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A GluD coming-of-age story

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Abstract

The GluD1 and GluD2 receptors form the GluD ionotropic glutamate receptor (iGluR) sub-family. Without known endogenous ligands, they have long been referred to as “orphan” and remained enigmatic functionally. Recent progress has, however, radically changed this view. Both GluD receptors express in wider brain regions than originally thought. Human genetic studies and analyses of knockout mice revealed their involvement in multiple neurodevelopmental and psychiatric disorders. The discovery of endogenous ligands, together with structural investigations, opened the way towards a mechanistic understanding of GluD signaling at central nervous system synapses. These studies have also prompted the hypothesis that all iGluRs, and potentially other neurotransmitter receptors, rely on the cooperative binding of extracellular small-molecule and protein ligands for physiological signaling.

Keywords

delta glutamate receptor; cerebellum; long-term depression; synapse formation; C1q family

Introduction

Ionotropic glutamate receptors (iGluRs) come in four flavors, AMPA, NMDA, kainate and delta (GluD). The GluD sub-type, GluD1 (GluRδ1) and GluD2 (GluRδ2), were identified more than 20 years ago [1–4]. Despite considerable sequence similarities to the other iGluRs in the putative **ligand-binding domains** (**LBDs**; see Glossary), GluD1 and GluD2 have been referred to as “orphan receptors” until recently, because their endogenous ligands were unknown. GluD2 is predominantly expressed in cerebellar Purkinje cells and plays crucial roles in motor coordination and motor learning [1–4]. In contrast, GluD1 is predominantly expressed in the inner ear and plays an essential role in high-frequency hearing [5]. Furthermore, various mutations in the genes encoding GluD1 and GluD2 in mice (*grid1*, *grid2*) and human (*GRID1, GRID2*) cause cognitive dysfunctions. Nevertheless, the mechanisms underlying the GluD1 and GluD2 functions in neural circuits have long remained elusive.

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In recent years, however, this situation has changed significantly, mostly due to the identification of two extracellular endogenous ligands for GluD2 in the cerebellum, D-serine and Cbln1. While D-serine binds to the LBD [6], Cbln1 binds to the **amino-terminal domain** (**ATD**) of GluD2 [7, 8]. Although endogenous ligands for GluD1 are still unknown, D-serine and Cbln family proteins (*i.e.*, Cbln1, Cbln2 and Cbln4) bind to GluD1 at least *in vitro* [7, 9]. GluD2 does not show any direct channel activity upon binding of known ligands (Box 1). Instead, GluD2 activation induces endocytosis of AMPA-type iGluRs during **long-term depression** (**LTD**), a form of synaptic plasticity involved in cerebellar motor learning. This metabotropic signaling mode, independent of channel activity, has recently captured the spotlight because it appears to be widespread among iGluRs, complementing their canonical ionotropic functions (Box 2). Endogenous ligands that bind to the ATD of other iGluRs have also been identified. Thus, the function of iGluRs may generally be regulated by cooperative binding of ligands, one at the ATD and another at the LBD (Box 3), and a mechanistic understanding of GluD1/D2 will likely provide broader insights into iGluR signaling at neuronal synapses. As it is often the case in science, answering one question triggers many others. However, this is a good time to summarize what has been learned about GluD receptors, celebrate their belated coming-of-age and overview future research directions.

GluD2 signaling

GluD2 is predominantly expressed in cerebellar Purkinje cells (PCs). However, it is now clear that GluD2 is widely present, albeit at lower levels, in multiple mouse brain regions including the cerebral cortex, hippocampus, striatum, thalamus, mesencephalon and in the retina [10–12]. Indeed, single-nucleotide polymorphisms in *GRID2* are significantly associated with schizophrenia-related endophenotypes, such as prepulse inhibition [13, 14]. Copy number variations in *GRID2* are also reported in autism spectrum disorder (ASD) [15, 16]. Furthermore, several human cases of *de novo* [17] and inherited [18–21] mutations in *GRID2* displayed not only cerebellar ataxia, but also intellectual disability and phenotypes unrelated to the cerebellum, such as paraplegia and retinal dystrophy. Located on chromosome 4 (q22), *GRID2* is also a candidate gene in “4q deletion syndrome,” [22] which is associated with neurodevelopmental disorders, such as ASD, intellectual disability and attention deficit hyperactivity disorder. Since the cerebellum itself is likely involved in non-motor cognitive functions [23–25], it remains to be determined whether and how GluD2 functions in extracerebellar regions. In the following sections, we focus on GluD2 signaling in the most well-characterized brain region, the cerebellum.

Synapse formation and maintenance

In the cerebellar cortex, GluD2 is highly expressed in distal dendrites of PCs that receive synaptic input from parallel fibers (PFs; axons of granule cells) (Figure 1A). Proximal dendrites that receive climbing fiber inputs (CFs; axons of inferior olivary nucleus) and molecular layer interneurons (MLI; stellate and basket cells) are completely devoid of GluD2. GluD2 binds to Cbln1, which is released from granule cells, via its ATD. At the same time, Cbln1 binds to its presynaptic receptor neurexin (Nrx) located on the PF terminals in a paracrine or autocrine manner (Figure 1B) [7, 8]. In *Grid2*-null mice, the number of PF–PC synapses is reduced by up to 60% of the wild-type phenotype. In addition,

loss of GluD2 in the adult mouse brain results in a gradual reduction of PF–PC synapses. Conversely, introduction of Cbln1 and GluD2 restored normal PF–PC synapses in adult *Cbln1*-null and *Grid2*-null mice, respectively [26]. In humans *GRID2* deletions also cause progressive cerebellar atrophy [17–20]. Therefore, Cbln1–GluD2 signaling is an important bidirectional **synaptic organizer** for both the formation and maintenance of PF–PC synapses throughout life.

