.05	t-test	4D
eIPSC amp. (pA)	control		1715	295	15/3			4E
eIPSC amp. (pA)	Nrx-2β^{D137A}		897	233	15/3	p<0.05	t-test	4E
eIPSC amp. (pA)	control		2620	417.35	16/3			4F
eIPSC amp. (pA)	Nrx-2β		851.4	141.8	11/3	p<0.01	t-test	4F
eIPSC amp. (pA)	Nrx-2β + DG		790.3	411.3	16/3	p<0.01	t-test	4F
eIPSC amp. (pA)	DG		2644	468	12/2	p>0.05	t-test	4F
eIPSC amp. (pA)	control		2106	318.7	14/3			4H
eIPSC amp. (pA)	Nrx-2β^{KDEL}		2295	358.3	13/3	p>0.05	t-test	4H
eIPSC amp. (pA)	control		2506	514.1	8/3			4H
eIPSC amp. (pA)	Nrx-2β		1162	112.1	8/3	p<0.01	t-test	4H
mIPSC freq. (Hz)		Ig-C	0.57	0.08	19/3			5A
mIPSC freq. (Hz)		IgNrx-1β	0.30	0.07	19/3	p<0.01	t-test	5A
mIPSC amp. (pA)		Ig-C	42.34	9.510	19/3			5A
mIPSC amp. (pA)		IgNrx-1β	44.86	8.948	19/3	p>0.05	t-test	5A
eIPSC amp. (pA)		Ig-C	3118	242	17/3			5B
eIPSC amp. (pA)		IgNrx-1β (0.05 µM)	2835	538	7/3	p>0.005		5B
eIPSC amp. (pA)		IgNrx-1β (0.1 µM)	2650	340	7/3	p>0.005		5B
eIPSC amp. (pA)		IgNrx-1β (0.5 µM)	1817	332	13/3	p<0.005		5B
eIPSC amp. (pA)		IgNrx-1β (1µM)	1703	169	12/3	p<0.001		5B
Sucrose (IPSC, nC)		Ig-C	7.1	0.8	14/3			5C
Sucrose (IPSC, nc)		IgNrx-1β	4.2	0.7	15/3	p<0.01	t-test	5C
eIPSC amp. (pA)		Ig-C (24h)	3258	209.6	19/3			5D
eIPSC amp. (pA)		Ig-C (48h)	3393	275.1	14/3			5D
eIPSC amp. (pA)		Ig-C (72h)	3378	191.5	19/3			5D
eIPSC amp. (pA)		Ig-C (96h)	3230	230.7	14/3			5D

Parameter	**Transfect with**	**Ig-Nrx**	**Value (mean)**	**Value (sem.)**	**n***	**p-value** (p<0.05)	**Stat. test**	**Fig.**
eIPSC amp. (pA)		IgNrx-1β (24h)	3123	210.8	14/3	p>0.05	t-test	5D
eIPSC amp. (pA)		IgNrx-1β (48h)	2901	197.8	15/3	p>0.05	t-test	5D
eIPSC amp. (pA)		IgNrx-1β (72h)	2548	155.4	19/3	p<0.05	t-test	5D
eIPSC amp. (pA)		IgNrx-1β (96h)	2090	129.95	15/3	p<0.01	t-test	5D
eIPSC amp. (pA)		Ig-C (24h)	1382	180.7	24/4			5E
eIPSC amp. (pA)		Ig-C (48h)	1904	329.2	18/3			5E
eIPSC amp. (pA)		Ig-C (72h)	2253	269.3	18/3			5E
eIPSC amp. (pA)		Ig-C (96h)	2991	269.6	18/3			5E
eIPSC amp. (pA)		IgNrx-1β (24h)	1479	136.2	26/4	p>0.05	t-test	5E
eIPSC amp. (pA)		IgNrx-1β (48h)	1402	232.4	18/3	p>0.05	t-test	5E
eIPSC amp. (pA)		IgNrx-1β (72h)	1495	145	22/3	p<0.05	t-test	5E
eIPSC amp. (pA)		IgNrx-1β (96h)	1618	201.8	18/3	p<0.01	t-test	5E
I_{GABA} amp. (pA)	control		1803	110.5	18/3			8A
I_{GABA} amp. (pA)	Nrx-2β		873.7	82.71	18/3	p<0.01	t-test	8A
I_{GABA} amp. (pA)	control (1µM GABA)		305.67	27.0196	19/4			8B
I_{GABA} amp. (pA)	control (5µM GABA)		1182.81	69.1361	19/4			8B
I_{GABA} amp. (pA)	control (10µM GABA)		1723.4	121.414	19/4			8B
I_{GABA} amp. (pA)	control (100µM GABA)		1770.66	98.135	19/4			8B
I_{GABA} amp. (pA)	control (200µM GABA)		1803	110.5	19/4			8B
I_{GABA} amp. (pA)	Nrx-2β (1µM GABA)		119.83	32.5226	17/4	p<0.05	t-test	8B
I_{GABA} amp. (pA)	Nrx-2β (5µM GABA)		567.705	69.4617	17/4	p<0.01	t-test	8B
I_{GABA} amp. (pA)	Nrx-2β (10µM GABA)		803.252	153.249	17/4	p<0.05	t-test	8B
I_{GABA} amp. (pA)	Nrx-2β (100µM GABA)		832.695	145.69	17/4	p<0.05	t-test	8B

