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Characterization of Trans-Neuronal Trafficking of Cbln1

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Abstract

Cbln1, a glycoprotein secreted from granule cells and GluRδ2 in the postsynaptic densities of Purkinje cells are components of an incompletely understood pathway essential for integrity and plasticity of parallel fiber-Purkinje cell synapses. We show that Cbln1 undergoes anterograde transport from granule cells to Purkinje cells and Bergmann glia, and enters the endolysosomal trafficking system, raising the possibility that Cbln1 exerts its activity on or within Purkinje cells and Bergmann glia. Cbln1 is absent in Purkinje cells and Bergmann glia of GluRδ2-null mice, suggesting a mechanistic convergence on Cbln1 trafficking. Ectopic expression of Cbln1 in Purkinje cells of L7-*cbln1* transgenic mice reveals Cbln1 undergoes anterograde and retrograde transneuronal trafficking even across synapses that lack GluRδ2, indicating it is not universally essential for Cbln1 transport. The L7-*cbln1* transgene also ameliorates the locomotor deficits of *cbln1*-null mice, indicating the presence and/or release of Cbln1 from the postsynaptic neuron has functional consequences.

Keywords

Purkinje cell; GluRδ2; endosome; lysosome; Bergmann glia

Introduction

Cbln1 is a glycoprotein belonging to the C1q and tumor necrosis factor superfamily of proteins and is secreted from cerebellar granule cells as homohexamers or in heteromeric complexes with another family member, Cbln3 (Bao et al., 2005, 2006; Iijima et al., 2007). Genetic elimination of *cbln1* in mice results in an ataxic phenotype caused by the disruption of synapses between the parallel fibers of granule cells and Purkinje cells (Hirai et al., 2005). Thus, in *cbln1*-null mice there are numerous, naked dendritic spines on Purkinje cells with enlarged postsynaptic densities (PSD), that lack any presynaptic contacts from granule cells (Hirai et al., 2005). In addition, multiple climbing fibers innervate single Purkinje cells in *cbln1*-null mice. One consequence of these abnormalities is that cerebellar long term depression (LTD) at the parallel fiber-Purkinje cell (PF-PC) synapse is no longer evident (Hirai et al., 2005). Furthermore, administration of recombinant Cbln1 improves ataxia and restores PF-PC synapse formation in *cbln1* knockout mice (Ito-Ishida et al., 2008). Thus despite the fact that Cbln1 was originally cloned (Urade et al., 1991) as the putative precursor of the

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hexadecapeptide, cerebellin (Slemmon et al., 1984) it has been proposed that, like TNF α and ACRP30, mature Cbln1 is the biologically active entity, and the proteolytic processing events that produce cerebellin serve to modify the overall structure, subunit composition and activity of Cbln1-containing complexes (Bao et al., 2005).

Compared with other mutations causing ataxia in mice, all ultrastructural, cellular and physiological abnormalities in *cbln1*-null mice are strikingly similar to those seen in mice lacking the gene encoding the $\delta 2$ glutamate receptor (GluR $\delta 2$) (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Hashimoto et al., 2001; Lalouette et al., 2001; Ichikawa et al., 2002). GluR $\delta 2$ is selectively localized to the dendritic spines and PSDs of Purkinje cells (Landsend et al., 1997). Although the cerebellar phenotypes of *cbln1*- and GluR $\delta 2$ -null mice are qualitatively similar there are quantitative differences. By generating mice lacking both *cbln1* and GluR $\delta 2$, the phenotypes were not additive but rather the GluR $\delta 2$ -null phenotype was dominant (Hirai et al., 2005). These results suggested that GluR $\delta 2$, which is in Purkinje cell postsynapses, and Cbln1, secreted from presynaptic granule cells, engage in a common trans-neuronal signaling pathway critical for synapse formation, maintenance and plasticity. However, the mechanisms that link Cbln1 and GluR $\delta 2$ in this process are unknown.

Earlier studies showed that cerebellin, a hexadecapeptide derived from Cbln1 (Slemmon et al., 1984; Urade et al., 1991) is concentrated in the synaptosome fraction of cerebellum (Slemmon et al., 1984) and undergoes calcium- and depolarization-dependent release (Burnet et al., 1988). Subsequent studies using antisera to various N-terminal epitopes of Cbln1 have shown it to be associated with parallel fibers, PF-PC synapses and Purkinje cell dendritic spines (Morgan et al., 1988; Mugnaini et al., 1988; Miura et al., 2009). We recently developed an antiserum to a distinct epitope in the C-terminus of Cbln1 that uniquely detects Cbln1 in endolysosomal vesicles of neurons in many brain regions (Wei et al., 2007) that also express *cbln1* mRNA (Miura et al., 2006). This suggested that Cbln1 undergoes intracellular vesicular trafficking and as it is a secreted protein that is present at PF-PC synapses (Miura et al., 2009), potentially transneuronal trafficking involving secretion and endocytosis into the endosome pathway in Purkinje cells. Here we use immunohistochemistry in wild type and genetically modified strains of mice to investigate the trafficking of Cbln1 in cerebellum and to establish its characteristics and whether GluR $\delta 2$ plays a role in this process.

Results

Localization of Cbln1-like immunoreactivity in mouse cerebellum

Immunohistochemistry revealed prominent punctate CLI at the junction of the granule and molecular layers (Figure 1A,C) with no detectable CLI evident in sections from littermate *cbln1*-null mice (Figure 1B,D). As a further proof of specificity immunohistochemistry was performed on sections through cerebellum from *cbln3*-null and *cbln1/cbln3*-double null mice. One feature of *cbln3*-null mice is an approximate 7-fold increase in Cbln1 protein in immunoblots from cerebellum (Bao et al., 2006). Reflecting this biochemical change, there was a dramatic increase in punctate CLI in the *cbln3*-null cerebellum (compare Figure 1C and 1E) and this was lost in *cbln1/cbln3*-double null cerebellum (Figure 1F).

The localization of the CLI was consistent with Purkinje cells and/or Bergmann glia, although neither cell types express *cbln1* mRNA (Pang et al., 2000; Hirai et al., 2005; Miura et al., 2006) or (β -galactosidase driven from the *cbln1* promoter in transgenic mice (Hirai et al., 2005; Wei et al., 2007). To confirm the cell types expressing CLI, sections were triple labeled for DAPI and Cbln1 and either the Purkinje cell marker, calbindin D-28K (Figure 2A–D) or the glia marker S100 (Figure 2E–H). Under confocal microscopy, punctate staining for Cbln1 was prominent in the cell bodies of calbindin-positive Purkinje cells in *cbln3*-null mice (Figure 2A,B) and was absent in *cbln1*-null cerebellum (Figure 2C,D). CLI was also prominent in

S100-positive Bergmann glia of *cbln3*-null (Figure 2E,F) but not *cbln1*-null (Figure 2G,H) mice.