Although various synaptic organizers have been identified, knockout of single, or even a combination of responsible genes does not typically result in loss of synapse as severe as observed in *Grid2*-null or *Cbln1*-null mice. How could Cbln1–GluD2 signaling achieve such a strong synaptogenic activity? Recent structural analyses have provided some clues. Cbln1 belongs to the **C1q-tumor necrosis factor superfamily**. Endogenous Cbln1 exists as a hexamer, a dimer of trimers linked by their amino-terminal cysteine-rich regions (CRR) [7]. Isothermal titration calorimetry (ITC) assays indicate that Cbln1 binds to Nrx containing a splice site 4 (SS4) insert [Nrx(+4)] with high affinity (K_D ~ 40 nM) [27, 28]. Although two monomeric Nrxs were proposed to bind to one hexameric Cbln1 [27], single particle electron microscopy analyses and ITC measurements support a 1:1 stoichiometry model [28, 29], in which one Nrx monomer binds to the CRR connecting two trimers of one Cbn1 hexamer, just like a person riding a bicycle (Figure 1C). The binding affinity between Cbln1 and Nrx(+4) is at least one order of magnitude higher than that between neuroligin and Nrx(+4) [30], indicating that Cbln1 will outcompete neuroligin as a binding partner for Nrx(+4) at PF terminals.

Like other iGluRs, GluD1 and GluD2 have dimeric ATDs [28]. The overall tetrameric GluD arrangement is largely driven by interactions in the transmembrane region, and further stabilized by low affinity “dimer-of-dimers” interactions between ATDs [28] and weak LBD dimers [31]. Surface plasmon resonance assays revealed that a trimeric Cbln1 globular domain, where one subunit forms the majority of the contacts, can bind a single GluD2 ATD with an affinity in the high μM range, representing the minimal units for the GluD2-Cbln1 interaction; however, avidity effects arising from the oligomeric nature of both partners increased the apparent affinity between the full-length hexameric Cbln1 and the tetrameric GluD2 into the nM range (~125 nM) (Figure 1D) [28]. Although other mechanisms may also be involved [29], such high affinity binding may be one of the reasons why Nrx–Cbln1–GluD2 forms a tight bridge over PF–PC synapses with a 2 (monomers) :2 (hexamers):1 (tetramer) stoichiometry (Figure 1E).

Regulation of presynaptic organization

Cbln1–GluD2 signaling leads to accumulation of neurexins and synaptic vesicle markers at presynaptic sites [32] (Figure 1B). However, while α-neurexins typically recruit N- and P/Q-type Ca^{2+} channels at presynaptic sites, GluD2 specifically regulates the function of R-type Ca^{2+} channels [33]. As a result, presynaptic long-term potentiation, which requires R-type Ca^{2+} channels on PF terminals, is impaired in *Grid2*-null mice [33]. Although the mechanism for this specific Ca^{2+} channel regulation by GluD2 remains to be determined, Nrx(+4), which are selectively recruited by Cbln1–GluD2 [8, 32], likely play a role.

Another key feature of the Cbln1–GluD2 presynaptic organizer complex is its ability to rapidly (within several hours) induce dynamic morphological changes at PF terminals [34]. Unlike most axons, PFs rapidly regenerate after surgical transection in adult wild-type mice and the PF–PC synaptic connections are fully restored after initial degeneration, and a subsequent hypertrophic phase [35]. However, transected PFs fail to regenerate synapses on *Grid2*-null PCs [35], highlighting that the rapid axonal remodeling induced by GluD2, via Cbln1, might be related to the unusual regeneration capacity of PFs.

Regulation of postsynaptic organization

As expected for postsynaptic organizers, Cbln1–GluD2 signaling triggers the accumulation of multiple molecules, including homer, shank and PSD93 (Figure 1B), at PF–PC synapses. In *Cbln1*-null mice, the density of postsynaptic GluD2 receptors decreases to approximately 50% of the wild-type phenotype [7]. An interesting consequence of Cbln1–GluD2 signaling is that it suppresses accumulation of postsynaptic AMPA receptors (Figure 1B). The density of AMPA receptors is 4–6 times lower at PF–PC synapses compared to CF–PC synapses, which do not express GluD2. Conversely, AMPA receptors increase by 3–5 folds at PF synapses, but not at CF ones, in *Grid2*-null PCs [36]. Since GluD2 is also expressed in wild-type MLIs at low levels as discussed later, the AMPA receptor density also increases at PF–MLI synapses in *Grid2*-null mice [36]. An increase in postsynaptic AMPA receptors density could be caused by the loss of LTD in *Grid2*-null PCs. However, this phenomenon did not occur in mice lacking metabotropic glutamate receptor 1 (mGlu1), in which LTD at PF–PC synapses (PF–LTD) was impaired [36]. Alternatively, the increased density of postsynaptic AMPA receptors could be a homeostatic response to the reduced number of PF–PC synapses in *Grid2*-null mice. However, a homeostatic mechanism cannot explain the same observation at PF–MLI synapses, because their number is unaffected in *Grid2*-null mice [12]. Interestingly, the constitutive inclusion of the SS4 sequence in presynaptic Nrx3 causes a reduction in postsynaptic AMPA receptor numbers in hippocampal neurons [37]. Conversely, a constitutive SS4 excision in Nrx3 increased the densities of both AMPA receptors and leucine-rich repeat transmembrane proteins (LRRTMs) in postsynaptic membranes. Thus, we postulate that AMPA receptors and LRRTMs are recruited through a similar SS4-dependent mechanism to PF–PC synapses in *Grid2*-null mice, where the Nrx(+4)-Cbln1-GluD2 signaling is disrupted.

GluD2 also regulates formation and maintenance of CF–PC synapses (Figure 1A). Immature PCs are normally innervated by multiple CFs, but redundant CFs are gradually eliminated until a one-to-one relationship is established. However, *Grid2*-null PCs remain innervated by supernumerary CFs even in adulthood. Since a similar disruption of the elimination process has been observed in *mGlu1*-null mice, it is likely that reduced PF synapses associated with decreased mGlu1 activity may indirectly cause this phenotype. While many mutant mice show sustained CF innervation of PCs, *Grid2*-null mice are unique in that surplus CFs innervate distal dendrites of PCs, which are normally occupied by PF synapses. Ablation of *Grid2* in adulthood also induces CF innervation to the distal PC dendrites [38]. Moreover, in *Grid2*-null mice, surplus CFs often innervate distal dendrites of PCs from different microdomains, leading to aberrantly clustered CF firing [39]. Although the underlying

mechanisms remain unclear, these findings suggest that GluD2 indirectly prevents CF invasion by strengthening PF–PC synapses at distal dendrites.

