nature neuroscience

Trans-synaptic adhesion between NGL-3 and LAR regulates the formation of excitatory synapses

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Synaptic adhesion molecules regulate multiple steps of synapse formation and maturation. The great diversity of neuronal synapses predicts the presence of a large number of adhesion molecules that control synapse formation through trans-synaptic and heterophilic adhesion. We identified a previously unknown trans-synaptic interaction between netrin-G ligand–3 (NGL-3), a postsynaptic density (PSD) 95–interacting postsynaptic adhesion molecule, and leukocyte common antigen-related (LAR), a receptor protein tyrosine phosphatase. NGL-3 and LAR expressed in heterologous cells induced pre- and postsynaptic differentiation in contacting axons and dendrites of cocultured rat hippocampal neurons, respectively. Neuronal overexpression of NGL-3 increased presynaptic contacts on dendrites of transfected neurons. Direct aggregation of NGL-3 on dendrites induced coclustering of excitatory postsynaptic proteins. Knockdown of NGL-3 reduced the number and function of excitatory synapses. Competitive inhibition by soluble LAR reduced NGL-3–induced presynaptic differentiation. These results suggest that the trans-synaptic adhesion between NGL-3 and LAR regulates excitatory synapse formation in a bidirectional manner.

Synaptogenesis involves a number of molecular processes, including axon-dendrite recognition, formation of nascent synapses and synapse maturation through recruitment of synaptic proteins. Synaptic cell adhesion molecules have been implicated in each of these processes[1–6]. Adhesion molecules capable of inducing early synaptic differentiation include neuroligin[7,8], neurexin[7,9], SynCAM[10,11], NGL[12,13] and EphB receptors[14].

The heterophilic and trans-synaptic adhesion between postsynaptic neuroligins and presynaptic neurexins is one of the most extensively studied synaptic adhesions[7]. Neuroligin and neurexin expressed in non-neural cells induce pre- and postsynaptic differentiation, respectively, in a bidirectional manner[8,9]. The interaction between neuroligin and neurexin is regulated by alternative splicing[15–17]. Neuroligin interacts with the postsynaptic scaffolding protein PSD-95 (ref. 18), which is thought to couple synaptic adhesion to postsynaptic protein clustering. Consistent with these important characteristics of the neuroligin-neurexin interaction, defects in neuroligin function are associated with human autism[19,20]. However, considering the great diversity of neuronal synapses, it is probable that additional cell adhesion molecule interactions that regulate synapse formation and functions remain to be discovered.

Recent studies have identified the NGL family of PSD-95–interacting postsynaptic adhesion molecules, which contains three known members, NGL-1, NGL-2 and NGL-3 (refs. 12,13). NGL associates with netrin-G/laminet[12], a family of glycosylphosphatidylinositol (GPI)-anchored adhesion molecules[21–23], in an isoform-specific manner; NGL-1 and NGL-2 associate with netrin-G1 and netrin-G2, respectively[12,13]. In transgenic mice with netrin-G1 deficiency, NGL-1, but not NGL-2, shows a diffuse dendritic distribution; likewise, NGL-2 is selectively dispersed in netrin-G2–deficient mice[24], suggesting that these interactions regulate axon-dependent localization of NGL-1 and NGL-2 in specific segments of dendrites. In support of the role for NGLs in synapse formation, NGL-2 induces presynaptic differentiation in contacting axons when expressed in non-neural cells[13]. Netrin-Gs show extensive alternative splicing[21–23], which may regulate the netrin-G–NGL-1/2 interaction. Mice that are deficient in netrin-G2 or NGL-2 show abnormal auditory responses[25]. Single-nucleotide polymorphism analysis associated both netrin-G1 and netrin-G2 with schizophrenia[26]. In contrast with the increasing studies of NGL-1/2 and their ligands, the third member of the NGL family (NGL-3), which does not bind netrin-G1 or netrin-G2, has remained an orphan receptor.

LAR is well-known for its involvement in axon guidance and presynaptic differentiation[27,28]. LAR contains three Ig-like domains and eight fibronectin type III domains in the extracellular region and two phosphatase domains in the intracellular region. The membrane-proximal phosphatase domain is catalytically active, whereas the membrane-distal phosphatase domain is inactive and associates with various cytoplasmic proteins, including liprin-α[29] and β-catenin[30]. *C. elegans* and *Drosophila* homologs of LAR are involved in the regulation of synaptic growth and active zone assembly at the neuromuscular junction (NMJ)[31–33]. Mammalian LAR has been shown to regulate excitatory synaptic development and maintenance[34].

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Received 23 December 2008; accepted 22 January 2009; published online 1 March 2009; doi:10.1038/nn.2279

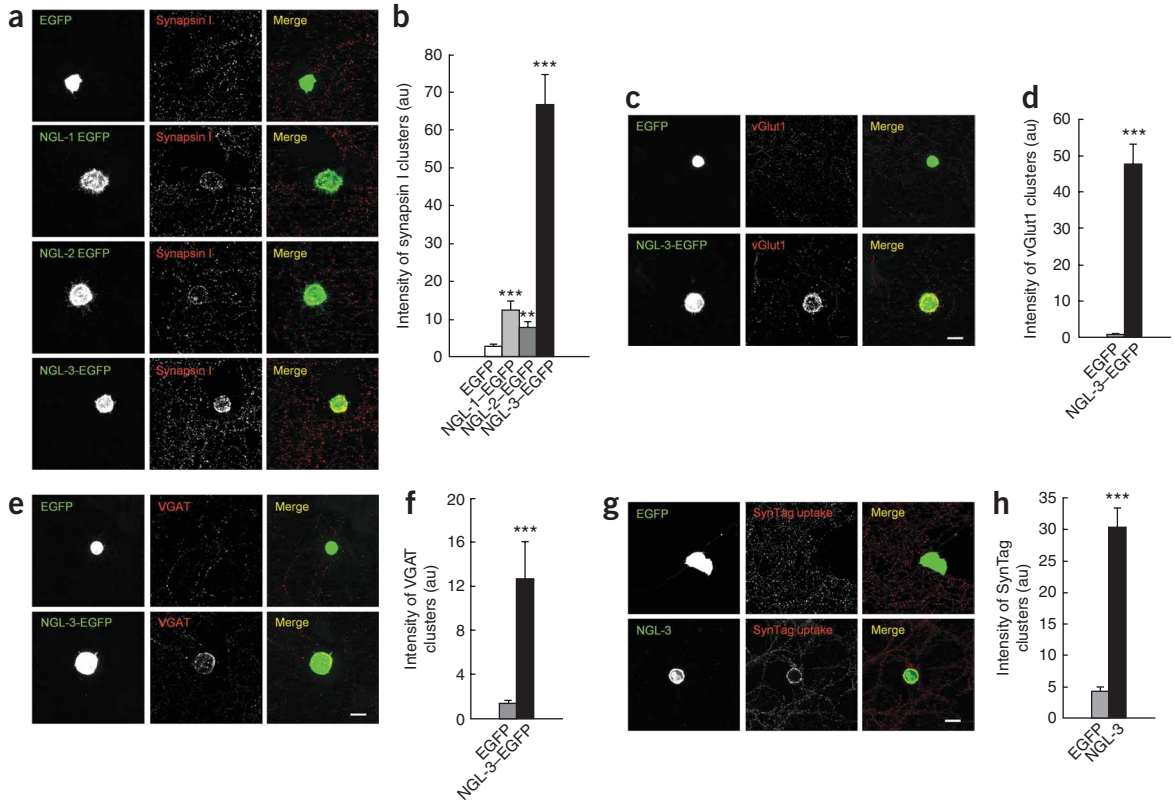

Figure 1 NGL-3 expressed in non-neural cells induces functional presynaptic differentiation in contacting axons. (**a**) Induction of presynaptic differentiation by NGL family proteins. HEK293T cells expressing EGFP alone, NGL-1–EGFP (C-terminally tagged), NGL-2–EGFP or NGL-3–EGFP were cocultured with hippocampal neurons (10–13 DIV) and stained for synapsin I. (**b**) Quantification of the integrated intensity of synapsin I clusters induced by NGLs (mean ± s.e.m.; $n = 32$ for EGFP, 29 for NGL-1–EGFP, 32 for NGL-2–EGFP and 29 for NGL-3–EGFP; ** $P < 0.01$, *** $P < 0.001$, Student's t test; au, arbitrary units). (**c**,**d**) NGL-3 induced clustering of vGlut1, an excitatory presynaptic marker. HEK293T cells expressing NGL-3–EGFP or EGFP alone were cocultured with neurons (10–13 DIV) and stained for vGlut1 (mean ± s.e.m.; $n = 34$ for EGFP and 37 for NGL-3, ***$P < 0.001$, Student's t test). (**e**,**f**) NGL-3 induced clustering of VGAT, an inhibitory presynaptic marker. Cocultured cells prepared as in **c** and **d** were stained for VGAT (mean ± s.e.m.; $n = 31$ for EGFP and 33 for NGL-3, ***$P < 0.001$, Student's t test). (**g**,**h**) NGL-3 induced functional presynaptic differentiation. Cocultured cells were incubated with antibodies to synaptotagmin I (SynTag) luminal domain to visualize functional presynaptic nerve terminals (mean ± s.e.m.; $n = 27$ for EGFP and NGL-3, ***$P < 0.001$, Student's t test). Scale bars represent 20 μm.

