Neuroligin-1 controls synaptic abundance of NMDA-type glutamate receptors through extracellular coupling

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Despite the pivotal functions of the NMDA receptor (NMDAR) for neural circuit development and synaptic plasticity, the molecular mechanisms underlying the dynamics of NMDAR trafficking are poorly understood. The cell adhesion molecule neuroligin-1 (NL1) modifies NMDAR-dependent synaptic transmission and synaptic plasticity, but it is unclear whether NL1 controls synaptic accumulation or function of the receptors. Here, we provide evidence that NL1 regulates the abundance of NMDARs at postsynaptic sites. This function relies on extracellular, NL1 isoform-specific sequences that facilitate biochemical interactions between NL1 and the NMDAR GluN1 subunit. Our work uncovers NL1 isoform-specific *cis*-interactions with ionotropic glutamate receptors as a key mechanism for controlling synaptic properties.

synapse | neurotranmitter receptor | neurexin

NMDA receptors (NMDARs) are key regulators of the development of neural circuits and synaptic plasticity (1, 2). In humans, perturbation of NMDAR function results in psychotic conditions, and genetic animal models with altered NMDAR activity exhibit phenotypes related to cognitive disorders such as schizophrenia and autism (3–5). Activity-dependent recruitment of NMDARs to synapses controls certain forms of synaptic plasticity (6, 7). However, the molecular mechanisms underlying the recruitment and physical tethering of NMDAR complexes at synapses are incompletely understood.

Neuroligin-1 (NL1), one of four postsynaptic neuroligin adhesion molecules (NL1, 2, 3, 4), contributes to NMDAR regulation (8, 9). In cultured neurons, overexpression of NL1 promotes clustering of synaptic NMDARs (8), and *NL1* KO mice show decreases in NMDAR-dependent excitatory postsynaptic currents (NMDAR EPSCs) (9–11). A major question in understanding neuroligin function is how specific isoforms couple to specific neurotransmitter receptors (12, 13). NMDARs were recovered in coimmunoprecipitations with NL proteins, indicating a potential complex formation, although in those experiments, no NL isoform-specificity was apparent (14). One candidate link between NLs and glutamate receptors is through postsynaptic scaffolding molecules such as postsynaptic density 95 (PSD95) (15, 16). However, all NL isoforms contain PSD95 binding sites, and NMDARs and PSD95 were recruited to NL1 with different time courses (14).

Our results demonstrate that NL1 controls synaptic abundance of NMDAR via NL1-specific extracellular determinants. Loss of these interactions results in impairment of NMDAR-mediated transmission and synaptic plasticity. Our findings uncover an unexpected mode of NL1-NMDAR coupling and demonstrate a key role for the NL1 adhesion protein in the physical incorporation and retention of NMDAR at glutamatergic synapses.

Results

NL1-Specific Recruitment of NMDARs Does Not Require PSD95. We examined the specificity of molecular coupling of NL isoforms (NL1, 2, 3) to NMDARs by NL overexpression in cultured hippocampal neurons. NL1 increased the density of clusters of the NMDAR subunits GluN1, GluN2A, and GluN2B (Fig. 1 *A* and *B*; Fig. S1*A*, minor effect on intensity and size of NMDAR-immunoreactive puncta; Table S1). For NL1-expressing cells, more than 70% of GluN1 clusters were apposed to vGluT1-positive structures (3,633 puncta counted from 10 cells). NL1 overexpression significantly elevated the NMDAR to AMPA receptor (AMPAR) ratios of evoked EPSCs (Fig.1*C*), consistent with an NL1-mediated recruitment of NMDARs to synapses. We examined NL1-triggered NMDAR clustering in cells where PSD95 expression was suppressed by RNA interference. NMDAR clustering was still observed in the absence of detectable PSD95 protein (Fig. 1 *D* and *E*; Fig. S1*B* for knock-down efficiency). Furthermore, a NL1 mutant (NL1 ΔC) lacking the interaction site for PSD95 and other PDZ domain–mediated interactions still stimulated NMDAR clustering as WT NL1 (Fig. 1 *D* and *E*). Although NL2 and NL3 failed to recruit NMDARs (Fig. 1 *A* and *B*), they did increase vGluT1 and PSD95 clustering (Fig. S1*C*). Therefore, NL-PSD95 interactions appear to be neither necessary nor sufficient to recruit NMDARs to postsynaptic sites.

