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Cell

Trans-Synaptic Interaction of GluRδ2 and Neurexin through Cbln1 Mediates Synapse Formation in the Cerebellum

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DOI 10.1016/j.cell.2010.04.035

SUMMARY

Elucidation of molecular mechanisms that regulate synapse formation is required for the understanding of neural wiring, higher brain functions, and mental disorders. Despite the wealth of in vitro information, fundamental questions about how glutamatergic synapses are formed in the mammalian brain remain unanswered. Glutamate receptor (GluR) δ2 is essential for cerebellar synapse formation in vivo. Here, we show that the N-terminal domain (NTD) of GluRδ2 interacts with presynaptic neurexins (NRXNs) through cerebellin 1 precursor protein (Cbln1). The synaptogenic activity of GluRδ2 is abolished in cerebellar primary cultures from Cbln1 knockout mice and is restored by recombinant Cbln1. Knockdown of NRXNs in cerebellar granule cells also hinders the synaptogenic activity of GluRδ2. Both the NTD of GluRδ2 and the extracellular domain of NRXN1β suppressed the synaptogenic activity of Cbln1 in cerebellar primary cultures and in vivo. These results suggest that GluRδ2 mediates cerebellar synapse formation by interacting with presynaptic NRXNs through Cbln1.

INTRODUCTION

Synapse formation is the key step in the development of neuronal networks. Precise synaptic connections between nerve cells in the brain provide the basis of perception, learning, memory, and cognition. Thus, elucidation of molecular mechanisms that regulate the formation and modulation of central synapses is essential for the understanding of neural wiring, brain functions, and mental disorders such as schizophrenia, autism, and mental retardation. Excitatory synapse formation in the brain requires the coordinate assembly of large numbers of protein complexes and specialized membrane domains required for synaptic transmission (Scheiffele, 2003; Kim and Sheng, 2004; Waites et al., 2005; Dalva et al., 2007; McAllister, 2007). Over the past few decades, a number of factors have been identified that play roles in synapse morphogenesis and synaptic plasticity. *Trans*-synaptic cell adhesion molecules represented by neurexins (NRXNs) and neuroligins (NLGNs) are thought to mediate target recognition and induction of pre- and postsynaptic specializations (Scheiffele, 2003; Dalva et al., 2007; Südhof, 2008). Cell culture studies indicate that NRXNs and NLGNs could act bidirectionally to induce pre- and postsynaptic assembly, thus controlling synapse formation (Scheiffele et al., 2000; Graf et al., 2004; Dean et al., 2003; Chih et al., 2005). However, phenotypic analyses of NLGN1, NLGN2, and NLGN3 triple-knockout mice and NRXN1α, NRXN2α, and NRXN3α triple-knockout mice suggest that these molecules are dispensable for synapse formation in vivo (Missler et al., 2003; Varoqueaux et al., 2006). Thus, despite the wealth of information, fundamental questions about how glutamatergic synapses are formed in the mammalian brain remain unanswered (Waites et al., 2005; McAllister, 2007).

On the other hand, there is clear in vivo evidence that GluRδ2, a member of the δ-type glutamate receptor (GluR), plays an essential role in cerebellar Purkinje cell (PC) synapse formation (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Takeuchi et al., 2005). The cerebellum receives two excitatory afferents, the climbing fiber (CF) and the mossy fiber-parallel fiber (PF) pathway, both converging onto PCs that are the sole neurons sending outputs from the cerebellar cortex. GluRδ2 is selectively expressed in cerebellar PCs (Araki et al., 1993; Lomeli et al., 1993) and is exclusively localized at PF-PC synapses (Takayama et al., 1996; Landsend et al., 1997). We found that a significant number of PC spines lack synaptic contacts with PF terminals and that some of residual PF-PC synapses show mismatching between pre- and postsynaptic specializations in conventional and conditional GluRδ2 knockout mice (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Takeuchi et al., 2005). These studies indicate

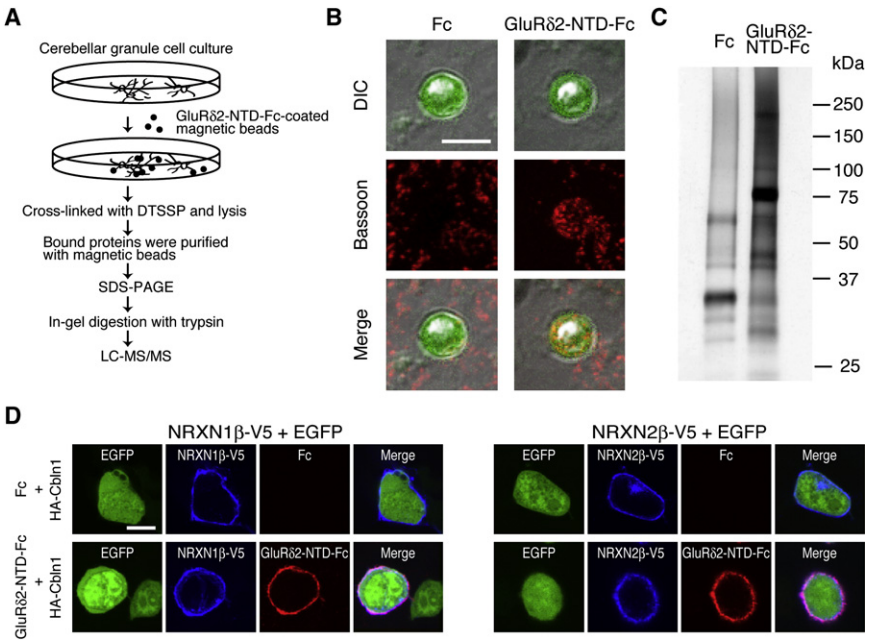

Figure 1. Identification of Presynaptic Proteins Interacting with the NTD of GluRδ2

(A) Schema for screening presynaptic proteins interacting with the NTD of GluRδ2.

(B) Induction of presynaptic differentiation of cerebellar GCs by GluRδ2-NTD-Fc-coated magnetic beads. After induction of presynaptic differentiation, the culture was treated with crosslinker DTSSP. Beads were visualized by differential interference contrast (DIC).

(C) SDS-PAGE analysis of crosslinked proteins by silver staining.

(D) Binding of GluRδ2-NTD-Fc to HEK293T cells transfected with NRXN1β-V5 or NRXN2β-V5 together with EGFP in the presence of HA-Cbln1. Scale bars represent 5 μm in (B) and 10 μm in (D). See also Figure S1 and Table S1.

that the formation and maintenance of PF-PC synapses are critically dependent on GluRδ2 in vivo. Thus, elucidation of the mechanism how GluRδ2 regulates PF-PC synaptic connection should provide a clue to understand synapse formation in the brain. Based on the direct relationship between the density of postsynaptic GluRδ2 and the size of presynaptic active zones in GluRδ2 mutant mice generated by inducible Cre-mediated ablation, we have proposed that GluRδ2 makes a physical linkage between the active zone and postsynaptic density (PSD) by direct or indirect interaction with an active zone component (Takeuchi et al., 2005). Indirect interaction through PSD proteins appears to be less likely since the C-terminal truncation of GluRδ2 has little effect on PF-PC synapse formation, while the mutation impairs cerebellar LTD and motor learning (Uemura et al., 2007), in agreement with the critical role of Delphilin interacting with the C-terminal of GluRδ2 in LTD and motor learning (Takeuchi et al., 2008). On the other hand, the synaptogenic activity of GluRδ2 is reproduced in vitro using primary cultures of cerebellar granule cells (GCs), and the extracellular N-terminal domain (NTD) of GluRδ2 is essential and sufficient to induce presynaptic differentiation in vitro (Uemura and Mishina, 2008). Thus, it is likely that GluRδ2 regulates synapse formation by direct interaction between its NTD and presynaptic protein(s). Here, we isolate GluRδ2-interacting molecules by crosslinking the NTD of GluRδ2 with cell surface proteins of cerebellar GCs after induction of presynaptic differentiation. Binding studies show that postsynaptic GluRδ2 interacts with presynaptic NRXNs through cerebellin 1 precursor protein (Cbln1). Inducible ablation of Cbln1 in the adult brain impairs PF-PC synaptic connections as found for GluRδ2 (Takeuchi et al., 2005). The synaptogenic activity of GluRδ2 is hindered by knockout of Cbln1 and by small interfering RNA (siRNA)-mediated knockdown of NRXNs. Furthermore, the synaptogenic activity of Cbln1 in cerebellar primary cultures and in vivo was abolished by the NTD of GluRδ2 and the extracellular domain (ECD) of NRXN1β. These results suggest that the *trans*-synaptic interaction of postsynaptic GluRδ2 and presynaptic NRXNs through Cbln1 mediates PF-PC synapse formation in the cerebellum.