Several extracellular ligands for LAR have been identified. LAR associates with the laminin-nidogen complex, a major component of the extracellular matrix, and regulates extracellular matrix–dependent morphological changes in non-neural cells[35]. In *Drosophila*, LAR associates with the heparan sulfate proteoglycans Syndecan and Dallylike at the NMJ[36,37]. Syndecan is a transmembrane protein that regulates LAR-dependent presynaptic growth and Dallylike is a GPI-anchored protein that inhibits LAR's ability to regulate active zone size at the NMJ[36,37]. LAR associates with a small (~11 kDa) ectodomain isoform of LAR, LARFN5C, in a homophilic manner to promote neurite outgrowth in mouse hippocampal neurons[38]. However, transmembrane ligands of LAR that adhere to LAR in a trans-synaptic manner at interneuronal synapses have not been identified in either invertebrates or vertebrates.

Here, we identified a previously unknown interaction between NGL-3 and LAR. NGL-3 and LAR induced pre- and postsynaptic differentiation in contacting axons and dendrites, respectively, when they were expressed in non-neural cells. Data from direct aggregation, knockdown and competitive inhibition experiments suggest that the trans-synaptic adhesion between NGL-3 and LAR regulates excitatory synapse formation.

RESULTS

NGL-3 in HEK293 cells induces presynaptic differentiation

The synaptogenic activity of an adhesion molecule can be studied in a mixed-culture or coculture assay in which an adhesion molecule expressed in non-neural cells is tested for its ability to induce presynaptic differentiation in contacting axons of cocultured neurons[8,39]. Accordingly, we used this mixed-culture assay to test for possible NGL-3 synaptogenic activity. NGL-3–expressing HEK293T cells that were cocultured with hippocampal neurons induced a strong clustering of synapsin I, a presynaptic vesicle marker, in contacting axons, whereas control cells expressing enhanced green fluorescent protein (EGFP) alone induced minimal synapsin I clustering (**Fig. 1a**,**b**). The extent of NGL-3–induced synapsin I clustering was much greater than that induced by NGL-1 or NGL-2 (**Fig. 1a**,**b**). This difference was not attributable to differential expression of the three NGL isoforms because all three were expressed on the surface of HEK293T cells at similar levels (**Supplementary Fig. 1** online).

NGL-3–expressing HEK293T cells also induced the clustering of both vGlut1 and VGAT, which are excitatory and inhibitory presynaptic markers, respectively (**Fig. 1c–f**). NGL-3 was more efficient in inducing vGlut1 clustering than VGAT clustering; the extent of vGlut1 clustering relative to the EGFP control was about sixfold greater than that of

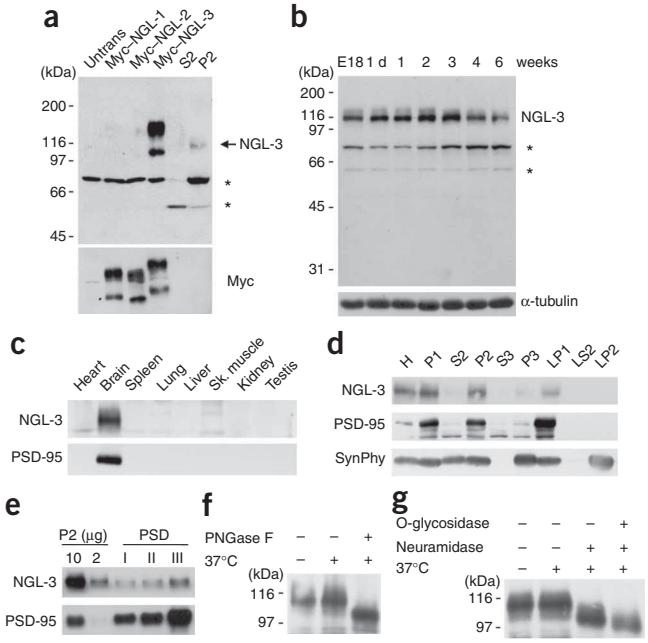

Figure 2 Expression patterns of NGL-3 proteins in rat brain. (**a**) NGL-3 antibodies selectively recognized NGL-3, but not NGL-1 or NGL-2 (lanes 1–4), and detected a 115-kDa band (arrow) in brain samples. The immunoblot was also probed with antibody to Myc for normalization. P2, crude synaptosomes; S2, supernatant after P2 precipitation. Asterisks indicate nonspecific bands. (**b**) NGL-3 protein expression gradually increased during the first 3 weeks of postnatal rat brain development. Whole rat brain extracts from different developmental stages were used. α-tubulin was visualized for normalization. Asterisks indicate nonspecific bands. (**c**) NGL-3 proteins were mainly expressed in the brain. PSD-95 was visualized for comparison. Sk. muscle, skeletal muscle. (**d**) Distribution of NGL-3 in subcellular fractions of adult rat brain. Note that NGL-3 proteins were mainly detected in synaptic fractions, including P2 and LP1. PSD-95 and synaptophysin (SynPhy) were probed for comparison. H, homogenates; LP1, synaptosomal membranes; LP2, synaptic vesicle-enriched fraction; LS2, synaptosomal cytosol; P1, crude nuclear fraction; P3, light membranes; S3, cytosol. (**e**) NGL-3 was detected in PSD fractions of rat brain (3 weeks), with a strong enrichment in the PSD III fraction. (**f**) N-glycosylation of NGL-3. The crude synaptosomal fraction of adult rat brain was subjected to PNGase F digestion, followed by immunoblot assay. (**g**) O-glycosylation and sialylation of NGL-3, evidenced by O-glycosidase and neuramidase digestion.

VGAT clustering (**Fig. 1d**,**f**). NGL-3 did not induce clustering of PSD-95 or gephyrin, which are excitatory and inhibitory postsynaptic markers, respectively (**Supplementary Fig. 2** online), indicating that the NGL-3–dependent presynaptic protein clustering was not induced by interneural synapses. In contacting axons of cocultured neurons, NGL-3 induced the uptake of antibodies that were directed against the luminal domain of the synaptic vesicle protein synaptotagmin I, which is present during the recycling of presynaptic vesicles (**Fig. 1g**,**h**), thus indicating that NGL-3 induces functional presynaptic differentiation. These results suggest that NGL-3 can induce functional presynaptic differentiation at both excitatory and inhibitory synapses.

Expression patterns of NGL-3 proteins

Previous northern blot and *in situ* hybridization results have shown that NGL-3 mRNAs are mainly expressed in the brain and are widespread in various brain regions[13]. The expression patterns of NGL proteins have been studied using an antibody that recognizes all three NGL isoforms (pan-NGL)[13]. To study the specific expression patterns of NGL-3 proteins, we generated an NGL-3 antibody that does not cross-react with NGL-1 or NGL-2 (**Fig. 2a**). The NGL-3 antibody detected a single band (~115 kDa) in rat brain (**Fig. 2a**). The apparent molecular weight (115 kDa) of NGL-3, which falls into the upper end of the size range of the three NGL proteins (95–115 kDa)[13], is consistent with the longer length of NGL-3 (709 amino acids in rat) relative to NGL-1 (640 amino acids) and NGL-2 (652 amino acids). NGL-3 expressed in heterologous cells showed two bands (~140 and 100 kDa), which differed from the size of NGL-3 expressed in the brain. This may reflect differential postsynaptic modification (that is, glycosylation) because NGL-3 proteins expressed in heterologous cells (140 and 100 kDa) showed higher and lower levels of glycosylation, respectively, compared with NGL-3 in the brain (**Supplementary Fig. 3** online).