Subcellular localization of Cbln1-like immunoreactivity in Purkinje cells

The punctate pattern of CLI in Purkinje cells is reminiscent of its localization to vesicular structures in neurons elsewhere in brain (Wei et al., 2007). To study its sub-cellular localization in Purkinje cells, cerebellar sections were labeled by triple immunofluorescence for Cbln1, calbindin-D28K, and markers of endoplasmic reticulum (KDEL, Morishita et al., 2005) (Figure 3A–D), Golgi apparatus (GM130, Kerov et al., 2005) (Figure 3E–H), late endosomes (mannose-6-phosphate receptor, Morishita et al., 2005) (Figure 3I–L) and lysosomes (cathepsin D, Fukaya et al., 2003) (Figure 3M–P). Using confocal laser microscopy, CLI was observed to co-localize with mannose 6-phosphate receptor-positive (Figure 3I–L) and cathepsin D-positive (Figure 3M–P) structures: indicating that it was associated with a subset of endosomes/lysosomes. There was minimal co-localization of CLI with the ER marker, KDEL (Figure 3A–D) or Golgi marker, GM130 (Figure 3E–H).

In neurons that constitutively express Cbln1, punctate CLI is predominantly co-localized with cathepsin D (~80%) and to a much lesser extent mannose-6-phosphate receptor (~5%) and minimally if at all with KDEL and GM130 (Wei et al., 2007). In Purkinje cells, which do not express Cbln1, CLI co-localized with cathepsin D to about the same extent (80%) as in other neuronal types, however, many more of the puncta were mannose-6-phosphate receptor-positive (~60%). This suggests that Cbln1 is released from granule cells and internalized by Purkinje cells via a mechanism that involves endocytosis and endosome trafficking and maturation to lysosomes in the Purkinje cell soma.

Trans-neuronal trafficking of Cbln1 in L7-*cbln1* transgenic mice

To confirm that Cbln1 can be trafficked between neurons, it was ectopically expressed in cerebellar Purkinje cells of transgenic mice using the Purkinje cell-specific L7 promoter (Oberdick et al., 1990) (Figure 4A). The *cbln1* insert was not modified with any epitope-tags or reporter sequences as these might compromise trafficking and/or activity. It also contains its native signal peptide and should undergo secretion, as it does when transfected into cultured cells (Bao et al., 2005; 2006; Iijima et al., 2007). Multiple lines of L7-*cbln1* transgenic mice were derived (Figure 4B) and analyzed but for brevity one (TgC) is described here. In order to unambiguously identify CLI derived from the L7-*cbln1* transgene it was crossed onto a *cbln1*-null background: this mouse is referred to as *cbln1*^{-/-}/TgL7-*cbln1*.

To confirm authentic Cbln1 was produced in *cbln1*^{-/-}/TgL7-*cbln1* mice, immunoblotting was performed (Figure 4C). In cerebellum from 2 month-old wild type mice (W) and wild type mice harboring the L7-*cbln1* transgene (T/W), mature Cbln1 (arrow) as well as multiple submonomeric degradation products were detected (Figure 4C). In *cbln1*-null (KO) mouse cerebellar extracts, no authentic mature protein or sub-monomeric fragments were observed. In *cbln1*^{-/-}/TgL7-*cbln1* mice (T/KO) the same spectrum of CLI bands were observed as in wild type animals, although the level of mature protein was somewhat less (Figure 4C). We have previously reported the presence of substantial levels of partially glycosylated Cbln1 and sub-monomeric degradation products of Cbln1 both in vivo and in vitro (Bao et al., 2005; 2006; Hirai et al., 2005). Thus, even when ectopically expressed in Purkinje cells, the pattern of degradation of Cbln1 and the size of the mature protein is the same. As mature Cbln1 is generated during passage through the endoplasmic reticulum and Golgi (Bao et al., 2006) it suggests that the transgenic protein is being trafficked through the secretory pathway in Purkinje cells.

To confirm Purkinje cell specific expression of the transgene in *cbln1*^{-/-}/TgL7-*cbln1* mice, *in situ* hybridization was performed (Figure 4D, panels a, b, e, f). Using an L7-specific probe that detects both endogenous L7 expression as well as sites of expression of the L7-*cbln1* transgene revealed hybridization exclusively associated with the Purkinje cell layer (Figure 4D, panels a,e). Similarly using a *cbln1*-specific probe (Pang et al., 2000) in *cbln1*^{-/-}/TgL7-*cbln1* mice showed prominent *cbln1* hybridization in cerebellar Purkinje cells whereas there was no detectable signal in normal sites of *cbln1* expression, such as cerebellar granule cells and cerebellar nuclear neurons (Figure 4D, panels b,f). Hybridization with their respective sense probes showed no detectable signal for L7 or *cbln1* mRNA, confirming specificity of labeling (Figure 4D, insets in panels a and b, respectively). Panels c and g show counterstained sections for orientation. Together these data establish that the L7 transgene and its *cbln1* product were only expressed in cerebellar Purkinje cells with no detectable ectopic sites of L7 or *cbln1* expression.

To further authenticate and characterize Cbln1 expression in the transgenic mice, immunohistochemistry for Cbln1 in *cbln1*^{-/-}/TgL7-*cbln1* mice was performed (Figure 4D, d,h). Prominent punctate CLI was evident in cerebellar Purkinje cells (Figure 4D, panels d,h). This staining was largely confined to the soma and proximal dendrites and was much higher than in wild type mice (compare Figure 1 and Figure 4). However, fine punctate staining could also be seen over the granule cell layer (Figure 4D, h) and so additional experiments were performed to identify the cell types containing CLI.

