Supporting Information

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SI Methods

Expression Vectors. ***MDGA1.*** pGW1-MDGA1 encodes full-length human MDGA1 cloned (amino acids 1–955) into the pGW1 vector (British Biotechnology). pGW1-HA-MDGA1 is an HA-epitope–tagged construct of mature, full-length human MDGA1 cloned into the pGW1 vector. L-304 MDGA1 encodes full-length human MDGA1 (amino acids 1–955) cloned into the L-304 vector, a lentiviral vector in which gene expression is driven by a ubiquitin promoter (see Fig. S5*A* for details). pDisplay-MDGA1-Ig1-6 encodes six Ig domains of human MDGA1 (amino acids 37–638) cloned into a pDisplay vector at BglII and SalI sites. pDisplay-MDGA1-Ig1-3 encodes the first three Ig domains (Ig1–3) of human MDGA1 (amino acids 37–330) cloned into a pDisplay vector at BglII and SalI sites. pDisplay-MDGA1-Ig4-6 encodes the first three Ig domains (Ig4–6) of human MDGA1 (amino acids 334–638) cloned into a pDisplay vector at Bg*l*II and SalI sites. pDisplay-MDGA1-FNIII encodes a single fibronectin type III repeat of human MDGA1 (amino acids 627–745) cloned into a pDisplay vector at XmaI and SacII sites. pDisplay-MDGA1-MAM encodes a MAM domain of human MDGA1 (amino acids 734–932) cloned into a pDisplay vector at *Bgl*II and SalI sites. pcDNA3.1-MDGA1-ECD-HA-His_8 encodes a full extracellular region of human MDGA1 (amino acids 1–932) fused to HA epitope and octahistidines.

MDGA2. pGW1-MDGA2 encodes full-length mouse MDGA2 (amino acids 1–956) cloned into the pGW1 vector. pGW1-HA-MDGA2 is an HA-epitope–tagged construct of mature, full-length mouse MDGA2 cloned into the pGW1 vector. pDisplay-MDGA2-Ig encodes six Ig domains of mouse MDGA2 (amino acids 39–633) cloned into a pDisplay vector at XmaI and SacII sites. pGEM7zf-MDGA2 contains nucleotides 2310–3029 of rat MDGA2.

Neuroligins and neurexins. pCMV-IgC-NL1 encodes the extracellular domains of rat NL1 (amino acids 1–636), and pCMV-IgC-NL2 encodes the extracellular domains of rat NL2 (amino acids 1–614). pCMV-IgC-NL2-32 is identical to pCMV-IgC-NL2, except for the mutations E281A, L374A, N375A, and D377N, which render it neurexin-binding defective (1, 2). pCMV5-NL2-mVenus encodes full-length rat NL2 fused to mVenus of pCMV5-NL2 (full-length NL2 cloned into the pCMV5 vector). pGEM7zf-NL2 contains nucleotides 2306–3061 of rat NL2 (GenBank accession number NM_053992). pCMV5-NL1-mVenus and pCMV-IgC were described previously (3, 4). pCMV5-neurexin-1β^{SS4-}-mCherry is a vector expressing full-length neurexin-1β (Nrx1β) lacking an insert in splice #4 fused to mCherry, as previously described (4). The entire coding sequences of NL1, NL2, and NL3 were amplified by RT-PCR using mRNAs prepared from mouse brains and cloned into the pcDNA3.1(+) vector (Invitrogen) to generate pNL1, pNL2, and pNL3 plasmids, respectively. Splice variants of neuroligins without specific splice segments (A1, A2, or B for NL1; A2 for NL2; A1 or A2 for NL3) were generated by PCR-based mutagenesis and cloned into the pCDNA3.1(+) vector to generate pNL1 (ΔA1, A2, or B), pNL2 (ΔA2), and pNL3 (ΔA1 or A2) plasmids.