Expression of NGL-3 proteins was gradually increased during the first 3 weeks of postnatal rat brain development (**Fig. 2b**) and mainly detected in the brain, but not in other tissues (**Fig. 2c**). NGL-3 was mainly detected in synaptic fractions, including the crude synaptosomal and synaptic plasma membrane fractions (**Fig. 2d**). NGL-3 was detected in PSD fractions, including the most detergent-resistant PSD III fraction (**Fig. 2e**), indicative of a tight association of NGL-3 with the PSD. PNGase F, O-glycosidase and neuramidase reduced the apparent molecular weight of NGL-3, indicating that NGL-3 is N- and O-glycosylated and sialylated (**Fig. 2f**,**g**).

Neuronal NGL-3 overexpression induces presynaptic contacts

We next tested the effects of NGL-3 overexpression in cultured neurons. NGL-3 overexpression in cultured hippocampal neurons substantially increased presynaptic contacts, as measured by the integrated intensity of synapsin I clusters on dendrites (**Fig. 3a**,**b**). NGL-3 also induced a strong increase in excitatory presynaptic contacts, as measured by vGlut1 clusters (**Fig. 3c**,**d**). In contrast, inhibitory presynaptic contacts (VGAT clusters) were not induced by NGL-3 overexpression (**Fig. 3e**,**f**). These results suggest that NGL-3 expression in neurons selectively induces excitatory, but not inhibitory, presynaptic differentiation in contact axons, which contrasts with our findings that both excitatory and inhibitory presynaptic differentiation are induced by NGL-3 in the mixed-culture experiments (**Fig. 1**).

However, the number of excitatory synapses, defined as vGlut1-positive PSD-95 clusters, was reduced by NGL-3 overexpression, mainly as the result of a decrease in the number of PSD-95 clusters (**Supplementary Fig. 4** online). It is possible that NGL-3 overexpression causes the dispersal of NGL-3–associated proteins, including PSD-95, to extrasynaptic sites in a dominant-negative manner, which have been observed in neurons overexpressing neuroligin-2 and SALM2 (refs. 9,40).

NGL-3 aggregation clusters postsynaptic proteins

Because NGL-3 has the ability to induce presynaptic differentiation, we reasoned that it may also promote postsynaptic differentiation by recruiting various postsynaptic proteins. To test this hypothesis, we expressed N-terminally EGFP-tagged NGL-3 in cultured hippocampal neurons and induced direct clustering of EGFP–NGL-3 on the dendritic surface by incubating the neurons with beads coated with antibodies to EGFP. Direct NGL-3 aggregation induced secondary clustering of postsynaptic proteins, including PSD-95, GKAP (a postsynaptic scaffold), Shank (a postsynaptic scaffold), GluR2 (an AMPA receptor subunit) and NR1 (an NMDA receptor subunit), but not gephyrin (**Fig. 4**). Direct aggregation of a control membrane protein containing EGFP alone in the extracellular region (EGFP-pDis) did not

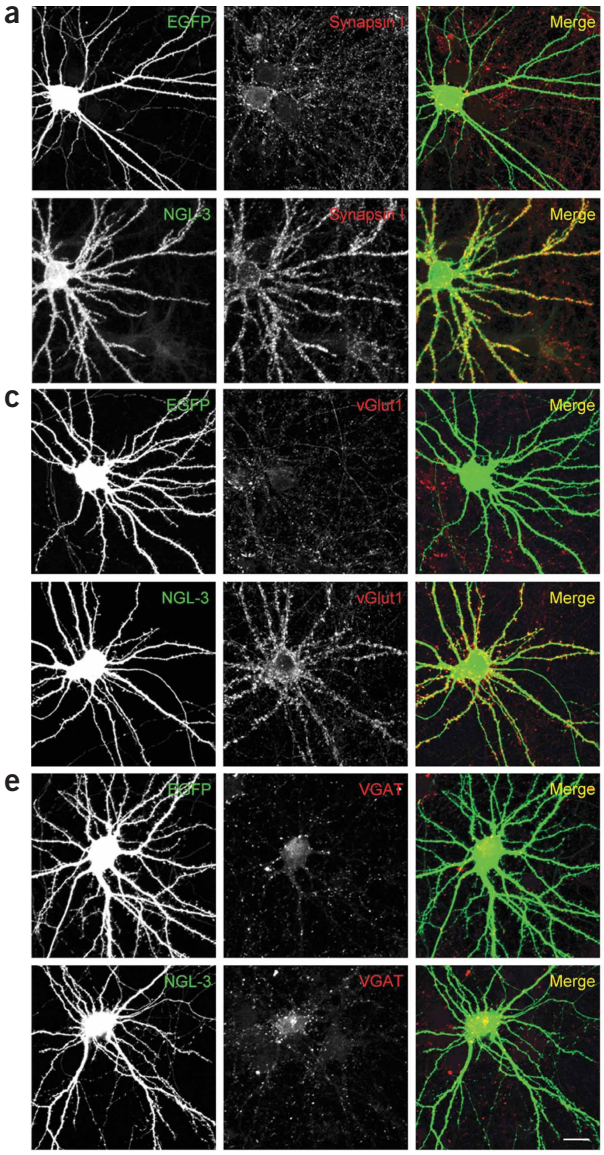

Figure 3 Overexpression of NGL-3 in cultured neurons increases excitatory, but not inhibitory, presynaptic contacts on dendrites of transfected neurons. (**a**–**f**) Cultured hippocampal neurons were transfected with NGL-3 and EGFP or EGFP alone (12–15 DIV) and immunostained for synapsin I (**a**), vGlut1 (**c**), VGAT (**e**) or EGFP (**a**,**c**,**e**). For quantification, integrated fluorescence intensities of presynaptic protein clusters along the dendrites were normalized to the dendrite area (synapsin I: $n = 10$ for EGFP and 11 for NGL-3, $^{**}P < 0.01$, Student's t test, **b**; vGlut1: $n = 12$ for EGFP and 10 for NGL-3, $^{***}P < 0.001$, **d**; VGAT: $n = 9$ for EGFP and 10 for NGL-3, $P = 0.3$, **f**). Data are presented as mean ± s.e.m. Scale bar represents 20 μm.

induce PSD-95 coclustering (**Supplementary Fig. 5** online). A quantitative analysis showed that a large fraction of NGL-3 clusters colocalized with excitatory postsynaptic protein clusters, but not with gephyrin clusters (95.8 ± 2.7%, $n = 41$ for PSD-95; 0.0%, $n = 38$ for gephyrin; 94.6 ± 2.6%, $n = 45$ for GKAP; 89.6 ± 5.4%, $n = 25$ for Shank; 83.9 ± 6.8%, $n = 30$ for GluR2; 98.5 ± 4.5%, $n = 44$ for NR1; 0.0%, $n = 33$ for PSD-95 by EGFP-pDis). In addition, the fluorescent intensities of the colocalized proteins normalized to nearby dendrites were 2.36 ± 0.18 for PSD-95 ($n = 46$), 0.93 ± 0.04 for gephyrin ($n = 35$), 2.06 ± 0.07 for GKAP ($n = 47$), 3.26 ± 0.27 for Shank ($n = 24$), 1.64 ± 0.09 for GluR2 ($n = 44$), 2.42 ± 0.10 for NR1 ($n = 40$) and 0.95 ± 0.99 for PSD-95 by EGFP-pDis ($n = 33$). Postsynaptic protein clusters induced by NGL-3 aggregation were negative for synapsin I or synaptophysin (**Fig. 4**), indicating that the postsynaptic protein clusters were not induced by interneuronal synapses. These results suggest that NGL-3 clustering on dendrites induces excitatory, but not inhibitory, postsynaptic differentiation.