Parameter	**Transfect with**	**Ig-Nrx**	**Value (mean)**	**Value (sem.)**	**n***	**p-value** (p<0.05)	**Stat. test**	**Fig.**
I_{GABA} amp. (pA)	Nrx-2β (200μM GABA)		873.7	82.71	17/4	p<0.01	t-test	8B
I_{GABA} amp. (pA)		Ig-C	2349.33	180.667	15/3			8C
I_{GABA} amp. (pA)		IgNrx-2β	1612	191	16/3	p<0.05	t-test	8C
GABAA-α1 levels	control		100	0	13			8D
GABAA-α1 levels	Nrx-2β		78	7	13	p<0.05	t-test	8D
mRNA level (GAD65)	Nrx-2β		88	25	3	p>0.05	t-test	S1C
mRNA level (VGAT)	Nrx-2β		75	24	3	p>0.05	t-test	S1C
mRNA level (GABAA-α1)	Nrx-2β		96	22	3	p>0.05	t-test	S1C
Synapse area (μm^2)	Control		0.80	0.01	62/4			S2C
Synapse area (μm^2)	Nrx-2β		0.82	0.01	62/4	p>0.05	t-test	S2C
Synapse number (per 50 μm)	Control		14.3	0.8	62/4			S2C
Synapse number (per 50 μm)	Nrx-2β		12.1	0.8	62/4	p>0.05	t-test	S2C
Synapse area (μm^2)	Control		0.76	0.01	64/4			S2E
Synapse area (μm^2)	Nrx-2β		0.77	0.01	64/4	p>0.05	t-test	S2E
Synapse number (per 50 μm)	Control		17.8	0.7	64/4			S2E
Synapse number (per 50 μm)	Nrx-2β		16.0	0.7	64/4	p>0.05	t-test	S2E
mIPSC amp. (pA)	Control		27.11	2.409	15/3			S4C
mIPSC amp. (pA)	Nrx TKD		33.62	2.541	15/3	p>0.05	t-test	S4C
eIPSC amp. (pA)	control		2486	428	9/3			S3
eIPSC amp. (pA)	Nrx-2β		880	199	10/3	p<0.01	t-test	S3
eIPSC amp. (pA, +CNQX)	control		2327	210	14/3			S3
eIPSC amp. (pA, +CNQX)	Nrx-2β		867	107	14/3	p<0.01	t-test	S3
eIPSC amp. (pA, +APV)	control		2447	293	21/3			S3
eIPSC amp. (pA, +APV)	Nrx-2β		687	188	24/3	p<0.001	t-test	S3
eIPSC amp. (pA, +PTX)	control		1956	332	12/3			S3
eIPSC amp. (pA, +PTX)	Nrx-2β		947	167	14/3	p<0.05	t-test	S3
eIPSC amp. (pA, +CNQX+APV+PTX)	control		2345	401	18/3			S3

