Trans-synaptic interaction of GluRdelta2 and Neurexin through Cbln1 mediates synapse formation in the cerebellum

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**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
that the formation and maintenance of PF-PC synapses are critically dependent on GluR\textsubscript{2} in vivo. Thus, elucidation of the mechanism how GluR\textsubscript{2} 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 GluR\textsubscript{2} and the size of presynaptic active zones in GluR\textsubscript{2} mutant mice generated by inducible Cre-mediated ablation, we have proposed that GluR\textsubscript{2} 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 GluR\textsubscript{2} 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 GluR\textsubscript{2} in LTD and motor learning (Takeuchi et al., 2008). On the other hand, the synaptogenic activity of GluR\textsubscript{2} is reproduced in vitro using primary cultures of cerebellar granule cells (GCs), and the extracellular N-terminal domain (NTD) of GluR\textsubscript{2} is essential and sufficient to induce presynaptic differentiation in vitro (Uemura and Mishina, 2008). Thus, it is likely that GluR\textsubscript{2} regulates synapse formation by direct interaction between its NTD and presynaptic protein(s). Here, we isolate GluR\textsubscript{2}-interacting molecules by crosslinking the NTD of GluR\textsubscript{2} with cell surface proteins of cerebellar GCs after induction of presynaptic differentiation. Binding studies show that postsynaptic GluR\textsubscript{2} interacts with synaptic NRXNs through cerebellin 1 precursor protein (Cbln1). Inducible ablation of Cbln1 in the adult brain impairs PF-PC synaptic connections as found for GluR\textsubscript{2} (Takeuchi et al., 2005). The synaptogenic activity of GluR\textsubscript{2} 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 GluR\textsubscript{2} and the extracellular domain (ECD) of NRXN1\textsubscript{b}. These results suggest that the trans-synaptic interaction of postsynaptic GluR\textsubscript{2} and presynaptic NRXNs through Cbln1 mediates PF-PC synapse formation in the cerebellum.

**RESULTS**

**Isolation of Presynaptic Proteins Interacting with the NTD of GluR\textsubscript{2}**

To look for GluR\textsubscript{2}-interacting proteins, we employed primary cultures of cerebellar GCs with which the synaptogenic activity of GluR\textsubscript{2} 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 GluR\textsubscript{2} (GluR\textsubscript{2}-NTD) fused to the Fc-domain of human immunoglobulin G (GluR\textsubscript{2}-NTD-Fc), and then surface proteins of cerebellar GC axons were crosslinked to GluR\textsubscript{2}-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 GluR\textsubscript{2}-NTD-Fc, but not on the surface of control beads coated with Fc alone (Figure 1B). Surface proteins of cerebellar GCs crosslinked to GluR\textsubscript{2}-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 GluR\textsubscript{2}-NTD-Fc 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 \(\alpha\) (PTP\(\alpha\)), and Cbln1 as possible GluR\textsubscript{2}-interacting proteins (Table S1 available online). Most of these proteins are known to accumulate at the presynaptic terminals and some of
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 GluRβ2.

**GluRβ2 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 PTPα. The transfected cells were incubated with soluble GluRβ2-NTD-Fc to test their ability to interact with GluRβ2. No significant signals for GluRβ2-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 GluRβ2-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 GluRβ2 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 GluRβ2-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 GluRβ2-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 PTPα showed any significant binding signals even in the presence of HA-Cbln1 (Figure S1B).

To determine whether heterophilic binding between the NTD of GluRβ2 and the ECD of NRXN1β mediates cell adhesion, we incubated HEK293T cells transfected with GluRβ2 and EGFP with those transfected with HA-NRXN1β and RFP in the presence of HA-Cbln1. Neither HEK293T cells expressing GluRβ2 nor those expressing HA-NRXN1β showed homophilic aggregation in the presence of HA-Cbln1. (B) Binding of NRXN1β-EC-D-Fc to HEK293T cells transfected with GluRβ2 in the presence of HA-Cbln1. (C) Cell aggregation assay of HEK293T cells transfected with GluRβ2 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.
**GluRd2 Is a Receptor for Cbln1**

We then tested for biochemical association between GluRd2 and NRXN in the presence of Cbln1. GluRd2-NTD-Fc and NRXN1β-ECD-AMH were incubated together with HA-Cbln1. Protein A coprecipitated NRXN1β-ECD-AMH and HA-Cbln1 together with GluRd2-NTD-Fc (Figure 3A). However, NRXN1β(S4)-ECD-AMH was not coprecipitated by Protein A with GluRd2-NTD-Fc and HA-Cbln1 (Figure S3A). These observations suggest that the NTD of postsynaptic GluRd2 can interact with the ECD of presynaptic β-NRXNs in the presence of Cbln1.

