Calsyntenin-3 interacts with both α- and β-neurexins in the regulation of excitatory synaptic innervation in specific Schaffer collateral pathways

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Calsyntenin-3 (Clstn3) is a postsynaptic adhesion molecule that induces presynaptic differentiation via presynaptic neurexins (Nrxns), but whether Nrxns directly bind to Clstn3 has been a matter of debate. Here, using LC–MS/MS–based protein analysis, confocal microscopy, RNAscope assays, and electrophysiological recordings, we show that β-Nrxns directly interact via their NLS domain with Clstn3 and Clstn3 cadherin domains. Expression of splice site 4 (SS4) insert–positive β-Nrxns variants, but not insert–negative variants, reversed the impaired Clstn3 synaptogenic activity observed in Nrxn-deficient neurons. Consistently, Clstn3 selectively formed complexes with SS4–positive Nrxns in vivo. Neuron-specific Clstn3 deletion caused significant reductions in number of excitatory synaptic inputs. Moreover, expression of Clstn3 cadherin domains in CA1 neurons of Clstn3 conditional knockout mice rescued structural deficits in excitatory synapses, especially within the stratum radiatum layer. Collectively, our results suggest that Clstn3 links to SS4–positive Nrxns to induce presynaptic differentiation and orchestrate excitatory synapse development in specific hippocampal neural circuits, including Schaffer collateral afferents.

Synaptogenic adhesion molecules, a class of synaptic transmembrane proteins that induce synaptic differentiation in vitro (1–3), are central to various aspects of synapse development, but their precise roles in synapse assembly, validation, and/or plasticity in vivo are only beginning to be revealed (1, 4, 5). Presynaptic neurexins (Nrxns) and leukocyte common antigen-related receptor protein-tyrosine phosphatases (LAR-RPTPs) are among the synaptogenic adhesion molecules that have emerged as key platforms that facilitate convergence of diverse signals from multifarious postsynaptogenic ligands at mammalian synapses (5, 6). Of particular note is the fact that, although Nrxns and LAR-RPTPs are evolutionarily conserved, only a subset of their ligands in mammals has homologs in invertebrate species that also play crucial roles in various aspects of central nervous system development, hinting at the possibility that Nrxns and LAR-RPTPs serve fundamental functions through these selective adhesion pathways.

Calsyntenins (Clstns) are evolutionarily conserved synaptogenic adhesion proteins of the cadherin superfamily that are expressed most highly in the brain (7). Synaptic functions of the three vertebrate Clstn family members have recently been reported. For example, juvenile Clstn1-deficient mice exhibit compromised excitatory synaptic transmission, possibly because of disrupted targeting of N-methyl-D-aspartate (NMDA) receptor subunits (8). They also show increased synaptic levels of GluN2B subunit-containing NMDA receptors, enhanced long-term potentiation (LTP), and greater filopodia-like dendritic protrusions in the hippocampus, but decreased dendritic arborization, suggesting that Clstn1 mediates dendritic transport of NMDA receptor subunits and regulates spine maturation during early development (8). In addition, Clstn1 regulates guidance receptor trafficking by shutting Rab11-positive vesicles, leading to switching of commissural axon responsiveness (9). Clstn1 also contributes to peripheral sensory axon arborization, branching, endosomal dynamics, and microtubule polarity in zebrafish (10, 11). Clstn2, on the other hand, plays a nonredundant role in inhibitory synapse development and influences a subset of cognitive abilities (12, 13). Clstn3 was identified as a postsynaptogenic adhesion molecule that acts through presynaptic Nrxns (14, 15). However, in contrast to the report of Pettem et al. (14), we did not detect direct interactions between Clstn3 and α-Nrxns.

In the present study, we revisited these issues. Strikingly, utilizing newly engineered Nrxn1 expression vectors to increase Nrxn1β expression levels, we found that recombinant Clstn3 bound both Nrxn1β and Nrxn1α, with a slight preference for splice site 4 (SS4) insert–positive variants, requiring an Nrxn splice variant containing an insert at SS4 as a functional receptor for its presynaptic differentiation-inducing activity. Conditional

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deletion of Clstn3 in neurons led to drastic reductions in excitatory synapse structures. Finally, adeno-associated virus-mediated expression of Nrxn-binding CST-3 cadherin domains was sufficient to rescue decreased excitatory synapse puncta density in CA1 stratum radiatum layers of Clstn3-deficient mice. Viewed together, our results revise our previous molecular model, showing that Clstn3 directly interacts with SS4-positive Nrxn splice variants to induce presynaptic differentiation, and suggesting synapse-organizing functions of Clstn3 that may control specific synaptic inputs from Schaffer-collateral afferents in the hippocampus.

**Results**

**Clstn3 directly binds β-Nrxn**

Our previous cell surface-binding assays used a variety of Nrnxnα deletion variants derived from the bovine Nrnxnα gene or Nrnxnβ variants derived from the rat Nrnxnβ gene (15). These vectors have long been used to characterize Nrnxn interactions with neuroligins (NLs) and other ligands, including leucine-rich repeat transmembrane neuronal proteins (LRTMs), neurexophins, and latrophilin-1 (16–18). However, using Nrnxn vectors constructed from mouse Nrxn genes, Craig and colleagues (14) reported that Nrnxnα, but not Nrnxnβ, binds to Clstn3. To resolve this discrepancy, we performed affinity chromatography of solubilized mouse synaptosomes using immobilized recombinant IgClstn3 followed by MS. Intriguingly, among the captured proteins was a tryptic peptide unique to Nrnxnβ (in addition to Nrnxnα peptides) (Fig. 1, A–D; Table S1). To confirm binding between Clstn3 and Nrnxnβ, we engineered mouse Nrnxnβ expression constructs and performed cell surface-binding assays. We found robust binding of IgClstn3 to HEK293T cells expressing C terminally FLAG-tagged mNrnxn1β lacking (mNrnxn1β-SS4-FLAG) or containing (mNrnxn1β+SS4-FLAG) the SS4 insert (Fig. 1, E and F). In assays measuring dimeric ligand binding to mNrnxnβ-expressing cell surfaces, IgClstn3 interacted with both mNrnxn1β-SS4 and mNrnxn1β+SS4 with nanomolar affinity (Fig. 1, G and H). Q<sub>a</sub> values calculated by Scatchard analyses were 51.39 ± 5.26 nM for Nrnxn1β-SS4 and 105.94 ± 7.89 nM for Nrnxn1β<sup>SS4</sup> (Fig. 1, G and H). These findings indicate that Clstn3 binds Nrnxnβ with high affinity and exhibits a slight preference for SS4 insert–positive splice variants. To investigate differences between bovine and rat Nrnxn1 plasmids (bNrnxn1α and rNrnxn1β) used in our previous studies and the mouse Nrnxn1 plasmids (mNrnxn1α and mNrnxn1β) used in the current study, we compared total protein expression levels produced by the different vectors by immunoblot analysis using serially diluted lysates from HEK293T cells transfected with expression plasmids for mNrnxn and bNrnxn1α or rNrnxn1β (Fig. S1). Surprisingly, expression levels of mNrnxn vectors were ∼100-fold higher than those of bNrnxn and rNrnxn vectors (Fig. S1A). This difference in expression was not likely attributable to differences in the position of the FLAG epitope between rNrnxn1β (N terminus) and our original mNrnxn1β construct, as evidenced by the comparably robust binding of a newly generated N terminally FLAG-tagged mNrnxn1β<sup>+SS4</sup> (FLAG-mNrnxn1β<sup>+SS4</sup>) and our original C terminally FLAG-tagged mNrnxn1β<sup>+SS4</sup> (mNrnxn1β<sup>+SS4</sup>-FLAG) to IgClstn3 in cell surface-binding assays (Fig. S1, B and C).