In additional experiments, we induced dendritic NGL-3 clustering by preclustered EGFP antibodies, instead of by EGFP antibody–coated beads. This primary NGL-3 clustering on dendrites induced coclustering of excitatory postsynaptic proteins (**Supplementary Fig. 6** online), similar to our results with bead-induced protein clustering (**Fig. 4**). In quantitative analysis, a large fraction of NGL-3 clusters colocalized with excitatory postsynaptic protein clusters, but minimally colocalized with gephyrin clusters (**Supplementary Fig. 6**). In addition, the fluorescence intensities of the colocalized excitatory postsynaptic protein clusters, normalized to nearby dendrites, were greater than that of gephyrin (**Supplementary Fig. 6**). Collectively, these results suggest that NGL-3 is sufficient to induce dendritic clustering of excitatory, but not inhibitory, postsynaptic proteins.

NGL-3 knockdown reduces excitatory synapse number

We next tested whether knockdown of NGL-3 in neurons would lead to a loss of excitatory synapses by RNA interference. We generated two independent shRNA constructs for NGL-3 knockdown (shNGL-3 #1 and shNGL-3 #2), which reduced NGL-3 expression in HEK293T cells by 61% and 46%, respectively (**Supplementary Fig. 7** online). In hippocampal neurons, shNGL-3 #1 and shNGL-3 #2 also reduced the expression of exogenous NGL-3 by 53% and 69%, respectively (**Supplementary Fig. 7**).

Knockdown of endogenous NGL-3 expression in cultured neurons could not be tested because we lacked NGL-3 antibodies that are suitable for immunostaining. In cultured hippocampal neurons, NGL-3 knockdown by the two shNGL-3 constructs reduced the number of excitatory synapses, defined as vGlut1-positive PSD-95 clusters, compared with control neurons transfected with empty shRNA vector (sh-vec) (**Fig. 5a**,**b**). In contrast, NGL-3 knockdown did not affect the number of inhibitory synapses, defined as VGAT-positive gephyrin clusters (**Fig. 5c**,**d**). A variant of shNGL-3 #1 with point mutations that do not induce NGL-3 knockdown in heterologous cells and neurons (shNGL-3 #1*; **Supplementary Fig. 7**) did not reduce excitatory synapse number relative to sh-vec (**Fig. 5a**,**b**). The reduction in excitatory synapse number by shNGL-3 #1 could be rescued by coexpression of a NGL-3 expression construct that is resistant to shNGL-3 #1 (NGL-3*) (**Fig. 5a**,**b** and **Supplementary Fig. 7**), further supporting the specific action of shNGL-3 #1. Functionally, NGL-3 knockdown reduced the frequency, but not the

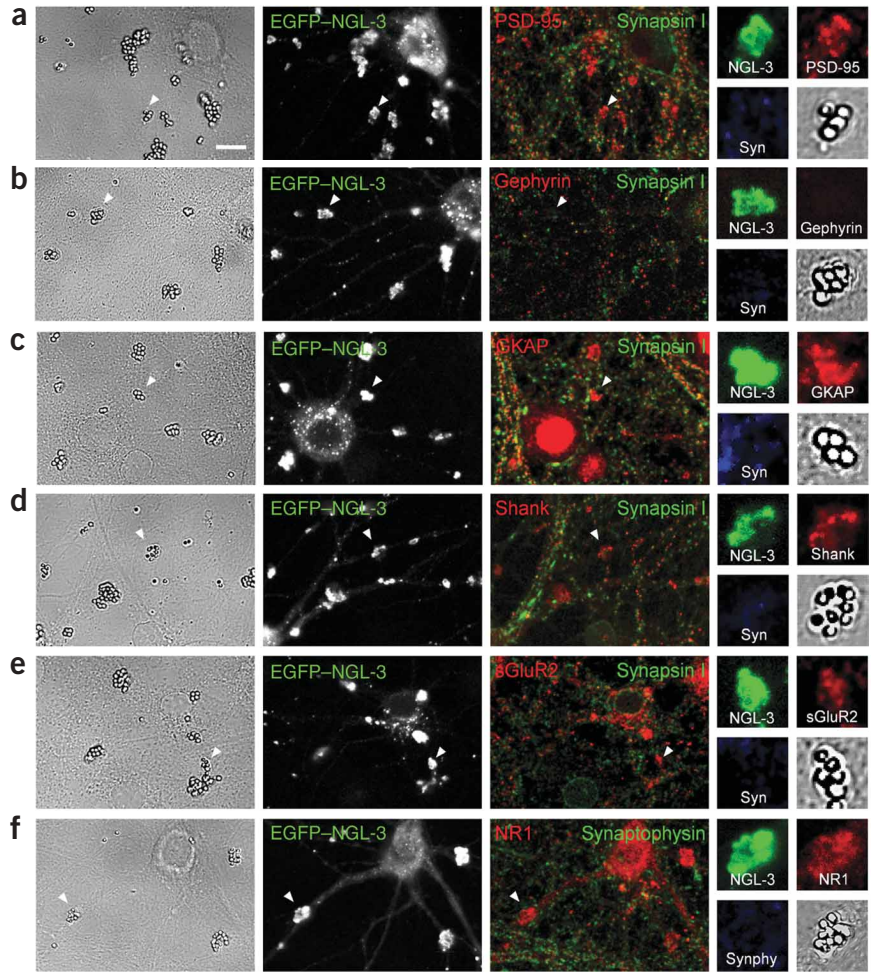

Figure 4 Direct aggregation of NGL-3 on the surface of dendrites induces coclustering of excitatory postsynaptic proteins. (**a**–**f**) Cultured hippocampal neurons expressing N-terminally EGFP-tagged NGL-3 (14–16 DIV) were incubated with EGFP-coated beads and visualized at 17 DIV by triple immunofluorescence staining for EGFP (NGL-3), synapsin I (**a**–**e**) or synaptophysin (**f**) and the indicated postsynaptic proteins. sGluR2, surface GluR2. Arrowheads indicate enlarged beads and protein clusters. Scale bar represents 10 μm.

amplitude, of miniature excitatory postsynaptic currents (mEPSCs) to a greater extent than the morphological reduction (**Fig. 5e–g**). These results suggest that NGL-3 is required for the morphological and functional maintenance of excitatory synapses.

NGL-3 interacts with LAR

NGL-3 probably induces presynaptic differentiation in contacting axons by interacting with a presynaptic ligand. To identify a specific ligand for NGL-3, we systematically screened NGL-3–expressing L cells for their ability to coaggregate with a panel of L cells expressing specific synaptic membrane proteins (examples in **Supplementary Fig. 8** online). This led us to the identification of LAR as a specific ligand for NGL-3.

In additional experiments, LAR-expressing cells selectively aggregated with NGL-3–expressing cells, but not with those expressing NGL-1 or NGL-2 (**Fig. 6a**,**b**). Removal of extracellular calcium did not reduce the aggregation between NGL-3– and LAR-expressing cells (**Fig. 6c**,**d**). There was no homophilic adhesion between cells expressing NGL-3 or LAR (**Supplementary Fig. 8**). To further confirm the adhesion of NGL-3 and LAR, we incubated HEK293T cells expressing the three NGL isoforms with LAR-ecto-Fc, a soluble LAR protein formed by fusion of the ectodomain of LAR with the immunoglobulin Fc domain. LAR-ecto-Fc selectively bound to NGL-3, but not to NGL-1 or NGL-2 (**Fig. 6e**). These results suggest that LAR selectively interacts with NGL-3 in a calcium-independent manner and that this interaction can mediate cell adhesion.

Deletion of the leucine-rich repeat (LRR) domain in the extracellular region of NGL-3 (NGL-3ΔLRR), but not the Ig domain (NGL-3ΔIg), abolished the interaction between NGL-3 and LAR in the cell adhesion and soluble LAR binding assays (**Supplementary Fig. 9** online). In addition, NGL-3ΔLRR, but not NGL-3ΔIg, failed to induce presynaptic differentiation in contacting axons of cocultured neurons when expressed in heterologous cells (**Supplementary Fig. 9**). Surface expression levels of these NGL-3 variants were comparable, as determined by biotinylation assays (wild type, 22.0 ± 3.0; ΔLRR, 20.1 ± 2.6; ΔIg, 24.3 ± 1.5 arbitrary units). These results indicate that the LRR domain of NGL-3 is important for the NGL-3–LAR interaction.