Extracellular Coupling Between NL1 and NMDARs. Given the ability of NL1 but not NL2 to recruit NMDARs, we constructed cDNAs encoding chimeric NL1-NL2 proteins with the aim to identify NL1-specific sequences required for NMDAR coupling. In the chimeric NL1-2, the extracellular cholinesterase (ChE) domain of NL1 was transplanted onto NL2, whereas in NL2-1, the ChE domain of NL2 was transplanted onto NL1 (Fig. 2*B*). Notably, the NL1-2 chimaera retained the ability to recruit NMDARs, whereas NMDAR distribution was unchanged in NL2-1–expressing neurons (Fig. 2 *A* and *B*, for NL1-2, 3,052 GluN1 clusters counted, 63.3% colocalized with vGluT1). These observations were substantiated by our electrophysiological recordings. To avoid complications due to the presence of endogenous NL1, we introduced chimeric NLs into cultured hippocampal

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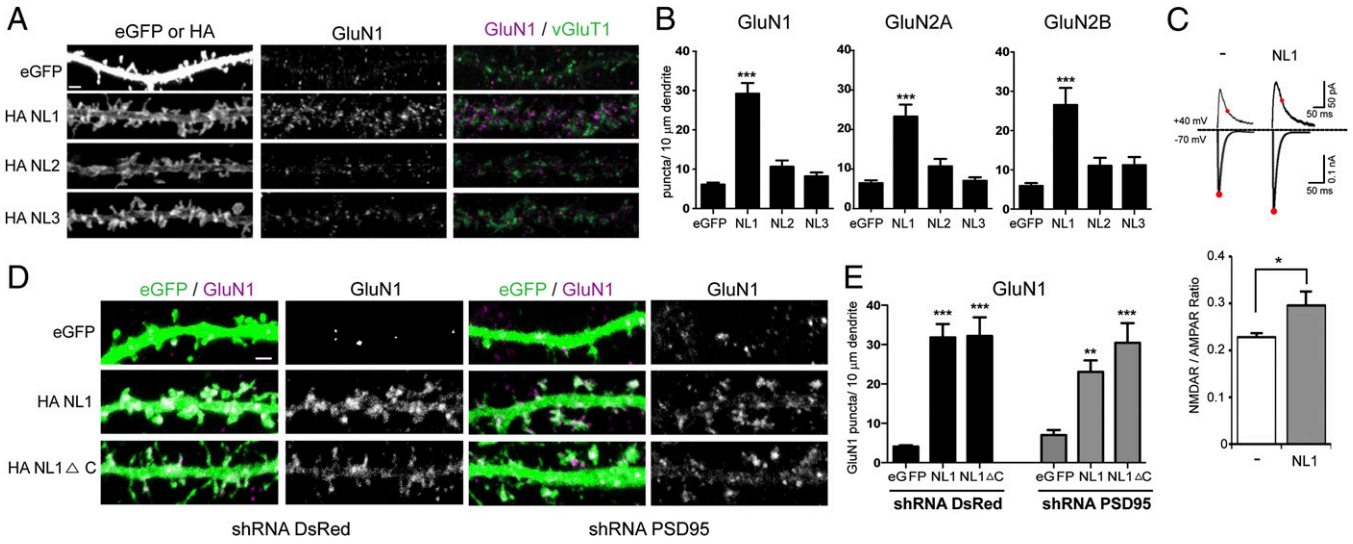

Fig. 1. NL1-specific recruitment of NMDARs. (*A*) Hippocampal neurons transfected with eGFP or NL expression vectors encoding HA-tagged NL1, NL2, or NL3. Dendritic segments of cells triple immunostained with anti-eGFP or anti-HA, anti-GluN1, and anti-vGluT1 antibodies. (Scale bar: 5 µm.) (*B*) Quantification of cells expressing eGFP, NL1, NL2, or NL3 ($n \geq 10$ for each group, *P* values for comparisons to eGFP controls). (*C*) EPSCs were elicited at WT hippocampal neurons nontransfected (–) or transfected with NL1. Sample traces of EPSCs evoked by local extracellular stimulation, with the measurement of NMDAR (*Upper*, at +40 mV, small red circles) and AMPAR (*Lower*, at −70 mV, large red circles) EPSCs. A summary histogram for mean NMDAR/AMPAR ratios ($n \geq 18$ cells for each group, $*P < 0.05$). (*D*) Hippocampal neurons were cotransfected with an expression construct for eGFP, HA-tagged NL1 (HA NL1), or a C-terminal deletion mutant of NL1 (HA NL1ΔC) and negative control (shRNA DsRed) or shRNA to PSD95 (shRNA PSD95). (Scale bar: 5 µm.) (*E*) Quantification in neurons cotransfected with eGFP, NL1, or NL1ΔC and either shRNA DsRed or shRNA PSD95 ($n \geq 10$ for each group, *P* values for comparisons to eGFP controls, $**P < 0.01$, $***P < 0.001$).

neurons prepared from *NL1* KO mice and then elicited EPSCs by extracellular stimulation near neurons (Fig. S2*A*), as in the previous studies (10, 17). The reintroduction of NL1 in *NL1* KO neurons restored NMDAR/AMPAR ratios, confirming that the synaptic transmission phenotype in *NL1* KO neurons can be fully rescued (Fig. S2*B*). Importantly, NMDAR/AMPAR ratios were elevated by the introduction of NL1-2 but not NL2-1, which demonstrated that the ChE domain of NL1 is indispensible for normal synaptic transmission in glutamatergic synapses (Fig. S2 *A* and *B*). Both chimaeras similarly elevated the number of vGluT1-positive terminals on the transfected cells compared with eGFP-expressing control cells (Table S2). Note that the recruitment of vGluT1 by NL2 is likely a consequence of overexpression, considering that endogenous NL2 is largely restricted to GABAergic synapses (18). However, this activity further confirmed efficient surface transport and functionality of the chimeric proteins.

