**Trans-Synaptic Signaling through the Glutamate Receptor Delta-1 Mediates Inhibitory Synapse Formation in Cortical Pyramidal Neurons**

**Highlights**

- GluD1 mediates the formation and specification of inhibitory synapses in the cortex
- GluD1 forms trans-synaptic interactions via Cbln4, a protein secreted by SST+ INs
- GluD1 elicits agonist-dependent postsynaptic signaling via ARHGEF12 and PPP1R12A
- GluD1 is a maverick iGluR operating through non-ionotropic mechanisms

**Authors**

Matteo Fossati, Nora Assendorp, Olivier Gemin, ..., Guillaume Arras, Damarys Loew, Cécile Charrier

**Correspondence**

cecile.charrier@ens.fr

**In Brief**

Fossati et al. show that, in stark contrast to other iGluRs, the glutamate receptor delta-1 (GluD1) operates at inhibitory, rather than excitatory, synapses in the cortex. GluD1 mediates trans-synaptic signaling governing the formation of synapses between specific interneurons and pyramidal neurons.
**Trans-Synaptic Signaling through the Glutamate Receptor Delta-1 Mediates Inhibitory Synapse Formation in Cortical Pyramidal Neurons**

Matteo Fossati,1,3,4 Nora Assendorp,1 Olivier Gemin,1 Sabrina Colasse,1 Florent Dingli,2 Guillaume Arras,2 Damarys Loew,2 and Cécile Charrier1,5,*

1Institut de Biologie de l’Ecole Normale Supérieure (IBENS), Ecole Normale Supérieure, CNRS, INSERM, PSL Research University, 75005 Paris, France
2Institut Curie, PSL Research University, Centre de Recherche, Laboratoire de Spectrométrie de Masse Protéomique, 75248 Paris Cedex 05, France
3Present address: CNR – Institute of Neuroscience, 20129 Milano, Italy
4Present address: Humanitas Clinical and Research Center, 20089 Rozzano MI, Italy
5Lead Contact
*Correspondence: cecile.charrier@ens.fr
https://doi.org/10.1016/j.neuron.2019.09.027

**SUMMARY**

Fine orchestration of excitatory and inhibitory synaptic development is required for normal brain function, and alterations may cause neurodevelopmental disorders. Using sparse molecular manipulations in intact brain circuits, we show that the glutamate receptor delta-1 (GluD1), a member of ionotropic glutamate receptors (iGluRs), is a postsynaptic organizer of inhibitory synapses in cortical pyramidal neurons. GluD1 is selectively required for the formation of inhibitory synapses and regulates GABAergic synaptic transmission accordingly. At inhibitory synapses, GluD1 interacts with cerebellin-4, an extracellular scaffolding protein secreted by somatostatin-expressing interneurons, which bridges postsynaptic GluD1 and presynaptic neurexins. When binding to its agonist glycine or D-serine, GluD1 elicits non-ionotropic postsynaptic signaling involving the guanine nucleotide exchange factor ARHGEF12 and the regulatory subunit of protein phosphatase 1 PPP1R12A. Thus, GluD1 defines a trans-synaptic interaction regulating postsynaptic signaling pathways for the proper establishment of cortical inhibitory connectivity and challenges the dichotomy between iGluRs and inhibitory synaptic molecules.

**INTRODUCTION**

Synapses constitute the elementary functional units of the brain. They convey excitatory or inhibitory signals that need to be coordinated in space and time for optimal brain function (Mullins et al., 2016; Nelson and Valakh, 2015). Excitatory and inhibitory synapses in the mammalian brain mainly use glutamate and gamma-aminobutyric acid (GABA) as a neurotransmitter, respectively. They are multi-molecular nanomachines composed of almost exclusive sets of proteins (Krueger-Burg et al., 2017; Sheng and Kim, 2011; Tyagarajan and Fritschy, 2014). Yet they share the same basic organization, which ensures the efficacy and fine-tuning of synaptic transmission. The organization of synapses relies on transient, highly regulated interactions between various categories of proteins (neurotransmitter receptors, scaffolding proteins, adhesion proteins, signaling molecules, and cytoskeleton elements) and accommodates a great level of molecular diversity (Choquet and Triller, 2013; Emes and Grant, 2012; Sheng and Hoogenraad, 2007; Tyagarajan and Fritschy, 2014; Ziv and Fisher-Lavie, 2014). The molecular diversity of synapses enables the establishment of complex neuronal networks: it specifies their functional properties and shapes the transfer of information between neurons throughout the brain. Hence, synaptic dysfunctions cause a wide range of neurodevelopmental and psychiatric disorders, such as epilepsy, autism, or schizophrenia (Bourgeron, 2015; Mullins et al., 2016; Nelson and Valakh, 2015; Ting et al., 2012; Zoghbi and Bear, 2012). Trans-synaptic molecular interactions critically contribute to both the development and diversification of synaptic connections. They instruct the formation of synapses following initial contact (McAllister, 2007; Missler et al., 2012), match pre- and post-synaptic neurons (Berns et al., 2018; de Wit and Ghosh, 2016), control the recruitment of neurotransmitter receptors and postsynaptic scaffolding proteins (Aoto et al., 2013; Bemben et al., 2015; Fukata et al., 2006; Lovero et al., 2015; Mondin et al., 2011; Nam and Chen, 2005; Poulopoulos et al., 2009), and regulate synaptic plasticity (Bemben et al., 2015; Jang et al., 2017; Tai et al., 2008; Yuzaki and Ariecescu, 2017). Nonetheless, frequent discrepancies between in vitro and in vivo studies have made the role of some trans-synaptic molecular interactions difficult to precisely delineate. Furthermore, the scarcity of information on how trans-synaptic signals are transduced in the post-synaptic neuron stymies our understanding of the molecular logic governing the assembly of synaptic connections.

Individual synaptic proteins may operate through a diversity of modalities. Recently, it has emerged that ionotropic glutamate receptors (iGluRs), which are the main excitatory...
neurotransmitter receptors in the CNS, do not solely operate through ionotropic mechanisms (Dore et al., 2015; Rodríguez-Moreno and Sihra, 2007; Yuzaki and Aricescu, 2017). At least some iGluRs engage in trans-synaptic interactions along with conventional cell adhesion molecules (CAMs) (Matsuda et al., 2010, 2016; Uemura et al., 2010) or mediate non-ionotropic signaling critical for synaptic development and plasticity (Sabie et al., 2014; Carter and Jahr, 2016; Dore et al., 2015; Grabauskas et al., 2007; Hayashi et al., 1999; Kakegawa et al., 2011; Lerma and Marques, 2013; Nabavi et al., 2013; Saglietti et al., 2007; Stein et al., 2015). Alternative functions of iGluRs are best characterized for the glutamate receptor delta-2 (GluD2), an iGluR of the delta subfamily (comprising GluD1 and GluD2 receptor subunits, encoded by the genes grid1 and grid2) predominantly expressed in the cerebellum (Araki et al., 1993). GluD2 is confined in the postsynaptic membrane of excitatory synapses between parallel fibers (PFs) and Purkinje cells (PCs). It contributes to synaptic adhesion by interacting with presynaptic neurexins containing an insert in the splice site 4 through the extracellular scaffolding proteins cerebrellins (Cbins), presynaptically secreted molecules that belong to the C1q family of the classical complement pathway (Sudhof, 2017; Uemura et al., 2010; Yuzaki, 2017, 2018). This trans-synaptic interaction controls the specification and maintenance of PF-PC synapses. In addition, activation of GluD2 by its agonist initiates signaling cascades regulating the local accumulation of 3-aminooxy-5-methyl-4-isoxazolidinone acid (AMPA) receptors and long-term depression (Kakegawa et al., 2011; Yuzaki and Aricescu, 2017). GluD1 can also form triads with Cbns and neurexins (Yasumura et al., 2012; Yuzaki and Aricescu, 2017). It is widely expressed in the neocortex, hippocampus, stratum, and cerebellum, where its expression is strongly upregulated during the period of synaptogenesis and remains high in adults (Hepp et al., 2015; Kondo et al., 2014). GluD1 has been implicated in the formation of excitatory synapses in the cerebellum (Kondo et al., 2014) and hippocampus (Tao et al., 2018) and in pruning in the hippocampus and medial prefrontal cortex (Gupta et al., 2015). Other studies have suggested a role in the firing of dopaminergic neurons or at inhibitory synapses (Benamer et al., 2018; Ryu et al., 2012; Yasumura et al., 2012). Notwithstanding, GluD1 function remains poorly understood.

In the present study, we have investigated the role of GluD1 in the development of excitatory and inhibitory synapses in the somato-sensory cortex. By depleting GluD1 in vivo in a few layer 2/3 cortical pyramidal neurons (CPNs) using sparse in utero electroporation (IUE), we demonstrate that GluD1 regulates the formation of inhibitory synapses in dendrites as well as inhibitory synaptic transmission. In contrast, GluD1 is dispensable for the formation and maintenance of excitatory synapses in CPNs. Using an in vivo structure/function analysis, we demonstrate that the regulation of inhibitory synapses by GluD1 requires trans-synaptic interaction via CblN4, an extracellular scaffolding protein secreted by somatostatin-expressing (SST+* interneurons (INs) (Favuzzi et al., 2019), activation of the receptor by its endogenous agonists glycine and D-Serine, and post-synaptic signaling via the intracellular C-terminal tail of the receptor. Using mass spectrometry, we characterize GluD1 interaction in developing synapses. We show that GluD1 serves as a hub for molecules implicated in inhibitory synaptogenesis, and we identify two major partners of GluD1, the signaling molecules guanine nucleotide exchange factor 12 (ARHGEF12) and protein phosphatase 1 regulatory subunit 12A (PPP1R12A), as critical regulators of inhibitory synapse formation in CPNs. Together, our results define a trans-synaptic signaling pathway centered on an atypical iGluR for the formation and specification of cortical inhibitory circuits.