**Cadherin domains of Clstn3 mediate direct binding to β-Nrxn**

In addition to cell surface–binding assays, in vitro pulldown assays clearly showed binding of Clstn3 to IgNmNrxn1β<sup>+SS4</sup> and IgNmNrxn1α<sup>+SS4</sup>. To identify a minimal Clstn3 domain involved in Nrnxn binding, we used three different FLAG-tagged Clstn3 constructs: Full (full-length Clstn3), Cad (containing tandem cadherin domains, the transmembrane segment and intracellular residues of Clstn3), and ΔCad (full-length Clstn3 lacking the tandem cadherin domains) (see Fig. 2A for schematic diagram of Clstn3 constructs). IgNmNrxn1α<sup>+SS4</sup> and IgNmNrxn1β<sup>+SS4</sup> both pulled down Clstn3 Cad, but not Clstn3 ΔCad (Fig. 2, B and C), suggesting that cadherin domains of Clstn3 mediate binding to Nrnxns. The results of these and aforementioned cell-based surface-binding assays do not exclude the possibility that intermediate(s) expressed in HEK293T cells may bridge indirect associations of Nrnxns with Clstn3. However, binding assays performed using purified recombinant IgNm1 (IgNm1β<sup>SS4</sup>, IgNm1β<sup>+SS4</sup>, IgNm1α<sup>SS4</sup>, or IgNm1α<sup>+SS4</sup>) and recombinant His-HA-Clstn3 (Clstn3 Ecto, Clstn3 Cad, or Clstn3 ΔCad) (Fig. 2D) showed that His-HA-Clstn3 Ecto directly bound to recombinant α- and β-Nrxns (Fig. 2, E and F). Moreover, Clstn3 Cad, but not Clstn3 ΔCad, directly bound to recombinant β-Nrxns (Fig. 2F). These data suggest that cadherin domains of Clstn3 mediate direct interactions with Nrnxns, consistent with our previous observation that the cadherin domains of Clstn3 are necessary and sufficient to induce presynaptic differentiation (15).

**Clstn3 binds to an LNS domain of mNrnxn1β in a Ca<sup>2+</sup>-dependent manner**

We next investigated which Nrnxn sequences mediate Clstn3 binding. To this end, we generated the following FLAG-tagged mNrnxn1β-deletion constructs: mNrnxn1β ΔHRD, which lacks a β-Nrxn-unique histidine-rich sequence; mNrnxn1β ΔLNS, which lacks a β-Nrxn LNS domain; and ΔStalk1, which lacks the entire stalk region (Fig. 3A). We found that IgClstn3 bound comparably to HEK293T cells expressing mNrnxn1β ΔHRD or mNrnxn1β WT, but did not bind HEK293T cells expressing mNrnxn1β ΔLNS (Fig. 3, B and C), indicating that the Nrnxn1β LNS domain is necessary for Clstn3 binding. Interestingly, deletion of the entire stalk region (mNrnxn1β ΔStalk1) diminished Clstn3 binding as well as NL-2 binding (Fig. 3, B and C). The stalk region contains ∼40 residues with several putative O-linked glycosylated sites and a short cysteine-loop sequence composed of two conserved cysteines flanking an 8-residue acidic sequence (19). We thus hypothesized that O-linked glycosylation of mNrnxnβ regulates Clstn3 binding and that mNrnxn1β ΔStalk1 displayed weak Clstn3 binding because it lacks O-linked glycosylation. To test this, we generated mNrnxn1β constructs containing point mutations or deletions in the conserved stalk region. Clstn3 binding was retained in mNrnxn1β constructs in which putative O-glycosylated threonines
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or serines were replaced with glycines (mNrxn1α –SS4 or SS4 or Nrxn1α –SS4), or IgC (negative control) proteins pulled down FLAG-Clstn3 Full and FLAG-Clstn3 Cad, but not FLAG-Clstn3 ΔCad. Input, 5%; C, IgNrxn1α –SS4; IgNrxn1β –SS4, or IgC used for pulldown assays were analyzed by direct comparison of bands revealed by parallel Ponceau S staining. D, diagrams of His-HA-tagged extracellular C lstn3 variants used in direct-binding assays. E and F, purified His-tagged C lstn3 proteins were incubated with purified IgNrxn proteins, as indicated. Precipitates obtained using Talon resin were analyzed by immunoblotting with human IgG or HA antibodies.

or serines were replaced with glycines (mNrxn1ΔCHO), the cysteine-loop sequence was deleted (mNrxn1ΔCysL1), the stalk region was partially deleted (mNrxn1ΔΔStalk2), or the conserved serine residue for attaching heparan sulfate chains was replaced with alanine (mNrxn1ΔHS) (Fig. 4, A–D and F). However, C lstn3 did not bind Nrxn1α, a newly identified Nrxn1 isoform (19), or other heparan sulfate proteoglycans, such as glypicans or syndecans (Figs. 3, B and C, and 4, E and F). Control experiments showed that surface expression levels of individual mNrxn1β deletion constructs in HEK293T cells were comparable with those of WT protein (Fig. S2). These findings suggest that the Nrxn1β LNS domain is a C lstn3-binding site. We further found that treatment with the Ca2+ chelator EGTA prevented these interactions (Fig. S3).

C lstn3 requires specific Nrxn splice variants for presynaptic differentiation

To delineate the molecular mechanisms that link Nrxns to C lstn3 and support its synaptogenic activity, we performed heterologous synapse-formation assays in cultured hippocampal neurons in which all three Nrxns were down-regulated using small hairpin RNAs (15, 20). We found that Nrxns triple-knockdown (TKD) significantly reduced the synaptogenic activity of C lstn3 (Fig. 5, A and B) (15). Synaptogenic activity was completely restored by coexpression of Nrxn1α –SS4 or Nrxn1β –SS4, but not Nrxn1αSS4 or Nrxn1βSS4, suggesting that SS4-positive Nrxns act as functional receptors for C lstn3 (Fig. 5, A and B). Subsequent pull-down assays showed that IgM/Nrxn1α+SS4 and IgM/Nrxn1β+SS4, but not IgM/Nrxn1αSS4 or IgM/Nrxn1βSS4, were capable of capturing C lstn3 from mouse synaptosomal membrane fractions (Fig. 5, C and D). Moreover, re-expression of the respective Nrx splice variants rescued deficits in the synaptogenic activities of NL-1 (neuroligin-1) and LRRTM2 (leucine-rich repeat transmembrane neuronal 2) (Fig. 5, A and B). Subsequent pull-down assays showed that IgM/Nrxn1α+SS4 and IgM/Nrxn1β+SS4, but not IgM/Nrxn1αSS4 or IgM/Nrxn1βSS4, were capable of capturing C lstn3 from mouse synaptosomal membrane fractions (Fig. 5, C and D). Moreover, re-expression of Nrx1β+SS4 in Nrxn-TKD neurons triggered clustering of both the inhibitory presynaptic marker GAD67 (glutamic acid decarboxylase 67) and the excitatory presynaptic marker VGLUT1 (vesicular glutamate transporter...
1) in contacting axons of co-cultured neurons (Fig. S5), suggesting that Clstn3 exerts its synaptogenic activities through Nrxn SS4-positive splice variants at both excitatory and inhibitory synapses.