We hypothesized that NL1 might interact with the NMDAR complex through extracellular interactions in *cis*. NL1 antibodies coprecipitated the NMDAR subunit GluN1 from brain extracts but not AMPAR subunits GluA2/3 (Fig. 2*C*), identifying an NL1–NMDAR complex. To test whether formation of NL1–NMDAR complexes requires the presence of the synaptic NL-binding partner neurexin, we further explored NL1–NMDAR interactions in heterologous cells. We observed selective coimmunoprecipitation of NL1 with NMDARs, but not with AMPARs (Fig. 2*D*). NL2 did not yield any significant association with either glutamate receptor complex. Deletion of the C-terminal domain of NL1 (NL1ΔC) did not prevent the NL1–NMDAR complex formation. However, the interaction was abolished for a NL1 mutant where the ChE domain was exchanged with the homologous sequence of mouse acetylcholinesterase (NL1 swap). The NL1-2 chimeric protein retained the NMDAR association, and the association was strongly reduced for NL2-1 (Fig. 2*E*).

To further test whether NL1 associates with NMDAR subunits at the cell surface, we used an in situ proximity ligation assay (PLA), which enables determination of the proximity of proteins within a maximal distance of 30–40 nm (19, 20). As a positive control, we expressed NL1 proteins with two different epitope tags in COS7 cells. Consistent with the formation of NL1 oligomers, these proteins yielded abundant PLA signals (Fig. 2*G*; Fig. S2*D*). We deleted endoplasmic reticulum (ER) retention motifs of GluN1 (GluN1-TM1; Fig. S2*C*) so that the isolated subunit would be efficiently transported to the plasma membrane (21, 22). Coexpressed GluN1-TM1 and NL1 exhibited strong PLA signals. By contrast, very low PLA signals were detected between NL1 and GluN2A or NL1 and GluN2B. Moreover, coexpression of GluN1-TM1 and a NL1 mutant lacking the cholinesterase domain (ΔChE) or an unrelated cell surface protein (γ-protocadherin A3) yielded no significant PLA signals (Fig. 2 *F* and *G*; Fig. S2). These experiments identify the GluN1 subunit as the primary subunit for the association with NL1.

Synaptic Incorporation and Retention of NMDARs Controlled by NL1. The reduction of NMDAR-mediated transmission in *NL1* KO mice may result from decreased NMDAR expression, impaired synaptic incorporation, or altered synaptic receptor function. Total protein levels and cell surface levels of NMDAR subunits are not notably altered in *NL1* KO brains (Fig. S3 *A* and *B*). To assess a role for NL1 in the synaptic recruitment of endogenous NMDARs, we monitored incorporation of extrasynaptic NMDARs into synapses using a MK-801 wash-out paradigm. Synaptic NMDARs are selectively inactivated by synaptic stimulation in the presence of the open channel blocker MK-801. After MK-801 removal, recovery of NMDAR EPSCs reports on the recruitment of extrasynaptic (unblocked) NMDARs into synapses (23). We observed considerable recovery of NMDAR EPSCs after MK-801 clearance in acute brain slices, whereas *NL1* KO neurons exhibited virtually no recovery over the same time frame (Fig. 3 *A* and *C*). We then assessed rescue of this phenotype by introducing either NL1 or chimeric NLs into the hippocampus of *NL1* KO animals by stereotaxic injection of recombinant, dual-promoter lentiviruses (Fig. S4*A*) (24). Reintroduction of WT NL1 and NL1-2 but not NL2-1 restored recovery of NMDAR EPSCs that represents synaptic incorporation of NMDARs (Fig. 3*C*). The impaired NMDAR EPSC recovery most likely arises from a failure to incorporate extrasynaptic receptors or from immobility of the pool of NMDARs remaining in the *NL1* KO synapses.