To characterize the cells containing CLI in *cbln1*^{-/-}/TgL7-*cbln1* mice triple labeling was performed with DAPI and the anti-Cbln1 antibody and antisera to one of, the Purkinje cell marker, calbindin-D28K, the neuronal dendrite marker, MAP2, the glia marker, S100, the neuronal nucleus marker, NeuN, or the presynaptic marker, synaptophysin (Figure 5). In agreement with immunohistochemistry, punctate CLI was prominently co-localized with the calbindin-positive cell bodies of Purkinje cells (Figure 5A). CLI was also co-localized within calbindin-positive dendrites (Figure 5A, B) and axons (Figure 5C) of Purkinje cells, indicating that Cbln1 can be transported into both axons and dendrites. The latter was also confirmed by co-localization of CLI with the dendrite marker, MAP2 (Figure 5G,H). Substantial CLI was also evident in calbindin-negative (Figure 5A) S100-positive (Figure 5D) cells at the level of the Purkinje cell layer, indicating that Cbln1 is also trafficked from Purkinje cells to Bergmann glia. Besides Purkinje cell dendrites, CLI was also co-localized with NeuN-positive cells in the molecular layer (Figure 5E,F), suggesting that Cbln1 is released from Purkinje cell dendrites and accumulated by interneurons. Within the granule cell layer there were many calbindin-negative Cbln1-positive puncta (Figure 5A,C,D). Based on their abundance and proximity to DAPI-(Figure 5A,C,D) and NeuN-positive nuclei (Figure 5E) these are predominantly granule neurons. Indeed, the punta within the granule cell layer were often co-localized with the dendrite marker MAP2 (Figure 5G,I) whereas the CLI was never co-localized with synaptophysin (Figure 5J), indicating it was not associated with mossy fiber terminals. Together these data indicate that Cbln1 can be trafficked from Purkinje cells to the cell bodies of granule neurons, presumptively via retrograde synaptic transport at PF-PC synapses. CLI was also evident in the cerebellar nuclei of *cbln1*^{-/-}/TgL7-*cbln1* mice (Figure 5K-M), where it was associated with both cell bodies (Figure 5K,L) and MAP2-positive dendrites (Figure 5K,M) of nuclear neurons. As CLI is present in calbindin-positive axons this suggests that Cbln1 undergoes anterograde axonal trafficking in Purkinje cells and subsequent release and uptake into the cerebellar nuclear neurons onto which they synapse.

The data indicate that in *cbln1*^{-/-}/TgL7-*cbln1* mice, Cbln1 can undergo both anterograde and retrograde trans-cellular trafficking from Purkinje neurons to other intrinsic neurons of the cerebellum and Bergmann glia. However, given the anatomical proximity of some of the cell types, it cannot be excluded that some of the trafficking occurred via release and uptake from

the extracellular milieu rather than exclusively at synapses. Therefore, we assessed whether trafficking occurred to neurons whose cell bodies were more distant to Purkinje cells. Punctate CLI was evident in neurons within the inferior olivary nucleus (ION) of *cbln1*^{-/-}/Tg^{L7-cbln1} mice (Figure 6 A,B). There was no CLI in ION neurons in wild type (Figure 6C) or *cbln1*-null mice (Figure 6D). Furthermore, in situ hybridization showed that the L7-*cbln1* transgene was not ectopically expressed in the ION (dashed region, Figure 6E,F). Therefore, the most plausible source of the CLI in the ION is retrograde trans-synaptic trafficking from Purkinje cells via climbing fibers.

To identify the subcellular compartments containing CLI in *cbln1*^{-/-}/Tg^{L7-cbln1} mice, triple immunofluorescence was performed. Within Purkinje cells, CLI co-localized (~80%) with cathepsin D-positive structures (Figure 7A–C), whereas about 30% of puncta were co-labeled for the mannose-6-phosphate receptor (Figure 7D–F). Within the ION, approximately 85% of the CLI-positive puncta were co-labeled for cathepsin D (Figure 7G–I) while ~60% co-labeled for the mannose-6-phosphate receptor (Figure 7J–L). Thus, Cbln1 appears to be trafficked from Purkinje cells to ION neurons via a retrograde trans-synaptic mechanism at the climbing fiber-Purkinje cell synapse. The endocytosed Cbln1 enters the endosome vesicular trafficking pathway and is transported to the cell body where there is apparent maturation to a lysosome. This is qualitatively the same process that traffics Cbln1 in the anterograde direction from granule cells to the soma of Purkinje cells.

Localization of Cbln1-like immunoreactivity in the cerebellum of *GluRδ2*^{-/-} mice

As Cbln1 and GluRδ2 appear to function in a common trans-neuronal signaling pathway, we asked whether postsynaptic GluRδ2 influenced the trafficking of Cbln1. As shown in Figure 8, the punctate CLI seen in Purkinje cells and Bergmann glia in wild type mice (Figure 8A,C) is absent in *GluRδ2*-null mice (Figure 8B,D). As ~40% of Purkinje cell spines lack presynaptic contacts in *GluRδ2*-null mice, it is possible that this reduces CLI to an undetectable level. Therefore, to ensure the absence of CLI was not due to a limitation in sensitivity we assessed expression of CLI in *cbln3*/GluRδ2-double null mice. In *cbln3*-null mice there was a large increase in CLI within the granule cell layer and Purkinje cells (Figure 1E and Figure 8E). However, in *cbln3*/GluRδ2-double null mice, there was still no CLI evident within Purkinje cells (Figure 8F). This result was confirmed using triple immunofluorescence for DAPI and anti-Cbln1 and either calbindin D-28K or S100 antisera (Figure 9). Punctate staining for Cbln1 was present in both calbindin- (Figure 9A–C) and S100-positive cells (Figure 9G–I) in cerebella of *cbln3* null mice. In contrast there was little or no co-labeling of CLI with calbindin (Figure 9D–F) or S100 (Figure 9J–L) in *cbln3*/GluRδ2-double null mice.

Counting of CLI-positive puncta in Purkinje cells from wild type (WT) *GluRδ2*-null (D2KO) *cbln3*-null (Cbln3KO) and *cbln3*/GluRδ2-double null (D2KO/Cbln3KO) animals showed the near absence of CLI in the *GluRδ2*-deficient strains (Figure 10A). The CLI present in Bergmann glia was also greatly reduced or eliminated in *GluRδ2*-null mice (Figure 8C–F; Figure 9 G–L). Like Purkinje cells, the CLI-positive puncta in S100-positive Bergmann glia were also cathepsin D-positive (Supplementary Figure 1E–H), although there was no co-localization with the mannose-6-phosphate receptor (Supplementary Figure 1A–D). However, in comparison to Purkinje and other neurons, we observed little staining for mannose-6-phosphate receptor in Bergmann glia (Supplementary Figure 1A–D). Therefore, it is unclear if the mannose-6-phosphate receptor is a good marker for late stage endosomes in Bergmann glia or if this cell type contains relatively few late endosomes. Nevertheless, Cbln1 accumulates in Bergmann glia in a *GluRδ2*-dependent manner.

These results are not the product of reduced expression or altered proteolysis/processing of Cbln1 in *GluRδ2*-null mice. First, roughly equivalent densities of CLI-positive puncta were evident in the granule cell layer of both strains (Figure 8, Figure 9). Second, immunoblotting

for Cbln1 showed no marked differences in the levels or profiles of Cbln1 between wild type and GluR δ 2-null mice (Figure 10B). Third, there was no difference in cerebellin peptide content between wild type and knockout animals (GluR δ 2 $^{+/+}$, 310 $\pm$ 56 fmol/mg tissue; GluR δ 2 $^{+/-}$, 340 $\pm$ 37 fmol/mg tissue; GluR δ 2 $^{-/-}$, 410 $\pm$ 59 fmol/mg tissue: mean $\pm$ SEM, n=5). Thus, loss of GluR δ 2 function does not alter the levels of expression or processing of Cbln1 but does result in a failure of trafficking of Cbln1 from granule cells to Purkinje cells and Bergmann glia.