Generation and characterization of Clstn3 conditional knockout (cKO) mice

To elucidate the physiological significance of Clstn3–Nrxn interactions in vivo, we used Clstn3<sup>tm<sub>1a</sub></sup>EUCOMM(Hmgu) mice in which a targeting cassette harboring FRT, lacZ, and loxP sites was inserted between exon 7 and exon 8, resulting in a "knock-out-first" lacZ-reporter-tagged Clstn3<sup>tm1a</sup> insertion allele with conditional potential (21). We then crossed Clstn3<sup>tm1a/tm1a</sup> mice with an FLPe knock-in strain to remove contaminating transgenes and the neomycin resistance cassette to generate Clstn3<sup>tm1c</sup>EUCOMM(Hmgu) mice. To decide which Cre driver lines to cross, for the current study, we first analyzed Clstn3 expression patterns in the mouse brain. For this, we performed RNA-scope-based in situ hybridization analysis using a Clstn3-specific probe. This analysis showed that Clstn3 mRNA was strongly expressed in most pyramidal cell layers and interneurons in the mouse hippocampus (Fig. 6A), with particularly prominent expression observed in pyramidal cell layers in the CA3 region (Fig. 6, A and C). We further found that Clstn3 mRNA was also expressed in parvalbumin (PV)- and somatostatin (SST)-positive GABAergic interneurons in the hippocampus, as previously reported (14) (Fig. 6B). Moreover, immunofluorescence analyses using a Clstn3-specific antibody (JK091) showed that the expression pattern of the Clstn3 protein was similar to that of Clstn3 mRNA in the hippocampus (Fig. 6D). Based on these results, the Nestin-Cre (Nestin–Clstn3<sup>Crt</sup>) driver line was chosen to further cross with Clstn3<sup>tm1c</sup>EUCOMM(Hmgu) mice to generate Clstn3-cKO mice (Fig. 7, A and B). Loss of Clstn3 protein in Nestin–Clstn3

Figure 3. Clstn3 binds to LNS domain of β-Nrxns. A, diagrams of Nrxn1β and Nrxn1γ constructs used in B, C, cell surface-binding assays. HEK293T cells expressing FLAG-tagged Nrxn1β<sup>SS4</sup> WT, its deletion variants (ΔHRD, ΔLNS, or ΔStalk1), or FLAG-tagged Nrxn1γ WT were incubated with IgClstn3, IgNL-2, or IgC, and analyzed by immunofluorescence imaging for Ig-fusion proteins (red) and FLAG (green). All binding reactions were performed in 2 mM CaCl<sub>2</sub> and 2 mM MgCl<sub>2</sub>. Scale bars: 10 μm (applies to all images). C, quantification of cell surface binding in B. Data are mean ± S.E. (**, p < 0.01; ****, p < 0.0001; nonparametric Kruskal-Wallis test with Dunn’s post hoc test; number of cells analyzed = 9–19). p values for IgC binding: WT versus ΔHRD, p = 0.0841; WT versus ΔLNS, p = 0.9999; WT versus ΔStalk1, p = 0.1214; WT versus Nrxn1γ, p = 0.0616. p value for IgClstn3 binding: WT versus ΔHRD, p = 0.9581; WT versus ΔLNS, p = 0.0001; WT versus ΔStalk1, p < 0.0001; WT versus Nrxn1γ, p = 0.0616. p values for IgNL-2 binding: WT versus ΔHRD, p = 0.4968; WT versus ΔLNS, p < 0.0001; WT versus ΔStalk1, p = 0.0033; WT versus Nrxn1γ, p < 0.0001.
mice was verified by the absence of detectable Clstn3 immunoreactivity to Clstn3-specific antibodies (JK091; Fig. 7, G and H, and Fig. S6). These mice were largely indistinguishable from control littermates (Clstn3f/f; Ctrl) in terms of birth rate, although Nestin-Clstn3 mice weighed marginally (but significantly) less at both postnatal day 30 (P30) and P54, which is a generalized metabolic phenotype of the Nestin-Cre driver line (Fig. 7C, Table S2). In addition, gross morphology (as assessed by Nissl staining) and neuron numbers (as assessed by NeuN staining) in Nestin-Clstn3 mice were comparable with those of control littermates (Fig. 7, D–F). Semi-quantitative immunoblot analyses showed that the relative expression levels of various synaptic proteins in the
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hippocampus (Fig. 7G) and cortex (Fig. 7H) of Nestin-Clstn3 mice were unchanged compared with littermate controls (Fig. 7, G–I). Collectively, these data suggest that Clstn3 is not essential for mouse survival or breeding, and does not affect the expression levels of other synaptic proteins.

Cadherin domains of Clstn3 are required for restoration of the impaired excitatory innervations observed in Clstn3-cKO hippocampal neurons

We next analyzed the effect of Clstn3 deletion on the intensity of excitatory and inhibitory synaptic puncta in various layers of the hippocampal CA1 region using Nestin-Clstn3 (Figs. 8 and 9). We found a significant decrease in the intensity of VGLUT1 and PSD-95 (excitatory postsynaptic marker) puncta in most layers of hippocampal CA1 regions (stratum oriens (SO) and stratum radiatum (SR)), but not in the stratum lacunosum moleculare (SLM) layer, in Clstn3-cKO (Fig. 8, B–E). In contrast, the intensity of GAD67, GAD65, and GABA, Ry2 puncta was unchanged in all examined layers of hippocampal CA1 regions (SO, SR, stratum pyramidal (SP), and SLM) (Fig. 9, A–D). To investigate the physiological significance of Clstn3–Nrxn interactions in vivo, we constructed AAVs encoding full-length Clstn3 (Full), cadherin domains plus intracellular regions (Cad), or a cadherin domain-deleted Clstn3 fragment (ΔCad) (Fig. 8A, Fig. S7). We then stereotactically injected AAVs expressing the indicated Clstn3 protein into the CA1 hippocampal of ~9-week-old Nestin-Clstn3 mice and performed quantitative immunofluorescence analyses after 2 weeks using antibodies to VGLUT1 or PSD-95 (Fig. 8, A–E). Expression of Clstn3 Full restored the decreased VGLUT1 and PSD-95 puncta density to WT mouse levels in SO and SR layers of Nestin-Clstn3 mice (Fig. 8, B–E). Strikingly, expression of Clstn3 Cad, but not Clstn3 ΔCad, also restored the decreased VGLUT1 puncta density in the SR layer, but neither Clstn3 Cad nor Clstn3 ΔCad expression exerted a rescue effect in the SO layer of Nestin-Clstn3 mice (Fig. 8, B–E). These results suggest that Clstn3 organizes the development of hippocampal CA1 excitatory synapses, and likely acts through cadherin domains-mediated interactions with presynaptic Nrxns to control the specific excitatory synaptic projections involving the SR layer.

To complement these anatomical analyses, we performed whole cell electrophysiological recordings of miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) in brain slices from Nestin-Clstn3 and littermate WT mice (Fig. S8). Surprisingly, no differences in the amplitude or frequency of mEPSCs and mIPSCs were detected between Nestin-Clstn3 and Ctrl mice (Fig. S8). These results suggest that Clstn3 is not required for basal excitatory synaptic transmission in CA1 hippocampal pyramidal neurons.

Discussion

The present study was initiated with the goal of reconciling discrepancies surrounding molecular mechanisms of Clstn3-mediated synapse development. For this, we revisited the most critical (and controversial) issues using newly engineered Nrxns expression vectors and recombinant proteins, and a newly developed Clstn3-cKO mice. Taken together with our previous findings, the three principal observations of the current study provide plausible explanations for discrepancies surrounding the molecular mechanisms of Clstn3-mediated synapse development.