RESULTS

Isolation of Presynaptic Proteins Interacting with the NTD of GluRδ2

To look for GluRδ2-interacting proteins, we employed primary cultures of cerebellar GCs with which the synaptogenic activity of GluRδ2 can be reproduced in vitro (Uemura and Mishina, 2008). The presynaptic differentiation of cerebellar GCs was induced by treatment with magnetic beads coated with the NTD of GluRδ2 (GluRδ2-NTD) fused to the Fc-domain of human immunoglobulin G (GluRδ2-NTD-Fc), and then surface proteins of cerebellar GC axons were crosslinked to GluRδ2-NTD using nonpermeable 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP) for identification by mass spectrometric analysis (Figure 1A). After incubation for 2 days, cultured cerebellar GCs extended their axons, and numerous punctate staining signals for active zone protein Bassoon accumulated on the surface of magnetic beads coated with GluRδ2-NTD-Fc, but not on the surface of control beads coated with Fc alone (Figure 1B). Surface proteins of cerebellar GCs crosslinked to GluRδ2-NTD were isolated from detergent-treated cultures through magnetic beads and were subjected to SDS-PAGE. Silver staining showed stronger signals including several prominent bands in the preparation from GluRδ2-NTD-Fc-coated beads compared with those from control Fc-coated beads (Figure 1C). Comparative analysis of the isolated proteins by liquid chromatography-tandem mass spectrometry (LC-MS/MS) identified NRXN1, NRXN2, FAT2, protein tyrosine phosphatase σ (PTPσ), and Cbln1 as possible GluRδ2-interacting proteins (Table S1 available online). Most of these proteins are known to accumulate at the presynaptic terminals and some of

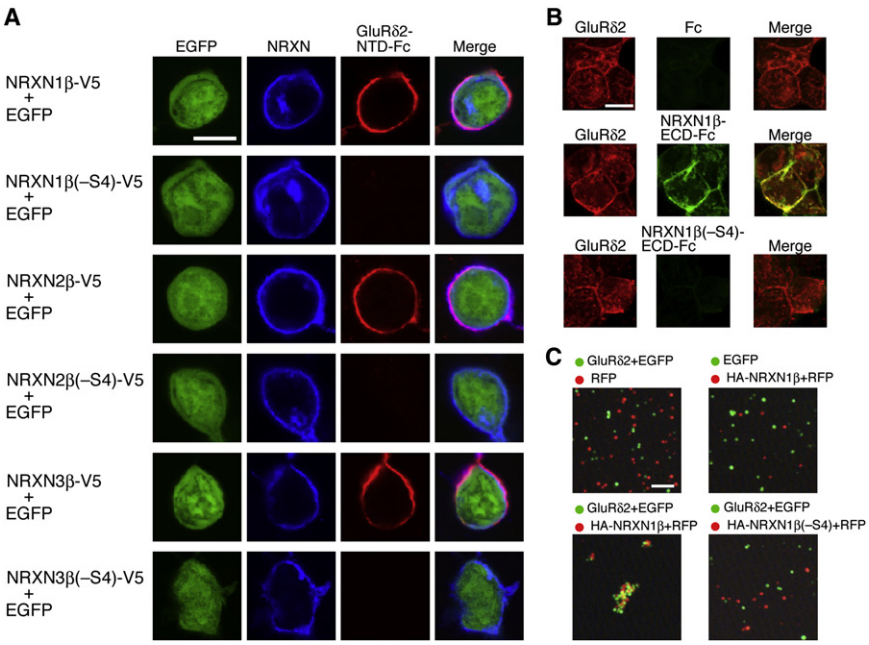

Figure 2. Selective Interaction of GluRδ2 with NRXN Variants Containing S4 in the Presence of Cbln1

(A) Binding of GluRδ2-NTD-Fc to HEK293T cells transfected with NRXN1β-V5, NRXN2β-V5 or NRXN3β-V5 together with EGFP in the presence of HA-Cbln1 but not to those transfected with NRXN1β(–S4)-V5, NRXN2β(–S4)-V5 or NRXN3β(–S4)-V5.

(B) Binding of NRXN1β-ECD-Fc to HEK293T cells transfected with GluRδ2 in the presence of HA-Cbln1.

(C) Cell aggregation assay of HEK293T cells transfected with GluRδ2 and EGFP and those with NRXN1β and RFP in the presence of HA-Cbln1.

Scale bars represent 10 μm in (A) and (B) and 100 μm in (C). See also Figure S2.

them are implicated in synaptogenesis. Thus, the crosslinking procedures appear to be effective to isolate presynaptic proteins interacting with or being close to the NTD of GluRδ2.

GluRδ2 Interacts with β-NRXN in the Presence of Cbln1

We expressed each of the isolated presynaptic membrane proteins in HEK293T cells by transfection of expression vectors for NRXN1β tagged with V5 epitope at the C terminus (NRXN1β-V5), NRXN2β-V5, FAT2 tagged with Myc at the C terminus (FAT2-Myc), and PTPσ. The transfected cells were incubated with soluble GluRδ2-NTD-Fc to test their ability to interact with GluRδ2. No significant signals for GluRδ2-NTD-Fc were detectable on the surface of any of the transfected HEK293T cells by immunocytochemistry with anti-Fc antibody (Figure S1A). HEK293T cells transfected with a mixture of all the expression vectors also showed no significant signals for GluRδ2-NTD-Fc. Among the isolated proteins, Cbln1 remained untested. Cbln1, originally identified as a precursor of cerebellin by Morgan and colleagues (Slemmon et al., 1984), is a glycoprotein secreted from cerebellar GCs (Bao et al., 2005). Interestingly, Cbln1 knockout mice phenotypically mimic GluRδ2 knockout mice (Kashiwabuchi et al., 1995; Kishimoto et al., 2001; Mishina, 2003), showing impairments of PF-PC synapse formation, LTD, and motor learning (Hirai et al., 2005). When recombinant Cbln1 tagged with hemagglutinin (HA) at the N terminus (HA-Cbln1) was added to the culture, we detected significant immunofluorescent signals for GluRδ2-NTD-Fc on the surface of HEK293T cells transfected with expression vectors for the presynaptic membrane proteins. After a series of selections, we found robust immunofluorescent signals for GluRδ2-NTD-Fc on the surface of HEK293T cells transfected with NRXN1β-V5 or NRXN2β-V5 in the presence of HA-Cbln1 (Figure 1D). Neither FAT2-Myc nor PTPσ showed any significant binding signals even in the presence of HA-Cbln1 (Figure S1B).

Splice Variant-Selective Interaction of GluRδ2 and NRXN

It is known that presynaptic NRXNs bind to postsynaptic NLGNs, forming *trans*-synaptic cell adhesion complexes (Ichtchenko et al., 1995; Scheiffele et al., 2000; Graf et al., 2004), and NLGNs preferentially bind to NRXNs lacking splice segment 4 (S4) (Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2006). We thus examined whether the splice segment of NRXNs affects the interaction with GluRδ2. HEK293T cells transfected with NRXN1β-V5 showed robust signals for GluRδ2-NTD-Fc in the presence of HA-Cbln1; however, signals for GluRδ2-NTD-Fc were hardly detectable on the surface of HEK293T cells transfected with a splice variant lacking S4 [NRXN1β(–S4)-V5] (Figure 2A). The NTD of GluRδ2 also bound to HEK293T cells expressing NRXN2β-V5 or NRXN3β-V5 in the presence of HA-Cbln1 but not to those expressing NRXN2β(–S4)-V5 or NRXN3β(–S4)-V5. Consistently, robust signals for the ECD of NRXN1β tagged with Fc (NRXN1β-ECD-Fc) but not for NRXN1β(–S4)-ECD-Fc were found on the surface of HEK293T cells transfected with GluRδ2 in the presence of HA-Cbln1 (Figure 2B). Thus, GluRδ2 selectively interacts with NRXN variants containing S4, in contrast to NLGNs showing higher affinity for NRXNs lacking S4. Interestingly, RT-PCR analysis showed that NRXN variants containing S4 were expressed in the cerebellum but those lacking S4 were hardly detectable, while both variants were found in the cerebral cortex and hippocampus (Figure S2).