Miscellaneous. pGW1-PTPRT is a vector expressing full-length human protein tyrosine phosphatase receptor type T (PTPRT), as previously described (5). pRK5-GluA1 is a vector expressing full-length rat glutamate receptor 1 (GluA1), as previously described (6). pDisplay-HA-Slitrk3 is an HA-epitope-tagged construct of full-length mouse SLIT and NTRK-like family, member 3 (Slitrk3) cloned into the pDisplay vector. L-309 NL2 KD is a lentiviral knockdown (KD) vector expressing small hairpin interfering RNA against NL2 (sh-NL2) under the control of an H1 promoter, created by cloning oligonucleotides targeting nucleotides 2123–2141 of mouse NL2 (NM_198862) into the L-309 vector. L-315 MDGA DKD is a lentiviral double-knockdown (DKD) vector expressing both sh-MDGA1 and sh-MDGA2 sequences under dual H1 promoters (Fig. S5*A*), created by cloning oligonucleotides targeting nucleotides 2848–2866 of mouse MDGA1 (NM_001081160; GTCTCTTTCTTCTACCACA) and nucleotides 1933–1951 of mouse MDGA2 (NM_001193266; AGGTGAAGCTAAAGAACAA) into the L-315 vector. These sequences are identical to those of the rat homologs [rat MDGA1 (XM_241623) and rat MDGA2 (NM_199269)].

Antibodies. The following antibodies were obtained from commercial sources: monoclonal anti-HA (Covance), rabbit polyclonal anti-HA (Sigma), rabbit polyclonal anti-MDGA1 (Millipore), monoclonal anti-eGFP; Invitrogen), monoclonal anti-MAP2 (microtubule-associated protein 2) (Sigma), rabbit polyclonal anti-synapsin I (Millipore), guinea pig polyclonal anti-VGLUT1 (vesicular glutamate transporter 1) (Chemicon), rabbit polyclonal anti-VGAT (vesicular GABA transporter) (Synaptic Systems), monoclonal anti-contactin/F3 (NeuroMab), and monoclonal anti–L1-CAM (Abcam). The following antibodies were described previously: rabbit polyclonal anti-NL2 (L068) (7), rabbit polyclonal anti-GluA1 (1193) (8), and rabbit polyclonal anti-PSD-95 (postsynaptic density 95 kDa; 1690) (9).

In Situ Hybridization. Riboprobes for MDGA1 (pBluescript II MDGA1) were described previously (10). Riboprobes for MDGA1, MDGA2, and NL2 were transcribed in vitro in the presence of ^{35}S-UTP (Amersham). Rat brain sections as indicated in Fig. S1 were hybridized overnight at 52 °C with labeled probe (1.2×10^6 cpm per slide). On the next day, brain sections were treated with RNase A (Boehringer-Mannheim) for 30 min at 37 °C, washed, dehydrated in a graded series of ethanol, and air-dried, as described previously (11). Hybridized radioactivity for MDGAs was visualized by exposure to Biomax film (Kodak).

Artificial Synapse Formation Assays. HEK293T cells were transfected with NL1-mVenus, NL2-mVenus, or mVenus using FuGene-6 (Roche). After 24 h, transfected HEK293T cells were trypsinized, seeded onto cultured hippocampal neurons at 9 d in vitro (DIV9), further cocultured for 48 h, and double immunostained with antibodies against GFP and synapsin I at DIV11. All images were acquired by confocal microscopy (LSM510; Zeiss). For quantification, the contours of transfected HEK293T cells were chosen as the region of interest. The fluorescence intensity of synapsin puncta normalized to each HEK293T cell area was quantified for both red and green channels using MetaMorph Software (Molecular Devices). Statistical significance was determined using Student's *t* test; all data are expressed as means ± SEM.

Cell-Surface Binding Assays. IgC-fusion proteins and MDGA1 recombinant protein (MDGA1-ECD-HA-His) were produced from the cell supernatant of transfected HEK293T cells as previously described (4). Soluble IgC proteins then were purified by elution from protein A-Sepharose beads (Amersham Pharmacia), and soluble MDGA1 protein was purified by Talon metal affinity resin (Clontech) following the standard protocol and dialyzed against PBS overnight. The experiments were performed as previously described (12). Transfected HEK293T cells expressing NL1-mVenus, NL2-mVenus, or mVenus were incubated with 0.2 μM IgC, IgNL1, IgNL2, or IgNL2-32, as indicated in Figs. 1 and 2. Transfected HEK293T cells expressing NL1, NL1(ΔA1, A2, B), NL2, NL2(ΔA2), NL3, NL3(ΔA1, A2), and eYFP were

incubated with 0.2 μM MDGA1-ECD-HA-His, as indicated in Fig. 2*A*. For the competition experiments shown in Fig. S6, transfected HEK293T cells expressing NL1-mVenus or NL2-mVenus were incubated with 0.2 μM IgC or IgNrx1β^{SS4-}. Then 50 μg/mL of recombinant MDGA1 protein was added simultaneously as indicated in Fig. S6. Calcium dependency was examined by including 10 mM EGTA in reactions. Images were acquired using a confocal microscope (LSM510; Zeiss and TCS2; Leica).