Cbln1–GluD2 signaling also has a suppressive role on MLI–PC synapse formation and function (Figure 1B). The density of MLI–PC inhibitory synapses is increased in *Cbln1*-null mice [40]. Amplitude and frequency of the miniature inhibitory postsynaptic current (mIPSC) were also increased in *Cbln1*-null PCs. Incubation with recombinant Cbln1 restored these phenotypes in a manner dependent on GluD2. Since GluD2 is not expressed at MLI–PC synapse, it is likely that Cbln1–GluD2 signaling at PF–PC synapses indirectly prevents MLI–PC synapse formation. As will be described later in detail, functions of MLI–PC are at least partly regulated by the protein tyrosine phosphatase activity downstream of Cbln1–GluD2 signaling [41].

Regulation of synaptic plasticity

PF-LTD is thought to mediate motor learning, although its exact role remains contentious [26]. Like LTD in other brain regions, PF-LTD is mediated by clathrin-dependent endocytosis of postsynaptic AMPA receptors. Phosphorylation of serine 880 (S880) of the GluA2 subunit of AMPA receptors by protein kinase C (PKC) is the initial step of PF-LTD, allowing AMPA receptors to be freed from their anchoring protein glutamate receptor interacting protein 1 (GRIP1). AMPA receptors then laterally diffuse to the perisynaptic endocytic zones where clathrin accumulates during LTD. PF-LTD is unique in that it absolutely requires GluD2.

The C-terminal domain (CTD) of GluD2 is essential for PF-LTD. Among various proteins that bind to the CTD, protein tyrosine phosphatase PTPMEG likely plays a key role in PF-LTD induction. While the virus-mediated expression of GluD2$^{\Delta CTD}$, which lacks the CTD, could not restore LTD in *Grid2*-null PCs, GluD2$^{\Delta CTD}$ was able to restore LTD when it was directly fused to the PTPMEG phosphatase domain, in a manner dependent on its phosphatase activities [42]. Substrate-trapping assays revealed that GluA2 tyrosine 876 (Y876) is a substrate for PTPMEG. Interestingly, phosphorylation of GluA2-Y876 hindered subsequent phosphorylation at GluA2-S880 by PKC *in vitro* [42]. Thus, in *Grid2*-null or *PTPMEG*-null PCs, LTD-inducing stimuli failed to phosphorylate S880 because GluA2-Y876 is already phosphorylated (Figure 2A). Therefore, GluD2 likely serves as a master switch, regulating LTD induction by coordinating unique interactions between two phosphorylation sites of GluA2. A recent computational model taking into account of the dual regulation by two GluA2 phosphorylation sites revealed how PTPMEG cooperates with PKC to drive LTD expression [43].

Agonist binding induces large relative D1–D2 domain motions and “closure” of the iGluR LBDs, most likely responsible for the subsequent ion channel opening. Although D-serine triggers similar conformational changes upon GluD2 LBD binding, no channel activity was observed [31]. Instead, application of D-serine induces AMPA receptor endocytosis in a manner dependent on the CTD of GluD2 and phosphorylation by PKC [6] (Figure 2B). Since burst stimulation of PFs leads to the release of D-serine from Bergman glia in immature cerebellum, PF-LTD induction and motor learning are facilitated through this pathway [6]. Interestingly, D-serine-dependent PF-LTD is impaired in *Cbln1*-null PCs or

Grid2-null PCs expressing mutant GluD2 that cannot bind Cbln1. Similarly, insertion of a glycosylated linker between the ATD and LBD layers of GluD2 disrupted D-serine-dependent PF-LTD [28] (Box 3). Therefore, D-serine binding to the LBD and Cbln1 binding to the ATD likely cooperate to induce conformational changes to GluD2, activating intracellular signaling leading to AMPA receptor phosphorylation and endocytosis.

Conjunctive stimulation of CFs and PFs can induce PF-LTD independent of D-serine binding. Indeed, D-serine becomes undetectable in mature cerebellum because expression of D-amino acid oxidase, an enzyme that degrades D-serine, significantly increases in adulthood. Nevertheless, D-serine-independent PF-LTD is impaired in *Cbln1*-null mice. Since D-serine-independent PF-LTD is normally induced in heterozygous *Grid2*-null mice, ~50% reduction of postsynaptic GluD2 in *Cbln1*-null mice cannot account for the impaired PF-LTD. Although the precise mechanism remains to be clarified, Cbln1 binding to the ATD of GluD2 is necessary to maintain basal tyrosine phosphorylation levels, and D-serine binding to the LBD may further enhance the PTPMEG association with the GluD2 C-terminus.

Burst stimulation of PFs induces slow excitatory postsynaptic currents (slowEPSCs), which are mediated by mGlu1 located at perisynaptic sites (Figure 2C). In *Grid2*-null mice, the slow EPSC amplitude is reduced [42, 44] or the time to reach the peak amplitude slowed [45]. Similarly, the slowEPSC amplitude is reduced in *Cbln1*-null mice [40]. Although slowEPSCs could be partially mediated by ion flow through GluD2 channels [44], they are largely carried by C3-type transient receptor potential channels (TRPC3) and completely abolished in *Trpc3*-null mice [46]. The reduction of slowEPSCs could partly reflect a redistribution of TRPC3 and mGlu1 in *Grid2*-null mice [45]. However, reduced slowEPSC amplitudes in *Grid2*-null [42] or *Cbln1*-null [40] PCs were significantly rescued by a 20-min treatment with a *Src* family tyrosine kinase inhibitor. Similarly, increased mIPSC amplitude in *Cbln1*-null PCs were restored by inhibition of *Src* [40] (Figure 2D). Although it remains unclear how mGlu1–TRPC3 coupling and GABA responses are regulated by tyrosine phosphorylation, Cbln1–GluD2 signaling likely modulates these synaptic responses by reducing postsynaptic protein tyrosine phosphorylation levels via PTPMEG.