First, we found that Clstn3 binds directly to β-Nrxns (Figs. 1 and 2). This interaction was only detected using newly engineered Nrxn1β constructs that were expressed at ~100-fold higher levels (Figs. 1 and 2; Fig. S1). Because Nrxn1βΔStalk1 displayed a significant decrease binding to Clstn3 and this juxtamembrane sequence is heavily glycosylated, we initially speculated that glycosylation patterns contributed to Clstn3 binding (Figs. 2 and 3); however, removal of glycosylated residues did not impair binding (Fig. 4). Instead, we conclude that this sequence may influence the orientation and/or conformation of the LNS domain in a manner that is critical for its interaction with Clstn3. Structural studies have suggested that a β-Nrxn LNS domain containing an SS4 insertion exists as a dynamic equilibrium between two conformational states: one in which the splice insert forms a protruded α-helix that supports binding to cerebrellins (Cblns), and one in which the additional residues adopt a β-sheet conformation and restores binding to NLs and LRRTMs (22). Because Clstn3 exhibits a slight preference for binding SS4-positive Nrxns, it is tempting to speculate that restoration of downstream residues promoted a conformational change in the SS4 insert, leading to Clstn3 binding. As noted above, the current study, unlike our previous study and that of Pettem et al. (14), employed newly engineered Nrxn recombinant proteins to provide the first demonstration of direct interactions with recombinant Clstn3 proteins (Fig. 2). Moreover, in keeping with results from the current study, it

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Figure 4. No Effect of Nrxn1β O-glycosylation on Clstn3–Nrxn1β interactions. A, diagrams of Nrxn1 constructs used in the cell surface–labeling assays presented in B, 8, cell surface–labeling assays. HEK293T cells expressing FLAG-tagged Nrxn1βSS4 WT or its deletion variants (ΔCHO, ΔCysl, or ΔStalk2) were incubated with IgC (control), IgClstn3, or IgNL-2 and analyzed by immunofluorescence imaging for Ig-fusion proteins (red) and FLAG (green). All binding reactions were performed in 2 mM CaCl2 and 2 mM MgCl2. Scale bars: 10 μm for all images. C, quantification of cell-surface binding in 8. Data are mean ± S.E. (number of cells analyzed = 11–16), p values for IgC binding: WT versus ΔCHO, p = 0.0816; WT versus ΔCysl, p = 0.1732; WT versus ΔStalk2, p = 0.0651, p value for IgClstn3 binding: WT versus ΔCHO, p = 0.0724; WT versus ΔCysl, p = 0.4738; WT versus ΔStalk2, p = 0.9999, p values for IgNL-2 binding: WT versus ΔCHO, p > 0.9999; WT versus ΔCysl, p > 0.9999; WT versus ΔStalk2, p > 0.9999. D, cell surface–labeling assays. HEK293T cells expressing FLAG-tagged Nrxn1βSS4 WT, its heparan sulfate binding defective mutant (ΔHS), or PTPr were incubated with IgC (control), IgClstn3, or IgNL-2 and analyzed by immunofluorescence imaging for Ig-fusion proteins (red) and FLAG (green). All binding reactions were performed in 2 mM CaCl2 and 2 mM MgCl2. Scale bars: 10 μm for all images. E, cell surface–labeling assays. HEK293T cells expressing HA-tagged Glypcan-4, Myc-tagged Syndecan-2, or Syndecan-3 were incubated with IgC (control), or IgClstn3 and analyzed by immunofluorescence imaging for Ig-fusion proteins (red) and HA/Myc (green). All binding reactions were performed in 2 mM CaCl2 and 2 mM MgCl2. Scale bars: 10 μm for all images. F, quantification of cell-surface binding in D and E. Data are mean ± S.E. (number of cells analyzed = 12–28). (***, p < 0.001; nonparametric Kruskal-Wallis test with Dunn’s post hoc test). p value for IgNL-2 binding: WT versus ΔHS, p = 0.3713, p value for IgClstn3 binding: WT versus ΔHS, p = 0.9999; WT versus PTPr, p < 0.0001; WT versus Glypcan-4, p = 0.0001; WT versus Syndecan-2, p = 0.0001; WT versus Syndecan-3, p = 0.0001. p values for IgC binding: WT versus ΔHS, p > 0.9999; WT versus PTPr, p > 0.9999; WT versus Glypcan-4, p > 0.9999; WT versus Syndecan-2, p > 0.9999; WT versus Syndecan-3, p = 0.1694.
was previously reported that β1-Nrxn binds immobilized Clstn3 with a complex binding mode (23). Although it was claimed that Clstn3 interacts with α-Nrxns (but not β-Nrxns), and we previously failed to detect the interaction of Nrxns with Clstn3 (14, 15), we would argue that conclusions based on binding experiments employing a limited set of methodologies could unintentionally be misleading, as was the case for our previous report (15).
Second, re-expressing select Nrxn1 variants in Nrxn-deficient neurons rescued impaired Clstn3 synaptogenic activity (Fig. 5). Previously, we reported that presynaptic Nrxns serve as functional receptors for postsynaptic Clstn3, but do not directly interact with them (15). Our new data indicate that our previous model should incorporate direct binding of $\beta$-Nrxns to...
Clstn3 (Figs. 1–5). In support of this interpretation, BAM-2, a Nrxn-related *Caenorhabditis elegans* homolog, was recently reported to bind CASY-1 and mediate neural circuit wiring of male-specific hook-sensory HOA neurons in *C. elegans* (24). Intriguingly, CASY-1 does not directly interact with NRX-1, a canonical *C. elegans* Nrxn ortholog (24). Although BAM-2 exhibits considerable sequence homology/similarity with α-Nrxns (25), whether these vertebrate and worm genes are functionally homologous remains to be determined. Nrxns interact with various postsynaptogenic proteins, including NLs, LRRTMs, Cblns, and latrophilins (3). These interactions are dynamically modulated by the alternative splicing status of

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**A** Clstn3 cKO design/strategy

**B** Genotyping of Nestin-Clstn3 mice

**C** P30 (M)  P30 (F)  P54 (M)  P54 (F)

**D** Nissl staining (P70)

**E** NeuN staining (P70)

**F** Neuron density (number/mm²)

**G** Hippocampus

**H** Cortex

**I**

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Nrxns, mainly at the canonical SS4 splice site. Strikingly, the synapse-promoting activity of Clstn3 required Nrxn splice variants containing the SS4 insert (Fig. 5), as has been shown for other Nrxn ligands (i.e. Cblns) (3). These observations are consistent with slightly stronger binding of Nrxn1β-SS4 to Clstn3, which contrasts with the exclusive binding of Nrxn1β-SS4 to Cblns (Fig. 1, G and H). Pulldown experiments in mouse brains also showed that greater enrichment of Clstn3 using IgNrxn1α-SS4 was than obtained using IgNrxn1α-SS4 (Fig. 5, C and D) (15). Additionally, the shed ectodomain of Clstn3 suppresses the synaptogenic activity of NL-2 and LRRTM2, suggesting that Clstn3 competes with these Nrxn ligands (14). However, LRRTM2 interacts only with Nrxn-SS4—negative splice variants (16), whereas Clstn3 activity requires Nrxn-SS4—positive splice variants; moreover, a β-Nrxn LNS domain (identical to the sixth LNS domain—sixth LNS domain in α-Nrxns) is sufficient for Clstn3 binding. Although we still failed to detect clear interactions of α-Nrxns with Clstn3 in cell surface-binding assays, we observed robust Nrxn1α–Clstn3 interactions in brain pulldown and direct binding assays (Figs. 2E and 5D). It is possible that the surface-binding assays employed here were too stringent to observe this interaction, or the cell surface presentation of α-Nrxns was not optimal for binding. Thus, we conclude that Clstn3 binds to both α- and β-Nrxns through a common LNS domain, but a subset of Clstn3 synaptic functions might require other unidentified ligands in vivo. Moreover, given the activity-dependent regulation of Nrxn alternative splicing at the SS4 site (26), future studies should investigate whether Clstn3–Nrxn interactions could be finetuned depending on distinct activity patterns in vivo. Furthermore, given a recent report that different SS4-positive splice variants of two different Nrxns (Nrxn1 versus Nrxn3) differentially control different postsynaptic responses (27), it is possible that Clstn3 preferentially partners with a specific Nrxn in vivo.