Quantitative Cell-Surface Binding Assay for K_d Calculation. HEK293T cells were cultured in 96-well plates until they reached 50–60% confluence. The cells then were transfected using calcium phosphate and 0.2 μg of the NL2 and NL2(ΔA2) plasmids per well. The cells were treated with various concentrations (3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 nM) of MDGA1-ECD-HA-His. After three washings with PBS, the cells were fixed with 4% (wt/vol) paraformaldehyde for 10 min, blocked with 5% (wt/vol) goat serum for 1 h, and incubated with rabbit anti-HA antibody at 4 °C for overnight. Cells were washed three times with PBS and incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit antibody to label surface-bound MDGA1 recombinant proteins. Cells were washed again three times with PBS and treated with TMB reagent (Bio-Rad). The absorbance was read at 450 nm. The results were plotted and analyzed by Michaelis–Menten function using GraphPad Prism6.

Cell-Adhesion Assays. HEK293T cells were transfected with the expression vectors, as listed in Fig. S3. Twenty-four hours after transfection, the cells were detached, mixed, and incubated at 4 °C with gentle agitation. The extent of cell aggregation was plotted as the ratio of N_0/N_{60}, where N_0 is the total number of cellular particles at the beginning of incubation (0 min), and N_{60} is the total number of cellular particles after 60 min of incubation.

In Vivo Pull-Down Assays. P2 membrane fractions (300 μg) prepared from adult (6-wk-old) rats were mixed with 10 μg of IgNL2 or IgC (control) and incubated at 4 °C for 4 h. The antibodies used for immunoblotting were anti-MDGA1 (1 μg/mL), anti-GluA1 (1193; 1:1,000), anti-contactin/F3 (1 μg/mL), and anti–L1-CAM (1 μg/mL).

Production and Characterization of Recombinant Lentiviruses. Recombinant lentiviruses were produced by transfection of human HEK293T cells with pRRE, pVSVg, pREV, and various lentiviral vectors, as indicated in the figures. Viruses were harvested 48 h posttransfection by collecting the cell-culture medium and briefly centrifuging at 1,000 × *g* to remove cellular debris. Cultured mouse cortical neurons were infected at DIV3 and harvested at DIV13–14 to measure the efficiency of gene knockdown, using quantitative RT-PCR (qRT-PCR), as previously described (13).

Primary Neuron Culture, Immunocytochemistry, Image Acquisition, and Quantitative Analysis. Cultured hippocampal neurons were prepared from embryonic day 18 (E18) rat or mouse brain as described previously (2). For the experiments shown in the Fig. 3, cultured neurons were (*i*) infected with L-309 (control) or L-309 NL2 KD viruses at DIV2 or (*ii*) infected with L-315 (control) or L-315 MDGA DKD or coinfected with L-315 MDGA DKD and L-304 MDGA1 (a rescue vector) at DIV4. At DIV14, cells were immunostained with antibodies against eGFP and VGLUT1 or VGAT, as indicated. The antibodies used for immunocytochemistry were anti-MAP2 (1:2,000), anti-EGFP (1:500), anti-synapsin I (1:1,000), anti-VGLUT1 (1:1,000), and anti-VGAT (1:500). Synapse density was measured as previously described (13). In brief, z-stacked confocal microscopic images were analyzed with respect to the density of synaptic markers using MetaMorph software (Molecular Devices). Total numbers of fluorescence puncta were normalized to dendritic length (per 50 μm). Primary dendrite numbers, defined as dendrites emanating directly from soma, were analyzed manually using MetaMorph software. Dendritic branching numbers and neuronal cell body areas were analyzed using MetaMorph software as previously described (13). All analyses were performed in a blind manner.