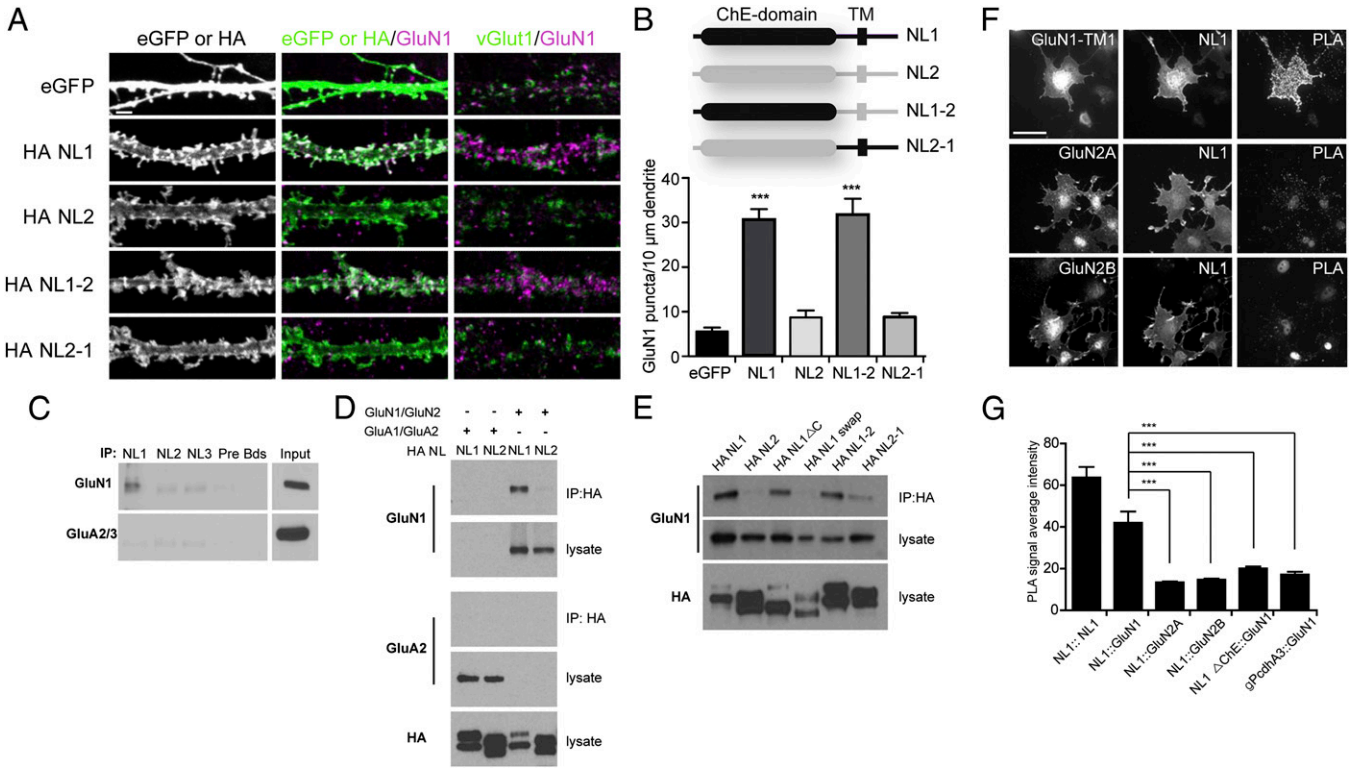

Fig. 2. NL1 cholinesterase domains are determinants of selectivity for NMDARs. (*A*) Dendritic segments of hippocampal neurons transfected with eGFP or HA-tagged NL1, NL2, NL1-2, or NL2-1, triple immunostained with anti-eGFP or anti-HA, anti-GluN1, and anti-vGluT1 antibodies. (Scale bar: 5 µm.) (*B*) Schematic depiction of NL1-NL2 chimeric proteins containing NL1 (black) and NL2 (gray) sequences and quantification of GluN1 puncta density ($n \geq 10$ for each group, *P* values for comparisons to eGFP controls). (*C*) NL1, NL2, or NL3 was immunoprecipitated from total brain lysates probed with GluN1 or GluA2/3 antibodies. Preimmune serum (Pre) and protein A beads without antibody (Bds) were used as negative controls. The panels shown for input (10%) and immunoprecipitate are derived from the same blotting membrane and identical exposure times. (*D*) HEK293 cells were cotransfected with HA-tagged NLs (NL1 or NL2) and NMDAR (GluN1/GluN2A/GluN2B) or AMPAR (GluA1/GluA2) expression vectors. Protein complexes immunoprecipitated (IP) with anti-HA antibodies probed with anti-GluN1 or anti-GluA2 antibodies. Comparable expression of transfected proteins confirmed by analysis of the cell lysates. (*E*) HEK293 cells cotransfected with HA-tagged NL variants and NMDAR (GluN1/GluN2A/GluN2B) expression vectors. Protein complexes immunoprecipitated (IP) with anti-HA antibodies probed in Western blotting with anti-GluN1 or anti-HA antibodies. (*F*) COS7 cells cotransfected with cDNAs encoding GluN subunits together with NL1. Images show PLA signal from nonpermeabilized cells and subsequent immunofluorescence detection of the individual proteins performed after completion of the PLA reaction and cell permeabilization. (Scale bar: 50 µm.) (*G*) Quantification of average intensity of PLA signals (mean and SEM, $n > 40$ cells per condition per experiment, ***$P < 0.001$).