Third, re-expressing Clstn3 cadherin domains was sufficient to completely rescue the impaired excitatory synapse structures in hippocampal CA1 neurons from Nestin–Clstn3 mice (Fig. 8), but only in the SR layer. In contrast, the structural deficits in other hippocampal CA1 layers were rescued by expression of full-length Clstn3 protein, but not by expression of partial Clstn3 proteins (Fig. 8). Although it is possible that other unidentified proteins could also bind to Clstn3 cadherin domains, it is likely that presynaptic neurexins and postsynaptic Clstn3 control the properties of excitatory synapse development in specific Schaffer-collateral projections within the SR layer. A recent paper highlighted the fact that the distinctive features of two different Schaffer-collateral projections differentially regulate mushroom spine density and high-magnitude LTP in the SO layer, organized by heterophilic type II cadherins (28). Moreover, Nrxn genes show differential, but overlapping, isoform- and region-dependent expression in different classes of neurons, and undergo highly distinctive, cell type-specific alternative splicing (29, 30). One plausible scenario would be that CA3 neurons projecting to CA1 neurons in the SR layer, but not the SO layer, express higher levels of SS4-positive Nrxns at their nerve terminals. A recent extensive chromogenic and fluorescent in situ hybridization analysis showed that the levels of Nrxn mRNAs in hippocampal subfields are significantly lower in GABAergic neurons than excitatory neurons (31), which might account for preferential deficits on excitatory synapses in CA1 hippocampal regions of Clstn3 KO mice. Another possible scenario is that major glutamatergic axon fibers of Schaffer-collateral (SC) pathways are targeted to CA1 pyramidal neurons in the SR layer, whereas a subspecialization of axon fibers of SC pathways innervates both bistratified GABAergic interneurons in the SO layer and CA1 pyramidal neurons. Additional studies will also be required to determine the identity of neurons in vivo that are responsible for Nrxn–Clstn3 interactions in other neural circuits inside and outside of the hippocampus. Notably, in stark contrast to the previous report that decreased inhibitory synapse structure and transmission in Clstn3-KO mice (14), we found that inhibitory synapses in CA1 hippocampal layers and the cortex were morphologically normal in our Clstn3-KO mice (Fig. 9). The reason for this discrepancy is currently unclear, but it is likely that Clstn family proteins are functionally redundant in the maintenance of basal synaptic transmission; alternatively, Clstn3 may be specifically required for certain forms of synaptic plasticity. A notable difference between the study of Pettet et al. (14) and the current study is that the former targeted exons 2 and 3 of the mouse Clstn3 gene, whereas our study targeted exon 8 (Fig. 7). In addition,
Pettem et al. (14) employed an EIIa-Cre driver line that permits germ line deletion in both excitatory and inhibitory neurons by driving expression of Cre recombinase in the early mouse embryo, whereas we used a pan-neuronal Nestin-Cre driver line was that presumed to be similar to EIIa-Cre. It is also possible that specifically deleting Clstn3 in GABAergic inhibitory neurons may produce marked deficits in GABAergic synapse development, as previously documented (14), but this did not clearly manifest in our Clstn3-cKO mice. A more systematic, follow-up investigation to probe cell type-specific contributions of Clstn3 to distinct anatomical and electrophysiological phenotypes should address these important, but puzzling, observations. Future studies should also probe how Nrxn–Clstn3 complexes mediate synaptic specificity involving the SR layer of the hippocampal CA1 region. A complete understanding of how Clstn3 functions will also require investigation of the potential contributions of other Clstn3 domains (e.g. LNS domain, intracellular region) to...
the functions of Clstn3, as was similarly shown in *C. elegans* (24, 32, 33). In summary, the present study unambiguously establishes that Clstn3 plays a role in specifying the properties of a specific hippocampal CA1 neural circuit, in part by regulating excitatory synapse development through formation of physical complexes with specific Nrxn splice variants.
**Experimental procedures**

**Plasmids**

Nrxn rescue vectors were generated by PCR amplification of full-length sequences of mouse Nrxn1β-SS4, Nrxn1β-ΔNS4, rat Nrxn1β-SS4, rat Nrxn1α-SS4, bovine Nrxn1α-SS4, and bovine Nrxn1α-ΔSS4, followed by digestion with *NheI* and *BsrGI* and cloning into the TKD vector (L-313 vector). Three nucleotides (underlined) in the GTGCCCTCTCTATGACAATCC sequence were then mutated to render it small hairpin RNA-resistant. pGW1-FLAG-mNrxn1β-SS4 and pGW1-FLAG-mNrxn1α-SS4 were generated by PCR amplification of full-length mNrxn1β and mNrxn1α, respectively, followed by digestion with *NheI* and cloning into the pGW1-FLAG vector. pCAGG-mNrxn1α-ΔSS4 and mNrxn1α-ΔSS4 were generated by PCR amplification of full-length mNrxn1α and mNrxn1α-ΔSS4, respectively, followed by digestion with *KpnI* and cloning into the pGW1-FLAG vector. pCAGG-mNrxn1β-ΔSS4 and pGW1-FLAG-mNrxn1β-ΔSS4 were generated by PCR amplification of full-length mNrxn1β and mNrxn1β-ΔSS4, respectively. pGW1-FLAG-Clstn3 and mNrxn1α were generated by PCR amplification of two Clstn3 fragments (aa 298–346), followed by digestion with *EcoRI* and cloning into a pCMV-Flag vector. pGW1-FLAG-mNrxn1β-ΔSS4 and pGW1-FLAG-Clstn3-ΔCad were generated by PCR amplification of full-length mNrxn1β and mNrxn1α-ΔSS4 fused to a His6 tag and HA tag and cloning into the pGW1-FLAG vector. The following constructs were previously described: L-315-Nrxn-TKD (20); pCMV5-mVenUS-NL-1 (34); pCMV-IgC-Clstn3 (15); pCMV-IgC-NL-2 (34); pDisplay-LRRTM2 (35); pCAGG-mNrxn1α-ΔSS4-FLAG, pCAGG-mNrxn1α-ΔSS4-FLAG, pCAGG-mNrxn1β-ΔSS4-FLAG, pCAGG-mNrxn1β-ΔSS4-FLAG, and pCMV-IgC-mNrxn1α-ΔSS4 (36). pAAV2-CAG-Clstn3 Full, pAAV2-CAG-Clstn3 Cad, and pAAV2-CAG-Clstn3 Cad were generated by PCR amplification using pGW1-FLAG-Clstn3 Full, pGW1-FLAG-Clstn3 Cad, and pGW1-Flag-Clstn3 Cad as templates, respectively, followed by digestion with *EcoRV* and HindIII and cloning into a pAAV2-CAG vector.