Electrophysiology. Neurons were visualized with an Olympus IX70 inverted epi-fluorescence microscope. Whole-cell patch-clamp recording was performed on cultured cortical pyramidal neurons (DIV 13–15), which were transfected with GFP alone or GFP together with MDGA1 cDNAs (1:1). The external bath solution was artificial cerebrospinal fluid, including (in mM): 124 NaCl, 3 KCl, 1.3 NaH_2PO_4, 10 dextrose, 2 $MgCl_2$, 2 $CaCl_2$, 10 Hepes (pH 7.4). This solution was supplemented with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μM), amino-5-phosphonovaleric acid (APV) (50 μM), and tetrodotoxin (TTX; 1 μM) to isolate miniature inhibitory postsynaptic currents (mIPSCs) or with bicuculline (20 μM) and TTX (1 μM) to isolate miniature excitatory postsynaptic currents (mEPSCs). All experiments were performed at room temperature (22–25 °C). Patch electrodes were made from borosilicate glass and had a resistance of 4–8 MΩ when filled with the following (in mM): 140 potassium gluconate, 4 NaCl, 0.5 $CaCl_2$, 10 Hepes, 5 EGTA, 2 MgATP, 0.4 Na_3GTP (pH 7.2–7.3, osmolality 285–295 mOsmol·kg^{-1}). For mIPSCs recordings, potassium gluconate was replaced by 140 mM CsCl. Neurons were voltage clamped at −65 mV using an Axon multiclamp 700B amplifier (Axon Instruments). Series resistance (<20 MΩ) was monitored regularly during recordings, and cells were rejected if resistance changed >10% during the experiment or if capacitance was lower than 30 pF. Data were filtered at 2 kHz, digitized, and stored on a computer using pClamp10.0 software. Spontaneous responses (mEPSCs and mIPSCs) were analyzed using the Mini Analysis Program (Synaptosoft), and statistical differences were evaluated by the two-tailed unpaired Student's *t* test. For detailed electrophysiological parameters, see Table S2.

Statistics. All data are expressed as means ± SEMs and represent the results of at least three independent experiments. Statistical significance was determined by Student's *t* test.

Miscellaneous. SDS/PAGE and immunoblotting experiments were performed as previously described (4, 13).

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13. Ko J, Soler-Llavina GJ, Fuccillo MV, Malenka RC, Südhof TC (2011) Neuroligins/LRRTMs prevent activity- and Ca^{2+}/calmodulin-dependent synapse elimination in cultured neurons. *J Cell Biol* 194(2):323–334.

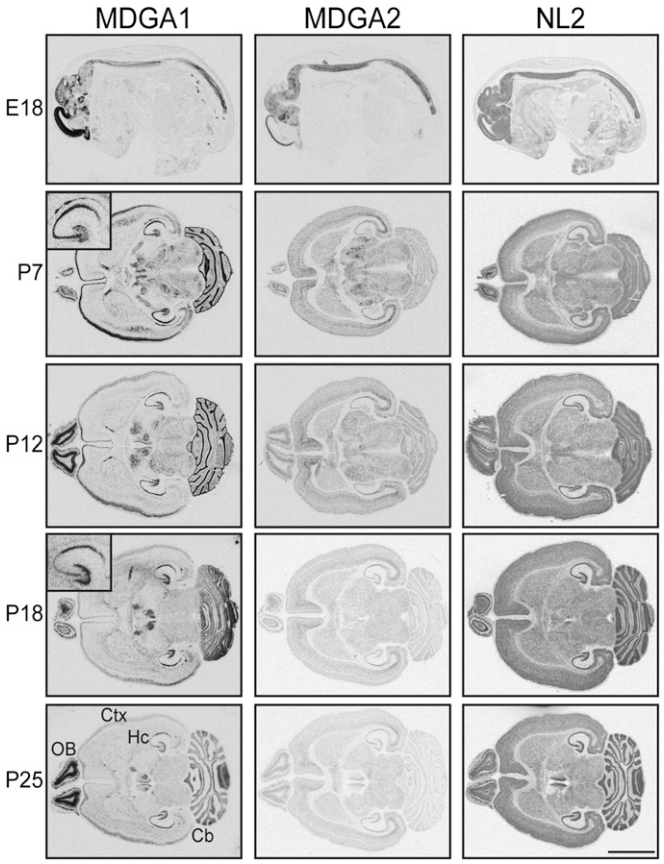

Fig. S1. Expression of MDGA mRNAs in the developing and adult rat brains. mRNA distribution patterns from rat brain sections. Sections from embryonic day 18 (E18), postnatal day 7 (P7), P12, P18, P25, and adult (6-wk-old) rat brains were probed with MDGA1, MDGA2, and NL2 cRNAs. (*Insets*) Hippocampal areas were magnified to highlight the mRNA distribution of MDGA1 in P7 and P18 brains. (Scale bar, 5 mm.) Cb, cerebellum; Ctx, cerebral cortex; Hc, hippocampus; OB, olfactory bulb.