Parameter	**Transfect with**	**Ig-Nrx**	**Value (mean)**	**Value (sem.)**	**n***	**p-value** (p<0.05)	**Stat. test**	**Fig.**
eIPSC amp. (pA, +CNQX+APV+PTX)	Nrx-2β		1004	240	15/3	p<0.05	t-test	S3
eIPSC amp. (pA, +TTX)	control		1797	412	12/3			S3
eIPSC amp. (pA, +TTX)	Nrx-2β		408	69	15/3	p<0.01	t-test	S3
eIPSC amp. (pA, control)			1894	257	7/2			S4A
eIPSC amp. (pA, NL-2 KO)			1784	336	7/2	p>0.05	t-test	S4A
eIPSC amp. (pA)		Ig-C	2099	239.2	25/3			S5C
eIPSC amp. (pA)		IgNrx-1β	1106	166.2	26/3	p<0.01	t-test	S5C
eEPSC amp. (pA)		Ig-C	448.2	59.16	15/3		t-test	S5D
eEPSC amp. (pA)		IgNrx-1β	406.2	57.46	15/3	p>0.05	t-test	S5D
Paired-pulse ratio		Ig-C	0.89	0.03	12/3		t-test	S5E
Paired-pulse ratio		IgNrx-1β	0.87	0.06	12/3	p>0.05	t-test	S5E
Normalized I_{GABA}	control (1µM GABA)		0.17	0.01				S8
Normalized I_{GABA}	control (5µM GABA)		0.66	0.04				S8
Normalized I_{GABA}	control (10µM GABA)		0.96	0.07				S8
Normalized I_{GABA}	control (100µM GABA)		0.98	0.05				S8
Normalized I_{GABA}	control (200µM GABA)		1	0.04				S8
Normalized I_{GABA}	Nrx-2β (1µM GABA)		0.14	0.02				S8
Normalized I_{GABA}	Nrx-2β (5µM GABA)		0.65	0.05				S8
Normalized I_{GABA}	Nrx-2β (10µM GABA)		0.92	0.11				S8

Table S4 (related to all Figures)

Summary of constructs/expressed proteins used in this study.

Name	Starting from… (residue)	Ending at… (residue)	Species	Notes
Nrx-2β (lentiviral and pCMV5 version)	MPPGGSGQGG…	…KKNKDKEYYV.	Rattus norveg.	
Ct-Nrx-1β (lentiviral and pCMV5 version)	Nrx 1β Signal peptide seq.+EVIRESSST…	…KKNKDKEYYV.	Rattus norveg.	
Nrx-2β$^{\Delta Ct}$	MPPGGSGQGG…	…LYAMYKYRNR.	Rattus norveg.	
Nrx-2β^{D137A}	MPPGGSGQGG…	…KKNKDKEYYV.	Rattus norveg.	Oligo (CZs0829): TCGGGGCAACCTGCAGCTGCACATT Oligo (CZs0829): AATGTGCAGCTGCAGGTATGCCCCGA
Nrx-1β	MYQRMLRCGA…	…KKNKDKEYYV.	Rattus norveg.	
Flag-Nrx1β	MSALLILALVGAAVA DYKDDDDKLAAA + Nrx1β mature protein	…KKNKDKEYYV.	Rattus norveg.	
Nrx-3β	MHLRIHARRN…	…QKNKDKEYYV.	Bos taurus	
IgNrx-1β	MLRCGAELGSPG…	…LVGEVPSSMTTE+Ig	Bos taurus	
IgNrx-2β	MPPGGSGQGG…	…GAVEVIRESS+Ig	Rattus norveg.	
IgNrx-2β^{D137A}	MPPGGSGQGG…	…GAVEVIRESS+Ig	Rattus norveg.	Oligo (CZs0829): TCGGGGCATACCTGCAGCTGCACATT Oligo (CZs0829): AATGTGCAGCTGCAGGTATGCCCCGA
GST-CASK	GST+DFSEDPTSSGL…	…QWVPVSWVY.	Rattus norveg.	aa. (328-909)
YFP-tagged GABA-Aα1 Receptor	MRKSPGLSDC…	…PQLKAPTPHQ.	Homo sapiens	Full length; gift from Dr. Robert L. Macdonald (Vanderbilt University)
GST-GABA-Aα1	GST+QPSLQDELKD…	…THFHLKRKIG+HA.	Homo sapiens	aa. (28-251)
GABA-Aα1 Receptor N-term.	QPSLQDELKD…	…THFHLKRKIG	Homo sapiens	aa. (28-251)
Nrx1β LNS	ASSLGAHHIHH…	EVPSSMTTES	Rattus	aa. (47-299)