Visually *cbln1* $^{-/-}$ /TgL7-*cbln1* mice had less marked locomotor deficits than *cbln1*-null littermates. To quantify this parameter we performed an accelerating rotor-rod analysis on gender-matched littermates of the respective genotypes aged 35–45 days of age (Figure 11). To further minimize confounders such as integration site and genetic background effects we analyzed two independent transgenic founder lines (lines C and D in Figure 4) crossed into the *cbln1*-null background. Multiple comparisons between groups using the Bonferroni post-hoc test showed that only the *cbln1*-null mice (KO) showed statistically significant differences in latency to fall (Figure 11A, B) when compared to wild type mice (WT), wild type mice harboring the L7-*cbln1* transgene (WT/Tg) or *cbln1*-null mice carrying the respective L7-*cbln1* transgene (KO/Tg). The presence of the L7-*cbln1* transgene from both strains significantly improved the rotor-rod scores of *cbln1*-null mice (p=0.002 for Tg line C; p=0.004 for Tg line D). Thus, Cbln1 generated in and secreted from Purkinje cells is biologically active and can ameliorate at least one of the phenotypic deficits in *cbln1*-null mice.

Discussion

We reported previously the strikingly similar phenotypes of *cbln1*-null mice and mice harboring loss of function mutations of the orphan GluR δ 2 receptor (Hirai et al., 2005). The common features of these mice suggested that Cbln1 secreted from presynaptic granule neurons and GluR δ 2 in Purkinje cell postsynapses were components of a trans-neuronal process essential for normal synaptic structure and function at PF-PC synapses. Although GluR δ 2 and *cbln1* interact at the genetic level, with GluR δ 2 being dominant over *cbln1* (Hirai et al., 2005) the molecular and cellular basis for this interrelationship is not understood. This study provides further mechanistic insight into this relationship by showing that loss of GluR δ 2 in Purkinje cells leads to a deficit in their ability to bind and/or take up Cbln1. In addition, we show that Cbln1 is taken up by Bergmann glia, and this process is also impaired in GluR δ 2-null mice. These data imply that Bergmann glia as well as Purkinje neurons may be the target of Cbln1 signaling and that GluR δ 2 is required in some fashion for both processes, even though the orphan receptor is not expressed in Bergmann glia.

The mechanistic role of GluR δ 2 in Cbln1 trafficking is likely an indirect one rather than a direct relationship such as ligand and receptor. Although GluR δ 2 could be a receptor for Cbln1 at PF-PC synapses it cannot be a unique receptor as Cbln1 is transported into cell types and neurons that do not express the orphan receptor, such as Bergmann glia, granule cells, deep nuclear neurons and neurons of the inferior olive. There is a range of morphological, biochemical and biological deficits in the PF-PC synapses of GluR δ 2-null mice (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Lalouette et al., 2001; Hirai et al., 2003) that might underlie the absence of trafficking of Cbln1. A prominent deficit is the presence of many naked dendritic spines on Purkinje cells that lack their presynaptic component (Kashiwabuchi et al., 1995; Kurihara et al., 1997). Therefore, the absence of Cbln1 in Purkinje cells in GluR δ 2-null mice could be the consequence of the increased anatomical distance between Purkinje cell spines and PF presynaptic components. However, only approximately 40% of Purkinje cell dendritic spines lack presynaptic components in GluR δ 2-null mice (Kashiwabuchi et al., 1995; Hirai et al., 2005) yet all Purkinje cells lack Cbln1 in these animals. Furthermore, even in GluR δ 2/*Cbln3* double-null mice that have a 5–10 fold increase in Cbln1 there is still no Cbln1 evident

in Purkinje cells; suggesting that the anatomical separation of 40% of pre- and postsynaptic components do not account for the lack of Cbln1 uptake. In addition to naked spines, the postsynaptic densities at the remaining intact PF-PC synapses are abnormal in GluR δ 2-null mice, and show enlargement beyond the zone of apposition (Kashiwabuchi et al, 1995; Kurihara et al., 1997). Thus, there may be gross disruption of other aspects of synaptic function in these animals. In particular, the intracellular domain of GluR δ 2 physically associates with a range of synaptic proteins within Purkinje cells (Hironaka et al., 2000; Miyagi et al., 2002; Uemura et al., 2004) and influences AMPA receptor trafficking and endocytosis at PF-PC synapses (Hirai et al., 2003). Therefore, it is possible that GluR δ 2 may be essential for the trafficking or turnover of an as yet unidentified Cbln1 receptor at the Purkinje cell synapse. Alternatively, GluR δ 2 may play a more promiscuous role in facilitating endocytosis at the PF-PC synapse. Nevertheless, whatever the precise mechanism, the demonstration that GluR δ 2 is required for the trans-synaptic trafficking of Cbln1 at PF-PC synapses provides a link between the two proteins that potentially underlies the common phenotypes of their respective null alleles. The finding that ectopic expression of Cbln1 in Purkinje cell improves the locomotor deficits in *cbln1*-null mice further underscores the possibility that the presence of Cbln1 in the postsynaptic neuron is biologically relevant.

We show that in wild type mice Cbln1 undergoes anterograde trafficking from granule cells into Purkinje cells. Furthermore, in transgenic mice Cbln1 can also undergo retrograde trans-synaptic transport from Purkinje cells to neurons in the inferior olivary nucleus as well as other neurons in the molecular and granule cell layers of the cerebellum. A number of neurotrophic factors including NGF (Hendry et al., 1974; Claude et al., 1982) and glial cell line-derived neurotrophic factor (GDNF) (Tomic et al., 1995) are trafficked in a retrograde manner. Anterograde trafficking has also been shown for neurotrophin-3 (von Bartheld et al., 1996; von Bartheld and Butowt, 2000; Butowt and von Bartheld, 2005). However, Cbln1 is unusual in that it is one of relatively few proteins that exhibit both anterograde and retrograde trans-synaptic trafficking, with other examples including the amyloid precursor protein (Simons et al., 1995; Yamazaki et al., 1995), BDNF (Altar and DiStefano, 1998; Magby et al., 2006), prion protein (Scott and Fraser, 1989; Bartz et al., 2003) and GDNF (Tomic et al., 1995; Rind and von Bartheld, 2002). Whether Cbln1 undergoes bidirectional trafficking at the same synapse, where Cbln1 released at the PF-PC synapse can be taken up postsynaptically by Purkinje cells and presynaptically by granule cells is presently uncertain. Such a mechanism would be analogous to the classical pre- and postsynaptic binding and uptake/reuptake of neurotransmitters. If true this might also imply the existence of distinct Cbln1 receptors or binding proteins on pre- and postsynaptic membranes as well as a pool of recycling Cbln1-containing vesicles in the presynaptic compartment. Indeed, it is also possible that the punctate CLI in granule cells of wild type mice represents Cbln1 that is derived from endocytosis and retrograde trafficking rather than being Cbln1 in the process of exocytosis. As our current transgenic mouse model expresses wild type Cbln1 we cannot discriminate transgenic from endogenous Cbln1 except when crossed onto a *cbln1*-null background (thereby eliminating an anterograde source of the protein). Therefore, to unambiguously demonstrate bidirectional trafficking of Cbln1 at the PF-PC synapse we will need to express a tagged version of Cbln1 in Purkinje cells of mice wild type for *cbln1*.