To determine whether heterophilic binding between the NTD of GluRδ2 and the ECD of NRXN1β mediates cell adhesion, we incubated HEK293T cells transfected with GluRδ2 and EGFP and those transfected with HA-NRXN1β and RFP in the presence of HA-Cbln1 (Figure 2C). The transfected cells aggregated into large clumps, which suggests that GluRδ2 and NRXN1β function as heterophilic cell adhesion molecules. Aggregation between GluRδ2- and NRXN1β-transfected cells was not observed in the absence of HA-Cbln1. Neither HEK293T cells expressing GluRδ2 nor those expressing HA-NRXN1β showed homophilic aggregation in the presence of HA-Cbln1.

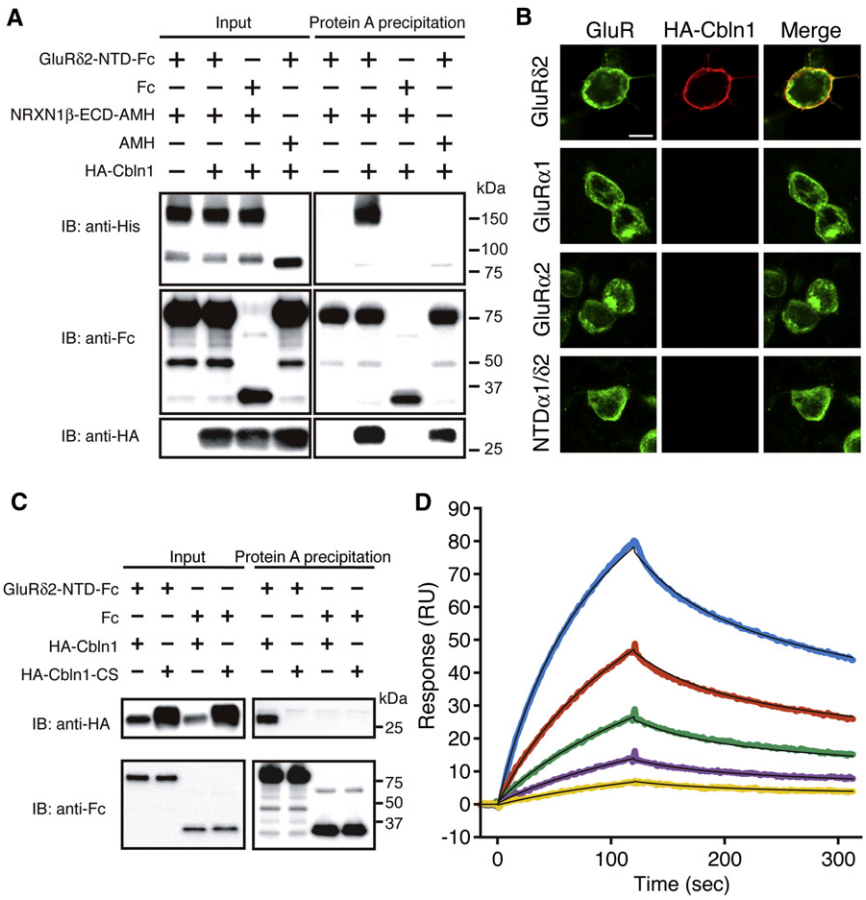

Figure 3. Direct Interaction between GluRδ2 and Cbln1

(A) Pulldown assay of the interaction of GluRδ2-NTD-Fc and NRXN1β-ECD-AMH in the presence of HA-Cbln1.

(B) Binding of soluble HA-Cbln1 to HEK293T cells transfected with GluRδ2 but not to those transfected with AMPA-type GluRs. The scale bar represents 10 μm.

(C) Pulldown assay of the interaction between GluRδ2-NTD-Fc and HA-Cbln1.

(D) SPR analysis. Interaction kinetics was measured by passing various concentrations (0.625, 1.25, 2.5, 5, and 10 μg/ml) of purified HA-Cbln1-His over GluRδ2-NTD-Fc captured on the surface of a sensor chip. Responses were fitted globally to a two-state reaction model with BIAevaluation 4.1 software. Thin black lines represent best-fit theoretical curves.

See also Figure S3.

GluRδ2 Is a Receptor for Cbln1

We then tested for biochemical association between GluRδ2 and NRXN in the presence of Cbln1. GluRδ2-NTD-Fc and NRXN1β-ECD tagged with alkaline phosphatase, Myc, and histidine epitopes at the C terminus (NRXN1β-ECD-AMH) were incubated together with HA-Cbln1. Protein A coprecipitated NRXN1β-ECD-AMH and HA-Cbln1 together with GluRδ2-NTD-Fc (Figure 3A). However, NRXN1β(–S4)-ECD-AMH was not coprecipitated by Protein A with GluRδ2-NTD-Fc and HA-Cbln1 (Figure S3A). These observations suggest that the NTD of postsynaptic GluRδ2 can interact with the ECD of presynaptic β-NRXNs in the presence of Cbln1.

Since GluRδ2 can interact with NRXNs only when Cbln1 is present, it appears that Cbln1 could bind to GluRδ2, NRXN, or both to stimulate their interaction. To clarify the issue, we expressed GluRδ2 in HEK293T cells and incubated the transfected cells with HA-Cbln1. Robust signals for HA-Cbln1 were found on the surface of the transfected HEK293T cells (Figure 3B). There were no detectable signals on the surface of HEK293T cells transfected with AMPA-type GluRα1 (GluR1) or GluRα2 (GluR2). Replacement of the NTD of GluRδ2 by that of GluRα1 abolished the binding signals. These results suggest that Cbln1 interacts selectively with the NTD of GluRδ2. To examine whether GluRδ2 binds directly to Cbln1, we incubated GluRδ2-NTD-Fc with HA-Cbln1. Protein A coprecipitated HA-Cbln1 together with GluRδ2-NTD-Fc (Figure 3C). Thus, we conclude that Cbln1 can bind directly to the NTD of GluRδ2. HA-Cbln1 hardly bound to GluRα1-NTD-Fc or GluRα2-NTD-Fc (Figure S3B).

To quantify the interaction between GluRδ2 and Cbln1, we employed surface plasmon resonance (SPR) binding assays. Decreasing concentrations of Cbln1 tagged with HA and His epitopes at the N and C termini, respectively (HA-Cbln1-His), were injected over GluRδ2-NTD-Fc-tethered chip surface (Figure 3D). Analysis of the association and dissociation phases of the sensorgrams by a two-state reaction model showed a dissociation constant (K_D) of 16.5 nM, indicating a high-affinity interaction between the NTD of GluRδ2 and Cbln1.

Thus, it is possible that Cbln1 may allosterically alter GluRδ2 to acquire NRXN binding ability. Alternatively, Cbln1 may also bind to NRXNs as a linker of GluRδ2 and NRXNs.

NRXN Is Another Receptor for Cbln1

To examine the issue, we expressed NRXN1β-V5 in HEK293T cells and incubated the transfected cells with HA-Cbln1. Robust signals for HA-Cbln1 were found on the surface of the transfected HEK293T cells (Figure 4A). There were no detectable signals on the surface of HEK293T cells transfected with NRXN1β(–S4)-V5. We also detected robust signals for HA-Cbln1 on the surface of HEK293T cells transfected with NRXN2β-V5 and NRXN3β-V5 but not of those transfected with their variants lacking S4 (Figure S4). These results suggest that Cbln1 interacts with NRXNs containing S4. To examine whether Cbln1 binds directly to NRXN1β, we incubated NRXN1β-ECD-AMH with HA-Cbln1. Anti-Myc antibody coimmunoprecipitated HA-Cbln1 together with NRXN1β-ECD-AMH (Figure 4B). Thus, Cbln1 can directly bind to the ECD of NRXN1β.