RESULTS

GluD1 Is Selectively Required for the Formation of Inhibitory Synapses

In order to assess the role of GluD1 in synaptic development, we used cortex-directed IUE at embryonic day (E)15.5. IUE at E15.5 allows the sparse and specific modification of layer 2/3 CPNs in their intact environment and the dissection of cell autonomous mechanisms operating at synapses in vivo (Figure 1A). We analyzed the consequences of GluD1 depletion or overexpression on excitatory and inhibitory synapses formed on oblique apical dendrites of layer 2/3 CPNs of the somato-sensory cortex using a morphometric approach (Figure 1A). We first used dendritic spines, the postsynaptic site of the majority of excitatory synaptic inputs in the brain (Bourne and Harris, 2008; Yuste, 2013), and clusters of PSD-95, a major scaffolding protein of excitatory synapses (Sheng and Hoogenraad, 2007), as a proxy for excitatory synapses (Figure 1B). We found that GluD1 depletion using short hairpin RNAs (shRNAs) (shGluD1; Figure S1A) did not affect the density of dendritic spines in juvenile (postnatal day [P]20–22) or adult (P > 69) mice (102% ± 3% and 105% ± 5% of control in juvenile and adult neurons respectively; Figures 1B–1D) or the density of endogenous PSD-95 clusters visualized using EGFP-tagged fibronectin intrabodies generated with mRNA display (FingR) (Gross et al., 2013) (94% ± 5% of control; Figures 1E and 1F). GluD1 overexpression, however, decreased spine density to 75% ± 4% of the control value (Figures 1B and 1C). These results suggest that GluD1 is not necessary for the formation or maintenance of excitatory synapses in layer 2/3 CPNs, though GluD1 may constrain their number if upregulated.

We then assessed the role of GluD1 at inhibitory synapses. To that aim, we expressed small amounts of fluorescent (EGFP-tagged) gephyrin (Figure 1A), the core component of inhibitory postsynaptic scaffolds (Krueger-Burg et al., 2017; Tyagarajan and Fritzsch, 2014). This approach has been shown to reliably label inhibitory synaptic contacts without affecting synaptic development or inhibitory neurotransmission (Chen et al., 2012; Fossati et al., 2016; van Versendaal et al., 2012). In juvenile mice, GluD1 knockdown (KD) using shRNAs decreased the density of gephyrin clusters compared to control neurons (77% ± 4% of control; Figures 1G and 1H; Figure S2). Normal gephyrin cluster density was rescued by co-electroporating shGluD1 with a KD-resistant GluD1 construct (GluD1*; Figures 1G and 1H; Figure S1A). Remarkably, the decrease in gephyrin cluster density induced by GluD1 KD was maintained in adults (79% ± 4% of control; Figure 1I), indicating that the loss of inhibitory synapses was not compensated over time. To further substantiate the role of GluD1 at inhibitory synapses, we knocked out grid1 in single cells using the CRISPR-Cas9 system. We expressed...
Figure 1. Selective Control of Inhibitory Synapse Density by GluD1 in CPNs

(A) Sparse labeling of layer 2/3 CPNs after in utero electroporation (IUE) with soluble tdTomato (red) and EGFP-gephyrin (EGFP-GPHN, green). Arrowheads in the enlarged area highlight inhibitory synapses in oblique apical dendrites. E15.5, embryonic day 15.5; P22: postnatal day 22. Scale bars: 100 µm (left) and 5 µm (right).

(B) Segments of dendrites expressing shControl or shGluD1 or overexpressing (OE) GluD1 along with mVenus to visualize dendritic spines in juvenile mice. Scale bar: 2 µm.

(C and D) Quantification of dendritic spine density in juvenile (C) and adult mice (D). Juveniles: nshControl = 38, nshGluD1 = 22, nGluD1 OE = 26. Adults: nshControl = 15, nshGluD1 = 13.

(E) Segments of dendrites expressing shControl or shGluD1 along with PSD95.FingR-EGFP in juvenile mice. Dashed lines define the contours of tdTomato fluorescence. Scale bar: 2 µm.

(F) Quantification of PSD-95 cluster density. nshControl = 21, nshGluD1 = 24.

(G) EGFP-gephyrin clusters in representative segments of dendrites expressing shControl, shGluD1, or shGluD1 together with shGluD1-resistant GluD1* in juvenile mice. Scale bar: 2 µm.

(H and I) Quantifications of gephyrin cluster density in juvenile (H) and adult mice (I). Juveniles: nshControl = 41, nshGluD1 = 30, nGluD1 OE = 32. Adults: nshControl = 11, nshGluD1 = 30.

(J) Segments of dendrites illustrating the effects of Crispr-mediated Grid1 knockout (KO) and GluD1 OE on gephyrin cluster density. Ctrl sgRNA, control sgRNA; KO sgRNA, Grid1-targeting sgRNAs. Scale bar: 2 µm.

(K) Quantification of gephyrin cluster density in the conditions described above. nshControl = 22, nshGluD1 = 27, and nGluD1 OE = 26.

Statistics: bars indicate mean ± SEM, ns: p > 0.05, **p < 0.01, ***p < 0.001. One-way ANOVA test followed by Tukey’s post test in (C) and (H); unpaired t test in (D), (F), and (K); and Mann–Whitney test for the comparison of GluD1 OE in K with corresponding control (shControl) in (H).

The enhanced specificity espCas9(1.1) (Slaymaker et al., 2016) and a combination of two guide RNAs (gRNAs) using IUE. In grid1 knockout (KO) neurons, the density of gephyrin clusters was decreased by 22% ± 5% compared to control neurons expressing espCas9(1,1) with mismatched gRNAs (Figures 1 J and 1K), which is consistent with GluD1 KD experiments with shRNAs. In line with these results, GluD1 overexpression increased the density of gephyrin clusters along dendrites by 33% ± 4% (Figures 1J and 1K).

To test the physiological consequences of GluD1 inactivation on synaptic transmission, we performed whole-cell patch-clamp recording in electroporated GluD1-depleted neurons and in neighboring non-electroporated control neurons (Figure 2A). We compared miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs, respectively) in brain slices from juvenile mice (Figure 2B). In line with the morphological data, GluD1 KD did not affect the amplitude or the frequency of mEPSCs (98% ± 8% and 100% ± 4% of control, respectively) (Figures 2B–2D). In contrast, GluD1 KD slightly increased the amplitude of mIPSCs and decreased their frequency by = 35% (Figures 2B, 2E, and 2F), which is consistent with the reduced gephyrin cluster density observed in the oblique dendrites of GluD1 KD and KO neurons. We conclude that GluD1 in CPNs is selectively required for the formation of inhibitory synapses. It regulates both the...
assembly of the gephyrin-based postsynaptic scaffold and inhibitory synaptic transmission.

**GluD1 Localizes to Inhibitory Postsynaptic Sites**

It is unexpected for a member of the iGluR family to control the formation of inhibitory synapses. Therefore, we asked whether GluD1 accumulates at inhibitory synapses. To answer this question, we performed immunohistochemistry in brain slices from juvenile mice. GluD1 fluorescent puncta were frequently associated with gephyrin clusters in the upper layers of the somato-sensory cortex (Figure 3A). To determine the precise subcellular localization of GluD1, we employed immuno-electron microscopy (EM). In cortical layer 2/3, inhibitory synapses represent only 10% of the total number of synapses, and they are “symmetrical” when observed in EM, meaning that they do not show the electron-dense post-synaptic differentiation facilitating the detection of excitatory synapses. To unambiguously identify inhibitory synapses, we performed double immunostaining of the vesicular GABA transporter (VGAT) and GluD1 (Figure 3B). We used diaminobenzidine (DAB) to reveal
VGAT. DAB oxidation forms electron-dense precipitates that largely stained VGAT-positive inhibitory presynaptic terminals. To visualize and precisely localize GluD1 in cortical tissue, we used nanogold particles and silver enhancement. Nanogold particles corresponding to GluD1 were detected in front of VGAT-positive presynaptic terminals and in intracellular compartments in dendrites (Figure 3B). Within synapses, GluD1 was frequently observed in postsynaptic membrane domains located at the edge or in the periphery of the active zone, which is consistent with the distribution profile of other synaptic adhesion molecules (Triller and Choquet, 2003; Uchida et al., 1996). To quantify the proportion of synapses containing GluD1, we electroporated mOrange-tagged GluD1 together with EGFP-tagged GPHN or PSD95 FingRs and a soluble blue fluorescent protein to visualize neuronal morphology (Figure 3C, blue filler not shown). In oblique apical dendrites from P22 neurons in utero electroporated with GluD1-mOrange and GPHN.FingR-EGFP or PSD95.FingR-EGFP, Arrowheads display the association between GluD1 and indicated synaptic markers. Scale bar: 2 μm.

(D) Scatterplot showing the fraction of gephyrin and PSD-95 clusters associated with GluD1-mOrange puncta (association index). Gephyrin, n = 31; PSD-95, n = 30. Bars indicate mean ± SEM.

Inhibitory Synapse Formation Requires GluD1 Binding to Cbln and Activation by Glycine or D-Serine

To determine the molecular basis for GluD1-mediated regulation of inhibitory synapses, we took advantage of the recent crystallographic analysis of the interactions between GluD, Cbln, and neurexin and the abundant literature on the structure/function of GluD2 (Cheng et al., 2016; Elegheert et al., 2016; Kakegawa et al., 2007, 2009; Kuroyanagi and Hirano, 2010; Yuzaki and Aricescu, 2017). GluDs, as all members of the iGluR family, are tetrameric receptors (Traynelis et al., 2010). Each subunit has a modular architecture. The extracellular region contains a distal N-terminal domain (NTD), followed by an agonist-binding domain (ABD). The NTD of the GluD receptor interacts with the extracellular scaffolding protein Cbln (Matsuda et al., 2010; Uemura et al., 2010), and their ABD binds to glycine and D-serine (but not glutamate) (Naur et al., 2007), as in some N-methyl-D-aspartate (NMDA) receptor subunits (Traynelis et al., 2010). Each subunit has a modular architecture. The extracellular region contains a distal N-terminal domain (NTD), followed by an agonist-binding domain (ABD). The NTD of the GluD receptor interacts with the extracellular scaffolding protein Cbln (Matsuda et al., 2010; Uemura et al., 2010), and their ABD binds to glycine and D-serine (but not glutamate) (Naur et al., 2007), as in some N-methyl-D-aspartate (NMDA) receptor subunits (Traynelis et al., 2010). Each subunit has a modular architecture.