Using a freeze-fracture replica immunoelectron microscopy with a pan-NL antibody (Fig. 4, note that no NL1-specific antibody usable for this application is currently available), NL immunoreactivity was detected accumulating over the postsynaptic membrane specialization of spine synapses of putative CA1 pyramidal cells. The particles on spines were closely intermingled with immunogold

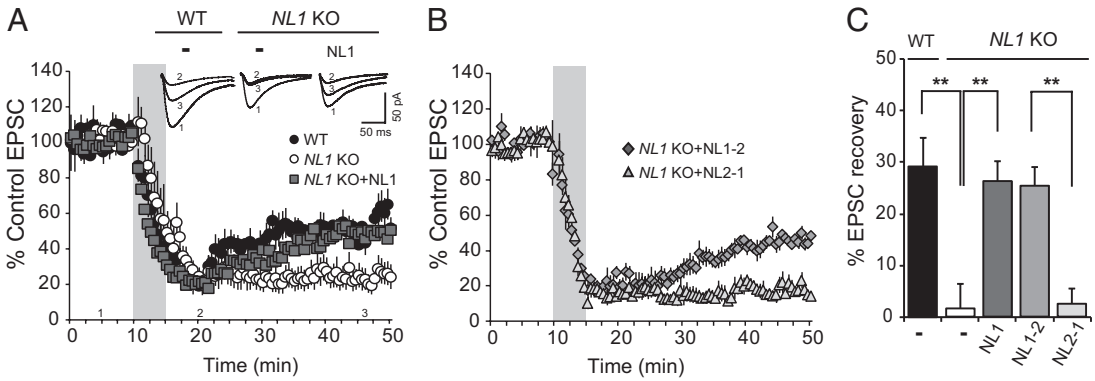

Fig. 3. Synaptic recruitment of endogenous NMDARs by NL1. (*A*) Hippocampal slices were perfused in 0.25 mM Mg^{2+} artificial cerebrospinal fluid (ACSF) containing 5 µM NBQX, and the perfusion rate was increased to 5 mL/min. Shaded bar indicates the period of MK-801 inclusion. (*Insets*) Representative traces of NMDAR EPSCs from CA1 pyramidal neurons of WT mice (–), noninfected (–), and infected neurons of *NL1* KO mice. The numbers of traces denote the time points when individual recordings were made. (*B*) MK-801 wash-out experiment after viral delivery of chimeric NLs into hippocampi of *NL1* KO mice. Shaded bar indicates the period of MK-801 inclusion. (*C*) Summary histogram of % EPSC recovery for each group ($n \geq 8$ for each group, see *SI Materials and Methods* for calculation of % EPSC recovery, **$P < 0.01$).