The interaction between NRXN1β and Cbln1 was examined by SPR analysis. Decreasing concentrations of HA-Cbln1-His

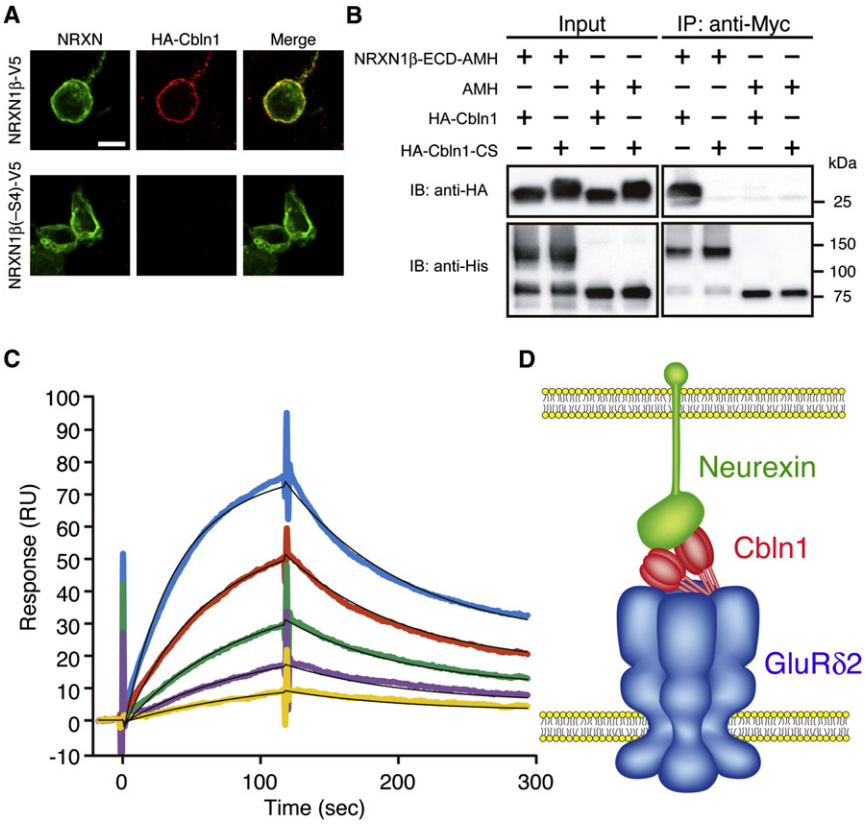

Figure 4. Direct Interaction between Cbln1 and NRXN1β

(A) Binding of HA-Cbln1 to HEK293T cells transfected with NRXN1β-V5 but not to those transfected with NRXN1β(–S4)-V5. The scale bar represents 10 μm.

(B) Pulldown assay of the interaction between NRXN1β-ECD-AMH and HA-Cbln1.

(C) SPR analysis. Interaction kinetics was measured by passing various concentrations (0.625, 1.25, 2.5, 5, and 10 μg/ml) of purified HA-Cbln1-His over NRXN1β-ECD-Fc captured on the surface of a sensor chip. Responses were fitted globally to a two-state reaction model with BIAevaluation 4.1 software. Thin black lines represent best-fit theoretical curves.

(D) A proposed model for *trans*-synaptic interaction between postsynaptic GluRδ2 and presynaptic NRXN through Cbln1.

See also Figure S4.

were injected over NRXN1β-ECD-Fc-tethered chip surface (Figure 4C). Sensorgrams showed specific binding of HA-Cbln1-His to NRXN1β-ECD-Fc. Analysis of the association and dissociation phases of the sensorgrams by a two-state reaction model showed a K_D of 0.17 nM, indicating a high affinity interaction between NRXN1β and Cbln1.

Based on these results, we propose a model for the *trans*-synaptic interaction of postsynaptic GluRδ2 with presynaptic NRXN through Cbln1 (Figure 4D).

Cbln1 Is Essential for PF-PC Synapse Formation

The synaptic connection between PF and PC is critically dependent on GluRδ2 not only during development but also in the adult stage (Kashiwabuchi et al., 1995; Takeuchi et al., 2005). Given that the interaction of GluRδ2 and Cbln1 is essential for PF-PC synapses, the ablation of Cbln1 in the adult cerebellum should also affect the synaptic connection. To test this hypothesis, we generated *Cbln1*$^{flox/flox}$ mice carrying *loxP* sites in the 5′ flanking region and intron 2 of the *Cbln1* gene using C57BL/6 embryonic stem cells (ESCs) (Mishina and Sakimura, 2007) and crossed with inducible and cerebellar GC-specific Cre mice (Tsujita et al., 1999) (Figure 5A and Figures S5A and S5B). Activation of Cre recombinase fused with progesterone receptor (CrePR) by intraperitoneal injection of RU-486 gradually abolished the expression of Cbln1 (28 kDa) in the adult cerebellum (Figure 5B). Inspection of PF-PC synapses by electron microscopy revealed the appearance of naked PC spines lacking presynaptic contacts and mismatched synapses with expanded PSD over active zone (Figure 5C and Figure S5C), suggesting the impairment of PF-PC synaptic connections. The numbers of free spines and aberrant synapses were increased as Cbln1 was decreased (Figure 5D). Thus, Cbln1 is required not only for synapse formation during development but also for its maintenance in the adult cerebellum. The similarity in the effect on PF-PC synaptic connections between GluRδ2 and Cbln1 conditional ablations supports the notion that their interaction is essential for PF-PC synapse formation in the cerebellum.

GluRδ2 Requires Cbln1 for Induction of Presynaptic Differentiation

To examine directly the role of GluRδ2-Cbln1 interaction in PF-PC synapse formation, we seeded HEK293T cells transfected with GluRδ2 and EGFP on the top of cultured cerebellar GCs prepared from wild-type and Cbln1 knockout mice. After 2 days of coculture, cells were immunostained with antibodies against GluRδ2, Bassoon, and vesicular glutamate transporter 1 (VGluT1). We detected numerous punctate staining signals for Bassoon and VGluT1 on the surface of HEK293T cells expressing GluRδ2 when cocultured with cerebellar GCs from wild-type mice (Figure 5E and Figure S5D). However, these punctate signals were hardly detectable for cerebellar GCs prepared from Cbln1 knockout mice. There were significant differences in the staining signals for Bassoon and VGluT1 between wild-type and knockout cultures (Tukey's test, $p < 0.01$) (Figure 5G and Figure S5E). Addition of HA-Cbln1 to primary cultures of cerebellar GCs from Cbln1 knockout mice restored the presynaptic differentiation induced by GluRδ2 expressed in HEK293T cells. It is suggested that Cbln1 forms a trimer through the C-terminal globular C1q domain and subsequently a hexamer through the cysteine residues 34 and 38 at the N terminus (Bao et al., 2005). HA-Cbln1 mutants in which serine