Neuron 104, 1081–1094, December 18, 2019
some phenotypes. Importantly, all mutants were properly trafficked to the cell surface (Figure S3A).

We first examined whether GluD1 function involves trans-synaptic interaction via Cbln. To that aim, we replaced endogenous GluD1 with a ΔNTD mutant lacking the whole NTD. In juvenile mice, gephyrin cluster density in neurons expressing this mutant was lower than in control (65% ± 4% of control), suggesting that the NTD is critical for GluD1 function (Figures 4A and 4C). We then specifically disrupted GluD1 interaction with Cbln by introducing two point mutations in the NTD (R341A/W343A, Figure S3B; residues corresponding to R345 and W347 in GluD2; Elegheert et al., 2016). Replacement of GluD1 with the R341A/W343A mutant also led to a lower density of gephyrin clusters (69% ± 4% of control; Figures 4A and 4C), indicating that GluD1 interaction with the extracellular scaffolding protein Cbln is required for inhibitory synapse formation. In contrast, preventing ion flux through the pore with a single point mutation (V617R) (Ady et al., 2014; Kakegawa et al., 2007; Robert et al., 2002) did not interfere with the formation of inhibitory synapses (Figures 4A and 4C). Collectively, these results demonstrate that the control of inhibitory synapse formation by GluD1 in CPNs requires trans-synaptic interactions via the CTD of the receptor.

GluD1 Specifies Synapses between SST+ INs and Layer 2/3 CPNs

We wondered if GluD1 mediates the formation of inhibitory synapses between layer 2/3 CPNs and specific classes of INs. In the cortex, distinct subtypes of INs express distinct isoforms of Cblns, with SST+ INs in upper cortical layers expressing Cbln4 and vasoactive intestinal peptide-positive (VIP+) INs expressing Cbln2 (Paul et al., 2017; Tasic et al., 2016). Therefore, we tested if Cbln2 and Cbln4 regulate the density of inhibitory synapses. To that end, adeno-associated viral vectors (AAVs) carrying an
shRNA directed against Cbln4, Cbln2, or a control shRNA (Figure S1D) were injected in vivo in the lateral ventricles of newborn pups previously electroporated in utero with EGFP-GPHN and tdTomato (Figures 5A and 5B). We then quantified the density of gephyrin clusters in sparse electroporated neurons surrounded by numerous infected cells (Figure 5B). In juvenile mice, Cbln4, but not Cbln2, inactivation significantly decreased the density of inhibitory synapses (85% ± 4% for shCbln4 and 102% ± 4% for shCbln2; Figure 5C). GluD1 interacted with Cbln4 (Figure 5D), and Cbln4 KD did not further decrease the density of gephyrin clusters in grid1 KO neurons (88% ± 5% of Grid1-KO/shCtrl neurons; Figure 5E), indicating that Cbln4 operates via GluD1. The role of Cbln4 at inhibitory synapses between Cbln4-expressing SST+ INs and CPNs has recently been characterized in more detail by Favuzzi et al. (2019). Taken together, these results indicate that GluD1 specifies inhibitory cortical connectivity by mediating synaptogenesis between Cbln4-expressing SST+ INs and CPNs. This is compatible with the partial colocalization of GluD1 and gephyrin (Figures 3C and 3D) in oblique apical dendrites, which are also contacted by other classes of interneurons (Fishell and Kepecs, 2019).

**Postsynaptic Signaling Controlling Inhibitory Synapse Formation**

To determine the postsynaptic mechanisms through which GluD1 regulates the formation of inhibitory synaptic machineries, we performed an unbiased proteomic screen aimed at identifying GluD1 interacting partners at synapses. We employed subcellular fractionations from P15 mouse brains to enrich our samples in proteins associated with synaptic membranes and efficiently immunoprecipitate GluD1 (Figure 6A). The proteins co-immunoprecipitated with GluD1 (gene name Grid1) were separated by liquid chromatography and identified using tandem mass spectrometry (LC-MS/MS) (Figures 6A and 6B; Table S1). We focused on the proteins that were the most represented in terms of the number of detections in LC-MS/MS biological triplicates relative to their molecular weight. GluD1 was strongly associated with regulators of GTPases (e.g., ARHGEF12 and SRGAP3) and regulators of protein phosphorylation (e.g., the serine/threonine phosphatase 1 regulatory subunit PPP1R12A, two pro-
implicated in building up inhibitory synapses. Neither the overexpression of a kinase-dead dominant-negative mutant of MRCKα (MRCKα KinD, with K106A substitution) (Leung et al., 1998) nor that of wild-type MRCKα (MRCKα WT) affected the density of gephyrin clusters (Figure 6D) in juvenile mice, indicating that MRCKα is not critical for the formation of inhibitory synapses. In contrast, depletion of PPP1R12A (also referred to as MYPT1), a targeting subunit of PP1, decreased the density of gephyrin clusters (74% ± 3% of the control), and normal gephyrin cluster density was rescued with the shRNA-resistant mutant PPP1R12A* (Figure 6E; Figure S1C), demonstrating that PPP1R12A is required for the formation of inhibitory synapses in dendrites. These data are in line with a previous study indicating that PP1 physically interacts with gephyrin and regulates the density of gephyrin clusters (Bausen et al., 2010).

Since both ARHGEF12 and PPP1R12A inactivation mimic GluD1 loss of function, our data suggest that GluD1 might signal through these two proteins to mediate the formation of inhibitory synapses. Using the CRISPR-Cas9 system combined with IUE to inactivate grid1 (Figures 1J and 1K), ppp1r12a, and arhgef12 (Figure 6F), we found that neither grid1/arhgef12 double KO nor grid1/ppp1r12a double KO further reduced the density of inhibitory synapses compared to single grid1 KO (104% ± 3% of Grid1-KO for Grid1-KO + Ppp1r12a-KO and 105% ± 4% of Grid1-KO for Grid1-KO + Arhgef12-KO; Figure 6G), indicating that GluD1 requires ARHGEF12 and PPP1R12A to operate at inhibitory synapses. Interestingly, other major partners of GluD1 (Figure 6B), such as the Rho GTPase-activating protein 32 (ARHGAP32/PX-RICS) (Nakamura et al., 2016; Uezu et al., 2016), SRCIN1 (p140cap) (Alfieri et al., 2017; Russo et al., 2019), and Ankyrin 3 (Ankyrin G) (Nelson et al., 2018), have been shown to interact with gephyrin and/or regulate inhibitory synaptogenesis. This supports the notion that GluD1 serves as a signaling hub for the formation and specification of inhibitory synapses (Figure 6H), and that the regulation of inhibitory synaptogenesis is a major function of GluD1 in the neocortex.

**DISCUSSION**

In the present study, we employed sparse in vivo molecular manipulations and proteomic approaches to characterize the role of GluD1 in synaptic development in layer 2/3 pyramidal neurons of the soma-sensory cortex. We demonstrate that GluD1 is a maverick among iGluRs, operating at inhibitory synapses rather than excitatory synapses. GluD1 is necessary for
the formation of specific inhibitory synapses along dendrites and regulates GABAergic synaptic transmission accordingly. In the upper layers of the cortex, GluD1 is enriched in the postsynaptic membrane of inhibitory synapses, lateral to the active zone, where it establishes trans-synaptic interactions via Cbln4, an extracellular scaffolding protein secreted by SST+ INs (Favuzzi et al., 2019), which, in turn, binds to presynaptic neurexins (Yuzuki, 2017; Zhong et al., 2017). When interacting with Cbln4 and binding to glycine or D-serine, GluD1 activates postsynaptic signaling pathways that do not depend on ion flux through its channel, but it involves intracellular interactions via its C-terminal tail, organizing the assembly of inhibitory postsynaptic machineries at contact sites with SST+ INs. These interactions involve ARHGEF12 and PPP1R12A, two molecules required for GluD1 function at inhibitory synapses and probably other molecules implicated at GABAergic synapses.

Region-Specific Function of GluD1 at Inhibitory Synapses

Although the repertoire of inhibitory synaptic proteins has recently expanded (Krueger-Burg et al., 2017), the molecular diversity of inhibitory synapses and the difficulty of investigating their biochemistry and their cell biology in vivo has obscured the mechanistic understanding of inhibitory synaptogenesis. Hence, few CAMs and signaling molecules have been shown to selectively control inhibitory synapse assembly (Krueger-Burg et al., 2017; Tyagarajan and Fritschy, 2014). These molecules show domain-specific functions at perisomatic (Früh et al., 2016; Poulopoulos et al., 2009), dendritic (Li et al., 2017), or axo-axonic synapses (Panzanelli et al., 2011). Their function also varies depending on brain areas. In particular, extensive studies of neuroligin 2 and collybistin (ARHGEF9), two proteins present in virtually all inhibitory synapses in the CNS, have highlighted fundamental differences between the cell biology of hippocampal and cortical inhibitory synapses (Gibson et al., 2009; Poulopoulos et al., 2009; Papadopoulos et al., 2007). Since the mechanisms of synaptogenesis in the forebrain are predominantly studied in hippocampal neurons, the molecular underpinning of inhibitory synapse formation in the cortex has remained enigmatic.