GluD1 signaling

GluD1 mRNA expression levels are generally higher in younger animals, especially in the striatum and the anteroventral thalamic nucleus. In the adult, GluD1 is most highly expressed in the inner ear and moderately in the hippocampus [5]. However, recent reexamination of adult mice brain using specific and sensitive histochemical probes revealed wide expression of GluD1, with high levels in higher brain regions, including the cerebral cortex, striatum, hippocampus, central nucleus of the amygdala and cerebellar cortex (Figure 3A) [11, 12]. In human, single-nucleotide polymorphisms in *GRID1* are significantly associated with schizophrenia [47, 48], depressive symptoms in schizophrenia [49], major depressive disorder [50], comorbid depressive syndrome and alcohol dependence [51]. Data mining analyses of large genome-wide association studies also indicated *GRID1* as a candidate gene for schizophrenia [52]. Common genetic variation in the promoter region of *GRID1* is associated with grey matter variation in prefrontal and anterior thalamic brain

areas in healthy subjects [53]. Copy number variation studies also reveal intronic deletions of *GRID1* [54–57] in ASD cases. Recurrent deletions in the chromosome 10q22-q23 region where *GRID1* is located were shown to be associated with behavioral and neurodevelopmental abnormalities, including cognitive impairment, ASD and hyperactivity [58]. Finally, GRID1 expression is commonly downregulated in induced pluripotent stem (iPS) cells derived from Rett patients who are associated with ASD [59]. Nevertheless, how and in which brain regions GluD1 signaling contributes to these disorders remains unclear. For example, not only *GRID1* mRNA but also miR-346, located in a *GRID1* intron, which targets several schizophrenia susceptibility genes, are reduced in schizophrenia patients [60], suggesting some symptoms could be caused by gene products other than *GRID1*. Here, we focus on what we have learned from *Grid1*-null mice regarding GluD1 signaling in the cerebellum, inner ear and the forebrain.

Synaptic organizer in the cerebellum

In the cerebellar cortex, GluD1 mRNA was selectively expressed in MLIs (Figure 3B) [12]. GluD1 proteins were enriched at PF–MLI synapses located on the somata of MLIs. Low levels of GluD1 and GluD2 are also found at PF–MLI synapses located on dendrites of MLIs. In *Grid1*-null mice, the density of PF–MLI synapses on MLI somata, as well as the size and number of MLIs, were significantly reduced. Therefore, like GluD2 in PCs regulating PF–PC synapse formation, GluD1 in MLIs is responsible for PF–MLI synapses. While survival of PCs was minimally affected by the loss of PF–PC synapses in *Grid2*-null mice [61], survival of MLIs may be more dependent on formation of PF–MLI synapses. Interestingly, the number of PF–MLI synapses is increased in *Grid2*-null mice, probably due to the upregulation of GluD1 (Figure 3B) [12]. GluD1 can induce synapse formation by binding to Cbln1 and Cbln2, which interact with Nrx(+4), *in vitro* [7, 9, 62]. Therefore, although precise mechanisms remain to be determined *in vivo*, Cbln1 released from PFs likely regulates the PF–MLI synaptogenesis by forming Nrx–Cbln1–GluD1 trans-synapic complexes.

Signaling in the inner ear

Laser capture microdissection of various inner ear cells revealed that GluD1 mRNA is highly expressed in the inner hair cells (IHCs), outer hair cells (OHCs), spiral ganglia, and vestibular hair cells, but not in other cells in adult mice [5] (Figure 3C). Auditory brainstem response (ABR) in *Grid1*-null mice showed significant hearing loss (20–45 dB) at high frequencies (> 16 kHz). In addition, thresholds of distortion product otoacoustic emission (DPOAE), which largely reflects OHC functions, is increased by ~20 dB. *Grid2*-null mice were more vulnerable to acoustic injury as measured by ABR and DPOAE. Thus, in analogy to the GluD2 role in regulating the PF-LTD in cerebellar PCs, GluD1 expressed in the spiral ganglion may modulate glutamatergic synaptic transmission between IHC and type I afferent fibers (Figure 3C), leading to hearing loss and increased vulnerability to acoustic injury by excitotoxicity. In this scenario, although its expression status in type II ganglion cells is unknown, GluD1 may also be involved in glutamatergic transmission between OHC and type II afferent fibers, which express kainate-type iGluRs [63].

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Interestingly, a 20–25 mV reduction in **endocochlear potential** (EP) was also observed in high-frequency cochlear regions in *Grid1*-null mice [5] (Figure 3C). Cochlear dysfunction caused by EP reduction can explain larger changes in ABR versus DPOAE thresholds in *Grid1*-null mice [5]. Since the EP itself is generated by cells that do not express GluD1, this receptor might be involved in forming a barrier together with a Cbln1-like extracelluar matrix to restrict K^+ leakage from the IHC or the OHC areas to maintain EP (Figure 3C).

Signaling in the forebrain

Behavioral analyses of *Grid1*-null mice revealed a variety of phenotypes [64, 65], which are relevant to the human genetic studies described above. These include higher spontaneous activity, lower anxiety-like behavior, depression, increased aggressiveness and reduced social interaction [64]. In addition, *Grid1*-null mice displayed deficits in reversal learning in the Morris water maze and fear conditioning [65]. By contrast, working memory was enhanced in the 8-arm radial maze and Y-maze tests in *Grid1*-null mice [65]. Forebrain-specific *Cbln1*-null mice also showed impaired fear conditioning and spatial memory [66]. Interestingly, a higher number of dendritic spines, lower expression levels of the GluA1 AMPA receptor subunit and a lower ratio between the GluN2A/GluN2B NMDA receptor subunits were commonly observed in the *Grid1*-null hippocampus and prefrontal cortex [64, 65, 67] (Figure 3D). Blockade of GluN2B activity restored the spine numbers in the *Grid1*-null hippocampus and prefrontal cortex [67]. These findings suggest that GluD1 may regulate the normal spine number in the prefrontal cortex and hippocampus, by suppressing GluN2B expression.