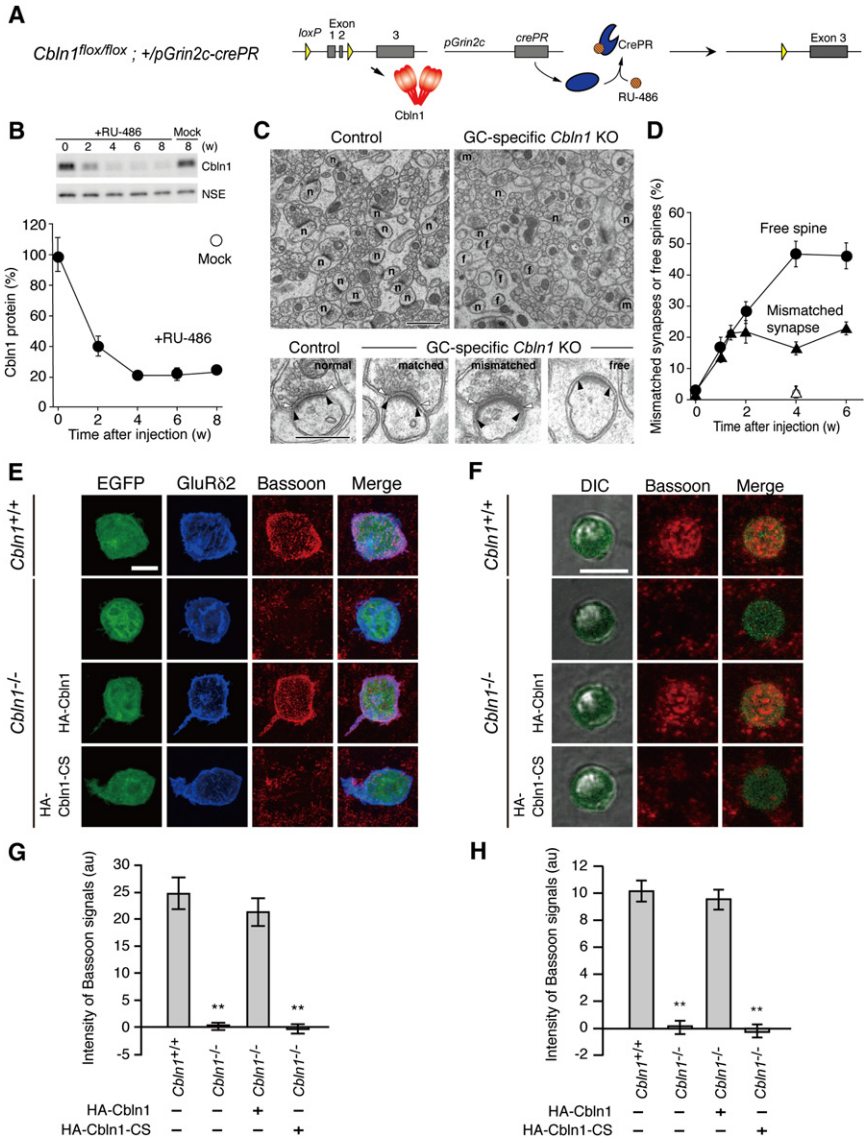

Figure 5. GluRδ2 Requires Cbln1 for Induction of Presynaptic Differentiation

(A) Schema for induction of cerebellar GC-specific *Cbln1* ablation.

(B) Decrease of Cbln1 after CrePR induction. Top: western blot analysis of Cbln1 and NSE in the cerebella before and after drug administration. Bottom: relative amounts of Cbln1 in drug- and mock-treated mice (two to three mice each).

(C) Electron micrographs of cerebella 4 weeks after RU-486 treatment. Drug-treated *Cbln1*$^{flox/flox}$ mice served as controls. n, normal synapses; m, mismatched synapses; f, free spines. Bottom left: a normal synapse in a control mouse. Bottom right: matched and mismatched synapses and a free spine in a GC-specific Cbln1 knockout mouse. Open and filled arrowheads indicate the edges of active zone and PSD, respectively.

(D) Emergence of mismatched synapses (triangles) and free spines (circles) in GC-specific *Cbln1* knockout (filled symbols) and control (open symbols) mice by drug treatment (two mice each).

(E and F) Induction of presynaptic differentiation of cultured cerebellar GCs prepared from *Cbln1*$^{+/+}$ or *Cbln1*$^{-/-}$ mice by GluRδ2 expressed on HEK293T cells (E) and by GluRδ2-NTD-Fc-coated on beads (F).

(G and H) Intensity of staining signals for Bassoon of cultured cerebellar GCs prepared from *Cbln1*$^{+/+}$ or *Cbln1*$^{-/-}$ mice on the surface of HEK293T cells transfected with GluRδ2 and EGFP (n = 10 each) (G) and on the surface of GluRδ2-NTD-Fc-coated beads (n = 20 each) (H).

All values represent mean ± SEM. **, $p < 0.01$; Tukey's test. Scale bars represent 1 μm in the top of (C), 0.5 μm in the bottom of (C), 10 μm in (E), and 5 μm in (F). See also Figure S5.

residues were substituted for these cysteine residues (HA-Cbln1-CS) failed to rescue the GluRδ2-induced presynaptic differentiation of cerebellar GCs from Cbln1 knockout mice. Similar results were obtained when the presynaptic differentiation of cerebellar GCs was induced by GluRδ2-NTD-Fc-coated beads (Figures 5F and 5H and Figures S5F and S5G). Consistently, HA-Cbln1-CS failed to interact with GluRδ2-NTD-Fc and NRXN1β-ECD-AMH (Figures 3C and 4B). These results suggest that GluRδ2 requires Cbln1 for its synaptogenic activity.

GluRδ2 Requires NRXN for Induction of Presynaptic Differentiation

We examined whether NRXNs are essential components of GluRδ2 and Cbln1-dependent presynaptic differentiation of cerebellar GCs by using soluble NRXN1β-ECD and siRNAs. Given that the synaptogenic activity of GluRδ2 requires NRXNs, recombinant NRXN1β-ECD may compete with endogenous NRXNs of GCs for interaction with GluRδ2 through Cbln1. Addition of NRXN1β-ECD-Fc to a coculture of GluRδ2-expressing HEK293T cells and cerebellar GCs significantly reduced the intensity of Bassoon signals ($p < 0.01$) (Figures 6A and 6B). This suggests that the interaction with NRXNs is indispensable for the synaptogenic activity of GluRδ2.

We next generated siRNAs directed against the mouse *Nrxn*1, *Nrxn*2, and *Nrxn*3, and tested their efficacy by cotransfection with NRXN expression vectors into HEK293T cells (Figures S6A and S6B). Introduction of a mixture of all the siRNAs into cerebellar GCs in culture suppressed the expression of endogenous *Nrxn*1, *Nrxn*2, and *Nrxn*3 messenger RNAs (mRNAs) (Figures S6C and S6D). When the mixture of siRNAs against *Nrxn*1, *Nrxn*2, and *Nrxn*3 was transfected into cerebellar GCs, we observed strong reduction of punctate staining signals for Bassoon on the surface of HEK293T cells expressing GluRδ2 (Figure 6C). Simultaneous knockdown of all three *Nrxn*s resulted in 72% reduction in the punctate staining signals for Bassoon on the surface of HEK293T cells expressing GluRδ2 ($p < 0.01$) (Figure 6D and Figure S6E). Transfection of an expression vector

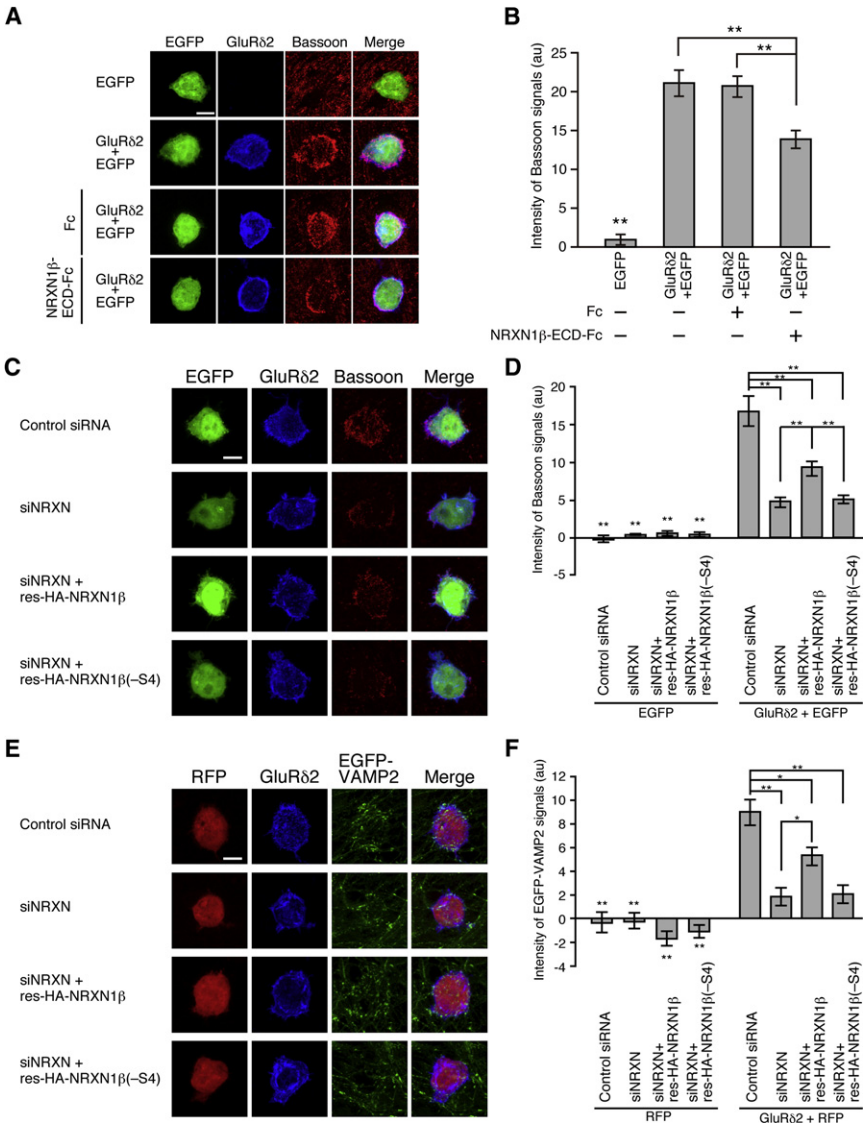

Figure 6. GluRδ2 Requires NRXN for Induction of Presynaptic Differentiation

(A) Inhibition of GluRδ2-induced presynaptic differentiation of cultured cerebellar GCs by NRXN1β-ECD-Fc.