Within Purkinje cells of wild type mice, Cbln1 immunoreactivity is associated with both mannose-6-phosphate receptor- and cathepsin D-positive structures, indicative of its localization to late endosomes and lysosomes, respectively. These and previous data suggest a process in which Cbln1 is released at PF-PC synapses whereupon it is internalized by Purkinje cells, presumptively by an endocytic mechanism, and enters the endosomal intracellular trafficking pathway. As the majority of the punctate Cbln1-like immunoreactivity is present in the soma, this also implies that the endosomes traffic from distal dendrites, the location of PF-PC synapses to the cell body while undergoing maturation to lysosomes. This process is

even more dramatic in the retrograde trafficking of Cbln1 from Purkinje cells of transgenic mice, via climbing fiber synapses and axons to the distant neuronal cell body in the inferior olive where it is also associated with mannose-6-phosphate receptor- and cathepsin-D-positive structures. While this phenomenon may reflect a mechanism to degrade Cbln1 it bears similarities to long range retrograde signaling of neurotrophins via signaling endosomes (Howe et al., 2001; Miller and Kaplan, 2001; Howe and Mobley, 2005; Cosker et al., 2008). Classically, neurotrophins released at synapses exert local signaling effects by activating their membrane receptors (Reichardt and Mobley, 2004). In addition, some neurotrophin-bound receptors are internalized into endosomes that are actively trafficked along axons to the cell body (Grimes et al., 1996; Grimes et al., 1997). During this transit the endosomes mature into signaling endosomes by recruitment of effector proteins to the cytoplasmic domains of the ligand activated neurotrophin receptors (Bronfman and Fainzilber, 2004). The signaling endosomes then initiate a signal transduction cascade in the neuronal cell body that mediates the long-range biological effects of neurotrophins (Tsui-Pierchala and Ginty, 1999; Watson et al., 2001; Delcroix et al., 2003; Kuruvilla et al., 2004). In this scenario the endosomal trafficking of Cbln1 from the synapse to the soma of Purkinje cells may provide a signal, potentially via activation of its putative receptor in the endosome, important for synaptic stability.

An unexpected finding was the presence of prominent Cbln1 immunoreactivity in Bergmann glia, that like Purkinje neurons do not express mRNA for *cbln1* (Pang et al., 2000; Hirai et al., 2005; Miura et al., 2006). Moreover, Bergmann glia are the only non-neuronal cell type that contain Cbln1 (Wei et al., 2007). As in neurons, the punctate staining of Cbln1 in Bergmann glia was co-localized with cathepsin D, indicating its presence in a lysosome compartment. Unlike Purkinje cells, Cbln1 was not markedly co-localized with the mannose-6-phosphate receptor in Bergmann glia, although in general these cells exhibited little immunoreactivity for this late endosome marker making interpretation of this observation difficult (Supplementary Figure 1). Bergmann glia play critical roles in the developing and adult cerebellum where they have important interactions with both granule cells and Purkinje neurons (Grosche et al., 1999; Castejón et al., 2002; Watanabe, 2002; Bellamy, 2006). In particular Bergmann glia intimately envelop Purkinje cell dendrites and synapses in the adult cerebellum and influence synaptic transmission at both PF-PC and climbing fiber-PC synapses (Yamada et al., 2000; Bellamy and Ogden, 2005; Bellamy and Ogden, 2006). Strikingly, Cbln1 immunoreactivity is greatly reduced or absent in Bergmann glia in GluRδ2-null mice even though they do not express the orphan receptor (Lansend et al., 1997). Given the intimate association of Bergmann glia with the PF-PC synapse, which is the location of GluRδ2, this suggests that Cbln1 is trafficked to Bergmann at these synapses and is not simply taken up from the extracellular space. This result also raises the possibility that Cbln1 plays some role in the biology of Bergmann glia. For example, Cbln1 may influence the ensheathment of PF-PC synapses by Bergmann glia, thereby modulating synaptic efficacy. Alternatively, Bergmann glia may be one route for elimination of Cbln1 at PF-PC synapses, perhaps serving to maintain a critical concentration of the protein in the synaptic cleft.

The ectopic expression of Cbln1 in Purkinje cells improved the locomotor deficits in *cbln1*-null mice. Furthermore, we observed trafficking of Cbln1 from Purkinje cells to granule cells and Bergmann glia in *cbln1*^{-/-}/TgLT7-*cbln1* mice. Together these data imply that the postsynaptic generation of Cbln1 supports, at least to some degree, the formation/stabilization of PF-PC synapses that are ensheathed by Bergmann glia, traffic Cbln1 and retain some level of normal physiological properties. In this regard they complement and extend an analysis in which recombinant Cbln1 was injected into the cerebellar subarachnoid space of *cbln1*-null mice and transiently restored PF-PC synaptic function (Ito-Ishida et al., 2008). As ours is a genetic rescue model it will now permit a more detailed analysis of the molecular, cellular and physiological sequelae that underlie this phenomenon.

These data provide a mechanistic link between Cbln1 and GluR δ 2 by showing that the orphan receptor is required in some manner for the trafficking of Cbln1 to both Purkinje cells and Bergmann glia. Furthermore, they raise the possibility that Cbln1 exerts its effects following internalization by Purkinje cells, perhaps via a signaling endosome mechanism and may also contribute to the biology of Bergmann glia. As Cbln1 can be trafficked in both anterograde and retrograde directions and is expressed in neurons in many brain regions it will be important to establish if it undergoes trafficking elsewhere in the nervous system.