It remains unclear how AMPA and NMDA receptor subunits are differentially regulated by GluD1. Indeed, in the *Grid1*-null amygdala, GluA1 and PSD95 levels were increased while GluN2B was unchanged [64] (Figure 3D). Since GluD1 induces synapse formation in hippocampal and cortical neurons *in vitro* [9, 62], and also at PF–MLI synapses *in vivo* [12], it also remains to be clarified why the number of synapses is increased in *Grid1*-null hippocampus and prefrontal cortex.

Mirroring the GluD2 association with mGlu1 pathway proteins [45], GluD1 was recently shown to coimmunoprecipitate with metabotropic glutamate receptor 5 (mGlu5) in the hippocampus [68]. Interestingly, mGlu1/5 agonist–induced chemical LTD is impaired in *Grid1*-null mice (Figure 3D). These findings suggest that like GluD2 at PF– PC synapses in the cerebellum, GluD1 may regulate AMPA receptor endocytosis in the hippocampus, although underlying intracellular mechanisms remain to be clarified.

Concluding remarks

Identification of two endogenous ligands and structural biological studies have significantly advanced our understanding about GluD2 signaling mechanisms in the cerebellum. GluD2 serves as a bidirectional synaptic organizer and facilitates synaptic adhesion by binding to Cbln1 at the ATD. Avidity effects arising from the oligomeric nature of these proteins strengthen the binding between Cbln1 and GluD2 to enable trans-synaptic anchoring of GluD2 to the Cbln1–Nrx complex. GluD2 also serves as a master switch to determine LTD induction by regulating AMPA receptor tyrosine phosphorylation levels. This process

requires an association between PTPMEG and the GluD2 CTD. Interestingly, D-serine binding to the LBD and Cbln1 binding to the ATD interacted with each other to induce GluD2 signaling leading to AMPA receptor endocytosis. Multiple synaptic cleft proteins have recently been identified as interaction partners for the ATDs of other iGluRs. Therefore, various metabotropic functions at synapses, such as cell adhesion and synaptic plasticity, may be regulated by such ATD-binding proteins. Although many questions remain to be answered (See Outstanding Questions), further understanding of GluD1 and GluD2 signaling in other brain regions is expected to provide important insights into these mechanisms.

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Box 1

Are GluD functional ion channels?

A GluD2 point mutation in the *lurcher* mice (GluD2^{Lc}), Ala654Thr, shows spontaneous channel activity (*i.e.* in the absence of ligand binding). GluD1 and GluD2 also showed glutamate-induced channel activities when their LBDs were swapped with those of a kainate or an AMPA receptor subunit [69]. Application of D-serine to GluD2^{Lc} caused reduction of the constitutive currents, probably reflecting the rapid desensitization caused by full D1–D2 closure at the LBD [31, 69]. These findings indicate that, like other iGluRs, GluD1 and GluD2 possess functional ion channel pores; however, ligand binding at the LBD does not effectively gate the channel opening, and may only result in desensitization-like conformation changes.

The channel pore region of GluD2 contains several highly conserved amino acid residues, responsible for ion selectivity and lining the permeation pathways in other iGluRs. Indeed, mutation of these residues modified or abolished ion permeability through GluD2^{Lc} channels. Nevertheless, back-expression of such mutant GluD2 versions in *Grid2*-null mice restored the wild-type phenotype, including PF-LTD and synapse formation [26]. These findings indicate that GluD2 does not require an active channel in order to perform its major functions at PF–PC synapses.

Recently, a GluD2 channel activity was proposed to occur upon glutamate binding to mGlu1. In heterologous cells co-expressing mGlu1 and GluD2, but not mGlu1 alone, an mGlu1 agonist induced slow currents, which were reduced by D-serine and NASPM, both inhibitors of GluD2^{Lc} currents [44]. The mGlu1 agonist–induced currents were dependent on Gαq, PKC, and phospholipase C activities [70]. Since PF-evoked slowEPSCs were also dependent on similar signaling pathways in wild-type mice, and were reduced in *Grid2*-null mice, GluD2 channel activity was proposed to contribute to slowEPSCs in PCs [44, 70]. However, the slowEPSCs were also completely abolished in *Trpc3*-null mice [46] and were insensitive to PKC. In addition, the reduced slowEPSCs in *Grid2*-null [42] PCs were significantly rescued by a short treatment with a *Src* family tyrosine kinase inhibitor. These findings indicate that slowEPSCs were mainly mediated by TRPC3, in a manner dependent on protein tyrosine phosphorylation levels regulated by GluD2. Whether a putative ionotropic GluD2 activity may play any physiological functions under some conditions, remains an open question.