(B) Intensity of staining signals for Bassoon of cultured cerebellar GCs on the surface of HEK293T cells transfected with GluRδ2 and EGFP in the presence or absence of NRXN1β-ECD-Fc (n = 5 each).

(C) Suppression of GluRδ2-induced presynaptic differentiation of cultured cerebellar GCs by a mixture of siRNAs against *Nrxn1*, *Nrxn2*, and *Nrxn3* and rescue by siRNA-resistant HA-NRXN1β (res-HA-NRXN1β).

(D) Effects of siRNA treatments on the intensity of staining signals for Bassoon of cultured cerebellar GCs on the surface of HEK293T cells transfected with GluRδ2 and EGFP (n = 20 each).

(E) Suppression of GluRδ2-induced EGFP-VAMP2 accumulation in cultured cerebellar GCs by a mixture of siRNAs against *Nrxn1*, *Nrxn2*, and *Nrxn3* and rescue by res-HA-NRXN1β.

(F) Effects of siRNA treatments on the intensity of staining signals for EGFP-VAMP2 in cultured cerebellar GCs (n = 20 each).

All values represent mean ± SEM. * and **, $p < 0.05$ and $p < 0.01$, respectively; Tukey's test. Scale bars represent 10 μm in (A), (C), and (E). See also Figure S6.

for an siRNA-resistant form of mouse HA-NRXN1β (res-HA-NRXN1β) together with the mixture of siRNAs partially restored the GluRδ2-induced presynaptic differentiation of cerebellar GCs as estimated by the accumulation of punctate staining signals for Bassoon ($p < 0.01$) (Figures 6C and 6D). On the other hand, cotransfection of res-HA-NRXN1β(–S4) failed to rescue the GluRδ2-induced presynaptic differentiation of cerebellar GCs from the suppression by the siRNAs against *Nrxn*s.

When an expression vector for vesicle-associated membrane protein-2 (VAMP-2) fused with EGFP at its N terminus (EGFP-VAMP2) as a synaptic vesicle marker was cotransfected into cerebellar GCs with the mixture of siRNAs against *Nrxn*1, *Nrxn*2, and *Nrxn*3, we observed strong reduction in the accumulation of EGFP-VAMP2 signals on the surface of HEK293T cells expressing GluRδ2 (Figure 6E). Simultaneous knockdown of all three *Nrxn*s resulted in 79% reduction in EGFP-VAMP2 signals on the surface of HEK293T cells expressing GluRδ2 ($p < 0.01$) (Figure 6F and Figure S6F). Transfection of res-HA-NRXN1β together with the mixture of siRNAs partially restored the GluRδ2-induced presynaptic differentiation of cerebellar GCs as estimated by the accumulation of EGFP-VAMP2 signals ($p < 0.01$) (Figures 6E and 6F). On the other hand, cotransfection of res-HA-NRXN1β(–S4) failed to rescue the GluRδ2-induced presynaptic differentiation of cerebellar GCs from the suppression by the siRNAs against *Nrxn*s. These results suggest that GluRδ2 requires NRXNs in addition to Cbln1 for the induction of presynaptic differentiation of cerebellar GCs.

Interaction of GluRδ2, Cbln1, and NRXN Is Essential for PF-PC Synapse Formation

Finally, we tested the effects of NRXN1β-ECD and GluRδ2-NTD on the synaptogenic activity of Cbln1 in cerebellar primary cultures and in vivo to examine the importance of the interaction between GluRδ2 and neurexin through Cbln1 in PF-PC synapse formation. In primary cultures of cerebellar neurons, numerous punctate staining signals for VGluT1 were found on the dendrites of PCs from wild-type mice, but VGluT1 signals on PC dendrites in cultures prepared from *Cbln1* knockout mice were significantly reduced ($p < 0.01$) (Figures 7A and 7B). Addition of HA-Cbln1 significantly restored the intensity of VGluT1 signals on PC dendrites in cultures from *Cbln1* knockout mice as described previously (Ito-Ishida et al., 2008). However, the synaptogenic activity of Cbln1 in primary cultures was significantly suppressed

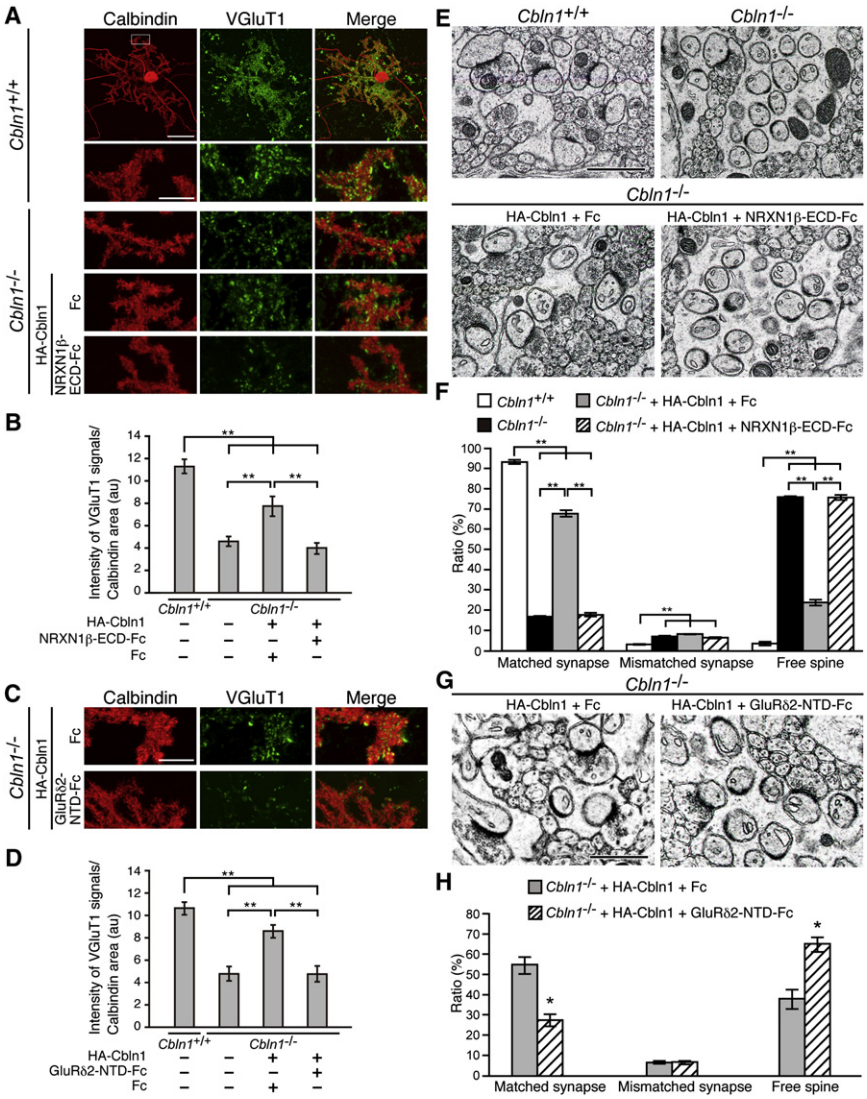

Figure 7. Suppression of Cbln1 Synaptogenic Activity by GluRδ2-NTD and NRXN1β-ECD

(A) Suppression of HA-Cbln1-induced accumulation of VGluT1 immunostaining signals on *Cbln1*$^{-/-}$ PC dendrites by NRXN1β-ECD-Fc. Top: *Cbln1*$^{+/+}$ cultures. Bottom: *Cbln1*$^{-/-}$ cultures incubated with HA-Cbln1 in the presence or absence of NRXN1β-ECD-Fc.