**Antibodies**

Fusion proteins of GSH-S-transferase and mouse Clstn3 (JK091; aa 21–244) were produced in BL21 Escherichia coli and purified using a GSH- Sepharose column (GE Healthcare, Chicago, IL, USA). Clstn1 peptides (JK025; CHQRT-MRDQTGKEN) and Clstn2 peptides (JK028; CSSQSS-PERSTWNTAGVNIWK) were synthesized and conjugated to keyhole limpet hemocyanin through a cysteine added to the N terminus of the peptide. Following immunization, rabbits with the immunogen antibodies were affinity-purified using a SulfoLink column (Pierce). The following commercially available antibodies were used: mouse monoclonal anti-α (clone HA-7; Covance), mouse monoclonal anti-MAG M2 (Sigma-Aldrich), rabbit polyclonal anti-FLAG (Sigma-Aldrich), goat polyclonal anti-EGFP (Rockland Immunocchemicals), mouse monoclonal anti-NL-1 (clone N97A/31; NeuronMab), rabbit polyclonal anti-NL-2 (Synaptic Systems, Gottingen, Germany), rabbit monoclonal anti-TrkB (clone C44H5; Cell Signaling Technology), guinea pig polyclonal anti-VGLUT1 (Millipore), mouse monoclonal anti-GAD67 (clone I1G0.3; Millipore), mouse monoclonal anti-P50-95 (clone K28/43; Thermo Fisher Scientific), mouse monoclonal anti-β-actin (clone AC-74; Sigma-Aldrich), mouse monoclonal anti-parvalbumin (clone PARV-19; Millipore), mouse monoclonal anti-gephyrin (clone 3B11; Synaptic Systems), rabbit polyclonal anti-GABAa receptor γ2 (Synaptic Systems); mouse monoclonal anti-GluN1 (clone 54.1; Millipore), rabbit polyclonal anti-GluN2A (Millipore), rabbit polyclonal anti-GluN2B (Millipore), goat anti-human IgG peroxidase (Sigma-Aldrich), and mouse monoclonal anti-NeuN (clone A60; Millipore). The following antibodies have been previously described: anti-P50-95 (JK016) (20), anti-synapsin (JK014) (37), anti-GAD65 (JK158) (38), anti-Clstn3 (JK001) (15), anti-GluA1 (1193) (39), and anti-GluA2 (1195) (39).

**Animals**

Clstn3<sup>Δnaal(EUCOMM)H019 mice, in which a targeting cassette harboring FRT, lacZ, and loxP sites is inserted between exon 7

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and exon 8, resulting in a “knockout-first” lacZ reporter-tagged Clstn3tm1a insertion allele with conditional potential, were obtained from the European Mouse Mutagenesis Consortium (EUCOMM). To generate conditional Clstn3 KO mice (Clstn3flox/flox), we first bred Clstn3tm1a/m1a mice in a C57BL/6j background with FLPO recombinase driver mice (C57BL/6N-Tg(CAG-Flpo)1Afst/Mmucd, MMRC) to generate Clstn3tm1a/m1a mice. Male homozygous Clstn3tm1a/m1a mice in a C57BL/6j genetic background were crossed with female heterozygous Clstn3tm1a/+ mice (for Nestin conditional) carrying the Cre transgene. A Nestin-Cre (003771) line in a C57BL/6N genetic background, generated by crossing the original Cre-driver lines purchased from the Jackson Laboratory with C57BL/6j for more than five generations, was obtained from Dr. Albert Chen (DUKE-NUS, Singapore). Cre-negative Clstn3flox/fox littermates were used as controls in all experiments. Mice were housed and bred at the animal facility of Daegeu Gyeongbuk Institute of Science and Technology (DGIST), and experimental use was approved by the Institutional Animal Care and Use Committee of DGIST (DGIST-IACUC-19052110-00). Mice were given ad libitum access to food and water, and were maintained in a controlled environment with a 12-h light/dark cycle. Mice were weaned at the age of P24–27, and mixed-genotype littermate mice were group-housed (4–5 mice per cage) until experiments. Genotyping was performed on genomic DNA extracted from tail biopsies using the 2× Taq PCR Smart mix 2 kit (Solgent Co.). Genotyping primers were as follows: Clstn3 forward, 5'-ACT TGA TCA GTC CTC CTG CAT CAG-3'; Clstn3 reverse, 5'-CTG AAG TTC AGG GTC AGC CTG TAA-3'; WT reverse, 5'-CCT TCC TCC TAC ATA GTT GGC AGT-3'; Cre (Nestin-Cre) forward, 5'-CCG CTT CCG CTT GGT CAC TGT-3'; WT reverse, 5'-CTG AGC AGC TGG TTC TGC TGC TCC T-3'; and Cre (Nestin-Cre) reverse, 5'-GAC CGG CAA AGC GAC AGA AGC A-3'. A PCR product of ~130 base pairs (bp) was obtained from WT DNA, whereas Clstn3flox produced a PCR product of ~300 bp. The Cre transgene from the Nestin-Cre mouse produced a PCR product of ~400 bp.

Production of recombinant lentiviruses and adeno-associated viruses (rAAVs)

Lentivirus production—Recombinant lentiviruses were produced as previously described (34). In brief, HEK293T cells were transfected with three plasmids: lentivirus vectors, psPAX2, and pMD2G, at a 2:2:1 ratio using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s protocol. After 72 h, lentiviruses were harvested by collecting the medium from transfected HEK293T cells and briefly centrifuging at 1,000 × g to remove cellular debris. Filtered media containing 5% sucrose were centrifuged at 117,969 × g for 2 h; supernatants were then removed and the virus pellet was washed with ice-cold PBS and resuspended in 80 μL of PBS.

AAV production—HEK293T cells were co-transfected with the indicated AAV vectors and pHelper and pRC1-DJ vectors. Seventy-two h later, transfected HEK293T cells were collected, lysed, and mixed with 40% PEG and 2.5 μl NaCl, and centrifuged at 2,000 × g for 30 min. The cell pellets were resuspended in HEPES buffer (20 mM HEPES; 115 mM NaCl, 1.2 mM CaCl2, 1.2 mM MgCl2, 2.4 mM KH2PO4) and an equal volume of chloroform was added. The mixture was centrifuged at 400 × g for 5 min, and concentrated three times with a Centriprep centrifugal filter (Millipore) at 1,220 × g for 5 min each and with an Amicon Ultra centrifugal filter (Millipore) at 16,000 × g for 10 min. Before titering AAVs, contaminating plasmid DNA was eliminated by treating 1 μl of concentrated, sterile-filtered AAVs with 1 μl of DNase I (Sigma-Aldrich) for 30 min at 37 °C. After treatment with 1 μl of stop solution (50 mM EDTA) for 10 min at 65 °C, 10 μg of protease K (Sigma-Aldrich) was added and AAVs were incubated for 1 h at 50 °C. Reactions were inactivated by incubating samples for 20 min at 95 °C. The final virus titer was quantified by quantitative RT-PCR detection of EGFP sequences and subsequent reference to a standard curve generated using the pAAV-U6-EGFP plasmid. All plasmids were purified using a Plasmid Maxi Kit (Qiagen GmbH).

In-gel digest

Gel bands were excised, after which proteins in bands were reduced by treating with DTT, alkylated by treating with indole-3 acetic acid, and then digested by treating with trypsin. Specifically, bands were washed with 10 μl ammonium bicarbonate and 50% acetonitrile, swollen in digestion buffer containing 50 μM ammonium bicarbonate, 5 μM CaCl2, and 1 μg of trypsin, and then incubated at 37 °C for 16 h. Peptides were recovered by two cycles of extraction with 50 μM ammonium bicarbonate and 100% acetonitrile. The resulting peptide extracts for each band were lyophilized and stored at −20 °C until mass spectrometric analysis.