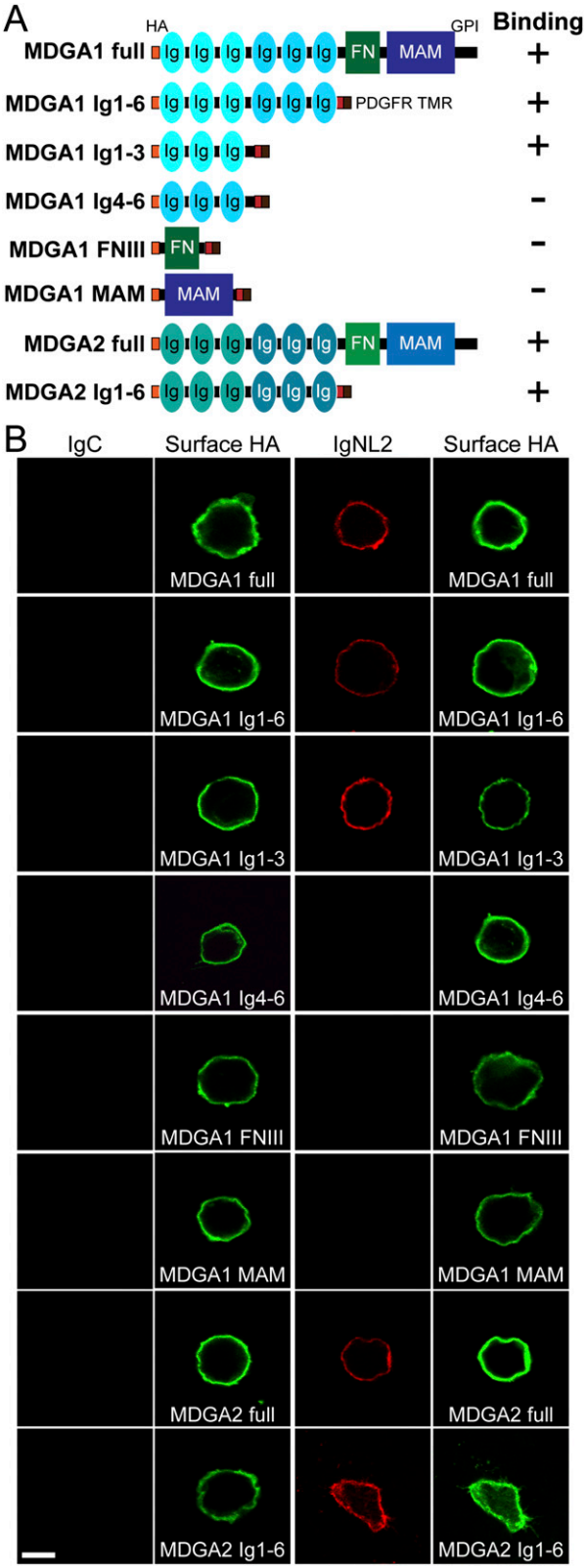

Fig. S2. Analysis of the NL2-binding domain of MDGAs using cell-surface binding assays. (*A*) Diagrams of MDGA expression vectors used in the cell-surface binding assays. The full-length MDGA1 or MDGA2 gene containing an HA epitope inserted after its signal peptide sequence was cloned into the GW1 vector (HA-MDGA1 full-length and HA-MDGA2 full-length, respectively). In pDisplay (pDis-) vectors, the GPI anchoring sequence of MDGA1 or MDGA2 was replaced with the transmembrane region (TMR) of the PDGF receptor. (*B*) Cell-surface binding assays. HEK293T cells expressing HA-tagged full-length MDGA1 (MDGA1 full), MDGA1 full Ig domain (MDGA1 Ig1–6), MDGA1 Ig1-3 domain (MDGA1 Ig1–3), MDGA1 Ig4-6 domain (MDGA1 Ig4–6), MDGA1 fibronectin type III repeat (MDGA1 FNIII), MDGA1 MAM domain (MDGA1 MAM), HA-tagged full-length MDGA2 (MDGA2 full), or MDGA2 Ig domain (MDGA2 Ig1–6) were incubated with IgC or IgNL2. Cells then were analyzed by immunofluorescence imaging for the Ig-fusion proteins (red) and surface-exposed HA-MDGA proteins (green). All binding reactions were performed in 2 mM $CaCl_2$ and 2 mM $MgCl_2$. (Scale bar, 5 μm.)