(B) Effect of NRXN1β-ECD-Fc on HA-Cbln1-induced VGluT1 staining signals on *Cbln1*$^{-/-}$ PC dendrites (n = 15 each).

(C) Suppression of HA-Cbln1-induced accumulation of VGluT1 immunostaining signals on *Cbln1*$^{-/-}$ PC dendrites by GluRδ2-NTD-Fc.

(D) Effect of GluRδ2-NTD-Fc on HA-Cbln1-induced VGluT1 staining signals on *Cbln1*$^{-/-}$ PC dendrites (n = 15 each).

(E) Electron micrographs of *Cbln1*$^{+/+}$ and *Cbln1*$^{-/-}$ cerebella (top) and *Cbln1*$^{-/-}$ cerebella 24 hr after injection of HA-Cbln1 with or without NRXN1β-ECD-Fc (bottom).

(F) Effect of NRXN1β-ECD-Fc on HA-Cbln1-induced restoration of PF-PC synaptic structures in *Cbln1*$^{-/-}$ cerebella (three or four mice each).

(G) Electron micrographs of *Cbln1*$^{-/-}$ cerebella 48 hr after injection of HA-Cbln1 with or without GluRδ2-NTD-Fc.

(H) Effect of GluRδ2-NTD-Fc on HA-Cbln1-induced restoration of PF-PC synaptic structures in *Cbln1*$^{-/-}$ cerebella (six mice each).

All values represent mean ± SEM. * and **, $p < 0.05$ and $p < 0.01$, respectively; Tukey's test or Student's t test. Scale bars represent 50 μm in the top of (A), 10 μm in the bottom of (A) and in (C), and 1 μm in (E) and (G).

by NRXN1β-ECD-Fc ($p < 0.01$) and GluRδ2-NTD-Fc ($p < 0.01$) (Figures 7A–7D). Injection of HA-Cbln1 in the cerebellum of *Cbln1* knockout mice significantly restored PF-PC connections as shown by the increase of matched PF-PC synapses and concomitant decrease of free spines (Figures 7E and 7F). The in vivo synatogenic activity of HA-Cbln1 was suppressed by NRXN1β-ECD-Fc ($p < 0.01$) and GluRδ2-NTD-Fc ($p < 0.05$) (Figures 7E–7H). These results suggest that the ternary interaction of postsynaptic GluRδ2, Cbln1, and presynaptic NRXN plays an essential role in PF-PC synapse formation in vivo.

DISCUSSION

Elucidation of molecular mechanisms that regulate the excitatory synapse formation in the brain is prerequisite for the understanding of neural wiring, higher brain functions, and mental disorders. GluRδ2 should be a clue to solve the issue because the analysis of conventional and conditional knockout mice provides evidence that GluRδ2 plays an essential role in vivo in the formation and maintenance of excitatory PF-PC synapses in the cerebellum (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Takeuchi et al., 2005). Furthermore, the synaptogenic activity of GluRδ2 can be reproduced in vitro (Uemura and Mishina, 2008). Here, we show that GluRδ2 mediates PF-PC synapse formation by interacting with presynaptic NRXNs through Cbln1.

Trans-Synaptic Triad of GluRδ2, Cbln1, and NRXN Is Essential for Excitatory Synapse Formation

Our results suggest that GluRδ2 requires both Cbln1 and NRXN for its synaptogenic activity. Consistently, both conventional and conditional Cbln1 knockout mice show impaired PF-PC synapse formation in the cerebellum as GluRδ2 knockout mice do (Figure 5) (Kashiwabuchi et al., 1995; Takeuchi et al., 2005; Hirai et al., 2005). Suppression of the synaptogenic activity of Cbln1 by GluRδ2-NTD and NRXN1β-ECD suggests that the ternary interaction of GluRδ2, Cbln1, and NRXN is essential for PF-PC synapse formation in vivo (Figure 7). Direct binding experiments show that GluRδ2 is a receptor for Cbln1 and NRXN is another receptor for Cbln1. The K_D value of Cbln1 for the NTD of GluRδ2 estimated by SPR binding assays is 16.5 nM and that for the ECD

of NRXN1β is 0.17 nM. These values suggest high-affinity interactions of GluRδ2, Cbln1, and NRXN as compared with K_D values (~200 to ~600 nM) reported for the interactions between NLGNs and NRXNs (Comoletti et al., 2003; Koehnke et al., 2008). Since Cbln1 is a ligand for both GluRδ2 and NRXN, we propose a model in which postsynaptic GluRδ2 interacts with presynaptic NRXN through Cbln1 and this ternary interaction provides a physical linkage between PSD and active zone (see Figure 4D). This model well explains our previous observations that the size of the presynaptic active zone shrank progressively concomitant with the decrease of postsynaptic GluRδ2 proteins upon inducible Cre-mediated GluRδ2 ablation (Takeuchi et al., 2005). Furthermore, it is reasonable that Cbln1 knockout mice phenotypically mimic GluRδ2 knockout mice.

On the other hand, it appears hard to reconcile our results with the observations that α-NRXN triple-knockout mice show no defects in the formation of the vast majority of synapses in vivo (Missler et al., 2003; Dudanova et al., 2007). However, β-NRXNs are intact in the α-NRXN knockout mice (Missler et al., 2003) and thus could support the synaptogenesis. Consistent with this possibility is our observation that impairment of the synaptogenic activity of GluRδ2 by knockdown of all three NRXNs can be rescued at least partially by NRXN1β, one of β-NRXNs. Furthermore, Li et al. (2007) showed that *Drosophila* neurexin plays a crucial role in the cytoarchitecture of synapses and adhesive interactions between pre- and postsynaptic compartments. In *Drosophila* neurexin mutants, presynaptic densities are not properly apposed to PSDs, reminiscent of mismatched synapses in GluRδ2 knockout mice (Figure 5) (Takeuchi et al., 2005). It is worthwhile to note that the density of inhibitory synapses was reduced in the brainstem of α-NRXN triple-knockout mice (Missler et al., 2003). Both α-NRXNs and β-NRXNs may have a general function of synapse formation and their roles may be differentially redundant.

Many *trans*-synaptic cell adhesion molecules interact in a homo- or heterophilic fashion across the synaptic cleft (Dalva et al., 2007). Thus, the triad of postsynaptic GluRδ2, Cbln1, and presynaptic NRXN essential for PF-PC synapse formation represents a new form of *trans*-synaptic adhesion interactions. Cbln1 secreted from presynaptic cerebellar GCs acts as a divalent ligand for both pre- and postsynaptic transmembrane receptors. Involvement of a soluble factor is also reported for *trans*-homophilic interaction of glial derived neurotrophic factor (GDNF) receptor molecules, which is triggered by GDNF probably through an allosteric mechanism (Ledda et al., 2007).

Synapse Formation in the Brain

GluRδ2 interacts selectively with NRXN variants containing S4 through Cbln1, whereas NLGNs bind preferentially to those without S4 (Boucard et al., 2005; Chih et al., 2006; Comoletti et al., 2006). Interestingly, NRXN variants containing S4 are preferentially expressed in the cerebellum (Figure S2). Enormous diversity of NRXNs produced by splicing may enable them to selectively interact not only with NLGN variants but also with multiple different molecules to ensure the specificity of large numbers of distinct synapses in the brain. Because GluRδ2 is selectively expressed in cerebellar PCs (Araki et al., 1993; Lomeli et al., 1993) and Cbln1 is predominantly expressed in GCs (Hirai et al., 2005; Miura et al., 2006), the ternary interaction of GluRδ2, Cbln1 and NRXN should be specific for cerebellar PF-PC synapses. Thus, the GluRδ2-Cbln1-NRXN triad may represent a combinatory "protein code" for synapse specificity determination in the brain.