Materials and methods

1. Animals and genotyping

The production, characterization and genotyping of *cbln1*-null (Hirai et al., 2005), *cbln3*-null (Bao et al., 2006) and GluR δ 2-null mice (Kashiwabuchi et al., 1995) have been described. The GluR δ 2 animals were originally obtained from Dr. M. Mishina and genotyped as described (Takeuchi et al., 2001). Mutant and wild type control animals were obtained from the same litters. Mice were maintained at St. Jude Children's Research Hospital and had free access to food and water. Investigational procedures conformed to all applicable federal rules and guidelines and were approved by the Institutional Animal Care and Use Committee.

2. Immunohistochemistry and in situ hybridization

Animals were anesthetized at postnatal day 30, using 0.1 mg/ml chloral hydrate and perfused transcardially with buffered 4% paraformaldehyde and the brains were dissected, fixed overnight in 4% paraformaldehyde, and processed for paraffin-embedding. Sagittal sections from cerebella were cut at a thickness of 5 μ m. The production and characterization of the polyclonal anti-Cbln1 E3 epitope antiserum was as described (Bao et al., 2005; Wei et al., 2007). Briefly, for immunohistochemical detection of Cbln1-like immunoreactivity, sections were first heat retrieved in 0.01M sodium citrate buffer (pH 6.0) containing 0.05% Tween-20 (Sigma, St. Louis, MO) for 20min at 98°C. Endogenous peroxidase activity was inhibited by incubating the sections in 3% hydrogen peroxide/water for 5 minutes. After blocking with 10% normal horse serum (Vector Labs, Burlingame, CA) in PBS, sections were incubated overnight at 4°C with anti-Cbln1 E3 antiserum (dilution, 1:1000 in blocking buffer). Immunocomplexes were revealed using a peroxidase-conjugated anti-rabbit kit and diaminobenzidine tetrahydrochloride (DAB) substrate (Dako, Carpinteria, CA). After immunostaining, the sections were counterstained with hematoxylin (Vector Labs, Burlingame, CA).

For double labeling, the E3 Cbln1 antiserum was combined with one of the following antisera: the glia marker, S100 (mouse antibody, 1:500; Chemicon, Phillipsburg, NJ) (Haglid et al., 1975), the neuron marker, Neu-N (mouse antiserum, 1:1000, Chemicon, Phillipsburg, NJ, USA) (Mullen et al., 1992), the Purkinje cell marker, calbindin D-28K (mouse antibody, 1:1000; Sigma-Aldrich, St Louis, MO) (Iacopino et al., 1990), or the neuronal dendrite marker, MAP2 (mouse antibody, 1:100; Sigma-Aldrich, St Louis, MO) (Bernhardt and Matus, 1984). The slices were incubated with a mixture of primary antibodies overnight at 4°C, then incubated for 1 h with Alexa 488-labeled donkey anti-mouse and Alexa 594-labeled donkey anti-rabbit secondary antibodies (1:200, Invitrogen, San Diego, CA). Sections were counterstained with DAPI (Invitrogen, San Diego, CA) and analyzed using confocal laser microscopy.

To study the sub-cellular localization of Cbln1, sections were incubated overnight with a mixture of rabbit anti-Cbln1 (1:1000) and antibodies to one of the following markers: cathepsin D (lysosome marker; goat antiserum, 1:300, Santa Cruz Biotechnology, Santa Cruz, CA) (Erickson & Blobel, 1979), mannose 6-phosphate receptor (late endosome/early lysosome marker; mouse antibody, 1:100, Affinity BioReagents, Golden, CO) (Brown & Farquhar, 1984), KDEL (endoplasmic reticulum marker; mouse antibody, 1:500, Stressgen Bioreagents,

Victoria, Canada) (Tooze et al., 1989, GM130 (Golgi marker, mouse antibody, 1:100, BD Biosciences, San Jose, CA) (Nakamura et al., 1995). Sections were incubated for 1 h with Alexa 488- or Alexa 594-labeled species-specific secondary antibodies (1:200, Invitrogen, San Diego, CA) as described previously (Wei et al., 2007). For triple staining, the sections were then incubated with either anti-calbindin D-28K (1:500) or anti-S100 (1:200), and then Alexa-647-labeled donkey anti-mouse secondary antibody (1:200, Invitrogen, San Diego, CA). Sections were analyzed using confocal laser microscopy.

In situ hybridization was performed on cryostat sections exactly as described previously for *cbln1* (Pang et al., 2000) and L7 (Wang et al., 2006). Sense and antisense riboprobes for *cbln1* and L7 were labeled with $\alpha^{32}\text{P}$ -UTP (Pang et al., 2000).

3. Generation of L7-*cbln1* transgenic mice

The entire coding region of *cbln1* was inserted into the unique BamHI site of pL7 Δ AUG (Oberdick et al., 1990). The resulting plasmid was digested with HindIII and EcoRI and the linearized construct separated from the vector by electrophoresis. This construct contained approximately 1 kb of DNA upstream of the L7 transcription start site and 2 kb of DNA downstream of the polyadenylation signal. The purified L7-*cbln1* DNA fragment was injected into fertilized oocytes from wild type FVB mice using standard techniques.

Mouse genotypes were determined by PCR of DNA samples prepared from tail clippings. The primers amplified the fusion region between L7 and *cbln1* and were: forward 5'-TGAGCCTCATGTTGAACGGG-3' and reverse 5'-GGAAGCTGAGTGCAGCAGGATC-3'. Thirty-five cycles of PCR were performed as follows: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Offspring positive for the L7-*cbln1* transgene were subsequently mated with wild type or *cbln1*^{+/-} mice to generate appropriate genotypes for analysis.

4. Quantitative analysis

Sagittal sections through cerebella from wild type, *cbln3*-null, GluR δ 2-null and GluR δ 2/*cbln3*-double null mice were immunostained for Cbln1 as described above. Four sections spanning from the cerebellar vermis to the hemispheres of each mouse were scored for the number of Cbln1-positive vesicle-like structures in the cell bodies of one hundred Purkinje neurons (total of four hundred Purkinje cells per mouse). The mean number of CLI-positive vesicle-like structures per one hundred Purkinje cells was compared between three independent mice of each genotype (n=3) using Student's t-test.

To quantify co-localization of Cbln1-like immunoreactivity with markers of intracellular structures double immunofluorescence and confocal microscopy was employed. Multiple fields from at least two sections through the cerebellum and brain stem from three mice of each genotype were analyzed using a 60X objective. At this magnification each field contained on average 6 cells of interest resulting in the analysis of approximately 70 cells per genotype. It was not possible to quantify co-labeling following Z-stack compression due to the overlap of signal, and so each optical section in each field was counted separately. The Z-stack consisted of five 1-micron optical sections that were first scored for CLI-positive structures and then for the number of CLI-positive structures that were double labeled for the respective marker. The data were calculated as the percent of CLI-positive structures that were co-labeled for the marker of interest. Data is presented in the text as the average percentage of co-labeled puncta for each cell type and genotype.