LC–MS/MS protein analysis

Peptides were analyzed using a nanoflow LC–MS/MS system consisting of an Easy nLC 1000 system (Thermo Fisher) and an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher) equipped with a nano-electrospray source. Peptide solutions (5-μl aliquots) were loaded onto a C18 trap column (20 × 75 μm, 3 μm particle size; Thermo Fisher) using an autosampler. Peptides were desalted and concentrated on the column at a flow rate of 5 μl/min. Trapped peptides were then separated on a 150-mm custom-built microcapillary column consisting of C18 (particle size, 3 μm; Aqua Science, Yokohama, Japan) packed in 100-μm silica tubing with a 6-μm inner diameter orifice. The mobile phases A and B were composed of 0 and 100% acetonitrile, respectively, and each contained 0.1% formic acid. The LC gradient began with 5% B for 5 min and was increased to 15% B over 5 min, 50% B over 55 min, and 95% B over 5 min, then remained at 95% B over 5 min, followed by 5% B for an additional 5 min. The column was re-equilibrated with 5% B for 15 min between runs. A voltage of 2.2 kV was applied to produce the electrospray. In each mass analysis duty cycle, one high-mass resolution (60,000) MS spectrum was acquired using the Orbitrap analyzer, followed by 10 data-dependent MS/MS scans using the linear ion trap analyzer. For MS/MS analysis, a normalized collision energy (35%) was used throughout the collision-induced dissociation phase. All MS and MS/MS spectra were acquired using the following parameters: no sheath and
auxiliary gas flow; ion-transfer tube temperature, 200 °C; activation Q, 0.25; and activation time, 20 ms. Dynamic exclusion was employed with a repeat count of 1, a repeat duration of 30 s, an exclusion list size of 500, an exclusion duration of 60 s, and an exclusion mass width of ±1.5 m/z.

**MS data analysis**

MS/MS spectra were analyzed using the following analysis protocol, referencing the UniProt mouse database (04-08-2020 release; 17,333 entries). Briefly, each protein’s reversed sequence was appended onto the database to calculate the false discovery rate. Peptides were identified using ProLuCID (version 1.3.5.1) (40) in Integrated Proteomics Pipeline software, IP2, with a precursor mass error of 25 ppm and a fragment ion mass error of 600 ppm. Trypsin was used as the protease, and two potential missed cleavages were allowed. Carbamidomethylation at cysteine was chosen as a static modification, and methionine oxidation was chosen as a variable modification. Protein lists consisting of two or more peptide assignments for protein identification (false-positive rate <0.01) were prepared by filtering and sorting output data files.

**Primary neuron culture, immunocytochemistry, image acquisition, and quantitative analysis**

The indicated analyses were performed using cultured, E18-derived, rat hippocampal neurons and confocal microscopy, as previously described (15, 41, 42). Rat hippocampal neurons were prepared from E18 rat brains and cultured on coverslips coated with poly-D-lysine in Neurobasal media supplemented with B-27 (Thermo Fisher), 0.5% fetal bovine serum, 0.5 mM GlutaMax (Thermo Fisher), and sodium pyruvate (Thermo Fisher). For immunocytochemistry, cultured neurons were fixed with 4% formaldehyde, 4% sucrose, permeabilized with 0.2% Triton X-100 in PBS, and immunostained with primary antibodies and Cy3- or FITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Images were acquired using a confocal microscope (LSM700; Carl Zeiss) equipped with a 63× objective lens. All image settings were kept constant. Z-stack images were converted to maximal projection. All images were quantitatively analyzed in a blinded manner using MetaMorph software (Molecular Devices).

**Cell surface–binding assays**

IgC-fusion proteins of Clstn3, IgNrxn1β splice variants, and IgC alone (Control) were produced in HEK293T cells. Soluble Ig-fused proteins were purified using protein A-Sepharose beads (GE Healthcare). Bound proteins were eluted with 0.1 M glycine (pH 2.5) and immediately neutralized with 1 M Tris-HCl (pH 8.0). Transfected HEK293T cells expressing the indicated plasmids were incubated with 10 µg/ml of Ig-fused proteins for 2 h at 37°C. Images were acquired using a confocal microscope (LSM700; Carl Zeiss).

**Pulldown assays**

HEK293T cells were transfected with pGW1-FLAG-Clstn3 Full, Cad, ΔCad, or pCMV-mVenus-NL-1, harvested 48 h later, and incubated for 1 h at 4°C in solubilization buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% octylphenoxypolyethoxylate, 1% sodium deoxycholate, 0.1% SDS, 5 mM CaCl₂, 5 mM MgCl₂). The suspension was centrifuged at 20,000 × g to remove insoluble debris, and each supernatant was mixed with 10 µg of IgC (control), IgNrxn1α +SS4, or IgNrxn1β +SS4 supplemented with protein A-Sepharose beads and incubated at 4°C for 2 h with gentle agitation. Protein A-Sepharose beads were washed three times with solubilization buffer, solubilized in SDS sample buffer, and loaded onto SDS-PAGE gels for immunoblot analyses. The antibodies used for immunoblotting were anti-FLAG (1 µg/ml), anti-EGFP (1:1000), anti-Clstn3 (JK091; 1 µg/ml), anti-NL-1 (1 µg/ml), anti-NL-2 (1 µg/ml), and anti-TrkC (1 µg/ml).

**Direct protein-interaction assays**

For direct interaction assays, 10 µg of IgC (control), IgNrxn1α +SS4, IgNrxn1α +SS4, IgNrxn1β +SS4, or IgNrxn1β +SS4 was incubated with 4 µg of purified His-HA-Clstn3 Full, His-HA-Clstn3 Cad, or His-HA-Clstn3 ΔCad for 2 h at 4°C in binding buffer (25 mM Tris, pH 7.5, 30 mM MgCl₂, 40 mM NaCl, and 0.5% Triton X-100). Talon metal affinity resins (Clontech) beads were then added to purified protein mixtures as indicated, and incubated for 2 h at 4°C. beads were washed three times with binding buffer, solubilized in SDS sample buffer, and loaded onto SDS-PAGE gels for immunoblot analyses. Anti-human IgG was used for immunoblotting.

**Heterologous synapse-formation assays**

Heterologous synapse-formation assays were performed using recombinant Clstn3 fusion proteins as previously described (43). Briefly, HEK293T cells were transfected with EGFP (negative control) or the indicated Clstn3 constructs using Lipofectamine 2000 (Thermo Fisher). After 48 h, the transfected HEK293T cells were trypsinized, seeded onto in vitro day 10 (DIV10) hippocampal neurons, co-cultured for an additional 72 h, and double-immunostained with antibodies against EGFP, HA, and the indicated synaptic markers (synapsin, VGLUT1, or GAD67). All images were acquired using a confocal microscope (LSM700; Zeiss). For quantification, the contours of transfected HEK293T cells were chosen as the region of interest. Fluorescence intensities of synaptic markers in each region of interest were quantified for both red and green channels using MetaMorph software (Molecular Devices). Normalized synapse density on transfected HEK293T cells was expressed as the ratio of red to green fluorescence.

**Semiquantitative immunoblot analysis**

For semiquantitative immunoblot analysis, brains from P42 WT, Clstn3⁻/⁻, or Clstn3⁻/⁻::Nestin-Cre (Nestin-Clstn3) mice were homogenized in 0.32 M sucrose, 1 mM MgCl₂ containing a protease inhibitor mixture (Thermo Fisher Scientific) using a

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Precellys Evolution tissue homogenizer (Bertin Co.). After centrifuging homogenates at 1,000 × g for 10 min, the supernatant was transferred to a fresh microcentrifuge tube and centrifuged at 15,000 × g for 30 min. The resulting synaptosome-enriched pellet (P2) was resuspended in lysis buffer and centrifuged at 20,800 × g, after which the supernatant was analyzed by Western blotting. Quantitation was performed using ImageJ software (National Institutes of Health).