Previous in vitro hemi-synapse formation assays suggested a synaptogenic activity of GluD1 in cortical neurons (Ryu et al., 2012; Yasumura et al., 2012). However, it was unclear whether the synaptogenic activity was selective for inhibitory synapses (Yasumura et al., 2012) or common to excitatory and inhibitory synapses (Ryu et al., 2012), and the role of endogenous GluD1 in cortical neurons in vivo remained unclear (Gupta et al., 2015). Here, we used in vivo single cell approaches based on sparse IUE to manipulate GluD1 expression and function in isolated layer 2/3 CPNs in the intact brain. Targeting a specific cell type allowed us to investigate a relatively homogeneous population of neurons and dissect cell-autonomous mechanisms with a subcellular resolution in spatially identified synapses along the dendritic tree. Moreover, sparse in vivo manipulations help avoid compensatory and adaptive changes at the network level, which might occur in KO mouse models. Our results provide direct evidence that GluD1 is necessary for the formation of specific cortical inhibitory synapses in vivo. While we do not exclude that GluD1 could regulate some properties of excitatory synapses in the cortex, we clearly show that GluD1 is not required for their formation, which is consistent with a previous study (Gupta et al., 2015). Therefore, GluD1 function in CPNs starkly contrasts with GluD1 function in cerebellar INs (Konno et al., 2014) and hippocampal pyramidal neurons (Tao et al., 2018), where GluD1 is required for the formation of excitatory synapses. This raises fundamental questions on the molecular basis underlying the region-specific function of GluD1 at excitatory or inhibitory synapses and the synaptic dysfunction associated with GluD1 mutations in brain disorders (Cooper et al., 2011; Fallin et al., 2005; Glessner et al., 2009; Guo et al., 2007; Treutlein et al., 2009).

An iGluR-Dependent Signaling Pathway at Inhibitory Synapses

It is unconventional for a member of the iGluR family to locate and operate at inhibitory synapses. We show that trans-synaptically engaged GluD1 binding to glycine or D-serine initiates postsynaptic signaling via non-ionotropic mechanisms, probably through conformational changes that are transmitted through the TMD, and controls intracellular interactions (Elegeheert et al., 2016). Whether GluD1 binds to glycine or D-serine in CPNs is unknown, but both glycine and D-serine may contribute to the regulation of inhibitory synapse formation. Glycine is present in the extracellular space, where it activates extrasynaptic NMDA receptors (Papouin et al., 2012) and mediates tonic inhibition in layer 2/3 CPNs (McCracken et al., 2017; Salling and Harrison, 2014). In the brainstem and spinal cord, where glycine is a major inhibitory neurotransmitter and GluD1 is highly expressed (https://www.gtexportal.org/home/gene/GRID1), presynaptic release of glycine may directly regulate the formation and maintenance of inhibitory synapses. D-serine is also present in the extracellular environment. It is synthesized through conversion of L-serine by serine racemase and released at least by the neuronal alanine-serine-cysteine transporter 1 (Asc-1) (Rosenberg et al., 2013). Ambient D-serine level is regulated by excitatory glutamatergic activity (Van Horn et al., 2017; Ma et al., 2014), and low D-serine levels are associated with epilepsy (Klatte et al., 2013) and schizophrenia-like behaviors (Ma et al., 2013), consistent with defects in synaptic inhibition and GluD1 function. So far, in the forebrain, the role of D-serine and, to some extent, glycine has been envisioned through the activation of NMDA receptors (Oliet and Mothet, 2009). The role of GluD1 in establishing the equilibrium between excitation and inhibition and the requirement of GluD1 activation by glycine/D-serine suggests that some functions initially attributed to NMDA receptors might instead depend on GluD1 signaling.

Proteomic and functional analyses of GluD1 interactome allowed us to identify signaling pathways controlling the postsynaptic organization of inhibitory machineries. We found that two major partners of GluD1, ARHGEF12 and PPP1R12A, are necessary for inhibitory synapse formation in layer 2/3 CPNs. Double inactivation experiments showed that ARHGEF12 and PPP1R12A operate in the same pathway as GluD1 and therefore also contribute to the specification of inhibitory connectivity between SST+ INs and layer 2/3 CPNs. ARHGEF12 contains a Dbl-homology (DH) domain mediating guanosine diphosphate
trans-synaptic interaction and signaling pathways coordinating the establishment of neocortical inhibitory circuitry, their interplay, their evolution in human, and their dysregulations in brain disorders will be a fertile area for future research.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **LEAD CONTACT AND MATERIALS AVAILABILITY**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Animals
  - Primary cultures of mouse cortical neurons
  - HEK293T cells

**METHOD DETAILS**

- Plasmids for protein expression
- shRNA and Crispr constructs and shRNA validation
- Lentivirus production and infection
- In utero electroporation, AAV injection and slice preparation
- Immunohistochemistry for confocal microscopy
- Confocal image acquisition
- Electron microscopy
- Electrophysiology
- Transfection and western blotting
- Cell surface biotinylation
- Subcellular fractionation
- Immunoprecipitation
- Proteomics

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2019.09.027.

**ACKNOWLEDGMENTS**

We thank members of A. Triller’s laboratory (IBENS, Paris, France), D. Stroebe, P. Paoletti, M. Casado, F. Selimi, and F. Polleux, for helpful discussions and comments on the manuscript; P. Rostaing for his help with electron microscopy; L. Tricoire for kindly providing anti-GluD1 antibody and HA-GluD1 plasmid; and B. Roska, D.B. Arnold, and F. Zhang for plasmids available through Addgene. This work was supported by INSERM, the Agence Nationale de la Recherche (ANR-13-PDOC-0003 and ANR-17-ERC3-0009 to C.C.), the European Research Council (ERC starting grant 803704 to C.C.), the Fyssen Foundation (to C.C.), the Research University, “Investments for the future”: NERF 2011-45; FRM DGE 20111123023; and FRC Rotary International France, the IBENS acute transgenesis facility, and the imaging unit of the Humanitas Clinical and Research Center.

**AUTHOR CONTRIBUTIONS**

M.F., C.C., N.A., O.G., and S.C. performed experiments and analyzed data. F.D. carried out the MS experimental work, G.A. analyzed MS data, and D. L. supervised MS and data analysis. M.F. and C.C. designed the study, interpreted the results, prepared the figures, and wrote the manuscript.

**DECLARATION OF INTEREST**

The authors declare no competing interests.
Hayashi, T., Umemori, H., Mishina, M., and Yamamoto, T. (1999). The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. Nature 397, 72–76.

Hepp, R., Hay, Y.A., Aguado, C., Lujan, R., Daunhinton, L., Potier, M.C., Nomura, S., Poirel, O., El Mestikawy, S., Lambolez, B., and Tricoire, L. (2015). Glutamate receptors of the delta family are widely expressed in the adult brain. Brain Struct. Funct. 220, 2797–2815.

Hyvönen, M., Macias, M.J., Nilges, M., Oschkinat, H., Saraste, M., and Wilmanns, M. (1995). Structure of the binding site for inositol phosphates in serine racemase binding elicits schizophrenia-like behavior via D-serine deple-

Jang, S., Lee, H., and Kim, E. (2017). Synaptic adhesion molecules and excitatory synaptic transmission. Curr. Opin. Neurobiol. 45, 45–50.

Kakegawa, W., Kohda, K., and Yuzaki, M. (2007). The δ2 ‘ionotropic’ glutamate receptor functions as a non-ionotropic receptor to control cerebellar synaptic plasticity. J. Physiol. 584, 89–96.

Kakegawa, W., Miyazaki, T., Emi, K., Matsuda, K., Kohda, K., Motohashi, J., Mishina, M., Kawahara, S., Watanabe, M., and Yuzaki, M. (2008). Differential regulation of synaptic plasticity and cerebellar motor learning by the C-terminal PDZ-binding motif of GluDeltaz2. J. Neurosci. 28, 1460–1468.

Kakegawa, W., Miyazaki, T., Kohda, K., Matsuda, K., Emi, K., Motohashi, J., Watanabe, M., and Yuzaki, M. (2009). The N-terminal domain of GluD2 (GluDeltaz2) recruits presynaptic terminals and regulates synaptogenesis in the cerebellum in vivo. J. Neurosci. 29, 5738–5748.

Kakegawa, W., Miyoshi, Y., Hamase, K., Matsuda, S., Matsuda, K., Kohda, K., Emi, K., Motohashi, J., Konno, K., Zatsu, K., and Yuzaki, M. (2011). D-serine regulates cerebellar LTD and motor coordination through the δ2 glutamate receptor. Nat. Neurosci. 14, 603–611.

Klatte, K., Kirschstein, T., Otte, D., Pothmann, L., Muller, L., Tokay, T., Kober, M., Uebachs, M., Zimmer, A., and Beck, H. (2013). Impaired D-serine-mediated cotransmission mediates cognitive dysfunction in epilepsy. J. Neurosci. 33, 13066–13080.

Kohda, K., Kakegawa, W., Matsuda, S., Yamamoto, T., Hirano, H., and Yuzaki, M. (2013). The δ2 glutamate receptor gates long-term depression by coordinating interactions between two AMPA receptor phosphorylation sites. Proc. Natl. Acad. Sci. USA 110, E948–E957.

Konno, K., Matsuda, K., Nakamoto, C., Uchigashima, M., Miyazaki, T., Yamasaki, M., Sakimura, K., Yuzaki, M., and Watanabe, M. (2014). Enriched expression of GluD1 in higher brain regions and its involvement in parallel fiber-interneuron synapse formation in the cerebellum. J. Neurosci. 34, 7412–7424.

Kruenger-Burg, D., Papadopoulos, T., and Brose, N. (2017). Organizers of inhibitory synapses come of age. Curr. Opin. Neurobiol. 45, 66–77.

Kuroyanagi, T., and Hirano, T. (2010). Flap loop of GluD2 binds to Cbln1 and regulates cerebellar LTD and motor coordination through the δ2 glutamate receptor. Nat. Neurosci. 13, 4959–4980.

Ma, T.M., Paul, B.D., Fu, C., Hu, S., Zhu, H., Blackshaw, S., Wolosker, H., and Snyder, S.H. (2014). Serine racemase regulated by binding to stargazin and PSD-95: potential N-methyl-D-aspartate–α-amino-3-hydroxy-5-methyl-4-isoxazo-lopipionic acid (NMDA-AMPA) glutamate neurotransmission cross-talk. J. Biol. Chem. 289, 29631–29641.

Matsuda, K., Miura, E., Miyazaki, T., Kakegawa, W., Emi, K., Narumi, S., Fukazawa, Y., Ito-Ishida, A., Kondo, T., Shigemoto, R., et al. (2010). Cbln1 is a Ligand for an Orphan Glutamate Receptor 2, a Bidirectional Synapse Organizer. Science 328, 363–368.