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Box 2

Non-ionotropic functions of iGluRs

Neurotransmitter receptors are classified into ionotropic and metabotropic. They either form ion channels to mediate rapid membrane potential changes, or exert their action though slower intracellular signaling pathways. Recently, the distinction between these classes become less clear because ionotropic receptors appear to also transmit signals via non-ionotropic mechanisms. In addition to classical metabotropic functions, certain iGluRs serve as synaptic scaffolds to regulate synapse formation and maintenance. Among iGluRs, the non-ionotropic functions of kainate receptors are best studied [71, 72]. Kainate receptors controls neurotransmitter release at presynaptic sites in a manner dependent on G-proteins and PKC. They also regulate intrinsic cell excitability by modulating potassium channels after membrane hyperpolarization. Furthermore, kainate receptors are shown to regulate neurite growth and synaptic maturation via both ionotropic and non-ionotropic pathways. AMPARs are also reported to activate *Lyn* [73] and mitogen-activated protein kinase (MAPK) in the hippocampus [74]. The ATD of GluA2 AMPAR subunit regulates formation of dendritic spines in hippocampal cultures by interacting with N-cadherin [75]. Recently, NMDA receptors were shown to regulate LTD induction and spine shrinkage in the hippocampus via non-ionotropic signaling pathways involving the p38 MAPK activation [76]. Furthermore, fluorescence resonance energy transfer measurement revealed that, upon ligand binding to the LBD, the CTD of NMDA receptors undergoes a rapid and transient conformational change in the absence of ion flow [77]. Oligomeric amyloid-β protein, which is believed to contribute to Alzheimer's disease, is also reported to activate non-ionotropic signaling pathways through NMDA receptors, to induce LTD at synapses [78]. Similarly, LTD was shown to be regulated by nonionotropic signaling of postsynaptic NMDA receptors in the barrel cortex of young rodents [79]. However, a highly sensitive Ca^{2+} imaging study indicated that the induction of LTD in the hippocampal CA1 region was dependent on ionotropic NMDA receptor signaling [80]. Thus, the role of ionotropic versus non-ionotropic NMDA receptor signaling in LTD remains contentious. The well-established non-ionotropic functions of GluD2 at PF–PC synapses, such as regulation of synapse formation and LTD induction, are expected to serve as a model system to clarify how similar processes might be regulated by other iGluRs.

Box 3

ATD–LBD allosteric interactions in iGluRs

All iGluR subunits consist of four domains: the ATD and the LBD in the extracellular region, the transmembrane domain (TMD), harboring the ion channel, and the intracellular CTD. In NMDA receptors, ATDs determine the channel kinetics of different subunit combinations. Furthermore, ligands such as ifenprodil and Zn^{2+} bind to the ATDs and modulate glutamate-gated currents [81]. Full-length NMDA receptor crystal structures revealed that their tetrameric ATD and LBD "layers" can come in close contact [82, 83]. Ligand binding at the ATDs is therefore likely to impact on the LBD conformations, and thus modulate the TMD channel opening. In contrast, a single-particle cryo-electron microscopy (cryo-EM) NMDA receptor analysis [84], as well as most AMPA and kainate receptor structures reported so far, have revealed little contact between the ATD and LBD layers [85, 86]. Nevertheless, the recently reported first structure of a heteromeric AMPA receptor, determined by cryo-EM, suggests that AMPA receptors can also adopt NMDA receptor-like tightly-packed conformations [87]. In addition, simulations of GluA2 dynamics indicate that its ATD and LBDs might also arrange in a broad range of relative conformations, more or less compact [88]. Although it is unclear whether small molecule ligands binding to the ATDs might function as allosteric modulators for all iGluR family members, it is now clear that most ATDs interact with protein molecules located in synaptic clefts. Such interactions may result in conformational changes that propagate to the downstream domains.

Recently, an allosteric interaction between ATD and LBD layers has been demonstrated for GluD2 [28]. Similar to other iGluRs, the GluD2 ATDs form high affinity dimers (~30 nM). While application of D-serine to PCs expressing wild-type GluD2 induced PF-LTD in cerebellar slices, it failed to do so in PCs expressing (i) GluD2 that cannot bind to Cbln1; (ii) GluD2 in which the ATD dimer interface was disrupted and (iii) GluD2 in which a glycosylated linker was inserted between the ATD and LBD layers [28]. In a trans-synaptic context, anchoring of GluD2 to the Nrx–Cbln1 complex is likely to limit large-scale motions of the ATD layer, allowing D-serine–induced conformational changes of the LBD to be efficiently transmitted downwards to the TMD and CTD. Interestingly, the ATDs of kainate receptors have also been shown to bind C1q-like proteins, which can interact with Nrx3, in the hippocampus [89]. Neuronal pentraxins, N-cadherin, olfactomedin/noelin, and very likely other molecules, also bind the ATDs of AMPA receptors. Thus, the trans-synaptic iGluR anchorning, and its impact on ATD–LBD allosteric interactions, may be broadly applicable to synaptic iGluRs.

Glossary

Amino-terminal domain (ATD): The ATD, also called N-terminal domain (NTD), constitutes ~50% of full-length iGluR subunits. The ATDs and the C-terminal domains show the highest levels of sequence variation among all iGluR domains. ATDs help fine-tune the specific assembly of tetrameric iGluRs by forming inter-subunit dimers with various affinities. In addition, multiple synaptic proteins, such as neuronal pentraxins, N-cadherin and C1q-like family members bind to the ATD.

C1q-tumor necrosis factor (TNF) superfamily: C1q family proteins are characterized by the globular C1q domain (gC1q) located at their C-terminus. The prototype member, C1q, is the target recognition protein of the classical complement pathway in the innate immune response. The gC1q domain forms homo-trimers and binds to various target proteins. The C1q family consists of at least 32 members in humans. The gC1q domain is structurally similar to the tumor necrosis factor α (TNFα).

Endocochlear potential (EP): The EP is the positive voltage of ~80 mV in the endolymphatic space of the cochlea. The EP enhances the sensitivity of hair cells by increasing the driving force to ~140 mV. The EP is generated by a combination of ion channels and pumps in various cells in the spiral ligament and the stria vascularis.

Ligand-binding domain (LBD): The LBD of iGluRs has a clamshell structure, composed of two lobes D1 and D2. Binding of small molecule ligands (such as glutamate, or D-serine) induces closure of the clamshell, leading to the ion channel opening.

Long-term depression (LTD): LTD at parallel fiber–Purkinje cell synapses is a form of synaptic plasticity thought to mediate motor learning in the cerebellum. It is caused by activity-dependent endocytosis of postsynaptic AMPA receptors following conjunctive activity of parallel and climbing fibers.