Since GluRd2 can interact with NRXNs only when Cbln1 is present, it appears that Cbln1 could bind to GluRd2, NRXN, or both to stimulate their interaction. To clarify the issue, we expressed GluRd2 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). 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 GluRd2 and Cbln1.

Thus, it is possible that Cbln1 may allosterically alter GluRd2 to acquire NRXN binding ability. Alternatively, Cbln1 may also bind to NRXNs as a linker of GluRd2 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 conclude that Cbln1 can bind directly to the NTD of GluRd2. HA-Cbln1 hardly bound to GluR2-NTD-Fc or GluR2-NTD-Fc (Figure S3B).

To quantify the interaction between GluRd2 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 GluRd2-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 GluRd2 and Cbln1.
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 GluRd2 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 GluRö2 not only during development but also in the adult stage (Kashiwabuchi et al., 1995; Takeuchi et al., 2005). Given that the interaction of GluRö2 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$^{llox/llox}$ mice carrying loxP sites in the 5' flanking region and intron 2 of the Cbln1 gene using C57BL/6 embryonic stem cells (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 GluRö2 and Cbln1 conditional ablations supports the notion that their interaction is essential for PF-PC synapse formation in the cerebellum.

GluRö2 Requires Cbln1 for Induction of Presynaptic Differentiation

To examine directly the role of GluRö2-Cbln1 interaction in PF-PC synapse formation, we seeded HEK293T cells transfected with GluRö2 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 GluRö2, Bassoon, and vesicular glutamate transporter 1 (VGluT1). We detected numerous punctate staining signals for Bassoon and VGluT1 on the surface of HEK293T cells expressing GluRö2 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 GluRö2 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
residues were substituted for these cysteine residues (HA-Cbln1-CS) failed to rescue the GluRd2-induced presynaptic differentiation of cerebellar GCs from Cbln1 knockout mice. Similar results were obtained when the presynaptic differentiation of cerebellar GCs was induced by GluRd2-NTD-Fc-coated beads (Figures 5F and 5H and Figures S5F and S5G). Consistently, HA-Cbln1-CS failed to interact with GluRd2-NTD-Fc and NRXN1b-ECD-AMH (Figures 3C and 4B). These results suggest that GluRd2 requires Cbln1 for its synaptogenic activity.

GluRd2 Requires NRXN for Induction of Presynaptic Differentiation

We examined whether NRXNs are essential components of GluRd2 and Cbln1-dependent presynaptic differentiation of cerebellar GCs by using soluble NRXN1b-ECD and siRNAs. Given that the synaptogenic activity of GluRd2 requires NRXNs, recombinant NRXN1b-ECD may compete with endogenous NRXNs of GCs for interaction with GluRd2 through Cbln1. Addition of NRXN1b-ECD-Fc to a coculture of GluRd2-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 GluRd2.

We next generated siRNAs directed against the mouse Nrxn1, Nrxn2, and Nrxn3, 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 suppressed the expression of endogenous Nrxn1, Nrxn2, and Nrxn3 messenger RNAs (mRNAs) (Figures S6C and S6D). When the mixture of siRNAs against Nrxn1, Nrxn2, and Nrxn3 was transfected into cerebellar GCs, we observed strong reduction of punctate staining signals for Bassoon on the surface of HEK293T cells expressing GluRd2 (Figure 6C). Simultaneous knockdown of all three Nrxns resulted in 72% reduction in the punctate staining signals for Bassoon on the surface of HEK293T cells expressing GluRd2 (p < 0.01) (Figure 6D and Figure S6E). Transfection of an expression vector...
for an siRNA-resistant form of mouse HA-NRXN1β (res-HA-NRXN1β) together with the mixture of siRNAs partially restored the GluR62-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 GluR62-induced presynaptic differentiation of cerebellar GCS from the suppression by the siRNAs against Nrxn5s. These results suggest that GluR62 requires NRXNs in addition to Cbln1 for the induction of presynaptic differentiation of cerebellar GCS.