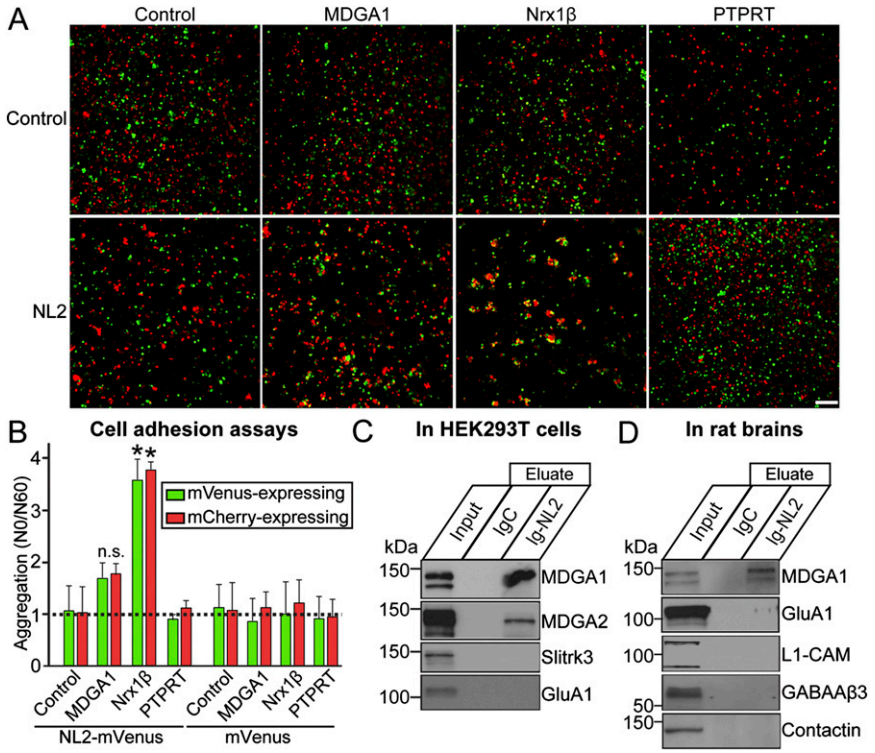

Fig. S3. Analysis of MDGA1–NL2 interaction using cell-adhesion assays and pull-down assays. (*A* and *B*) Representative images (*A*) and quantitation (*B*) of cell-adhesion assays. HEK293T cells expressing mVenus alone (Control) or an NL2-fusion protein (NL2-mVenus) were mixed with HEK293T cells expressing mCherry alone (Control) or mCherry-neurexin-1β fusion protein (Nrx1β) or coexpressing MDGA1 or PTPRT with mCherry. Cells then were imaged, and free cell numbers were counted immediately after the respective cell populations had been mixed (T_o) and again after 60 min (T_{60}), as indicated in representative images in *A*. (Scale bar, 100 μm.) (*C*) Immunoblot analysis of pull-downs of MDGAs expressed in HEK293 cells. Pull-downs were performed with IgC and IgNL2 using Slitrk3 or GluA1 as negative controls (input = 5% of total). Immunoblotting was performed using antibodies to HA (for MDGA1, MDGA2, and Slitrk3) or GluA1. Numbers at the left in this and subsequent panels indicate markers for molecular masses (kDa). (*D*) As in *C*, except that pulldown experiments were performed with solubilized rat brain synaptosomal fractions. Equivalent amounts of bound proteins were analyzed using the antibodies against proteins indicated on the right side of the panels (Input = 5% of total).

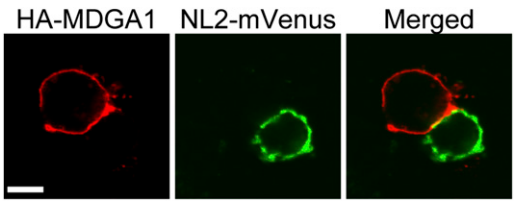

Fig. S4. The absence of *trans* interactions of MDGA1 with NL2. HEK293T cells expressing HA-MDGA1 were mixed with HEK293T cells expressing NL2 fusion protein (NL2-mVenus). Cells then were fixed, and surface-exposed MDGA1 and NL2 were visualized by indirect immunofluorescence confocal microscopy. Note that MDGA1-expressing cells did not induce any clustering with NL2-expressing cells. (Scale bar, 5 μm.)