Immunohistochemistry

Mice were transcardially perfused first with PBS and then with 4% paraformaldehyde. After post-fixation overnight, mouse brains were slowly sectioned at 40 μm using a vibratome (VT1200S; Leica) and washed with PBS. Brain sections were incubated in blocking solution containing 10% horse serum, 0.2% BSA, and 2% Triton X-100 for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibodies against VGLUT1 (1:200), GAD67 (1:100), GAD65 (1:300), GABAARγ2 (1:500), PSD-95 (1:100), Clstn3 (JK091; 1:100), or NeuN (1:500). After washing three times, sections were incubated with Cy3- or FITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. Sections were then washed extensively, and mounted on glass slides with Vectashield Mounting Medium (Vector Laboratories). Images were acquired by slide scanner microscopy (AxioScan.Z1; Zeiss).

Nissl staining

Mice were perfused first with PBS and then with 4% paraformaldehyde by cardiac injection. Fixed brain tissue was isolated, post-fixed for 12 h at 4 °C, and dehydrated in 30% sucrose for 1–2 days. Thereafter, brain tissue was embedded in OCT (optimal cutting temperature) compound and stored at room temperature. Sections were then washed extensively, and mounted on glass slides with Vectashield Mounting Medium (Vector Laboratories). Images were acquired by slide scanner microscopy (AxioScan.Z1; Zeiss).

Fluorescent in situ hybridization (RNAscope assay)

Frozen sections (14 μm thick) were cut coronally through the hippocampal formation and thaw-mounted onto Superfrost Plus microscope slides (Advanced Cell Diagnostics). Sections were fixed in 4% formaldehyde for 10 min, dehydrated in increasing concentrations of ethanol for 5 min, air dried, and then pretreated with protease for 10 min at room temperature. For RNA detection, sections were incubated in different amplifier solutions in a HybEZ hybridization oven (Advanced Cell Diagnostics) at 40 °C. Three synthetic oligonucleotides complementary to the sequence corresponding to nucleotide residues 302-1210 of Mm–Clstn3–tv1, 896-1986 of Mm–CaMKIIα–C2, 18-407 of Mm–SST–C3, and 2-885 of Mm–Pvalb–C3 (Advanced Cell Diagnostics) were used as probes. The labeled probes were conjugated to Alexa Fluor 488, Allo 550, or Allo 647, after which labeled probe mixtures were hybridized by incubating with slide-mounted sections for 2 h at 40 °C. Non-specifically hybridized probes were removed by washing the sections three times for 2 min each with 1× wash buffer at room temperature, followed by incubation with Amplifier 1-FL for 30 min, Amplifier 2-FL for 15 min, Amplifier 3-FL for 30 min, and Amplifier 4 Alt B-FL for 15 min at 40 °C. Each amplifier was removed by washing with 1× wash buffer for 2 min at room temperature. Slides were imaged with an LSM700 microscope (Zeiss) and analyzed using MetaMorph software (Molecular Devices).

Stereotaxic injection of rAAVs

Adult (~9-week-old) male mice were anesthetized with Avertin (400 mg/kg body weight) by intraperitoneal injection. rAAV solutions (titers ≥1 × 10^11 viral genomes/ml) were injected with a NanoFil syringe (World Precision Instruments) at a flow rate of 0.1 μl/min. The coordinates used for the CA1 region of the dorsal hippocampus were AP −2.5 mm, ML ±1.5 mm, DV +1.5 mm (from the dura). The site at DV +1.5 mm received a 1-μl injection. Injected mice were allowed to recover for at least 14 days following surgery prior to use in experiments.

Electrophysiology

Electrophysiological recordings were performed in acute hippocampal CA1 slices. Mice were anesthetized with isoflurane and decapitated. Their brains were rapidly removed and placed in ice-cold, oxygenated (95% O2 and 5% CO2) low Ca2+/high Mg2+ solution containing the following: 3.3 mM KCl, 1.3 mM NaH2PO4, 26 mM NaHCO3, 11 mM d-glucose, 0.5 mM CaCl2, 10 mM MgCl2, and 211 mM sucrose. Slices were equilibrated at 30 °C for at least 60 min in oxygenated artificial cerebrospinal fluid, consisting of the following: 124 mM NaCl, 3.3 mM KCl, 1.3 mM NaH2PO4, 26 mM NaHCO3, 11 mM d-glucose, 3.125 mM CaCl2, and 2.25 mM MgCl2. Slices were then transferred to a recording chamber, where they were maintained at 24–27 °C and perfused continuously with 95% O2 and 5% CO2-bubbled artificial cerebrospinal fluid. Whole cell recordings of miniature postsynaptic currents were performed on CA1 pyramidal neurons voltage clamped at −70 mV. Glass pipettes were filled with an internal solution containing the following: 130 mM CeMeSO4, 0.5 mM EGTA, 5 mM tetraethylammonium-Cl, 8 mM NaCl, 10 mM HEPES, 1 mM QX-314, 4 mM Mg-ATP, 0.4 mM Na-GTP, and 10 mM phosphocreatine-Na2 for mEPSCs; 130 mM CsCl, 1.1 mM EGTA, 2 mM MgCl2, 0.1 mM CaCl2, 10 mM NaCl, 10 mM HEPES, and 2 mM Na-ATP for mIPSCs. The osmolarity of the internal solution was 290–300 mOsm (pH 7.3; adjusted with CsOH). For mEPSC recordings, 1 μM TTX, 50 μM DL-2-amino-5-phosphonopentanoic acid, and 100 μM picrotoxin were added to block Na+ currents, NMDA receptors, and GABA receptors, respectively. For mIPSC
recordings, 1 μM TTX, 20 μM 6-cyano-7-nitroquinoline-2,3-dione, 50 μM DL-2-amino-5-phosphonopentanoic acid were added to block Na⁺ currents, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and NMDA receptors, respectively.

Statistical analysis

All data are expressed as mean ± S.E., and significance is indicated with asterisk (compared with a value from control group) or hashtag (compared with a value from experimental group). N values are denoted in the individual figure legends. For cell surface-binding and heterologous synapse-formation assays, assays used for analysis were obtained from 5 to 10 images from at least three independent experiments. The normality of data distributions was evaluated using the Shapiro-Wilk test, followed by nonparametric Kruskal-Wallis test with Dunn's multiple comparison test for post hoc group comparisons or ANOVA with Tukey's test, using cell numbers or the number of experiments as the basis for n.

Data availability

All data except MS data are contained within this article and the supporting information. All raw MS and supporting data files from the current study have been deposited to the repository of MassIVE with identifier PXD018433.

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Abbreviations—The abbreviations used are: Nrxn, neurexin; AAV, adeno-associated virus; Cad, cadherin; Clstn3, calstntenin-3; GAD65, glutamic acid decarboxylase 65; GAD67, glutamic acid decarboxylase 67; LAR-RPTPs, leucokocyte common antigen-related receptor protein-tyrosine phosphatases; LNS, lamin, neurexin, sex-hormone binding globulin; LTP, long-term potentiation; LRRTM, leucine-rich repeat transmembrane neuronal protein; NL, neoligrin; NMDA, N-methyl-d-aspartate; SLAM, stratum lacunosum molecular; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radium; SS4, splice site 4; TKD, triple-knockdown; VGLUT1, vesicular glutamate transporter 1; PV, parvalbumin; SST, somatostatin; mIPSC, miniature inhibitory postsynaptic current; mEPSC, miniature excitatory postsynaptic current; aa, amino acid(s); HA, hemagglutinin; DAPI, 4′[6-diamidino-2-phenylindole]; EGFP, enhanced green fluorescent protein; TTX, tetrodotoxin; P30, postnatal day 30; ANOVA, analysis of variance.

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