Matsuda, K., Bucisantoso, T., Mitakidis, N., Sugaya, Y., Miura, E., Kakegawa, W., Yamasaki, M., Konno, K., Uchigashima, M., Abe, M., et al. (2016). Transynaptic Modulation of Kainate Receptor Functions by C1q-like Proteins. Neuron 90, 752–767.

McAllister, A.K. (2007). Dynamic aspects of CNS synapse formation. Annu. Rev. Neurosci. 30, 425–450.

McCracken, L.M., Lowes, D.C., Salling, M.C., Carreau-Vollmer, C., Odean, N.N., Blednov, Y.A., Betz, H., Harris, R.A., and Harrison, N.L. (2017). Glycine receptor α3 and α2 subunits mediate tonic and exogenous agonist-induced currents in forebrain. Proc. Natl. Acad. Sci. USA 114, E7179–E7186.

Missaler, M., Sudoku, T.C., and Biederer, T. (2012). Synaptic cell adhesion. Cold Spring Harb. Perspect. Biol. 4, a005694–a005694.

Mondin, M., Labrousse, V., Hosy, E., Heine, M., Tessier, B., Levet, F., Poujol, C., Blanchet, C., Choquet, D., and Thomine, O. (2011). Neurexin-neuroligin adhesions capture surface-diffusing AMPA receptors through PSD-95 scaffold. J. Neurosci. 31, 13500–13515.

Mulkins, C., Fishell, G., and Tsien, R.W. (2016). Unifying Views of Autism Spectrum Disorders: A Consideration of Autoregulatory Feedback Loops. Neuron 89, 1131–1156.

Nabavi, S., Kessels, H.W., Alfonso, S., Aow, J., Fox, R., and Malinow, R. (2013). Metabotropic NMDA receptor function is required for NMDA receptor-dependent long-term depression. Proc. Natl. Acad. Sci. USA 110, 4027–4032.

Nakamura, T., Arima-Yoshida, F., Sakaue, F., Nasu-Nishimura, Y., Takeda, Y., Matsuura, K., Akshoomoff, N., Mattson, S.N., Grossfeld, P.D., Manabe, T., and Akiyama, T. (2016). PX-RICS-deficient mice mimic autism spectrum disorder in Jacobsen syndrome through impaired GABAA receptor trafficking. Nat. Commun. 7, 10861.

Nan, C.I., and Chen, L. (2005). Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. Proc. Natl. Acad. Sci. USA 102, 6137–6142.

Naur, P., Hansen, K.B., Kristensen, A.S., Dravid, S.M., Pickering, D.S., Olsen, L., Vestergaard, B., Egebjerg, J., Gajhede, M., Traynelis, S.F., and Kastrup, J.S. (2007). Ionotropic glutamate-like receptor delta2 binds D-serine and glycine. Proc. Natl. Acad. Sci. USA 104, 14116–14121.

Nelson, S.B., and Valakh, V. (2015). Excitatory/Inhibitory Balance and Circuit Homeostasis in Autism Spectrum Disorders. Neuron 87, 684–698.

Nelson, A.D., Caballero-Florán, R.N., Rodríguez Díaz, J.C., Hull, J.M., Yuan, Y., Li, J., Chen, K., Walder, K.K., Lopez-Santiago, L.F., Bennett, V., et al. (2018). Ankyrin-G regulates forebrain connectivity and network synchronization via interaction with GABARAP. Mol. Psychiatry. Published online November 30, 2018. https://doi.org/10.1038/s41380-018-0308-x.

Okada, H., Oueu, A., Mason, F.M., Soderblom, E.J., Moseley, M.A., 3rd, and Bennett, V., et al. (2018). Ankyrin-G regulates forebrain connectivity and network synchronization via interaction with GABARAP. Mol. Psychiatry. Published online November 30, 2018. https://doi.org/10.1038/s41380-018-0308-x.

Panzanelli, P., Gunn, B.G., Schlatter, M.C., Benke, D., Tagarajna, S.K., Scheiffele, P., Belelli, D., Lambert, J.J., Rudolph, U., and Fritschy, J.-M. (2011). Distinct mechanisms regulate GABAA receptor and gephyrin clustering at perisomatic and axo-axonic synapses on CA1 pyramidal cells. J. Physiol. 589, 4959–4980.
Paolelli, P., Bellone, C., and Zhou, Q. (2013). NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. Nat. Rev. Neurosci. 14, 383–400.

Papadopoulos, T., Korte, M., Eulenburg, V., Kubota, H., Retounskaias, M., Harvey, R.J., Harvey, K., O’Sullivan, G.A., Laube, B., Hülsmann, S., et al. (2007). Impaired GABAergic transmission and altered hippocampal synaptic plasticity in collybistin-deficient mice. EMBO J. 26, 3888–3899.

Papouin, T., Ladépéché, L., Ruel, J., Sacchi, S., Labasque, M., Hanini, M., Groc, L., Pollegioni, L., Mothet, J.-P., and Oliet, S.H.R. (2012). Synaptic and extrasynaptic NMDA receptors are gated by different endogenous coagonists. Cell 150, 633–646.

Paul, A., Crow, M., Raudales, R., He, M., Gillis, J., and Huang, Z.J. (2017). Transcriptional Architecture of Synaptic Communication Delinates GABAergic Neuron Identity. Cell 171, 522–539.e20.

Poulet, F., Carpentier, S., and Barillot, E. (2007). myProMS, a web server for management and validation of mass spectrometry-based proteomic data. Proteomics 7, 2553–2556.

Poulopoulos, A., Aramuni, G., Meyer, G., Soykan, T., Hoon, M., Papadopoulos, T., Zhang, M., Paarmann, I., Fuchs, C., Harvey, K., et al. (2009). Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. EMBO J. 28, 628–646.

Pribiag, H., and Stellwagen, D. (2013). TNF-z downregulates inhibitory neurotransmission through protein phosphatase 1-dependent trafficking of GABA(A) receptors. J. Neurosci. 33, 15879–15893.

Robert, A., Hyde, R., Hughes, T.E., and Howe, J.R. (2002). The expression of the glutamate receptor ion channels: structure, regulation, and function. Pharmacol. Rev. 62, 405–496.

Trembleau, A., Morales, M., and Bloom, F.E. (1994). Aggregation of vasoressin mRNA in a subset of axonal swellings of the median eminence and posterior pititary: light and electron microscopic evidence. J. Neurosci. 14, 39–53.

Trueblin, M., Muhleisen, T.W., Frank, J., Matthesien, M., Herms, S., Ludwig, K.U., Treutlein, T., Schmael, C., Strohmalier, J., Bösshenz, K.V., et al. (2009). Dissection of phenotype reveals possible association between schizophrenia and Glutamate Receptor Delta 1 (GRIK1) gene promoter. Schizophr. Res. 111, 123–130.

Triller, A., and Choquet, D. (2003). Synaptic structure and diffusion dynamics of synaptic receptors. Biol. Cell 95, 465–476.

Tyagarajan, S.K., and Fritschy, J.-M. (2014). Gephyrin: a master regulator of neuronal function? Nat. Rev. Neurosci. 15, 141–156.

Uchida, N., Honjo, Y., Johnson, K.R., Wheelock, M.J., and Takeichi, M. (1996). The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones. J. Cell Biol. 135, 767–779.

Uemura, T., Lee, S.-J., Yasumura, M., Takeuchi, T., Yoshida, T., Ra, M., Taguchi, R., Sakimura, K., and Mishina, M. (2010). Trans-synaptic interaction of GluRdelta2 and Neurexin through Cbln1 mediates synapse formation in the cerebellum. Cell 141, 1088–1097.

Uezu, A., Kanak, D.J., Bradshaw, T.W.A., Soderblom, E.J., Catavero, C.M., Burette, A.C., Weinberg, R.J., and Soderling, S.H. (2016). Identification of an elaborate complex mediating postsynaptic inhibition. Science 353, 1123–1129.

Van Horn, M.R., Strasser, A., Miraucourt, L.S., Pollegioni, L., and Ruthazer, E.S. (2017). The Gliotransmitter d-Serine Promotes Synapse Maturation and Axonal Stabilization In Vivo. J. Neurosci. 37, 6277–6288.

van Versendaal, D., Rajendran, R., Saeipour, M.H., Klooster, J., Smit-Rigter, L., Sommerjeier, J.P., De Zeeuw, C.I., Hofer, S.B., Heimel, J.A., and Levine, C.N. (2012). Elimination of inhibitory synapses is a major component of adult ocular dominance plasticity. Neuron 74, 374–383.

Vizcaino, J.A., Csordas, A., Del-Toro, N., Dianes, J.A., Grijss, J., Lavidas, I., Mayer, G., Perez-Riverol, Y., Reisinger, F., Ternent, T., et al. (2016). 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 44, D447–D456.

Wertz, A., Trenholm, S., Yonehara, K., Hillier, D., Raics, Z., Leinweber, M., Szalay, G., Ghanem, A., Keller, G., Rozsa, B., Conzelmann, K.K., and Roska, B. (2015). Single-cell-initiated monosynaptic tracing reveals layer-specific cortical network modules. Science 349, 70–74.

Yasumura, M., Yoshida, T., Lee, S.-J., Uemura, T., Joo, J.-Y., and Mishina, M. (2012). Glutamate receptor δ1 induces preferentially inhibitory presynaptic differentiation of cortical neurons by interacting with neurexins through cebellrin precursor protein subtypes. J. Neurochem. 121, 705–716.
Yuste, R. (2013). Electrical compartmentalization in dendritic spines. Annu. Rev. Neurosci. 36, 429–449.

Yuzaki, M. (2017). The C1q complement family of synaptic organizers: not just complementary. Curr. Opin. Neurobiol. 45, 9–15.

Yuzaki, M. (2018). Two Classes of Secreted Synaptic Organizers in the Central Nervous System. Annu. Rev. Physiol. 80, 243–262.

Yuzaki, M., and Aricescu, A.R. (2017). A GluD Coming-Of-Age Story. Trends Neurosci. 40, 138–150.

Zhong, C., Shen, J., Zhang, H., Li, G., Shen, S., Wang, F., Hu, K., Cao, L., He, Y., and Ding, J. (2017). Cbln1 and Cbln4 Are Structurally Similar but Differ in GluD2 Binding Interactions. Cell Rep. 20, 2328–2340.