We further tested the interaction between NGL-3 and LAR in a mixed-culture assay, in which NGL-3–expressing HEK293T cells were cocultured with neurons expressing exogenous LAR. NGL-3–expressing HEK293T cells induced a strong aggregation of Flag-tagged LAR (LAR-Flag) in contacting axons, whereas EGFP-expressing HEK293T cells (control) induced minimal LAR clustering (average fluorescence intensities of LAR clusters normalized to the HEK293T cell area: NGL-3 induced, 24.22 ± 5.98, $n = 12$; EGFP induced, 6.34 ± 0.95; $n = 14$; $P < 0.005$, Student's t test; **Fig. 6f**). Conversely, HEK293T cells expressing LAR, but not EGFP, induced clustering of Flag-tagged NGL-3 (NGL-3–Flag) in contacting dendrites (average fluorescence intensities of NGL-3 clusters: LAR induced, 62.27 ± 9.48, $n = 11$; EGFP induced, 21.37 ± 2.47, $n = 11$; $P < 0.001$, Student's t test; **Fig. 6g**). These results suggest that the interaction between NGL-3 and LAR may occur in a trans-synaptic manner.

We next determined the binding affinity for the interaction between NGL-3 and LAR. NGL-3–expressing HEK293T cells were incubated with increasing amounts of LAR-ecto-Fc and proteins bound to the cell surface were quantified by ELISA assays. The K_d value for the NGL-3–LAR interaction that we calculated by Scatchard analysis was 37.4 ± 2.1 nM (**Fig. 6h**).

Soluble LAR inhibits NGL-3–dependent presynaptic induction

If NGL-3–induced presynaptic differentiation in the mixed-culture assay is dependent on the interaction of NGL-3 with LAR expressed on the surface of axons, exogenously added soluble LAR fusion proteins (LAR-ecto-Fc), which compete with endogenous LAR for NGL-3 binding, should inhibit the NGL-3–induced presynaptic differentiation. Indeed, LAR-ecto-Fc reduced NGL-3–induced synapsin I

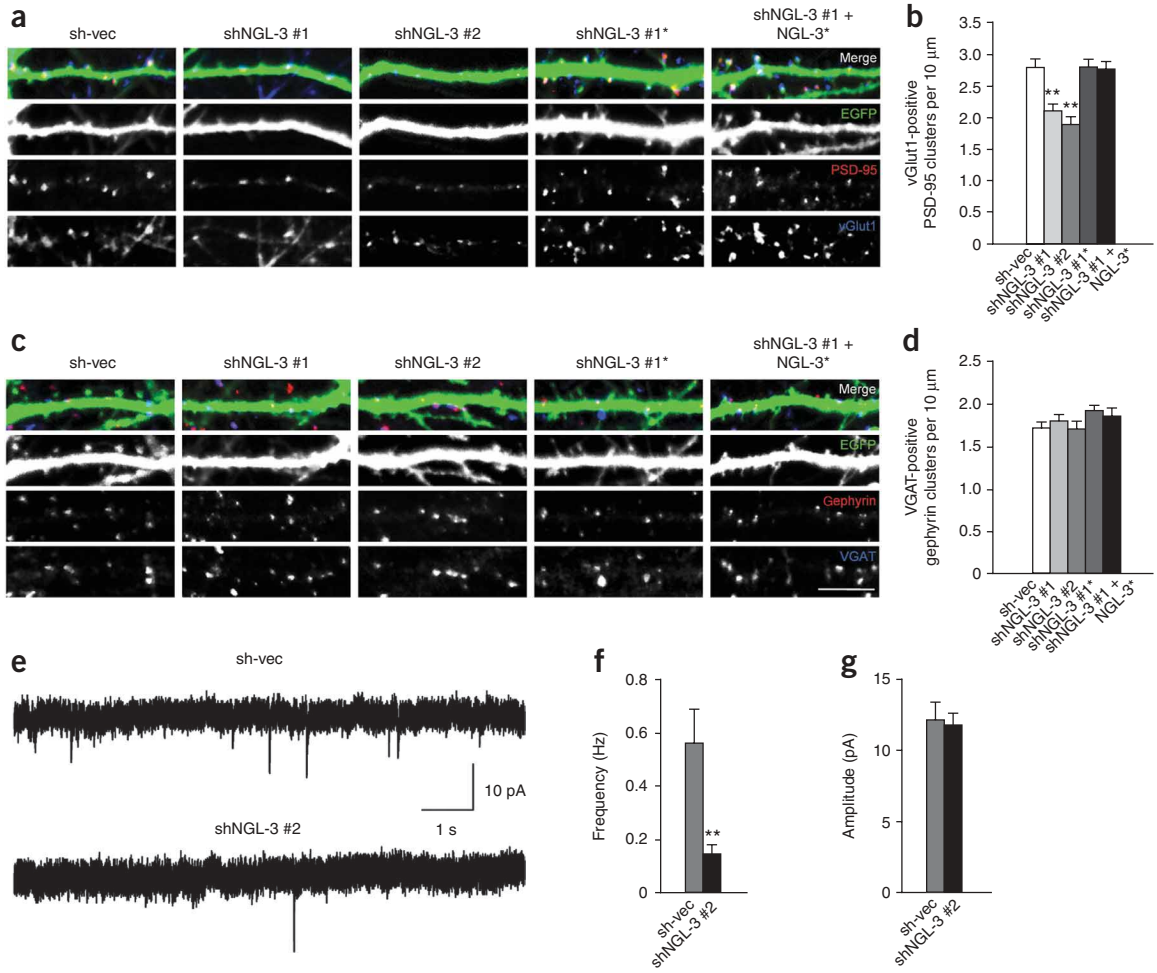

Figure 5 Knockdown of NGL-3 leads to decreases in the number and function of excitatory synapses. (**a**–**d**) NGL-3 knockdown reduced the number of excitatory synapses (vGlut1-positive PSD-95 clusters) without affecting inhibitory synapses (VGAT-positive gephyrin clusters). Cultured hippocampal neurons transfected with sh-vec, shNGL-3 #1 and #2, shNGL-3 #1* or shNGL-3 #1 + NGL-3* (10–14 DIV) were immunostained with the indicated antibodies (data are presented as mean ± s.e.m.; excitatory synapses: $n = 21$ for sh-vec, 22 for shNGL-3 #1, 19 for shNGL-3 #2, 17 for shNGL-3 #1* and 22 for shNGL-3 #1 + NGL-3*, $^*P < 0.005$, ANOVA; inhibitory synapses: $n = 20$ for sh-vec, 21 for shNGL-3 #1, 21 for shNGL-3 #2, 19 for shNGL-3 #1* and 20 for shNGL-3 #1 + NGL-3*). Scale bar represents 10 µm. (**e**–**g**) NGL-3 knockdown in cultured neurons (13–16 DIV) reduced the frequency (**f**), but not amplitude (**g**), of mEPSCs ($n = 12$ for sh-vec and 10 for shNGL-3 #2, $^{**}P < 0.01$, Student's t test).

clustering in contacting axons of cocultured neurons compared with Fc alone (control) (**Fig. 7a**,**b**).

However, when we added LAR-ecto-Fc to cultured neurons, it did not reduce the number of excitatory synapses (synapsin I–positive PSD-95 clusters; **Supplementary Fig. 10** online). It is possible that other trans-synaptic adhesions unaffected by LAR may be maintaining normal excitatory synapses when the LAR–NGL-3 interaction is inhibited, whereas the synapses formed in the mixed-culture assay, which rely on the LAR–NGL-3 interaction, but not other synaptic adhesions, may be more easily inhibited. These results suggest that the adhesion between NGL-3 and LAR is required for NGL-3–induced presynaptic differentiation.

LAR in HEK293T cells induces postsynaptic differentiation

If the trans-synaptic interaction between NGL-3 and LAR is involved in the formation of excitatory synapses, presynaptic LAR may also induce postsynaptic differentiation in contacting dendrites. Indeed, in a mixed-culture assay, LAR-expressing HEK293T cells induced the clustering of excitatory postsynaptic proteins PSD-95 and Shank, whereas EGFP alone induced minimal postsynaptic protein clustering (**Fig. 8a–d**). In addition, LAR expressed in HEK 293T cells did not induce gephyrin clusters in contacting dendrites (**Fig. 8e**,**f**). These results suggest that presynaptic LAR induces excitatory, but not inhibitory, postsynaptic protein clustering on contacting dendrites.