Domain			norvegicus	
NL1	MALPRCMWPNYV…	…PHPHPHSHSTTRV.	Rattus norvegicus	
NL2	MWLLALCLVGLAG…	…NNTLPHPHSTTRV.	Rattus norvegicus	
Nrx-2β^{KDEL}	MPPGGSGQGG…	…RESSSTEQKLISEEDLKDEL.	Rattus norvegicus	

Supplemental Experimental Procedures

Construction of Expression Vectors. Full-length cDNA for Neurexin-1α and -1β, -2β, and -3β (Sugita et al., 2001; Ushkaryov et al., 1994) were employed for this study (all in a pCMV5 backbone). FLAG tag (DYKDDDDK) was fused to neurexin-1β right after the signal peptide, and the original signal peptide was replaced with a trypsin signal peptide (sequence of N-terminus including Flag-tag: MSALLILALVGAAVADYKDDDDKLAAA). Nrx-2β$^{\Delta Ct}$ encodes the extracellular sequences, transmembrane region, and 7 residues of the cytoplasmic tail of neurexin-2β (ending with: MYKYRNR-Stop). Nrx-2β^{KDEL} encodes the extracellular sequences (ends at …RESSST) following myc-tag (EQKLISEEDL) and KDEL sequence (KDEL-Stop). Ct-Nrx-1β encodes the original neurexin signal peptide fused to the last 10 residues of the extracellular neurexin-2 sequence (starting with EVIRESSST), the transmembrane region, and the cytoplasmic tail of neurexin-1β. Constructs expressing neuroligin-1 and -2 (Chubykin et al., 2007), Ig-fused neurexin-1β (Boucard et al., 2005) and GST-CASK (Mukherjee et al., 2008) were described before. The vector encoding YFP-tagged $GABA_A$α1-receptor was a gift from Robert Macdonald (Gallagher et al., 2005). Ig-fused neurexin-2β was made similar to Ig-fused neurexin-1β (residues 1-359 of rat neurexin-2β fused to the human IgG Fc fragment; Ushkaryov et al., 1994). The lentiviral constructs used in this paper were based on the pFUGW backbone vector (Lois et al., 2002), and generated as described (Maximov and Sudhof, 2005). To allow visualization of infected neurons using fluorescent microscopy, IRES-EGFP was inserted into the pFUGW backbone, and used as control vector in lentiviral experiments. Full-length neurexin-2β cDNA (with or without an insert in splice site 4) was inserted into the BamHI site of pFUGW vector. Neurexin knockdown constructs were based on the lentiviral backbone vector described previously (Maximov et al., 2009). The expression of shRNA was driven by human H1 promoter, which is upstream of the ubiquitin (Ub) promoter driving EGFP expression in the lentiviral shuttle vector.

Production of recombinant Ig-fused neurexins. Recombinant Ig-fusion proteins were produced as essentially described (Boucard et al., 2005). Briefly, COS cells were transfected with the Ig constructs using FUGENE 6 (Roche Applied Science), and the media of transfected COS cells were harvested 3-4 d after transfection and cleared by centrifugation at 2,500 g. The supernatants were then adjusted to 10 mM HEPES-NaOH

pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 mg/l pepstatin, 1 mg/l leupeptin and 2 mg/l aprotinin, incubated overnight at 4 °C with protein A-Sepharose (Amersham Pharmacia Biotech), and washed three times with cold PBS before use in affinity chromatography experiments. Soluble Ig-proteins were obtained by elution from protein A-Sepharose beads with 0.02 M glycine pH 2.2 for 5 min on ice, followed by equilibration with 15 ml 50 mM HEPES-NaOH pH 7.5. The collected soluble proteins were concentrated to 500 µl using a centriprep concentrator (Millipore, UFC901024, 10 Kda cut-off), and then washed with PBS six times to reduce the amount of glycine in the final elution. The concentration of eluted recombinant protein was quantified using Coomassie Blue staining, and the protein solution was diluted to 1 µM using neuronal medium or HEK293 culture medium, then filtered with 0.22 µm syringe filter before applying to neuronal culture or HEK293 cells.