5. Immunoblotting and radioimmunoassay

Immunoblotting for Cbln1 was performed on cerebellar lysates using the E3 anti-Cbln1 antiserum exactly as described in Bao et al., 2006. For radioimmunoassay, cerebella were homogenized in 6M guanidine hydrochloride and desalted and the peptide fraction purified using a C18 SepPak cartridge (Waters Corporation, Milford, MA, USA) as described previously (Slemmon et al., 1984). The quantitative radioimmunoassay for cerebellin using iodinated synthetic cerebellin and the E2 anti-cerebellin peptide antiserum was as described (Morgan et al., 1988)

6. Rota-rod analysis

To quantify locomotor performance in *cbln1*-null and *cbln1*-null mice harboring an L7-*cbln1* transgene, an accelerating rotor-rod analysis was performed on gender-matched littermates of the respective genotypes aged 35–45 days of age. To further minimize confounders such as integration site and genetic background effects we analyzed two independent transgenic founder lines (lines C and D) crossed into the *cbln1*-null background. Littermates of all four genotypes (wild type and *cbln1*-null mice with or without an L7-*cbln1* transgene) were tested on an accelerating Rota-rod (San Diego Instruments, San Diego, CA). The Rota-rod was programmed to accelerate from 0 to 40 rpm in 4 min. Each mouse was tested daily over 5 consecutive days. The latency of the mice to fall from the rod was scored as an index of their motor coordination. The data from each genotype was pooled over the 5 days of testing and analyzed by one-way Anova and multiple comparisons between the groups was performed using the Bonferroni post-hoc test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Cbln1-like immunoreactivity (CLI) in adult mouse cerebellum. Immunohistochemistry with the E3 epitope antiserum revealed punctate CLI in wild type cerebellum (A) whereas there was no staining in cerebellum from *cbln1*-null mice (B). Panels (C) and (D) show enlarged views of the boxed regions in (A) and (B), respectively. Panel (E) reveals enhanced expression of CLI in an adult *cbln3*-null mouse cerebellum whereas there is no CLI in a *cbln1*-, *cbln3*-double null mouse (F). Scale bars: A and B, 50 μ m; C–F, 15 μ m.

Fig. 2.

Localization of Cbln1-like immunoreactivity (CLI) to Purkinje cells and Bergmann glia. Double immunofluorescence labeling was performed on *cbln3*-null (A, B, E, F) or *cbln1*-null (C, D, G, H) mouse cerebellum using the E3 Cbln1 antiserum (red) and either the Purkinje cell marker, calbindin-D28K (green, A–D) or the glia marker, S100 (green, E–H). Merged confocal images revealed that punctate CLI is co-localized with both calbindin- (A, B) and S100-positive (E, F) cells, indicative of its presence in Purkinje cells and Bergmann glia, respectively. There is no detectable CLI in *cbln1*-null cerebellum (C,D,G,H). DAPI is used to visualize nuclei. Scale bar: 20 μ m.

Fig. 3.

Localization of Cbln1-like immunoreactivity (CLI) to lysosomes and endosomes in Purkinje cells. Triple immunofluorescence labeling was performed on *cbln3*-null mouse cerebellum using the anti-E3 Cbln1 antiserum (red, A, E, I and M), anti-calbindin-D28K (blue, D, H, L and P), and markers (green) of endoplasmic reticulum (KDEL, B), Golgi apparatus (GM130, F), late endosomes (mannose-6-phosphate receptor, J), and lysosomes (cathepsin-D, N). Merged confocal images reveal that CLI is almost always co-localized with the lysosome marker (O). The majority of CLI is co-localized with this endosome marker (K) suggesting that CLI is present in endosomes and persists through their maturation into lysosomes. CLI is

not present in ER (C) or Golgi (G). Merged confocal images (D, H, L, P) with calbindin-D28K show the localization of CLI in Purkinje cells. Scale bar, 10 μ m.

Fig. 4. Generation and characterization of L7-*cbln1* transgenic mice. (A) Structure of the L7-*cbln1* transgene. A *cbln1* cDNA including its native initiator ATG and signal sequence was inserted into a unique BamHI site in the fourth exon of the L7 gene (see Materials and Methods for details). (B) RT-PCR of mRNA from cerebellum of wild type (Wt) and four transgenic (TgA-D) mouse lines showing reverse transcriptase-dependent (+/-RT) expression of the L7-*cbln1* recombinant mRNA. Lower panel shows actin loading control. (C) Immunoblotting of protein extracts from cerebella of wild type (W), transgenic line C on a wild type background (T/W), *cbln1*-null (KO), and transgenic line C on a *cbln1*-null background (T/KO) mice using an anti-Cbln1 E3 antiserum. The arrow shows the location of full length Cbln1. (D) In situ

hybridization (a, b, e, f) and immunohistochemistry (d, h) showing localization of L7-*cbln1* mRNA and protein, respectively, to cerebellar Purkinje cells of transgenic line C on a *cbln1*-null background. An L7-specific probe (a, e) and a *cbln1*-specific probe (b,f) were used for in situ hybridization. The inserted panels in (a) and (b) show the negative control using the respective sense probes and have no specific hybridization signal. (e) and (f) show enlarged views of (a) and (b), respectively. Cresyl violet stained sagittal sections (c, g) show the location of the positive signals in Purkinje cells in a *cbln1*^{-/-}L7-*cbln1* mouse. The L7 probe detects both endogenous L7 mRNA as well as recombinant RNA, and is used to detect ectopic expression of the transgene. As a *cbln1*-null mouse was used the *cbln1* probe can only detect recombinant mRNA generated from the transgene. (d) Using immunohistochemistry, intense, punctate CLI is detected in Purkinje cells. (h) An enlarged view of the boxed region in (d) confirms punctate cytoplasmic CLI in Purkinje cells and also reveals additional punctate CLI in the granule cell layer. Scale bar: d, 150 μ m; h, 50 μ m.