Synaptic organizer: Protein molecules that directly regulate the differentiation, formation and plasticity of synapses are referred to as synaptic organizers. Examples include postsynaptic leucine-rich repeat transmembrane neuronal proteins (LRRTMs), specifically binding to presynaptic neurexins (Nrxs) that do not contain the splice site 4 sequence [Nrx(–4)], Cbln1, which bridge pre-synaptic Nrxs containing the splice site 4 sequence [Nrx(+4)] with post-synaptic GluD2, or neuroligins, able to bind to both Nrx(+4) and Nrx(–4) with variable affinities. Typically, such interactions initiate bi-directional signaling in the pre- and post-synaptic neurons.

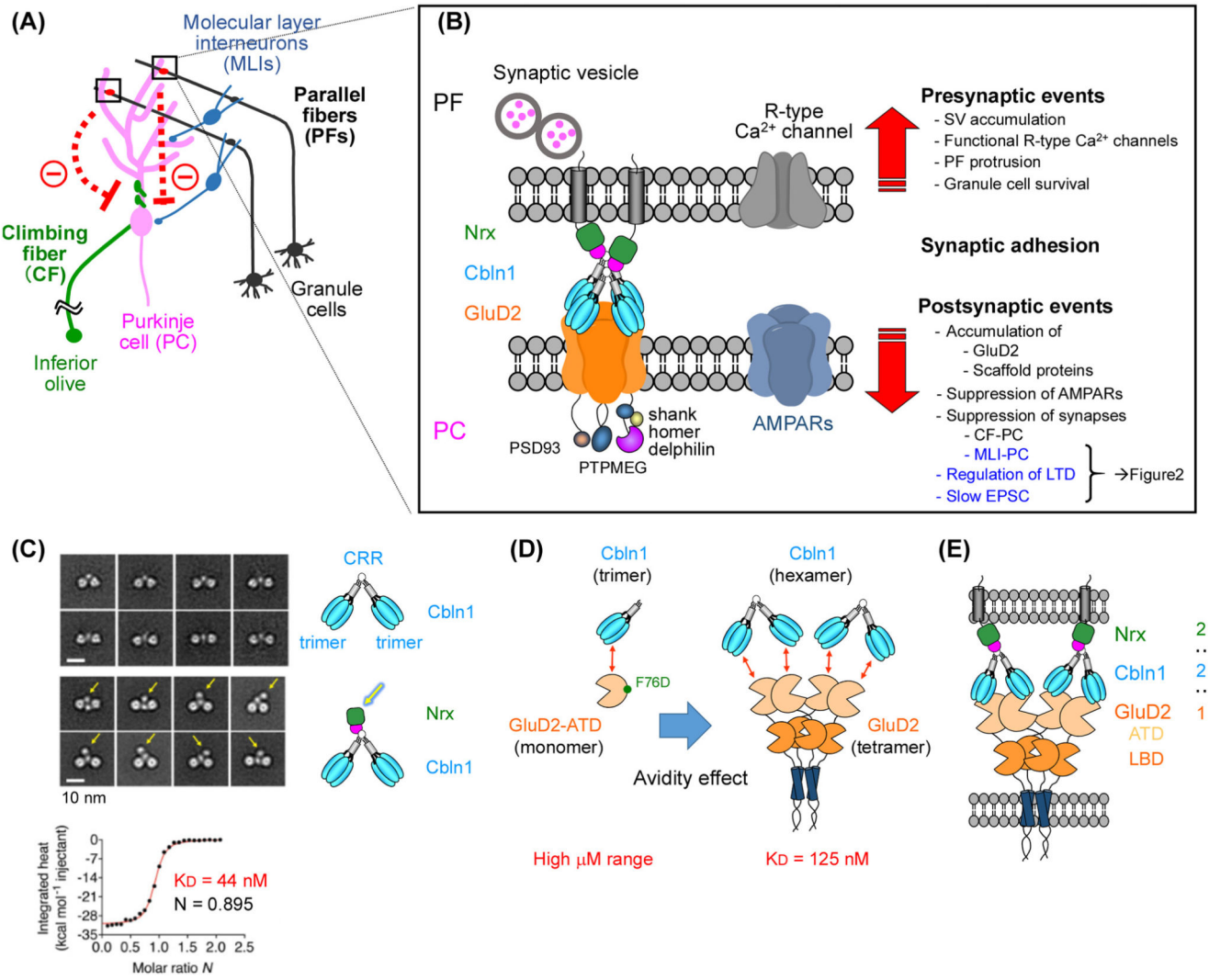

Figure 1. GluD2 as a synaptic organizer.

(A) Synapses regulated by GluD2 in cerebellar Purkinje cells (PCs). Postsynaptic GluD2 regulates parallel fiber (PF)–PC synapses directly (boxes), molecular layer interneuron (MLI)–PC and climbing fiber–PC synapses indirectly (dotted lines with minus signs). (B) GluD2 signaling at PF–PC synapses. GluD2 binds to Cbln1 and its presynaptic receptor neurexin (Nrx) on PFs and regulates synaptic adhesion as well as pre- (up arrow) and post-synaptic (down arrow) events. (C) The Nrx–Cbln1 interaction. Negative-stain electron microscopic class averages of Cbln1 alone (upper panels) and Cbln1 with Nrx (bottom panels) illustrate the dimer-of-trimers arrangement. Yellow arrows indicate the suggested position of Nrx binding to the cysteine-rich region (CRR) of Cbn1. Isothermal titration calorimetric analyses revealed the stoichiometry (N) and affinity (K_D) of this interaction. Reproduced from [28] with permission. (D) The Cbln1–GluD2 interaction. The weak, high micromolar, binding between Cbln1 trimer and the monomeric amino-terminal domain (ATD) of GluD2 (left; minimal interaction unit) is enhanced to an apparent K_D of 125 nM)

by the avidity effect in (right; oligomeric interaction). (E) The transsynaptic triad consisting of neurexin-Cbln1-GluD2 with 2 (monomers): 2 (hexamers): 1 (tetramer) stoichiometry