Ziv, N.E., and Fisher-Lavie, A. (2014). Presynaptic and postsynaptic scaffolds: dynamics fast and slow. Neuroscientist 20, 439–452.

Zoghbi, H.Y., and Bear, M.F. (2012). Synaptic dysfunction in neurodevelopmental disorders associated with autism and intellectual disabilities. Cold Spring Harb. Perspect. Biol. 4, a009886–a009886.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse anti-HA       | Covance | Cat# MMS-101P; RRID:AB_2314672 |
| Rabbit anti-GFP     | Thermo Fisher Scientific | Cat# A-6455; RRID:AB_221570 |
| Rabbit anti-Myc     | Cell Signaling Technology | Cat# 2272; RRID:AB_10692100 |
| Mouse anti-Myc      | Cell Signaling Technology | Cat# 2276; RRID:AB_331783 |
| Rabbit anti-GluD1   | Hepp et al., 2015 | N/A |
| Mouse anti-gephyrin | Synaptic Systems | Cat# 147 011; RRID:AB_887717 |
| Rabbit anti-Arhgef12| Thermo Fisher Scientific | Cat# PA5-39418; RRID:AB_2556008 |
| Rabbit anti-Ppp1r12a| Thermo Fisher Scientific | Cat# PA5-17164; RRID:AB_10978517 |
| Rabbit anti-RFP     | Rockland | Cat# 600-401-379; RRID:AB_2209751 |
| Rabbit anti-Srgap2  | Charrier et al., 2012 | N/A |
| Rabbit anti-GAPDH   | Synaptic Systems | Cat# 247 002; RRID:AB_10804053 |
| Mouse anti-VGAT     | Synaptic Systems | Cat# 131 011; RRID:AB_887872 |
| Goat anti-mouse HRP | Jackson ImmunoResearch Labs | Cat# 115-035-003; RRID:AB_10015289 |
| Goat anti-rabbit HRP| Jackson ImmunoResearch Labs | Cat# 111-035-144; RRID:AB_2307391 |
| Goat anti-rabbit Alexa 488 | Thermo Fisher Scientific | Cat# A27034; RRID:AB_2536097 |
| Goat anti-mouse Cy3 | Jackson ImmunoResearch Labs | Cat# 115-165-003; RRID:AB_2338680 |
| Goat biotinylated anti-mouse | Vector Laboratories | Cat# BA-9200; RRID:AB_2336171 |
| Nanogold goat anti-rabbit | Nanoprobes | Cat# 2003; RRID:AB_2687591 |
| **Bacterial and Virus Strains** |        |            |
| AAV2/1 H1-shControl.Syn-TagBFP | This paper | N/A |
| AAV2/1 H1-shCbln2.Syn-TagBFP | This paper | N/A |
| AAV2/1 H1-shCbln4.Syn-TagBFP | This paper | N/A |
| Lentivirus H1-shControl.Syn-EGFP | This paper | N/A |
| Lentivirus H1-shGluD1.Syn-EGFP | This paper | N/A |
| Lentivirus H1-shPpp1r12a.Syn-EGFP | This paper | N/A |
| Lentivirus H1-shPpp1r12a.Syn-EGFP | This paper | N/A |
| Lentivirus H1-shSrgap2.Syn-EGFP | This paper | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Tetrodotoxin        | Abcam   | Cat# ab120055 |
| NBQX                | Abcam   | Cat# ab120046 |
| D-AP5               | Abcam   | Cat# ab120003 |
| Gabazine            | Abcam   | Cat# ab120042 |
| **Experimental Models: Cell Lines** |        |            |
| HEK293T             | ATCC    | Cat# CRL-1573; RRID:CVCL_0045 |
| **Experimental Models: Organisms/Strains** |        |            |
| Mouse: Swiss/CD-1   | Janvier Labs | N/A |
| Mouse: C57BL/6J     | Janvier Labs | N/A |
| **Oligonucleotides** |        |            |
| shRNA oligos, see methods detail | This paper | N/A |
| gRNA oligos, see methods detail | This paper | N/A |
| **Recombinant DNA** |        |            |
| pCAG HA-GluD1       | This paper | N/A |
| pCAG HA-GluD1-mOrange | This paper | N/A |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Cécile Charrier (cecile.charrier@ens.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All animals were handled according to French and EU regulations (APAFIS#:1530-201508261508691v3). In utero electroporations were performed on pregnant Swiss females at E14.5-15.5 (Janvier labs). For viral injections in the lateral ventricles, newborn pups (P0) of undetermined sex were used. Primary cultures were prepared from timed pregnant C57BL/6J mice at E18.5 (Janvier labs). Juveniles correspond to mice between P20 and P22. Adults correspond to mice between P69 and P75. Mice were maintained in a 12 hr light/dark cycle with unlimited access to food and water.

Primary cultures of mouse cortical neurons

Primary cultures were performed as described previously (Charrier et al., 2012) with few modifications. After dissection and dissociation of mouse cortices from E18.5 embryos, neurons were plated on glass coverslips coated with poly-D-ornityline (80 μg/ml, Sigma) in MEM supplemented with sodium pyruvate, L-glutamine and 10% horse serum. Medium was changed 2-3 h after plating with Neurobasal supplemented with L-glutamine (2 mM), B27 (1X) and penicillin (2.5 units/ml) - streptomycin (2.5 μg/ml). Then, one third of the medium was changed every 5 days. Unless otherwise indicated, all products were from Life Technologies. Cells were maintained at 37°C in 5% CO2 until use.

HEK293T cells

HEK293T (CRL-1573 from ATCC) cells were cultured according to suggested protocols. Briefly, cells were maintained in DMEM (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and 1% Penicillin- Streptomycin (GIBCO) at 37°C, 5% CO2, and passaged by trypsin/EDTA digestion (GIBCO) upon reaching confluency.

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| pCAG_PSD95.FingR-EGFP-CCR5TC | Gross et al., 2013 | RRID:Addgene_46295 |
| pCAG_GPHN.FingR-EGFPCCRSTC | Gross et al., 2013 | RRID:Addgene_46296 |
| Mouse Ppp1r12a cDNA | Dharmacon | Cat# MMM1013-211691718 |
| Human MRCKα cDNA | Dharmacon | Cat# MHS6278-213663984 |
| pCDNA3 mRFP-ARHGEF12 | Bodmann et al., 2017 | N/A |
| Mouse Cbln2 cDNA | Dharmacon | Cat# MMM1013-202798518 |
| Mouse Cbln4 cDNA | Dharmacon | Cat# MMM1013-20279844 |
| pCAG 3xMyc-MRCKα | This paper | N/A |
| pCAG 3xMyc-Ppp1r12a | This paper | N/A |
| pCAG ARHGEF12 | This paper | N/A |
| pCAG Myc-Cbln2 | This paper | N/A |
| pCAG Myc-Cbln4 | This paper | N/A |
| pH1SCV2 vectors | Charrier et al., 2012 | N/A |
| pH1SCTdT2 vectors | Fossati et al., 2016 | N/A |
| Lenti H1-shRNA.Syn-EGFP vectors | This paper | N/A |
| pAAV H1-shRNA.Syn-TagBFP vectors | This paper | N/A |
| eSpCas9(1.1) vectors | Slaymaker et al., 2016 | RRID:Addgene_71814 |

Software and Algorithms

- GPP sgRNA Designer: Broad Institute, https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design
- myProMS v3.6: Poullet et al., 2007, http://bioinfo-out.curie.fr/myproms/proms.html
- Fiji: Schindelin et al., 2012, https://fiji.sc/; RRID:SCR_002285
- pCLAMP10: Molecular Devices, RRID:SCR_011323
- Prism 7: GraphPad, RRID:SCR_002798
METHOD DETAILS

Plasmids for protein expression

EGFP-GPHN was previously described (Fossati et al., 2016) and it was used to visualize inhibitory synapses. HA-tagged mouse grid1 (gift from Ludovic Tricoire, IBPS, Paris, France) was inserted into pCAG vector by PCR between XhoI and BsrGI to obtain pCAHA GluD1 or between XhoI and KpnI to obtain pCAHA GluD1-EGFP. EGFP was then replaced by mOrange between KpnI and NotI to generate pCAHA GluD1-mOrange. pCAG_PSD95.FingR-EGFP-CCRSTC and pCAG_GPHN.FingR-EGFP-CCRSTC were purchased from Addgene (plasmids #46295 and #46296, respectively). Mouse cDNA for Cbln1 (for Cbln2) or of Cbln4 (for Cbln4) and one Myc tag between AgeI and AgeI. The three constructs were Myc-tagged at the N terminus by inserting a DNA cassette containing a start codon, the signal sequence of Cbln1 (for Cbln2) or of Cbln4 (for Cbln4) and one Myc tag between EcoRI and AgeI.