Fig. 5. Localization of Cbln1-like immunoreactivity (CLI) in the *cbln1*^{-/-}/L7-*cbln1* mouse cerebellum. To identify the cell types containing CLI in *cbln1*^{-/-}/L7-*cbln1* mice double immunofluorescence labeling was performed. The anti-E3 Cbln1 antiserum (red) was used in combination with the markers (green) of Purkinje cells (calbindin-D28K, A–C), glia (S100, D), neurons (NeuN, E,F), dendrites (MAP2, G–I and K–M), and presynaptic terminals (synaptophysin, J). (A) shows co-localization of CLI with the soma of Purkinje cells. Enlarged views (B and C) of the boxed regions in (A) showing CLI associated with Purkinje cell dendrite (B) and axon (C). (D) co-localization of CLI with S100 in Bergmann glia. (E) shows co-localization of CLI with NeuN-positive nuclei. (F) is enlarged view of the boxed region in (E)

showing CLI in neurons in the molecular layer. (G) shows co-localization of CLI with MAP2 in molecular and internal granule cell layers. Enlarged view (H) of boxed region in G shows CLI in a Purkinje cell dendrite and enlarged view (I) of boxed region in (G) shows CLI co-localized with presumptive dendrites of granule neurons. (J) shows abundant punctate CLI in the granule cell layer that is not co-localized with the presynaptic marker, synaptophysin, which predominantly labels mossy fiber terminals. (K) shows co-localization of CLI with MAP2 in a deep cerebellar nucleus. (L) is an enlarged view of box in (M) and shows association of CLI with the cell body of a large nuclear neuron and panel (M) is an enlarged view of box in (K) showing association of CLI with a dendrite of a small nuclear neuron. DAPI staining is used throughout to provide additional orientation. Scale bar: A,G,K, 50 μm ; B,C,F,H,I,L,M, 7 μm ; D,E,J, 35 μm .

Fig. 6. Presence of Cbln1-like immunoreactivity (CLI) in the inferior olive of *cbln1*^{-/-}/L7-*cbln1* mice. (A) Immunohistochemistry reveals CLI in inferior olive neurons. (B) Enlarged view of boxed region in A showing punctate cytoplasmic CLI within inferior olive neurons. CLI staining is not detected in inferior olive neurons of wild type (C) or *cbln1*-null (D) mice. (E) Cresyl violet stained sagittal section shows the location of the inferior olive (circled region) in a *cbln1*^{-/-}/L7-*cbln1* mouse. (F) Serial sagittal section processed for in situ hybridization with a *cbln1*-specific probe does not detect *cbln1* mRNA in the inferior olive (circled region). Scale bars: A, 70 μm; B-D, 30 μm.

Fig. 7. Subcellular localization of Cbln1-like immunoreactivity (CLI) in Purkinje cells and inferior olive neurons of *cbln1*^{-/-}/*L7-cbln1* mice. (A–F) Purkinje cells, (G–L) inferior olive neurons. Double immunofluorescence labeling was performed using the E3 Cbln1 antiserum (red, A, D, G and J) and the markers, cathepsin D (B and H) for lysosomes or mannose-6-phosphate receptor (E and K) for late endosomes. Merged confocal images show that the majority of the CLI is co-localized with both cathepsin D (C and I), and mannose-6-phosphate receptor (F and L) in both classes of neurons. DAPI is used to visualize nuclei in the merged images. Scale bar: A–F, 10 μ m; G–L, 5 μ m.

Fig. 8.

Cbln1-like immunoreactivity (CLI) is dramatically decreased in the Purkinje cell layer of adult GluRδ2-deficient mice. Prominent CLI is detected in the Purkinje cell layer of wild type (A) but not GluRδ2-null cerebellum (B) using the E3 antiserum. (C) and (D) show enlarged views of the boxed regions in (A) and (B), respectively. Punctate CLI is present in the granule cell layers of wild type (C) and GluRδ2-null cerebellum (D), but little or no staining is evident in presumptive Purkinje cells and Bergmann glia of GluRδ2-deficient mice (D). To obtain a stronger signal for CLI, immunohistochemistry was performed on GluRδ2-null mice bred onto a *cbln3*-null background. Prominent CLI was present in the granule cell layers of *cbln3*-null (E) and *cbln3*/GluRδ2-double null (F) mice. However, no CLI was evident in presumptive

Bergman glia or Purkinje cells of *cbln3*/GluR δ 2-double null (F) mice. Scale bar: A and B, 50 μ m; C–F, 20 μ m.

Fig. 9.

Cbln1-like immunoreactivity (CLI) is absent in Purkinje cells and Bergmann glia of GluR δ 2-null mice. Double immunofluorescence labeling was performed on sections from *cbln3*-null (A–C and G–I) and *cbln3*/GluR δ 2-double null (D–F and J–L) mice using the E3 Cbln1 antiserum (red, A, D, G and J) and antisera to either the Purkinje cell marker calbindin D-28 (green, B and E) or the glia marker S100 (green, H and K). Merged confocal images show that the CLI associated with Purkinje cells (C) and Bergmann glia (I) in *cbln3*-null mice is absent in the equivalent cell populations (F and L, respectively) in *cbln3*/GluR δ 2-double null mice. DAPI is used to visualize nuclei. Scale bar: 10 μ m.

Fig. 10.

GluR δ 2 influences Cbln1 trafficking but not Cbln1 protein levels. (A) To quantify the influence of GluR δ 2 on CLI, the number of CLI-positive puncta were counted in the cell bodies of identified Purkinje neurons from 3 wild type (WT), GluR δ 2-null (D2KO), *cbln3*-null (Cbln3KO), and *cbln3*/GluR δ 2-double null mice. Data are presented as mean and SEM of the number of CLI-positive puncta per 100 Purkinje cells (PCs). Immunoreactive puncta decreased significantly (***) $p < 0.0001$, $n = 3$) in the GluR δ 2-null strains compared to their respective control littermates. (B) Immunoblotting of cerebellar extracts from wild type (WT) and GluR δ 2-null (D2KO) mice with the E3 antiserum revealed no obvious difference in the levels or pattern of Cbln1 and its degradation products.

Fig. 11.

Ectopic expression of Cbln1 in Purkinje cells of *cbln1*-null mice improves rota-rod performance. Gender balanced littermates of each genotype aged between 35–45 days old were tested on a standardized accelerating rota-rod. To reduce variability due to genetic background and integration site effects, two independent lines of L7-*cbln1* transgenic mice (TgC, and TgD, see Figure 4B for details) were crossed onto a *cbln1*-null background and offspring analyzed separately. Panel (A) shows results for transgenic line C (TgC) and panel (B) the data for line D (TgD). Wild type (WT) mice, wild type mice harboring the respective transgene (WT/TgC/D), *cbln1*-null (KO) mice and *cbln1*-null animals carrying the transgene (KO/TgC/D) were tested on 5 consecutive days. The latency to fall in minutes for all animals of a given genotype

was summed over the 5 trials and data presented as mean and SEM (error bars). The number of animals in each group (N) is shown. Multiple comparisons between groups using the Bonferroni post-hoc test revealed that the presence of TgC (panel A, *** $p=0.002$) and TgD (panel B, *** $p=0.004$) significantly improved the performance of *cbln1*-null animals. One-way ANOVA showed that only the *cbln1*-null group differed significantly from all other groups.