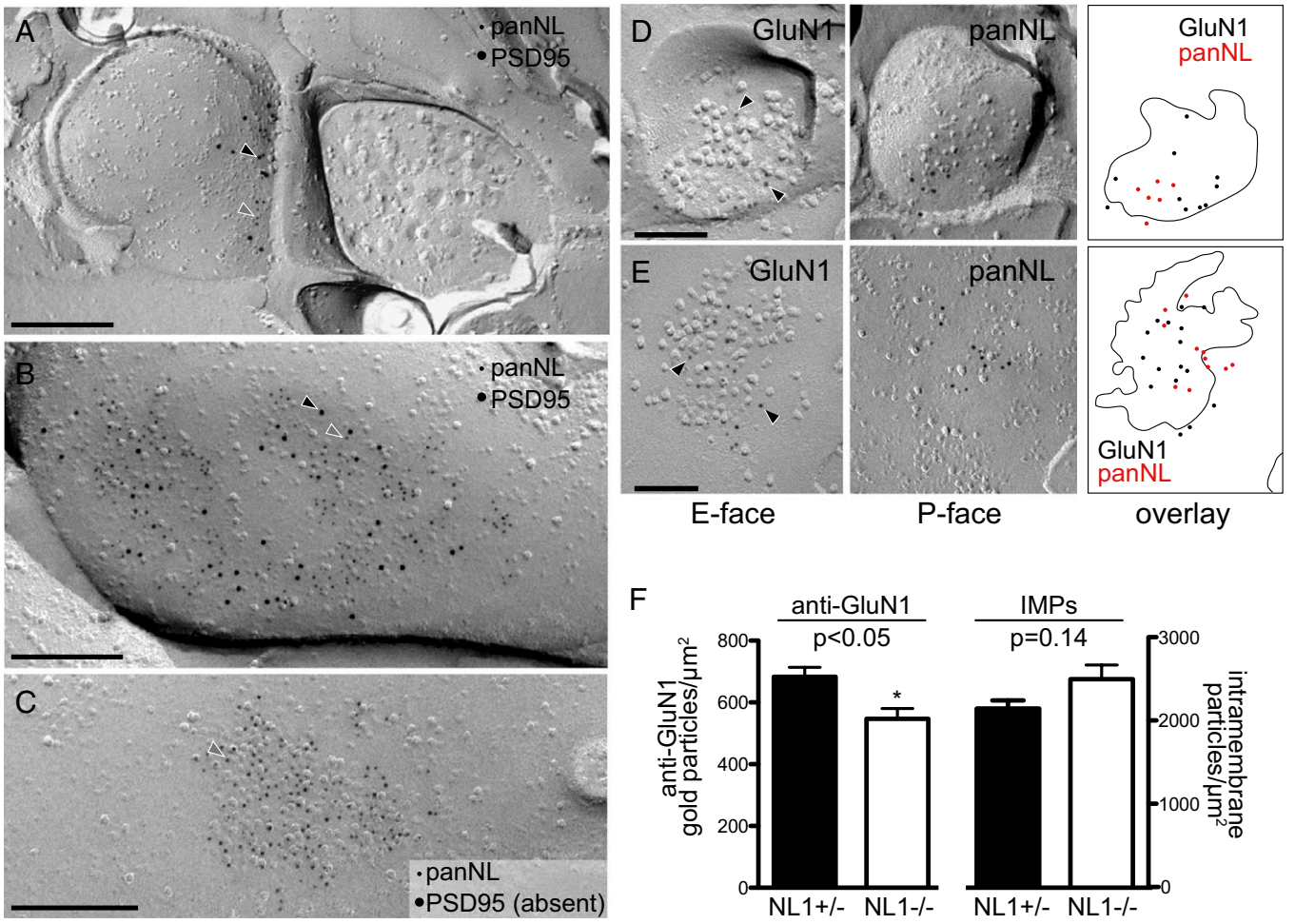

Fig. 4. Localization of NLs to the neurotransmitter receptor–containing PSD. (*A–E*) Synaptic localization of NLs detected with a panNL antibody that recognizes all NL isoforms (5-nm gold particles, one example in *A* marked with a gray arrowhead) colabeled with anti-PSD95 antibody (10-nm gold particles, one example in *A* marked with a black arrowhead; *A–C*) or GluN1 antibody (5-nm gold particles, examples marked with black arrowheads, *D* and *E*) in Stratum radiatum of mouse hippocampal CA1 area. Synaptic transmembrane protein complexes are visible as intramembrane particles (IMPs). P-face of a spine synapse with cross-fractured presynaptic terminal (*A*), a perforated glutamatergic synapse (*B*), and a PSD95-negative presumptive GABAergic synapse (*C*). *D* and *E* show E- and P-faces of single synapses, labeled with anti-GluN1 and anti-panNL antibodies, respectively (in both cases, the antigen is detected with 5-nm gold particles). The proximity of synaptic territory occupied by GluN1 and NL1 is illustrated by overlaying the position of gold particles with a delineation of a postsynaptic area (solid line) based on an IMP cluster on the E-face (*Right*). [Scale bars: 200 (*A–C*) and 100 nm (*D* and *E*).] (*F*) Quantitative assessment of anti-GluN1 labeling in CA1 reveals a significant reduction in GluN1 density at postsynaptic sites. In the same preparation, no significant change in the density of IMPs was detected ($n = 3$ pairs of heterozygous control and *NL1* KO animals, total of 100 synapses analyzed, mean ± SEM, $^*P < 0.05$).

particles for PSD95 in macular and perforated PSDs (Fig. 4 *A* and *B*, respectively). Immunoreactivity for NLs was also found on PSD95-negative presumptive GABAergic shaft synapses with intramembrane particle (IMP) clusters (Fig. 4*C*, these contacts lack 10-nm gold particles used to detect PSD95), consistent with the localization of NL2 and NL3 isoforms at some GABAergic synapses (18, 25, 26). Labeling experiments for GluN1 and NLs on corresponding faces [exoplasmic (E) and protoplasmic (P) faces] of single glutamatergic synapses further confirmed NL1-GluN1 colocalization (Fig. 4 *D* and *E*). Using quantitative immuno-gold labeling, we observed that the synaptic anti-GluN1 labeling density but not IMP density in the *NL1* KO hippocampus was significantly reduced (Fig. 4*F*). These findings demonstrate that endogenous NL proteins are central components of the postsynaptic GluN1-containing structures in vivo and that loss of NL1 results in a reduction in the number of NMDAR molecules at postsynaptic sites.

ChE Domain–Dependent Function of NL1 in Synaptic Transmission and Synaptic Plasticity. Initially we confirmed the previously reported decreases in NMDAR/AMPAR ratios and NMDAR EPSCs in *NL1* KO CA1 neurons compared with CA1 neurons of WT mice (Fig. S5). After infusion of viral vectors, simultaneous dual whole-cell recordings were made from infected and neighboring noninfected pyramidal cells of CA1 region, and EPSCs were elicited by electrical stimulation of the Schaffer collateral pathway (Fig. S4*C*). NL1 reexpressing CA1 neurons in *NL1* KO slices displayed increases in NMDAR EPSC amplitudes but no change in AMPAR EPSCs compared with noninfected control neurons (Fig. 5*A*). Thus, NMDAR-mediated currents at synapses lacking NL1 were successfully rescued to those levels of WT neurons by reintroduction of NL1 (Fig. 5*A*; Fig. S5*B*). We also observed the restoration of NMDAR EPSCs by expression of NL1-2 but not NL2-1 (Fig. 5 *B* and *C*).