Ablation of GluRδ2 causes the appearance of free spines as hallmarks of impaired synapse formation in cerebellar PCs (Kashiwabuchi et al., 1995). Despite the strong effect on synapse formation in vivo, approximately half of PF-PC synapses appear to remain preserved in GluRδ2 null mutant mice. There should be parallel mechanism(s) of cerebellar PF-PC synapse formation in addition to the GluRδ2-Cbln1-NRXN triad system. GluRδ1 may partly replace GluRδ2 since GluRδ1 has the activity to induce presynaptic differentiation of cerebellar GCs (Uemura and Mishina, 2008). Alternatively, NLGNs may act as postsynaptic partners of NRXNs to mediate cerebellar PF-PC synapse formation in place of GluRδ2 and Cbln1. However, genetic ablation of NLGN1, NLGN2, and NLGN3 does not result in a substantial loss of either excitatory or inhibitory synaptic contacts (Varoqueaux et al., 2006).

Our results suggest that Cbln1 selectively binds to the δ-type GluR among GluR subfamilies. The selectivity of Cbln1-GluR interaction is consistent with our previous observations that GluRδ1 and GluRδ2 but not AMPA-type GluRs induce presynaptic differentiation of cerebellar GCs (Uemura and Mishina, 2008). In contrast to highly selective expression of GluRδ2 in cerebellar PCs, GluRδ1 is widely expressed in the brain (Yamazaki et al., 1992; Lomeli et al., 1993). In addition, members of Cbln and NRXN families are widely distributed in the brain (Ullrich et al., 1995; Miura et al., 2006). It is possible that NRXNs may interact with GluRδ1 through Cblns and that this triad might be involved in synapse formation in the forebrain. Interestingly, both GluRδ1 and NRXN are implied in the pathogenesis of schizophrenia (Fallin et al., 2005; Rujescu et al., 2009).

Despite the wealth of information on the molecular mechanisms of glutamatergic synaptogenesis proposed by studies using cortical and hippocampal cell culture models, evidence for their relevance to synaptogenesis in vivo is lacking (Waites et al., 2005; McAllister, 2007). For example, numbers of studies have demonstrated a role for NRXNs and NLGNs in both excitatory and inhibitory synapse formation in vitro (Scheiffele et al., 2000; Graf et al., 2004; Prange et al., 2004; Chih et al., 2005). However, these in vitro results are in contrast to in vivo loss-of-function studies, which show dramatic functional impairments at both types of synapses in triple NLGN and α-NRXN null mutant mice but no significant defects in synaptogenesis (Missler et al., 2003; Varoqueaux et al., 2006; Dudanova et al., 2007). The discrepancy between in vitro and in vivo studies might reflect compensation by other synaptogenic factors or redundancy in the systems that control synapse formation (Dalva et al., 2007). Our finding that GluRδ2 mediates cerebellar PF-PC synapse formation by interacting with NRXN variants containing S4 through Cbln1 raises an intriguing possibility that presynaptic NRXNs in the forebrain may regulate synapse formation by interacting with postsynaptic molecules other than NLGNs.

EXPERIMENTAL PROCEDURES

Screening of Proteins Interacting with the NTD of GluRδ2

GluRδ2-NTD-Fc- and Fc-coated magnetic beads were added to cultured cerebellar GCs prepared from neonatal ICR mice at postnatal day 7 (P7) (Uemura and Mishina, 2008). After 2 days, cultures were crosslinked with 1 mM DTSSP (Pierce). After lysis of crosslinked neurons, bound proteins were purified by magnetic separator. Proteins separated by SDS-PAGE were stained with silver staining or negative gel stain MS kit (Wako) for in gel digestion. Gel lanes were excised into 12 individual fractions, and proteins in each fraction were reduced, alkylated, and digested with trypsin as described (Katayama et al., 2004). The resulting peptides were analyzed by LC-MS/MS with an ESI ion trap mass spectrometer (LTQ, Thermo Electron Corporation). Details are described in the Extended Experimental Procedures.

Construction of Expression Vectors and Preparation of Soluble Recombinant Proteins

Soluble recombinant proteins were prepared by transfection of respective expression vectors into the Freestyle 293 cells (Invitrogen). Details are described in the Extended Experimental Procedures.

Cell Cultures

Primary cerebellar cultures were prepared from neonatal mice at P0 and P7 as described (Uemura et al., 2004; Uemura and Mishina, 2008). Details of cell cultures, coculture assay, cell surface binding assay, and cell aggregation assay are described in the Extended Experimental Procedures.

Pulldown Assay

Soluble recombinant proteins were mixed and incubated with Protein A-Sepharose Fast Flow (GE Healthcare) or anti-Myc antibody-conjugated agarose (MBL). Bound proteins were analyzed by western blotting. Details are described in the Extended Experimental Procedures.

SPR Binding Analysis

SPR binding assays were conducted on a Biacore 3000 biosensor equipped with a sensor chip CM5 (GE Healthcare). Data analysis was performed with BIAevaluation software Ver. 4.1. The responses were fit to a two-state reaction model to calculate the K_D. Details are described in the Extended Experimental Procedures.

Generation of Floxed *Cbln1* Mice and Induction of GC-Specific Ablation of *Cbln1*

Floxed *Cbln1* mice were generated with C57BL/6 ESCs, and induction of CrePR-mediated gene ablation by RU-486 was carried out according to the procedures described previously (Takeuchi et al., 2005; Mishina and Sakimura, 2007). Details are described in the Extended Experimental Procedures.

Electron Microscopy

Cerebellar parasagittal sections were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in graded alcohols, and embedded in Epon 812. Electron micrographs were taken at a magnification of 4000× with an H-7100 electron microscope (Hitachi High-Technologies). Details are described in the Extended Experimental Procedures.

RNA Interference Experiment

We transfected a mixture of siRNAs (150 pmol each) with or without pCAG-EGFP-VAMP2 to cerebellar GCs by electroporation using Neucleofector and mouse Nucleofector kit (Amaxa Biosystems) with program G-013. For rescue experiments, an expression vector for siRNA-resistant HA-NRXN1β was cotransfetced with the mixture of siRNAs. The transfected GCs (DIV4) were cocultured with HEK293T cells transfected with pcGRD2 (Uemura and Mishina, 2008) and pEGFP-C1 (Clontech) or with pcGRD2 and pTagRFP (Evrogen) for 5 hr. Details are described in the Extended Experimental Procedures.

Assay of Synaptogenic Activity of Cbln1

The in vitro synaptogenic activity of HA-Cbln1 was examined in cultured cerebellar neurons prepared from *Cbln1*$^{-/-}$ mice (DIV20). The in vivo synaptogenic activity was examined by injection of HA-Cbln1 into the subarachnoid space above the rostrodorsal part of the cerebellum of *Cbln1*$^{-/-}$ mice (4–6 weeks of age). We examined the effects of NRXN1β-ECD-Fc and GluRδ2-NTD-Fc on the synaptogenic activity of HA-Cbln1 in vitro and in vivo. Details are described in the Extended Experimental Procedures.

Image Acquisition and Quantification

Image acquisition and quantification were performed as described previously (Uemura and Mishina, 2008). The outlines of distal dendrites of PCs were traced and the intensities of the VGluT1 signals within the traced regions were measured on the computer screen using ImageJ software. Statistical significance was evaluated by one-way ANOVA. When the interaction was significant, Tukey's post hoc test or Student's t test was used. Statistical significance was assumed when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.04.035.

ACKNOWLEDGMENTS

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We thank M. Watanabe for antibodies against Cbln1, GluRα1, GluRα2, GluRδ2, VGluT1 and calbindin, J. Miyazaki for CAG-*cre* transgenic mice and pCAGCS, M. Kataoka for pEGFP-VAMP2, and Y. Miwa for pEB6-CAGMCS. We are grateful to H. Sagara, T. Miyazaki, and Y. Mikami for helpful advice on electron microscopy, T. Shimizu and S. Tokuoka for use of Biacore 3000 biosensor, T. Fuse for advice on cerebellar culture, A. Maeda for technical assistance, K. Osaki and R. Natsume for help in knockout mouse generation, and Y. Nakajima for help in preparation of the manuscript.

Received: October 27, 2009
Revised: March 10, 2010
Accepted: April 9, 2010
Published online: May 27, 2010

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