For in utero knockdown experiments with shRNAs, we used the previously described pH1SCV2 and pH1SCTdT2 vectors (Charrier et al., 2012). An H1 promoter drives the expression of the shRNA and a CAG promoter that of myristoylated Venus (mVenus) or TdTomato, respectively. The vector pH1SCV2 was used for dendritic spine analysis, pH1SCTdT2 was co-expressed with EGFP to analyze inhibitory synapses. For shRNA validation on endogenous mouse grid1, arhgef12 and ppp1r12a, we used a lentiviral vector carrying the H1 promoter to drive shRNA expression and the synapsin promoter to drive EGFP expression (Fossati et al., 2016). For AAV-mediated in vivo knockdown of cbin2 and cbin4 the plasmid pAAV-eF1α-tdTomato-WPRE-pGHPa (Addgene, plasmid #67527; Wertz et al., 2015) was modified as follows: a DNA cassette containing the shRNA with the H1 promoter and EGFP with the synapsin promoter was amplified by PCR from the lentiviral vector described above and inserted into pAAV-eF1α-tdTomato-WPRE-pGHPa between MluI and EcoRI. EGFP was then replaced by TagBFP between EcoRI and NheI. AAVs (serotype 2/1) were produced by the Centre of vector production of INSERM (CPV, UMR1089, Nantes, France). shRNA (shControl) was described previously (Charrier et al., 2012). The following shRNAs targeted the corresponding seed sequences: shGluD1: 5′-GAAGATAGCTCAATCTTAT-3′; shArhgef12: 5′-GCAAGCCTGTTTCAAGAGATTG-3′; shPpp1r12a: 5′-GTGAATCTAGTG-3′; shCbln2: 5′-GTTATTGACAGAATGCT-3′; shCbln4: 5′-GCGTTCTGCTATTAGTG-3′; shPpp1r12a: 5′-GCCGTTCTGCTATTAGTG-3′. ShRNAs were validated as previously described (Charrier et al., 2012). Briefly, HEK293T cells were co-transfected with mouse HA-GluD1, Myc-Ppp1r12a, Myc-Cbln2, Myc-Cbln4 or human RFP-Arhgef12 together with the corresponding shRNA at 1:2 ratio. Two days after transfection, cells were collected and lysed in RIPA buffer (150 mM NaCl, 1.0% NP–40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Sigma-Aldrich) and further processed for western blot analysis of the relative protein expression levels. The knockdown of endogenous grid1, arhgef12 and ppp1r12a was further validated in primary cultures of cortical neurons infected with lentiviral vectors. Neurons infected at DIV (days in vitro) 4 were harvested at DIV21 and lysed in RIPA buffer under agitation for 1 h at 4 °C. 20 μg of total proteins for neurons infected with shGluD1 and shArhgef12 or 40 μg of total proteins for neurons infected with shPpp1r12a were separated by SDS-PAGE and further processed for western blot analysis. For rescue experiments, four point silent mutations were introduced in grid1 (c11939t_c11940c_c1195 g_a1196c), ppp1r12a (c1355t_a1356t_g1357c_t1358a) and ARHGEF12 (g1119a_a1120t_g1121c_c1122a) to resist to shRNA-mediated knockdown (mutants named GluD1*, Ppp1r12a* and Arhgef12*, respectively). To knock out grid1, arhgef12 and ppp1r12a with Crispr, we used an engineered spCas9 with enhanced specificity (espCas9(1.1), Addgene plasmid #71814) (Slaymaker et al., 2016). gRNAs were designed using the prediction software: https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design. Each gene was knocked-out using two gRNAs that were encoded by the same plasmid together with the espCas9(1.1). A DNA cassette containing the U6 promoter and one gRNA was inserted between XbaI and KpnI into espCas9(1.1) plasmid carrying a second gRNA. grid1: 5′-GGCAATAATCCGCTTCCAGG-3′ (targets exon 2) and 5′-GAAACTCAATACCCCTGTG-3′ (targets exon 8); arhgef12: 5′-GTCTACTATCACGGACAGGT-3′ (targets exon 1) and 5′-GGATCAATCAGCGGGCCGT-3′ (targets exon 11); ppp1r12a: 5′-GGTGAAGCCGAGCGACTCC-3′ (targets exon 1) and 5′-GTTGTGTTATAAGCCGCAGCT-3′ (targets exon 4).

Lentivirus production and infection

48 h after transfection of HEK293T cells, the viral supernatant was collected, centrifuged at 3,000 g for 5 min at 4 °C to remove cell debris, and ultracentrifuged at 25,000 g for 2 h on a 20% sucrose cushion. Viral pellets were resuspended in sterile PBS, aliquoted and stored at –80 °C. When indicated, cortical neurons were infected 4 days after plating with concentrated lentiviruses driving the expression of shRNA and EGFP.
**Immunohistochemistry for confocal microscopy**

Animals at postnatal day 21 were intracardially perfused with PBS and 4% paraformaldehyde (see above). After post-fixation, dissected brains were cryoprotected in 20% sucrose at 4°C for at least 16 h and then frozen at −80°C. 20 μm coronal sections were obtained using a cryostat and further processed for immunohistochemistry. Briefly, slices were incubated in 0.1% Triton X-100 and 0.25% fish gelatin (Sigma-Aldrich) in PBS to permeabilize and block unspecific staining. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 3 h at room temperature under gentle agitation. Both primary and secondary antibodies were diluted in 0.1% Triton X-100 and 0.125% fish gelatin in PBS. Coverslips were mounted on slides in Vectashield (Vector). Mouse anti-Gephyrin (Synaptic Systems Clone 7a, 1:400) and rabbit anti-GluD1 (kind gift from L. Tricoire, IBPS, Paris, France) (Benamer et al., 2018; Hepp et al., 2015) were used as primary antibodies. All secondary antibodies Alexa- (Invitrogen) and Cy3 (Jackson Immunoresearch) were used as secondary antibodies. Sections were mounted on slides in Vectashield and covered with a glass coverslip. Images were acquired in 1024x1024 mode using Leica TCS SP8 confocal laser scanning platforms controlled by the LAF AS software and equipped with a tunable white laser and hybrid detectors (Leica Microsystems) or, for slices infected with AAVs expressing TagFP in, 512x512 mode using an inverted microscope (Nikon Ti P; SF) equipped with a CSUX1-A1 Yokogawa spinning disc and an EMCCD camera and controlled by the Metamorph software (Molecular Devices). We used the following objective lenses: 10X PlanApo, NA 0.45 (identification of electroporated neurons and low magnification images) and 100X HC-PL APO, NA 1.44 CORR CS (Leica) or 100 X HC-PlanApo, NA 1.4 (Nikon) (Images of spines, gephyrin and PSD-95 clusters and association between GluD1, immunostained gephyrin and PS-D95 or Gephyrin.FingRs). Images were blindly acquired and analyzed. 2 stacks of images were acquired with spacing of 150 nm.

**Electron microscopy**

Anesthetized P21 mice were intracardially perfused with 2% paraformaldehyde (PFA) and 0.1% glutaraldehyde in phosphate-buffered saline (PBS) and post-fixed overnight at 4°C in 2% PFA. Coronal sections (200 μm) were obtained using a vibratome (see above) and cryoprotected overnight in 20% glycerol and 20% sucrose under gentle agitation at 4°C. They were permeabilized with 3 freeze-thawing cycles performed by floating them on liquid nitrogen in an aluminum cup. They were then extensively rinsed in PBS, and immersed for 20 min in 50 mM ammonium chloride and for 30 min in PBS with 0.1% gelatin (PBSg). For double detection of VGAT and GluD1, GluD1 labeling was performed first. Coronal sections were incubated for 60 h at 4°C with a rabbit anti-GluD1 antibody (1:1,000 dilution, see immunohistochemistry). Sections were rinsed extensively in PBSg and then incubated 6 h at room temperature with a goat anti-rabbit secondary antibody coupled with nanogold particles (Nanoprobe, 1:100). Gold particles were intensified for 5 min at 20°C with HQ silver kit (Nanoprobe) in a dark room. Gold labeling (Trembleau et al., 1994) was performed on the sections. They were then rinsed extensively in PBS and PBSg. For the detection of VGAT, sections were incubated for 48 h at 4°C with anti-VGAT mouse monoclonal antibody (Synaptic Systems, 1:100), rinsed extensively in PBSg and then incubated 4 h at room temperature with a biotinylated goat anti-mouse antibody (Vector Laboratories, 1:100). Detection of the biotinylated antibody was carried by the avidin–biotin complex method (Elite Vectastain kit, Vector; and Sigma fast DAB, Sigma-Aldrich). Antigen-antibody complexes were stabilized by dipping the sections for 5 min in 1% glutaraldehyde in PBS. Sections were then post-fixed for 1 h in 2% OsO4 in PBS at 4°C in the dark, dehydrated in graded ethanol and flat-embedded in epoxy resin (Araldite, Polysciences). Embedded sections were mounted orthogonally on a resin block and cut tangentially to the pial surface. To reach cortical layers 2/3, 200 sections...
of 1 µm thickness of tissue were removed from the onset of layer 1 using an UC6 ultramicrotome (Leica Microsystems). Ultrathin sections (70 nm, pale yellow) were contrasted with uranyl acetate and Reynolds lead citrate. Observations were performed with a Tecnai 11 electron microscope (Philips).

**Electrophysiology**

Acute coronal brain slices (300 µm thick) were obtained from juvenile (postnatal day 16-19) Swiss mice electroporated in utero with shGluD1 in pH1SCTdT2. Whole-cell patch-clamp recordings were performed in layer 2/3 cortical pyramidal neurons of the somatosensory cortex. Briefly, after decapitation the brain was quickly removed from the skull and placed in ice-cold (4°C) NaCl baths containing (in mM): 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 1.2 NaH2PO4, 24 NaHCO3, 25 glucose (pH 7.4). Slices were cut using the 7000 smz-2 tissue slicer (Campden Instrument). Slices recovered in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1 MgCl2, 2 NaH2PO4, 24 NaHCO3, and 25 glucose (pH 7.4), at 35°C for 10 min and then at room temperature for at least 45 min. For electrophysiological recordings, slices were transferred to a submerged recording chamber and continuously perfused at 33-34°C with oxygenated ACSF at a rate of 4-5 ml/min. Inhibitory and excitatory miniature post-synaptic miniature currents (mIPSCs and mEPSCs, respectively) were recorded at a holding potential of −60 mV in the presence of 0.5 µM TTX. mIPSCs were isolated by adding NBOX (10 µM) and D-APS (50 µM) to the ACSF. mEPSCs were isolated using gabazine (10 µM). mIPSCs were recorded using an intracellular solution containing (in mM): 150 KCl, 1.5 MgCl2, 10 HEPES, 1 EGTA, 2 NaATP, 0.5 NaGTP (pH adjusted to ~7.3 with KOH). mEPSCs were recorded using an intracellular solution containing (in mM): 144 K-gluconate, 7 KCl, 10 HEPES 10, 1 EGTA, 2 NaATP, 1.5 MgCl2, 2 NaATP, 0.5 NaGTP, (pH adjusted to ~7.3 with KOH). Access and input resistance were monitored by applying 5 mV hyperpolarizing steps of current. All drugs were obtained from Abcam.