Finally, we used a pairing protocol of presynaptic stimulation with simultaneous postsynaptic depolarization that triggers long-term potentiation (LTP) that requires activation of NMDARs (27). LTP was almost completely abolished in noninfected CA1 neurons of *NL1* KO mice, whereas the same protocol reliably induced LTP in those of WT mice (Fig. 5*D*; Fig. S6*A*). Importantly, reintroduction of NL1 and NL1-2 but not NL2-1 rescued paired-induced LTP (Fig. 5 *D–F*). However, forskolin-induced LTP that is normally independent of NMDAR function (28, 29)

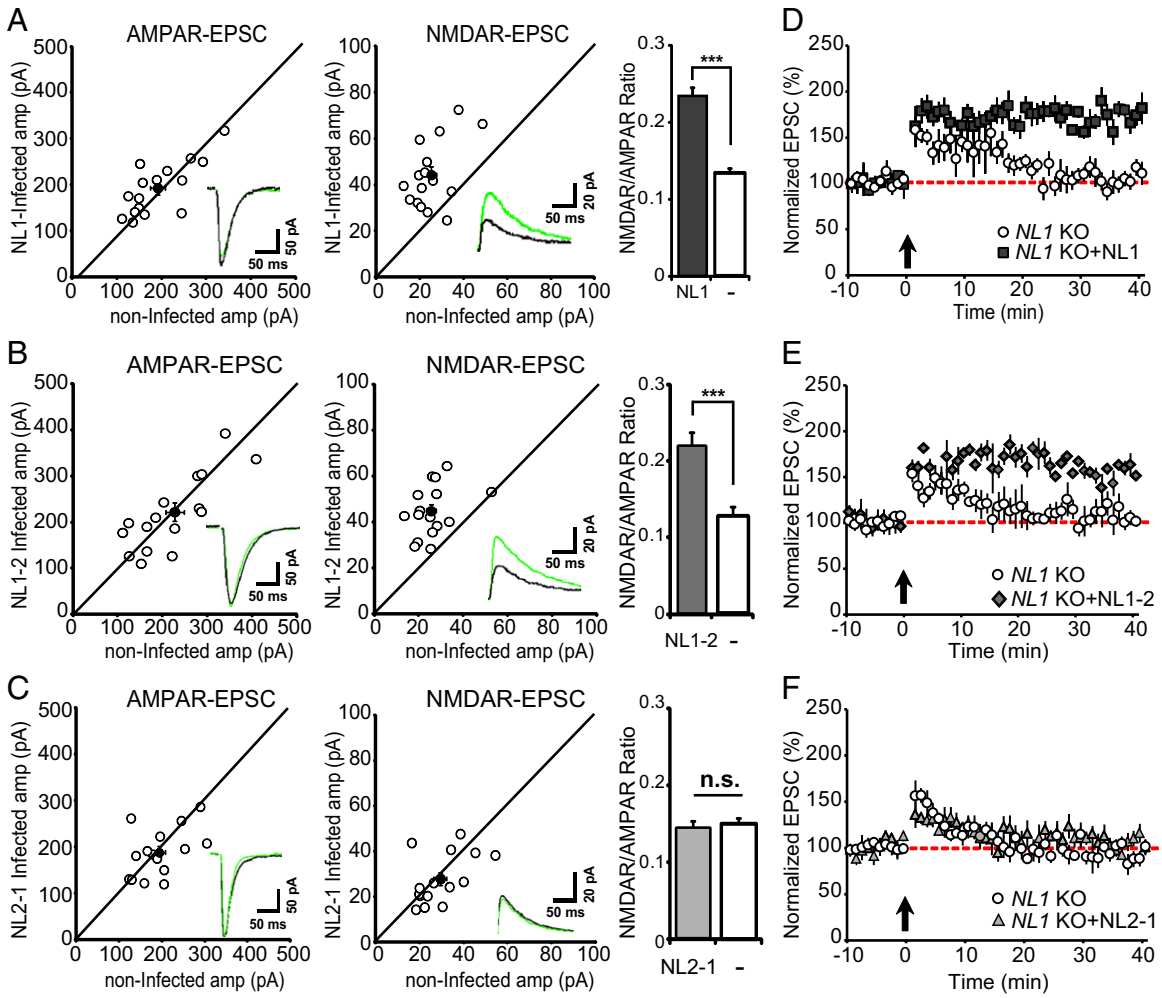

Fig. 5. ChE domain–dependent NL1-NMDAR coupling for normal synaptic properties. (*A–C*) Scatter plots show individual amplitudes of AMPAR (holding at −70 mV, *Left*) and NMDAR-EPSCs (holding at +40 mV, *Center*) for each recording pair and filled symbols indicate the means. *Insets* show representative traces of EPSCs from noninfected (black lines) or infected (green lines) *NL1* KO neurons. NMDAR/AMPAR ratios are presented for noninfected (–) or *NL1* KO neurons infected with lentiviruses encoding NL1, NL1-2, or NL2-1, respectively (*Right*). n.s., nonsignificant; ***$P < 0.001$. (*D–F*) Mean EPSC amplitudes before and after a pairing protocol (arrows denote the time point for the pairing) are shown after the normalization to prepairing levels for noninfected or *NL1* KO neurons expressing NL1, NL1-2, or NL2-1 ($n \geq 8$ pairs for each group).

remained intact in the *NL1* KO mice (Fig. S6*B*). These findings support the hypothesis that NL1-specific, ChE domain–mediated interactions underlie the synaptic incorporation and retention of NMDARs for normal synaptic transmission and synaptic plasticity in hippocampal CA1 neurons.