Affinity Chromatography. For brain affinity chromatography experiment, one rat brain was homogenized with 6-8 strokes in 10 ml of PBS, 0.1 mM EGTA, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin and 2 µg/ml protinin, and adjusted to 1% Triton X-100 final concentration. Proteins in the homogenate were extracted for 2 h at 4 °C, insoluble material was removed by centrifugation (2 h at 100,000g), and 1 mM $MgCl_2$ and 2 mM $CaCl_2$ were added for the pulldown assays (except where noted). For neurexin pull-downs, protein A-Sepharose containing Ig-fused neurexin-1β, -2 β or Ig-C control (~6, 9 and 5 µg protein) were added to 2 ml brain extract, and incubated overnight at 4 °C, then centrifuged (800g for 5 min), and washed five times with PBS containing 2 mM Ca^{2+} and 1 mM Mg^{2+}. For GST-CASK pulldowns, 5 ml brain extract was incubated with 50 µg immobilized GST-CASK PDZ-domain or GST alone overnight at 4 °C, beads were washed, and bound proteins were analyzed by SDS-PAGE and immunoblotting. For pulldowns from HEK293 cell lysates, cells were washed with PBS once, kept at -80°C overnight, thawed at 37°C for 1 min, and collect the cells and centrifuge at 2000g for 15 min at 4°C to get the cell pellet. Membrane proteins were extracted from the cell pellet for 1 h at 4 °C in 20 mM HEPES-NaOH pH 7.4 containing 1% Triton, 0.1 mM EDTA, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 100 mM NaCl with protease inhibitors (1 mM PMSF, 1 µg/ml Pepstatin, 1 µg/ml Leupeptin and 2 µg/ml Aprotinin). Insoluble material was removed by centrifugation (10,000 g for 30 min), and supernatant was used for various pulldowns. All

pulldown experiments were done over night at 4 °C, and bound proteins were eluted with sample buffer, and analyzed by immunoblotting with specific antibodies.

Surface Plasmon Resonance measurements. The recombinant extracellular N-terminal regions of the neurexin-1β (Araç et al., 2007) and of the rat $GABA_A\alpha1$-receptor (residues 28-251; see Suppl. Table 6) were expressed essentially as described (Araç et al., 2007); Garboczi et al., 1996). Surface plasmon resonance binding experiments were carried out at 25 ˚C in 10 mM Hepes-NaOH pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.005% (v/v) surfactant P20 on a BIAcore T100 instrument. Control (inactive Botulinum neurotoxin A) and the $GABA_A\alpha1$-receptor protein were covalently attached to carboxymethylated dextran on a research grade CM5 chip (BIAcore, Uppsala, Sweden) by NHS/EDC amine coupling, with resonance units of 400 or 1000 for the $GABA_A\alpha1$-receptor. The measured binding affinities were similar in both cases. Neurexin-1β was injected over the $GABA_A\alpha1$-receptor surfaces at increasing concentrations (range: 1.14 nM – 30 μM) in 3-fold increments, and each data point was measured twice. A flow rate of 50 μl/min was used to minimize sample diffusion and mass transport limitations. Reference data obtained using a flow-cell with immobilized control protein were subtracted from the sample flow channels to obtain specific neurexin-1β binding. $GABA_A\alpha1$-receptor surfaces were regenerated by injection of a regeneration solution (10 mM Hepes-NaOH pH7.4, 0.15 M NaCl, 1 mM EDTA and 0.005% P20). For analysis of steady-state binding affinities, the magnitude of the response at the end of each injection were measured, and fitted using a single-site stoichiometry model.

Neuronal cultures, calcium-phosphate transfection and lentiviral Infection. Primary hippocampal neurons were isolated from P1-3 pups of wild-type mice, and dissociated by papain digestion as described (Maximov et al., 2007). The neurons were plated on poly-D-lysine-coated glass coverslips and maintained at 37 °C for 13-15 days. Hippocampal neuronal cultures were transfected with various cDNA constructs at 10 days in vitro (DIV) using a calcium-phosphate transfection method modified from the ClonTech CalPhos transfection protocol. Briefly, the DNA/Ca^{2+}/HBS mixture was added to the culture for 20-25 min at 37 °C, and the cells were washed with MEM three times and kept in the incubator for 3-4 days before recording. Typically, 1-2 % of neurons were transfected as assessed by counting EGFP-positive neurons. In all transfection experiments, pCMV5