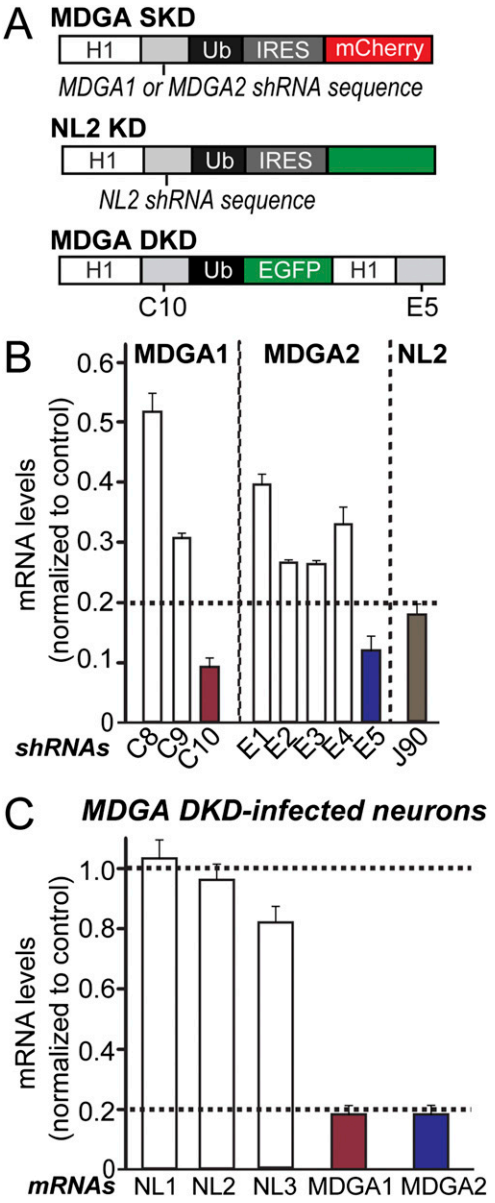

Fig. S5. Design of shRNAi vectors targeting MDGAs and NL2. (*A*) Design of lentiviral shRNA vectors for knockdown of MDGA1, MDGA2, or NL2. DKD, double knockdown;H1, human H1 promoter; IRES, internal ribosome entry sequence; SKD, single knockdown; Ub, ubiquitin promoter. (*B*) Levels of target mRNAs (MDGA1, MDGA2, and NL2) were measured by quantitative RT-PCR in cultured cortical neurons infected at DIV3 with lentiviruses expressing the indicated shRNAs. (*C*) Similarly, levels of target mRNAs (NL1, NL2, NL3, MDGA1, and MDGA2) were measured by quantitative RT-PCR in cultured hippocampal neurons infected at DIV3 with lentiviruses expressing MDGA DKD. The mRNA levels were determined at DIV12-13. The dotted line indicates the 80% knockdown cutoff level for tests of biological effects.

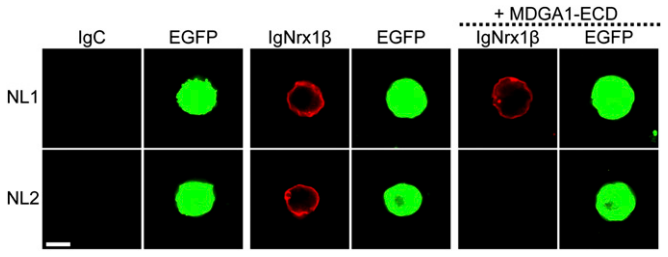

Fig. S6. Disruption of the interaction between NL2 and Nrx1β by excess recombinant MDGA1 protein in cell-surface binding assays. HEK293T cells were transfected with NL1-mVenus or NL2-mVenus, as indicated, and incubated with 5 μg/mL of control Ig-fusion protein (IgC) or Ig-fusion proteins of neurexin-1β^{SS4-} (IgNrx1β). Coincubation of IgNrx1β with 50 μg/mL MDGA1 recombinant proteins (see Fig. 2 *A* and *B* for details) specifically blocked the interaction between NL2 and Nrx1β but not between NL1 and Nrx1β. (Scale bar, 5 μm.)