Discussion

In this study, we addressed several key questions regarding the coupling of NMDARs to the NL adhesion complex. First, we demonstrate that NL1 regulates the abundance of NMDAR molecules at synapses. Second, we identify the molecular determinants that confer NL1 isoform specificity to NMDARs. Third, we demonstrate that these determinants exert critical physiological roles in NMDAR-mediated transmission and synaptic plasticity in the adult hippocampus.

NL1 Drives the Incorporation and Synaptic Retention of NMDARs. Although the impairment of NMDAR function in *NL1* KO mice has been reported (10, 30), it remained unclear whether NL1 was required for NMDAR function or played a structural role in NMDAR recruitment. Our findings support a function of NL1 for the physical recruitment of NMDAR to synapses. First, quantitative freeze-fracture replica immunoelectron microscopy demonstrates that the density of GluN1 immunoreactivity at hippocampal CA1 synapses is significantly reduced in the absence of NL1. Second, we discovered an impairment in the synaptic incorporation of endogenous NMDARs with the MK-801 wash-out paradigm (23). Combined, these results strongly support a structural role for NL1 in the synaptic recruitment and retention of NMDARs at glutamatergic synapses.

There is accumulating evidence that global ablation of NL1 expression (as in KO mice) and the down-regulation in subsets of cells exhibit different phenotypes. Whereas suppression of NL1 in subsets of cells results in a loss of synapses in vitro and in vivo (8, 31, 32), global KO has little impact on synapse density but rather regulates the density of NMDARs per synapse (10, 33, 34). The mechanistic underpinnings of these observations remain to be resolved, but it is likely that competitive mechanisms between cells with different NL1 expression levels contribute to these phenomena (35).

Neurotransmitter Receptor Selectivity of NL1 at Glutamatergic Synapses. In addition to NMDAR-mediated transmission, gain- and loss-of-function manipulations for NL1 resulted in altered activity of AMPARs (32, 36). In our biochemical analysis, we observed an association of NMDARs but not AMPARs with NL1. However, our results do not exclude a modification of AMPAR function by NL1 expression. Such modifications might

occur through additional auxiliary proteins interacting with the NL1 cytoplasmic tail (32, 36) or calcium signaling downstream of NL1–NMDAR complexes in the postsynaptic compartment. Indeed, trapping of AMPAR subunits in cultured neurons at sites of NL1 aggregation has been described, indicating that the postsynaptic NL complex provides a platform to further the recruitment of additional glutamatergic proteins (36, 37).

Neuroligin Isoform Specificity in the Association with Neurotransmitter Receptors. NL1 is localized almost exclusively to glutamatergic synapses, NL2 is restricted to GABA and glycinergic synapses, NL3 is detected at both glutamatergic and GABAergic sites, and NL4 is detected at a subset of glycinergic synapses in the retina (18, 25, 38–40). The mechanisms underlying this synaptic specificity of the NL isoforms and the selective association with neurotransmitter receptors at the respective synapses are poorly understood. For NL2, interactions through the cytoplasmic collybistin–gephyrin complex contribute to GABA-A receptor recruitment to perisomatic synapses (41). For NL1 at glutamatergic synapses, it has been puzzling that all NL isoforms bind indiscriminately to a broad range of glutamatergic scaffolding molecules that would connect them to glutamatergic neurotransmitter receptors without apparent selectivity (15, 42). Our findings indicate that not intracellular sequences but the extracellular ChE domain of NL1 plays an instructive role in selective coupling to NMDARs. The requirement for NMDAR coupling discovered here provides an interesting parallel to the requirement for NL1 extracellular sequences for hippocampal LTP (31), and a similar extracellular link has been previously demonstrated for NMDAR coupling to EphB receptor tyrosine kinases (43). In combination, these findings suggest that extracellular interactions represent a more broadly operating principle for NMDAR stabilization at synapses. This activity is unique to NL1 and not supported by NL2 or NL3 sequences. Therefore, the discovery of ChE domain–dependent coupling between NL1 and NMDARs resolves the conundrum of the nonselective interactions of glutamatergic scaffolding proteins with the cytoplasmic tails of all NL isoforms and strictly isoform-specific requirement for NL1 in NMDAR function. Thus, extracellular, NL1 isoform-specific interactions represent one of mechanisms for controlling synaptic abundance of NMDARs.

Materials and Methods

All animal care and use were in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committee of Pohang University of Science and Technology and the Cantonal Veterinary Office Basel-Stadt. Acute brain slices, dissociated neuronal cultures, lentiviruses, and immunoelectron microscopy were performed as described previously (8, 11, 24, 25). Detailed materials and methods are described in *SI Materials and Methods*.

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