LAR regulates excitatory synaptic development and maintenance partly through its association with AMPA receptors[34], suggesting that LAR is also present on the postsynaptic plasma membrane. We therefore tested whether LAR expressed in HEK293T cells is capable of inducing presynaptic differentiation in contacting axons. LAR-expressing HEK293T cells did not induce synapsin I clustering in contacting axons (**Fig. 8g**,**h**). This suggests that postsynaptic LAR has a different role, which is unrelated to the induction of presynaptic differentiation.

DISCUSSION

Here, we identified a previously unknown trans-synaptic adhesion between NGL-3 and LAR. In support of the synaptogenic function of this interaction, NGL-3 and LAR expressed in non-neural cells induced pre- and postsynaptic differentiation in contacting axons and

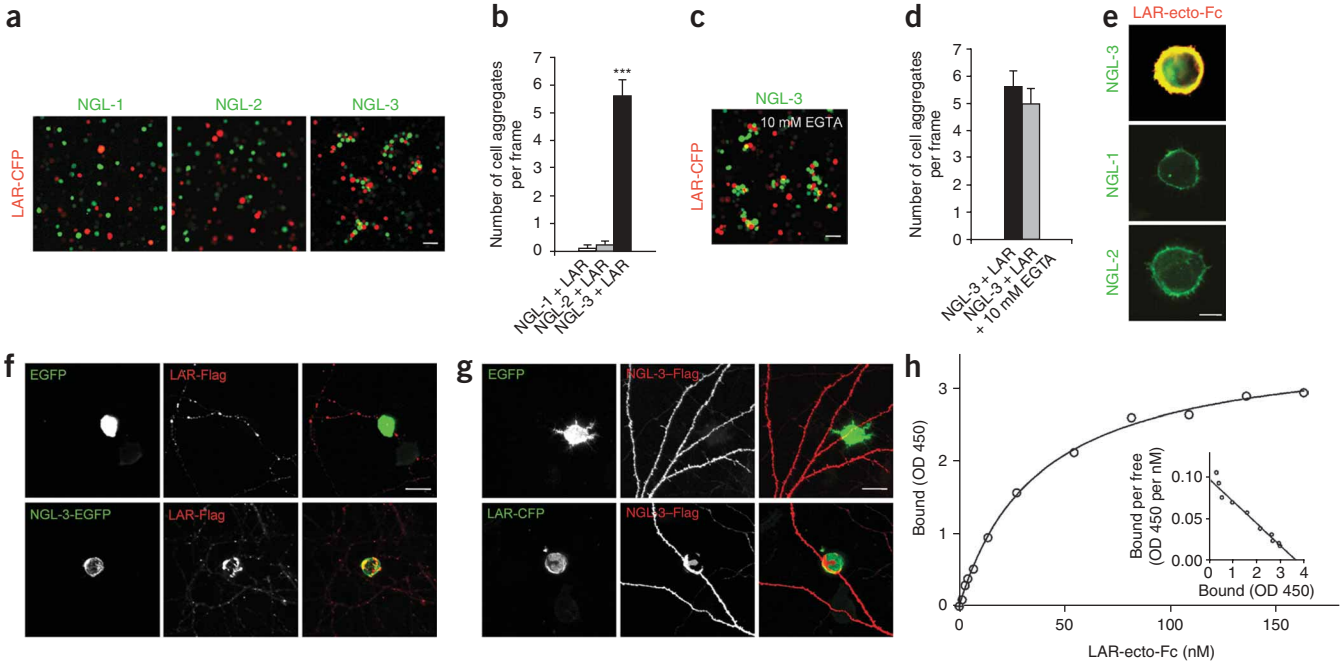

Figure 6 NGL-3 interacts with LAR. (**a**) NGL-3, but not NGL-1 or NGL-2, interacted with LAR in cell-aggregation assays. L cells cotransfected with NGLs and EGFP or LAR (C-terminally cyan fluorescent protein (CFP)-tagged) and dsRed were mixed to induce cell aggregation. Scale bar represents 50 μm. (**b**) Quantification of the cell aggregation in **a**. Cell aggregates were defined by four or more clustered cells containing at least one red or green cell (mean ± s.e.m., $n = 10$, $^{***}P < 0.001$, ANOVA). (**c**,**d**) Calcium-independent adhesion between NGL-3 and LAR. L cells expressing NGL-3 + EGFP or LAR-CFP + dsRed were mixed in the presence or absence of 10 mM EGTA (mean ± s.e.m., $n = 10$, $^{***}P < 0.001$, Student's t test). Scale bar represents 50 μm. (**e**) Selective association of LAR-ecto-Fc with NGL-3, but not with NGL-1 and NGL-2 expressed in HEK293T cells. Scale bar represents 10 μm. (**f**) NGL-3–expressing HEK293T cells induced LAR-Flag clustering on contacting axons of cocultured neurons. HEK293T cells expressing NGL-3–EGFP or EGFP alone were cocultured (14–15 DIV) with hippocampal neurons expressing LAR-Flag C1522S (C-terminally Flag-tagged, 13–14 DIV), followed by EGFP and Flag staining. The phosphatase-dead LAR C1522S mutant was used to minimize possible effects of phosphatase activity on transfected neurons. (**g**) LAR-expressing HEK293T cells induce NGL-3–Flag clustering on contacting dendrites of cocultured neurons. HEK293T cells expressing LAR-CFP or EGFP alone were cocultured (14–15 DIV) with hippocampal neurons expressing NGL-3–Flag (13–14 DIV). Scale bar represents 20 μm. (**h**) Saturation curve of LAR-ecto-Fc binding to NGL-3 expressed in HEK293T cells. A Scatchard plot analyzed by linear regression of the data is shown in the inset. The calculated K_d for the interaction was 37.4 ± 2.1 nM.

dendrites, respectively. Neuronal NGL-3 expression increased presynaptic contacts. Dendritic NGL-3 aggregation induced excitatory postsynaptic protein clustering. NGL-3 knockdown reduced excitatory synapse number and function. Soluble LAR inhibited NGL-3–induced presynaptic differentiation. These results suggest that the trans-synaptic adhesion between NGL-3 and LAR regulates excitatory synapse formation in a bidirectional manner.

We were unable to determine the ultrastructural synaptic localization of NGL-3 because of the lack of suitable NGL-3 antibodies. However, our previous electron microscopy results, obtained using a pan-NGL antibody, indicate that NGL isoforms are mainly postsynaptically localized at excitatory synapses[13]. The presynaptic localization of LAR in *C. elegans* and *Drosophila* has been well characterized[28,32,33,36], although the pre- or postsynaptic localization of mammalian LAR remains to be determined[34]. On the basis of the widespread distribution of both mRNAs, determined by *in situ* hybridization[13,41], the adhesion between NGL-3 and LAR probably occurs in various brain regions. Notably, NGL-3 proteins are mainly expressed in the brain (**Fig. 2b**), whereas LAR mRNAs, determined by Northern analysis, are expressed in various tissues[42], suggesting that the interaction between NGL-3 and LAR occurs primarily in the brain.

The three NGL isoforms share an identical domain structure and interact with PSD-95 through their extreme C termini[12,13]. However, their primary sequences are substantially different (especially in their

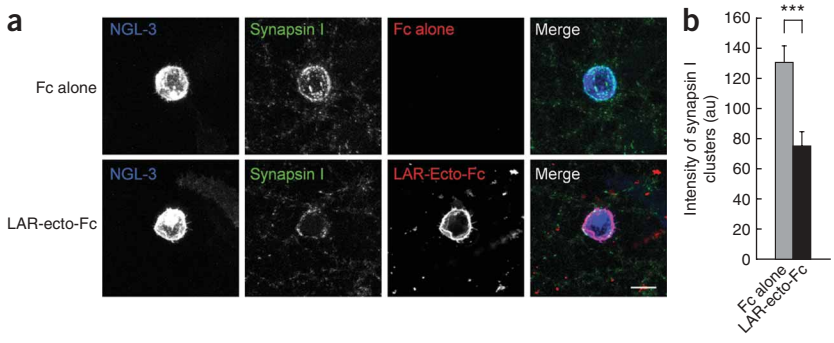

Figure 7 Soluble LAR reduces NGL-3–induced presynaptic differentiation. (**a**) Reduced NGL-3–induced presynaptic differentiation in the presence of soluble LAR. HEK293T cells expressing NGL-3–EGFP or EGFP alone were cocultured with hippocampal neurons (10–13 DIV) in the presence of LAR-ecto-Fc or Fc alone (10 μg ml^{-1}) and stained for EGFP (for NGL-3), synapsin I and human Fc (for LAR-ecto-Fc). Scale bar represents 20 μm. (**b**) Quantification of the integrated intensity of synapsin I clusters normalized to the cell area (mean ± s.e.m., $n = 20$ for Fc alone and 23 for LAR-ecto-Fc, $^{***}P < 0.001$, Student's t test).