**Interaction of GluR62, Cbln1, and NRXN Is Essential for PF-PC Synapse Formation**

Finally, we tested the effects of NRXN1β-ECD and GluR62-NTD on the synaptogenic activity of Cbln1 in cerebellar primary cultures and in vivo to examine the importance of the interaction between GluR62 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 together with the mixture of siRNAs partially restored the GluR62-induced presynaptic differentiation of cerebellar GCS as estimated by the accumulation of VGluT1 signals on the surface of HEK293T cells expressing GluR62 (Figure 6E). Simultaneous knockdown of all three Nrxns resulted in 79% reduction in EGFP-VAMP2 signals on the surface of HEK293T cells expressing GluR62 (p < 0.01) (Figure 6F and Figure S6F). Transfection of res-HA-NRXN1β GluR62 requires NRXNs in addition to Cbln1 for the induction of presynaptic differentiation of cerebellar GCS.
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

![Figure 7. Suppression of Cbln1 Synaptogenic Activity by GluRδ2-NTD and NRXN1β-ECD](image-url)
of NRXN1β is 0.17 nM. These values suggest high-affinity interactions of GluRβ2, Cbin1, and NRXN as compared with $K_D$ values (~200 to ~600 nM) reported for the interactions between NLGNs and NRXNs (Comoletti et al., 2003; Koehne et al., 2008).

Since Cbin1 is a ligand for both GluRβ2 and NRXN, we propose a model in which postsynaptic GluRβ2 interacts with presynaptic NRXN through Cbin1 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 GluRβ2 proteins upon inducible Cre-mediated GluRβ2 ablation (Takeuchi et al., 2005). Furthermore, it is reasonable that Cbin1 knockout mice phenotypically mimic GluRβ2 knockout mice.

On the other hand, it appears hard to reconcile our results with the observations that $\alpha$-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, $\beta$-NRXNs are intact in the $\alpha$-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 GluRβ2 by knockdown of all three NRXNs can be rescued at least partially by NRXN1β, one of $\beta$-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 GluRβ2 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 $\alpha$-NRXN triple-knockout mice (Missler et al., 2003). Both $\alpha$-NRXNs and $\beta$-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 GluRβ2, Cbin1, and presynaptic NRXN essential for PF-PC synapse formation represents a new form of trans-synaptic adhesion interactions. Cbin1 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**

GluRβ2 interacts selectively with NRXN variants containing S4 through Cbin1, 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 GluRβ2 is selectively expressed in cerebellar PCs (Araki et al., 1993; Lomeli et al., 1993) and Cbin1 is predominately expressed in GCs (Hirai et al., 2005; Miura et al., 2006), the ternary interaction of GluRβ2, Cbin1 and NRXN should be specific for cerebellar PF-PC synapses. Thus, the GluRβ2-Cbin1-NRXN triad may represent a combinatorial “protein code” for synapse specificity determination in the brain.

Ablation of GluRβ2 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 GluRβ2 null mutant mice. There should be parallel mechanism(s) of cerebellar PF-PC synapse formation in addition to the GluRβ2-Cbin1-NRXN triad system. GluRβ1 may partly replace GluRβ2 since GluRβ1 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 GluRβ2 and Cbin1. 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 Cbin1 selectively binds to the $\delta$-type GluR among GluR subfamilies. The selectivity of Cbin1-GluR interaction is consistent with our previous observations that GluRβ1 and GluRβ2 but not AMPA-type GluRs induce presynaptic differentiation of cerebellar GCs (Uemura and Mishina, 2008). In contrast to highly selective expression of GluRβ2 in cerebellar PCs, GluRβ1 is widely expressed in the brain (Yamazaki et al., 1992; Lomeli et al., 1993). In addition, members of Cbin and NRXN families are widely distributed in the brain (Ulrich et al., 1995; Miura et al., 2006). It is possible that NRXNs may interact with GluRβ1 through Cbins and that this triad might be involved in synapse formation in the forebrain. Interestingly, both GluRβ1 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 $\alpha$-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 GluRβ2 mediates cerebellar PF-PC synapse formation by interacting with NRXN variants containing S4 through Cbin1 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 GluR2
GluR2-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 an ESI ion trap mass spectrometer (LTQ, Thermo Electron Corporation). SPR binding assays were conducted on a Biacore 3000 biosensor equipped with a sensor chip CM5 (GE Healthcare). Data analysis was performed with the Biacore Evaluation 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 Nucleofector and mouse Nucleofector kit (Amaxa Biosystems) with program G-013. For rescue experiments, an expression vector for siRNA-resistant HA-NRXN1 was cotransfected 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<sup>b<sup>a</sup></sup> mice (DIV20). The in vitro synaptogenic activity was examined by injection of HA-Cbln1 into the subarachnoid space above the rostrodloral part of the cerebellum of Cbln1<sup>b<sup>a</sup></sup> mice (4–6 weeks of age). We examined the effects of NRXN1<sup>b<sup>a</sup></sup>-ECD-Fc and GluR2-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.

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