backbone vector was used as control, and vector expressing CFP was co-transfected with the indicated plasmids for visualization. All transfections used the same total amount of plasmids (3 µg plus 1 µg of CFP plasmid for visualization); for control transfections, empty pCMV5 vector was used to substitute for the expression vectors. In neurexin triple knockdown transfection experiments, three vectors encoding shRNA against neurexin-1, -2 and -3 (NRXN1_9, NRXN2_15 and NRXN3_29) were co-transfected with CFP-expressing vector using the calcium-phosphate transfection method. Lentiviruses were produced by transfecting human embryonic kidney HEK293 cells (CRL-11268, ATCC) with the respective pFUGW vectors and two helper plasmids (pVSVg and pCMVΔ8.9) using FUGENE 6. Viruses were harvested 48 hr after transfection by collecting the medium from transfected cells. Neurons were infected with 0.2-0.5 ml conditioned HEK293 cell medium for each 24-well of high-density neurons at DIV1-2, and the medium was exchanged to normal growth medium at DIV4, then kept until DIV13–15 for biochemical and electrophysiological analyses. To screen the efficiency of various neurexin knockdown constructs, mouse cortical neurons were infected at DIV4 and harvested on DIV13 for mRNA measurement. For all experiments, expression of exogenous proteins in neurons was confirmed by immunoblotting and CFP fluorescence. All mRNA measurements were performed using Taqman assays in an ABI qRT-PCR instrument.

Morphological studies. Immunofluorescence analyses of cultured neurons were performed essentially as described (Chubykin et al., 2007). Briefly, cultured neurons (14 DIV) were rinsed with PBS, fixed with 100% methanol at -20°C for 10 min, blocked in 3% milk, 0.1% saponin/PBS (PBSS) for 60 min, and then incubated with primary antibodies for 1 h at room temperature. After rinsing three times with PBBS, the neurons were incubated with fluorescent secondary antibodies for 30 min in the dark at room temperature,washed 5 times, and mounted with Vectashield mounting medium (H-1000, Vector labs). Confocal microscopy was performed with a Zeiss LSM 510 Meta microscope. Identical acquisition settings were applied to all samples of the experiment. Images of neurons were collected with 63x oil-immersion objective. Stacks of z-section images obtained by confocal microscopy were converted to maximal projection images and analyzed blindly using the NIH Image/ImageJ program.

Electrophysiological recordings. Electrophysiological recordings were performed as previously reported (Maximov et al., 2007). Neurons were patched and kept in voltage-clamp whole-cell configuration using a Multiclamp 700A amplifier (Molecular Devices). Synaptic responses were evoked by 0.5 ms current injection (90~100 µA) through a concentric bipolar electrode (FHC, CBAEC75) with an Isolated Pulse Stimulator (Model 2000, A-M Systems, Inc.). Data were digitized at 10 kHz with a 2 kHz low-pass filter. For whole-cell patch-clamp recordings, the pipette solution contained (in mM): 145 CsCl, 5 NaCl, 10 HEPES-CsOH pH 7.2, 5 EGTA, 5 QX-314, 4 MgATP and 0.3 Na_2GTP, 305 mOsm. The bath solution contained (in mM): 150 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-NaOH pH 7.4, 10 glucose, 315 mOsm. Evoked IPSCs were pharmacologically isolated by adding 10 µM CNQX to the bath solution. Evoked EPSCs were pharmacologically isolated by adding 100 µM picrotoxin to the bath solution. Tetrodotoxin (1 µM) was added to the bath to block evoked synaptic responses for mEPSC/mIPSC recordings. The readily-releasable pool was measured by background perfusion of hypertonic sucrose (0.5 M) into the bath solution at a speed of 2 ml/min. Series resistance was compensated to 60%–70%, and recordings with series resistances of >20 MΩ were rejected. Data were analyzed using Clampfit 9.02 (Molecular Devices) and Igor 4.0 (Wavemetics). Data are shown as means ± SEMs. Statistical significance was determined by the Student's t test except for the cumulative graphs in Suppl. Fig. 1, which used the Kolmogorov-Smirnov test.

Miscellaneous. SDS-PAGE and immunoblotting were performed using standard procedures. ^{125}I-labeled secondary antibodies were employed for quantitative analyses followed by PhosphorImager detection (Molecular Dynamics). Most antibodies employed were previously characterized (Ichtchenko et al., 1995; Song et al., 1999; Sugita et al., 2001; Ushkaryov et al., 1992). The antibodies that were used to detect $GABA_A\alpha1$ were purchased from Santa Cruz (sc-7348, 1:200) or Alomone Labs (AGA-001, 1:200). HEK293 cells stably expressing $GABA_A$ receptors were purchased from ATCC (CRL-2029).

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