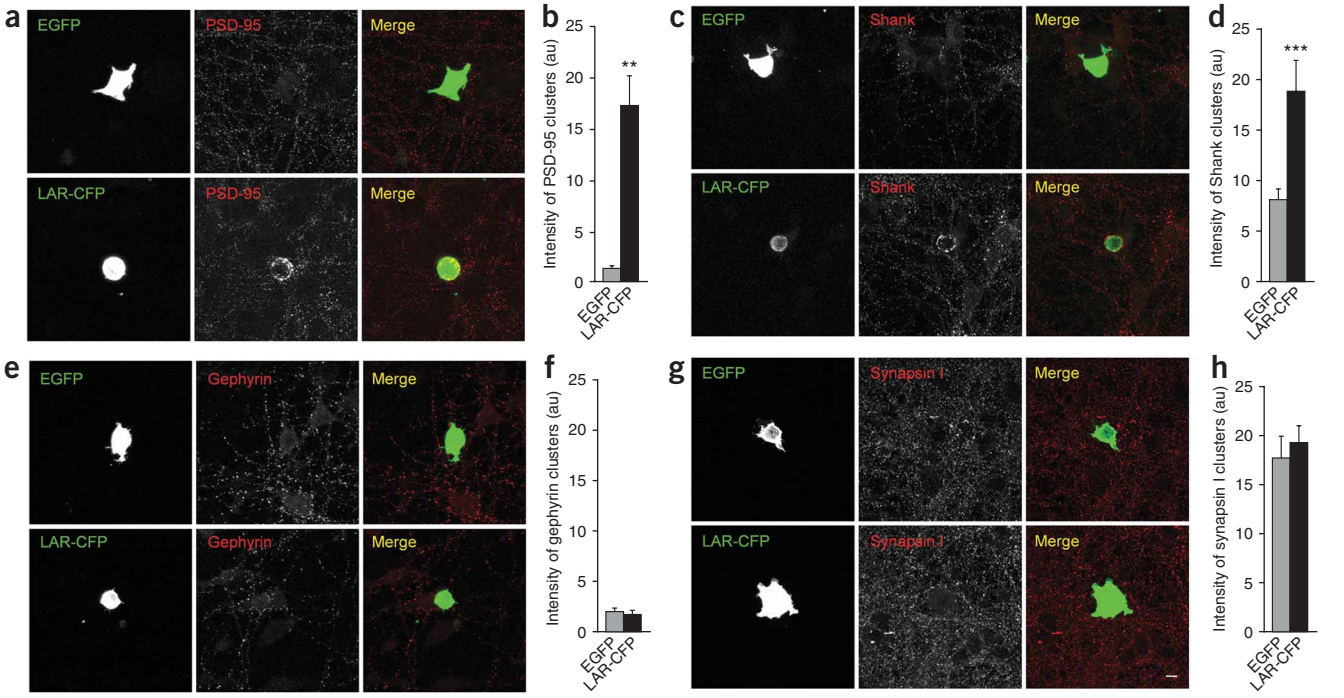

Figure 8 LAR expressed in non-neural cells induces excitatory postsynaptic differentiation in contacting dendrites. (**a**–**f**) LAR induced clustering of excitatory postsynaptic proteins in contacting dendrites. HEK293T cells expressing LAR-CFP or EGFP alone were cocultured with neurons (10–13 DIV) and stained for EGFP/CFP (**a**,**c**,**e**), PSD-95 (**a**), Shank (**c**, pan-Shank) and gephyrin (**e**). Scale bar represents 20 μm. We quantified the integrated intensity of postsynaptic protein clusters normalized to the cell area (mean ± s.e.m.; PSD-95: $n = 22$ for EGFP and 22 for LAR, $^{**}P < 0.01$, Student's t test, **b**; Shank: $n = 25$ for EGFP and 29 for LAR, $^{***}P < 0.001$, Student's t test, **d**; gephyrin: $n = 24$ for EGFP and 20 for LAR, **f**). (**g**) LAR did not induce the clustering of synapsin I in contacting axons. HEK293T cells expressing LAR-CFP or EGFP alone were cocultured with neurons (10–13 DIV) and stained for EGFP/CFP and synapsin I (**g**). Scale bar represents 20 μm. (**h**) Quantification of the integrated intensity of presynaptic protein clusters normalized to the cell area (mean ± s.e.m.; synapsin I: $n = 28$ for EGFP and 27 for LAR, $P = 0.59$, Student's t test).

cytoplasmic domains), suggesting that NGL isoforms have different functions. In support of this notion, NGLs interact with netrin-Gs in an isoform-specific manner: NGL-1 and NGL-2 interact with netrin-G1 and netrin-G2, respectively, whereas NGL-3 binds neither netrin-G1 nor netrin-G2 (ref. 13). Our data indicate that NGL-3, which to date has remained an orphan receptor, selectively associates with LAR, whereas NGL-1 and NGL-2 do not bind LAR. This suggests that the extracellular domains of NGLs, despite their close amino acid sequence identity relative to the cytoplasmic region, are functionally distinct. Consistently, NGL-3 was more efficient than NGL-1 and NGL-2 in inducing presynaptic differentiation in mixed-culture assays, although they had comparable surface expression levels.

Taken together with previous results, our data indicate that the two different types of NGL ligands, netrin-G1/2 and LAR, have both similar and contrasting features. Extensive alternative splicing is observed in both netrin-Gs[21–23] and LAR[43,44], suggesting that alternative splicing may regulate the NGL-3–LAR adhesion, as shown in the splicing-dependent LAR interaction with nidogen[35]. NGL-3 expression, however, is probably not regulated by alternative splicing, as the NGL-3 gene (*LRRC4B*) has only two coding exons. Netrin-Gs are unique in that they are GPI-anchored proteins[21,22]. In addition, netrin-G2 does not induce postsynaptic differentiation in contacting dendrites when it is expressed in non-neural cells[13]. These results suggest that netrin-Gs may require an additional co-receptor for its functional interaction with NGL-2. In contrast, LAR is a transmembrane protein with the ability to induce postsynaptic differentiation in contacting dendrites, suggesting that LAR alone may be sufficient to function as a presynaptic NGL-3 receptor.

The NGL-3–LAR interaction is similar to the well-known adhesion between neuroligin and neurexin in that a heterophilic trans-synaptic adhesion mediates synapse formation in a bidirectional manner[7–9]. In addition, when it is expressed in non-neural cells, NGL-3 induced both excitatory and inhibitory presynaptic differentiation in contacting axons, similar to neuroligins[9], which induce excitatory and inhibitory presynaptic differentiation by interacting with neurexins that are present in both glutamatergic and GABAergic axons[9]. Localization of LAR at excitatory synapses is supported by the role of LAR in the development and maintenance of excitatory synapses[34]. Whether LAR is present at inhibitory synapses remains to be determined. It should be noted that NGL-3 that is expressed in neurons selectively induces excitatory presynaptic contacts, contrary to the results from mixed cultured assays. This might be attributable to the interaction of NGL-3 with postsynaptic proteins that promote excitatory presynaptic differentiation in contacting axons.

LAR expressed in non-neural cells selectively induced excitatory postsynaptic protein clustering in contacting dendrites, unlike neurexin 1β, which induces both excitatory and inhibitory postsynaptic protein clustering[9]. Neurexin 1β induces excitatory and inhibitory postsynaptic protein clustering by interacting with both neuroligin 1 and neuroligin 2 (ref. 9), which have specific excitatory and inhibitory synaptic localizations, respectively[4,5]. The selective excitatory postsynaptic protein clustering by LAR may arise from LAR's association with NGL-3 at excitatory synapses. This is consistent with our observation that direct dendritic clustering of NGL-3 selectively induced excitatory postsynaptic protein clustering, and suggests that it is unlikely that there are unknown LAR-binding postsynaptic adhesion molecules at inhibitory synapses.