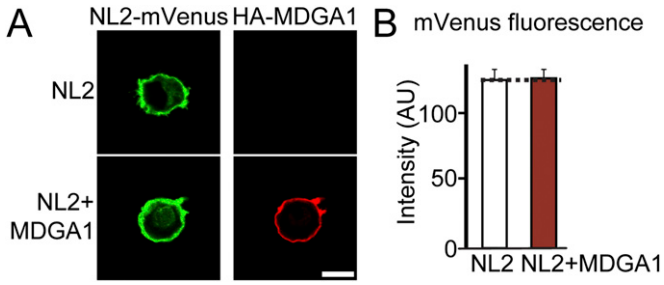

Fig. S7. Analysis of surface expression of NL2 in HEK293T cells expressing NL2 alone or coexpressing NL2 and MDGA1. (*A* and *B*) Representative images (*A*) of HEK293T cells expressing NL2 mVenus or coexpressing NL2 with HA-tagged full-length MDGA1 (NL2+MDGA1). Unpermeabilized HEK293T cells were labeled with anti-HA antibody and then imaged with a confocal microscope. Note that NL2-mVenus signals are comparable in both conditions, as quantified in *B*. (Scale bar, 5 μm.)

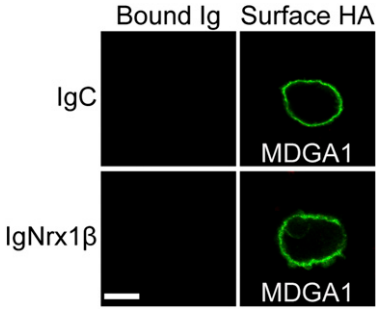

Fig. S8. Nrx1β δoes not interact with MDGA1. Nrx1β does not bind to MDGA1 in cell-surface labeling assays. HEK293T cells expressing HA-tagged full-length MDGA1 (MDGA1) were incubated with control Ig-fusion protein (IgC) or Ig-fusion proteins of neurexin-1$\beta^{S5^{4-}}$ (IgNrx1β). Cells then were analyzed by immunofluorescence imaging for the Ig-fusion proteins (Bound IgC) (red) and surface-exposed HA-MDGA1 (green). (Scale bar, 5 μm.)

Table S1. Candidate molecules expressed in HEK293T cells that were screened for binding of IgC-NL2 in cell-surface binding assays

Gene	Vector for cloning
LRRTM1	pDisplay (HA-tagged)
LRRTM2	pDisplay (HA-tagged)
LRRTM3	pDisplay (HA-tagged)
LRRTM4	pDisplay (HA-tagged)
MDGA1	pGW1 (untagged)
MDGA2	pGW1 (HA-tagged)
LAR	pECFP-N1
AMIGO1	pDisplay (HA-tagged)
AMIGO2	pDisplay (HA-tagged)
AMIGO3	pDisplay (HA-tagged)
FLRT1	pDisplay (HA-tagged)
FLRT2	pDisplay (HA-tagged)
FLRT3	pDisplay (HA-tagged)
LRTM1	pDisplay (HA-tagged)
LRTM2	pDisplay (HA-tagged)
Slitrk1	pDisplay (HA-tagged)
Slitrk2	pDisplay (HA-tagged)
Slitrk3	pDisplay (HA-tagged)
Slitrk4	pDisplay (HA-tagged)
Slitrk5	pDisplay (HA-tagged)
Slitrk6	pDisplay (HA-tagged)
LRRC24	pDisplay (HA-tagged)
Contactin-1	pDisplay (HA-tagged)
KIAA0644	pDisplay (HA-tagged)
Lingo4	pDisplay (HA-tagged)
LRRN1	pDisplay (HA-tagged)
LRRN3	pDisplay (HA-tagged)
TrkA	pDisplay (HA-tagged)
TrkB	pDisplay (HA-tagged)
PTPRT	pGW1 (untagged)

Table S2. Electrophysiological properties of eGFP-expressing (control) and MDGA1-expressing (MDGA1) neurons

	Miniature inhibitory postsynaptic currents (mIPSCs)				Miniature excitatory postsynaptic currents (mEPSCs)			
Groups/ parameters	Membrane capacitance (pF)	Membrane input resistance (MΩ)	Rise time (ms)	Decay time (ms)	Capacitance (pF)	Input resistance (MΩ)	Rise time (ms)	Decay time (ms)
Control	48.8 ± 2.6	278.7 ± 23.1	8.1 ± 0.5	12.9 ± 1.1	54.9 ± 5.89	258.2 ± 26.9	1.13 ± 0.05	1.24 ± 0.08
MDGA1	55.0 ± 3.9	272.1 ± 19.5	7.2 ± 0.3	9.7 ± 0.6	62.48 ± 4.1	233.9 ± 21.3	1.07 ± 0.03	1.23 ± 0.06

Number of neurons recorded for mIPSCs: Control, n = 15; MDGA1. n = 14. Number of neurons recorded for mEPSCs: Control, n = 11; /MDGA1. n = 13.