**Transfection and western blotting**

Transfection was performed using Jet-Prime (Polyplus Transfection) according to the manufacturer protocol. Western blotting was performed using the following primary antibodies: mouse anti-HA (HA.11 Clone 16B12 Monoclonal Antibody, Covance, 1:1,000), rabbit anti-GFP (Life Technologies, 1:2,000), rabbit anti-Myc (Cell Signaling Technology, 1:1,000), rabbit anti-GluD1 (gift from L. Tricoire, 1:1000), rabbit anti-Arhgef12 (ThermoFisher Scientific, 1:1,000), rabbit anti-Ppp1r12a (ThermoFisher Scientific, 1:1,000), rabbit anti-RFP (Rockland Immunocchemicals, 1:1,000), rabbit anti-SRGAP2 (1:2,000; Charrier et al., 2012; Fossati et al., 2016), rabbit anti-GAPDH (Synaptic Systems, 1:1,000). All HRP-conjugated secondary antibodies were used at 1:30,000 dilution (Jackson ImmunoResearch). Protein visualization was performed by chemiluminescence using LumiLight western blotting (Roche) or Clarity Western ECL (Biorad) substrates and ImageQuant LAS 4000 (GE Healthcare) or Chemidoc (Biorad) imagers.

**Cell surface biotinylation**

Transfected HEK cells or primary cultures of cortical neurones at 15 or 22-23 days in vitro (DIV) were washed 3 times in ice-cold PBS supplemented with 0.8 mM CaCl2 and 0.5 mM MgCl2 (PBS2+) and then incubated for 12 min at room temperature followed by further 12 min at 4°C with 1 mg/ml Sulfo-NHS-SS-Biotin (ThermoFisher Scientific) in PBS2+. After rinsing in ice-cold PBS2+, biotin was quenched in 50 mM glycine in PBS2+ for 10 min. Cells were scraped in NaCl-Tris buffer supplemented with protease inhibitory cocktail (Roche) and then lysed (150 mM NaCl, 50 mM TrisHCl, 2% Triton X-100, 2 mM EDTA, 1 mM PMFS, protease inhibitor cocktail) for 1 h at 4°C. Biotinylated proteins were pulled down by incubating cell lysates with neutravidin agarose beads (ThermoFisher Scientific) for 2 h at 4°C. After extensive washes, beads were resuspended in gel loading buffer (Sigma-Aldrich) and bound proteins were eluted with boiling. Relative cell surface expression levels were analyzed by western blotting. Inputs correspond to 20% of the cell surface fraction.

**Subcellular fractionation**

Subcellular fractionation was performed from Swiss P15 mouse brains. All steps were performed at 4°C. Briefly, brains were homogenized in ice-cold HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES pH 7.4, 5 mM EDTA, 5 mM EGTA, protease inhibitor cocktail, from Sigma) using a motor driven glass-teflon homogenizer. The homogenate was centrifuged at 3000 g for 15 min. The resulting supernatant was centrifuged at 38,400 g for 15 min, yielding the crude synaptosomal pellet. The pellet was then subjected to hypotonic shock and centrifuged at 38,400 g for 20 min. The resulting pellet was lysed for 1 h using HEPES-buffered NaCl (100 mM NaCl, 4 mM HEPES pH 7.4, 5 mM EDTA, 5 mM EGTA, protease inhibitor cocktail) supplemented with 1% CHAPS (Sigma) and centrifuged at 100,000 g for 1 h. The corresponding supernatant is referred to as synaptic fraction or synaptic membranes. Protein concentration was measured and protein samples were subjected to immunoprecipitation.

**Immunoprecipitation**

For HEK cells, 1 mg of total protein from each sample was diluted in NP-40 buffer (1% Igepal, 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, protease inhibitor cocktail) and incubated overnight at 4°C, with either 5 µg of mouse anti-HA antibody (HA.11 Clone 16B12 Monoclonal Antibody, Covance) or 5 µg of mouse anti-Myc antibody (clone 9B11 Monoclonal Antibody, Cell Signaling Technology) and 5 µg of mouse IgG as negative control. Protein G-agarose beads (ThermoFisher Scientific) were then added for 2 h at 4°C. After extensive washes (1% Igepal, 50 mM Tris pH 7.4, 200 mM NaCl, 2 mM EDTA, protease inhibitor cocktail), the beads were resuspended
in gel-loading buffer and bound proteins were released with boiling. Inputs correspond to 50 μg of proteins. Samples were subjected to western blot analysis. For brain samples and mass spectrometry analysis, the immunoprecipitations were performed using antibodies covalently cross-linked to protein G magnetic beads (Pierce). 36 μg of rabbit anti-GluD1 antibody, or total rabbit IgG in control condition, were incubated 1 h at room temperature and cross-linked with 20 mM DMP (dimethylpimelimidate, Pierce) in 0.2 M Sodium Borate pH 9. After 30 min, the reaction was blocked for 1 h with 0.2 M Ethanolamine pH 8. Eventual unbound antibody molecules were washed out by incubating beads for 5 min in 0.1 M glycine pH 3. The efficiency of cross-linking was checked by running samples on polyacrylamide 4%–15% gradient gels (Biorad) followed by Comassie Blue staining. 1 mg of total proteins from purified synaptic membranes were diluted in a HEPES-NaCl buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, protease inhibitor cocktail) supplemented with 1% CHAPS and incubated overnight at 4°C with 36 μg of rabbit anti-GluD1 antibody, or total rabbit IgG in control condition, covalently cross-linked to protein G magnetic beads. The beads were rinsed 3 times using HEPES-NaCl buffer supplemented with 0.1% CHAPS and further washed 3 times in a buffer containing 20 mM HEPES pH 7.4 and 150 mM NaCl. The samples were then subjected to mass spectrometry analysis (see below). GluD1-immunoprecipitation from brain extracts was repeated three times.

Proteomics
Proteins on magnetic beads were washed twice with 100 μL of 25 mM NH₄HCO₃ and we performed on-beads digestion with 0.2 μg of trypsin/LysC (Promega) for 1 h in 100 μL of 25 mM NH₄HCO₃. Samples were then loaded onto a homemade C18 StageTips for desalting (principle by stacking one 3M Empore SPE Extraction Disk Octadecyl (C18) and beads from SepPak C18 CartridgeWaters into a 200 μL micropipette tip). Peptides were eluted using 40/60 MeCN/H₂O + 0.1% formic acid and vacuum concentrated to dryness.

Online chromatography was performed with an RSLCnano system (Ultimate 3000, Thermo Scientific) coupled online to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Peptides were trapped on a C18 column (75 μm inner diameter × 2 cm; nanoViper Acclaim PepMap 100, Thermo Scientific) with buffer A (2/98 MeCN/H₂O in 0.1% formic acid) at a flow rate of 4.0 μL/min over 4 min. Separation was performed on a 50 cm × 75 μm C18 column (nanoViper Acclaim PepMap RSLC, 2 μm, 100Å, Thermo Scientific) regulated to a temperature of 55°C with a linear gradient of 5% to 25% buffer B (100% MeCN in 0.1% formic acid) at a flow rate of 300 nL/min over 100 min. Full-scan MS was acquired in the Orbitrap analyzer with a resolution set to 120,000 and ions from each full scan were HCD fragmented and analyzed in the linear ion trap.

QUANTIFICATION AND STATISTICAL ANALYSIS
Confocal image analysis
Gephyrin and PSD-95 clusters, dendritic spines and the association of GluD1 with Gephyrin and PSD95 were quantified in the proximal part of oblique dendrites directly originating from the apical trunk using Fiji (Schindelin et al., 2012; https://fiji.sc/). Only dendrites that were largely parallel to the plane of the slice and acquired from sections of comparable rostro-caudal position were analyzed (usually no more than 1 dendrite per neuron). The density of dendritic spines and gephyrin clusters along dendrites was calculated as described (Charrier et al., 2012; Fossati et al., 2016). Gephyrin and PSD-95 clusters were quantified over a dendrite of a minimal length of 60 μm. The length of the dendritic segment was measured on the z projection. The fraction of gephyrin and PSD-95 clusters associated with GluD1 (association index) was manually determined on individual dendrites. A gephyrin or PSD-95 cluster labeled with EGFP-tagged FingRs was considered associated with GluD1 if it overlapped with a GluD1-mOrange puncta.

mEPSC and mIPSC analysis
Data were sampled at 10 kHz and filtered at 2 kHz. Miniature currents were analyzed over 1 min periods using pClamp 10.0 (Molecular Devices). Cells showing > 20% change in access and input resistance upon application of 5 mV hyperpolarizing steps of current were excluded from the analysis. Overlapping events were excluded from amplitude analysis. Cumulative probability graphs were obtained by taking the first 200 events within the analyzed time window of each recorded cell.

Proteomic analysis
For protein identification, data were searched against the Mus musculus (Mouse) UniProt database using Sequest HF through proteome discoverer (version 2.1). Enzyme specificity was set to trypsin and a maximum of two missed cleavage site were allowed. Oxidized methionine, N-terminal acetylation, and carbamidomethyl cysteine were set as variable modifications. Maximum allowed mass deviation was set to 10 ppm for monoisotopic precursor ions and 0.6 Da for MS/MS peaks. The resulting files were further processed using myProMS (Poulet et al., 2007) v3.6 (work in progress). FDR calculation used Percolator and was set to 1% at the peptide level for the whole study.

Statistics
Data are a minimum of three independent experiments. For in utero electroporations and AAV injections, data were obtained from at least three experiments or three animals from two independent litters.

For statistical analysis, we first checked the normality of the distributions using the D’Agostino-Pearson normality test. In case of normal distributions, we used unpaired Student’s t test or one-way analysis of variance followed by Tukey’s post test. Non-normal
distributions were assessed using the non-parametric Mann-Whitney test or the Kruskal-Wallis test followed by the Dunn’s multiple comparison test. A test was considered significant when p < 0.05. Data represent the distribution (or the mean) of the mean value per cell in the main figures. The whiskers of the boxplots in Figure 2 are the minimal and maximal values. Statistical analyses were performed with Prism (GraphPad Software).

**DATA AND CODE AVAILABILITY**

The mass spectrometry dataset generated in this study has been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD010373.