How might NGL-3 and LAR lead to post- and presynaptic differentiation, respectively? One possible mechanism by which synaptic adhesion molecules contribute to synapse formation is by interacting with and promoting the synaptic localization of specific membrane and cytoplasmic proteins. PSD-95, which interacts with NGL-3, is an abundant postsynaptic protein that interacts with a variety of membrane, signaling and scaffolding/adaptor proteins of excitatory synapses. Therefore, PSD-95 may couple NGL-3–dependent synaptic adhesion to the localization of various postsynaptic proteins.

Liprin-α, which binds to the membrane-distal phosphatase domain of LAR, may act together with LAR to mediate NGL-3–dependent presynaptic differentiation. Liprin-α has been implicated in the regulation of active zone assembly and presynaptic development through its interactions with synaptic proteins, including ERC/ELKS, RIM and CASK[28]. Studies on liprin-α homologs in *C. elegans* (SYD-2) and *Drosophila* (Dliprin-α) have firmly established the roles of liprin-α in presynaptic differentiation[31,32,45,46].

Another LAR-interacting cytoplasmic protein that may contribute to LAR-dependent presynaptic differentiation is β-catenin, a component of the cadherin-catenin complex[30]. Presynaptic β-catenin localizes the reserve pool of vesicles to presynaptic sites through mechanisms involving interaction of its C terminus with synaptic PDZ proteins[47]. Notably, brain-derived neurotrophic factor activation of the TrkB receptor tyrosine kinase regulates tyrosine phosphorylation of β-catenin, which disrupts the cadherin–β-catenin interaction and promotes synaptic vesicle splitting and synapse formation[48]. Considering that LAR dephosphorylates β-catenin[30,34], LAR and TrkB may reciprocally regulate β-catenin–dependent presynaptic vesicle clustering.

One possibility is that the binding of NGL-3 to LAR may regulate the tyrosine phosphatase activity of LAR. Receptor tyrosine phosphatases can be negatively regulated by dimerization[49]. However, a study on the crystallographic structure of the phosphatase domain of LAR has suggested that the phosphatase activity of LAR is probably not regulated by dimerization[50].

In conclusion, our results suggest that the trans-synaptic interaction between NGL-3 and LAR regulates excitatory synapse formation in a bidirectional manner. Future investigations will aim to explore possible functional interactions between NGL-1/2–netrin-Gs and NGL-3–LAR adhesions.

METHODS

DNA constructs and antibodies. Full-length human NGL-1 (NM_020929, amino acids 1–641) and rat NGL-3 (XM_218615, amino acids 1–709) were subcloned into pEGFP-N1 (Clontech). Human LAR (amino acids 1–1881) was subcloned into pECFP-N1 (Clontech). For small interfering RNA knockdown, nucleotides 359–377 of rat NGL-3 (GCA AGA ATC TGG TGC GCA A), its point mutant (GCA AGT CTC TTG TGC GCA A) and nucleotides 1,254–1,272 (GCA CGA TGG CAC ACT CAA T) were subcloned into pSuper.gfp/neo (OligoEngine) to generate shNGL-3 #1, shNGL-3* and shNGL-3 #2, respectively. The extracellular domain of LAR (amino acids 1–1,235) was subcloned into pEGFP-N1, in which EGFP was replaced with human Fc. Other constructs, antibodies and reagents are described in the **Supplementary Methods** online.

Mixed-culture assay. Mixed-culture assays were carried out as described[39]. Briefly, primary hippocampal neuron cultures at 10 d *in vitro* (10 DIV) prepared from embryonic day 18–19 (E18–19) rats were cocultured with HEK293T cells expressing NGLs, LAR or EGFP, followed by immunostaining at 13 DIV. For the synaptotagmin I antibody uptake assay, neurons were incubated with antibodies to the synaptotagmin luminal domain at the dilution of 1:10 in isotonic depolarizing solution for 5 min. For competitive inhibition, LAR-ecto-Fc proteins (10 μg ml^{-1}) were added to NGL-3–expressing HEK293T cells that were cocultured with neurons for 3 d (10–13 DIV).

Transfection of neurons and immunocytochemistry. Cultured neurons were transfected using a mammalian transfection kit (Clontech) and fixed with 4% paraformaldehyde/4% sucrose (vol/vol), permeabilized with 0.2% Triton X-100 (vol/vol) in phosphate-buffered saline, incubated with primary antibodies and then incubated with Cy3-, Cy5- or FITC-conjugated secondary antibodies (Jackson ImmunoResearch). For the inhibition of endogenous synapses with soluble LAR, neurons were treated with LAR-ecto-Fc for 3 d (20 μg ml^{-1}, 7/8–10/11 DIV) and transfected with pEGFP-N1 for visualization (9/10–10/11 DIV).

Bead and antibody aggregation assays. Bead aggregation assays were performed as described previously[13]. Briefly, neutravidin-conjugated FluoSphere beads (Molecular Probes) were pre-incubated with biotin-conjugated antibodies to EGFP. The antibody-coated beads were added onto neurons expressing EGFP–NGL-3 (14–16 DIV) and cultured for 24 h. For antibody aggregation, antibodies to EGFP were clustered by FITC-conjugated antibodies to guinea pig in complete neurobasal medium and added to neurons at 16 DIV, followed by 24-h culture and immunofluorescence staining at 17 DIV.

Image acquisition and quantification. All *z*-stacked images were randomly acquired by confocal microscopy (LSM510, Zeiss) and analyzed with MetaMorph image analysis software (Universal Imaging). The density of synaptic protein clusters was measured from 15–30 neurons; the primary dendritic lengths of ~50 μm from the cell body were measured for each neuron. For quantification of images from coculture assays, all captured images were thresholded and the integrated intensity of the clusters on transfected HEK293T cells was normalized to the cell area. All values are presented as mean ± s.e.m. and analyzed by Student's *t* test or ANOVA Tukey's test.

Quantitative cell surface–binding assay. HEK293T cells transfected with NGL-3 were transferred to 96-well plates and grown for 24 h. After fixation in 4% paraformaldehyde/4% sucrose, cells were incubated with increasing concentrations of LAR-ecto-Fc for 1 h, incubated with horseradish peroxidase–conjugated antibodies to human Fc (Sigma, 1:10,000) and color reacted with TMB (Sigma).

Electrophysiology. Cultured pyramidal neurons from the hippocampus transfected with shNGL-3 (13–16 DIV) were whole-cell voltage clamped at −60 mV using an Axopatch 200B amplifier (Axon Instruments). The extracellular solution contained 145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 0.4 mM sodium ascorbate. The intracellular solution contained 100 mM potassium gluconate, 20 mM KCl, 10 mM HEPES, 8 mM NaCl, 4 mM magnesium ATP, 0.3 mM sodium GTP and 0.5 mM EGTA. For mEPSC measurement, tetrodotoxin (10 nM, Tocris) and bicuculline (10 μM, Tocris) were added into the extracellular solution. Synaptic currents were analyzed using a custom-written macro in Igor Pro (WaveMetrics).

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We would like to thank A.M. Craig for neurexin 1α, 2α, 3α and 1β cDNAs, T. Biederer for SynCAM 1, Y.-P. Hsueh for Syndecan-2, P. Maness for NCAM-140, J. Ko for quantitative analysis, J. Nam for SALM4, M.-H. Kim for the mini analysis program, and Y.-G. Oh, M.-S. Baek, M. Ryu and J.-G. Jung for the help with antibody generation. This work was supported by the National Creative Research Initiative Program of the Korean Ministry of Science and Technology (E.K.).

AUTHOR CONTRIBUTIONS

J.W. and S.-K.K. carried out the experiments, analyzed the data and wrote the manuscript. S.C. helped with the shRNA knockdown experiments. S.K., J.-R.L., A.W.D. and M.S. contributed reagents. E.K. supervised the project and wrote the manuscript. All authors discussed the results and commented on the manuscript.

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