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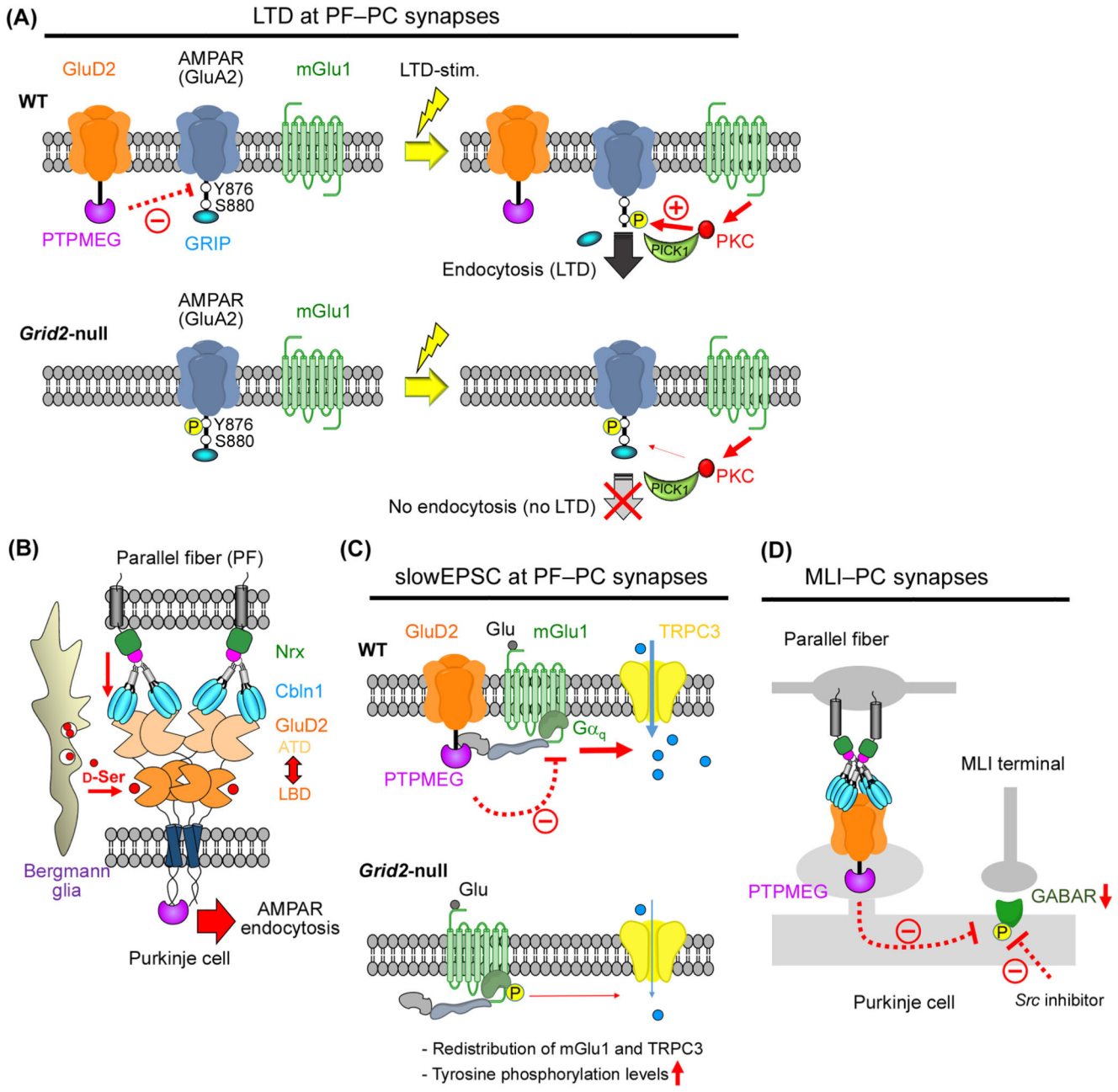

Figure 2. GluD2 as a regulator of functional synaptic transmission.

(A) A model for the GluD2 function in long-term depression (LTD) at PF–Purkinje cell (PC) synapses [42]. In wild-type (WT) mice (top), GluD2 maintains low phosphorylation levels at tyrosine 876 (Y876) of the GluA2 AMPA receptor subunit (dotted line with minus sign) through PTPMEG, a protein tyrosine phosphatase which binds to the GluD2 C-terminus. An LTD-inducing stimulation (LTD-stim.) activates mGlu1 to further reduce Y876 phosphorylation, allowing PKC to phosphorylate serine 880 (S880) of GluA2 (thick arrow with plus sign), a crucial step to replace the AMPA receptor anchoring protein from GRIP to PICK1 for AMPA receptor endocytosis during LTD. In *Grid2*-null mice, PTPMEG fails to

dephosphorylate Y876 of GluA2, thereby impairing S880 phosphorylation (thin arrow) and the LTD. (B) Allosteric interactions between the amino-terminal domain (ATD) and the ligand-binding domain (LBD) of GluD2. The interaction with Cbln1 anchors ATD to the presynaptic site via Nrx (down arrow), and allows the conformational change induced by D-serine binding (right arrow) to the LBD to be transmitted to postsynaptic sites to induce AMPA receptor endocytosis [28]. (C) A model for the GluD2 function in slow excitatory postsynaptic currents (slowEPSCs). Burst stimulation of parallel fibers activates mGlu1 and Gαq to induce slowEPSCs, which are mainly mediated by TRPC3 in adult wild-type mice (top). Low tyrosine phosphorylation levels in PCs, which are partly mediated by PTPMEG (dotted line with minus sign), increase slowEPSC amplitudes by unknown mechanisms. In *Grid2*-null mice, slowEPSCs are reduced because mGlu1, which is anchored at the perisynaptic site via GluD2 and its interacting proteins (shown as shaded irregular structures), and TRPC3 are redistributed [45]. Alternatively, tyrosine phosphorylation levels are increased [42] by the loss of PTPMEG associated with GluD2 (bottom). (D) A model for the GluD2 function in inhibitory synapses formed between molecular layer interneuron (MLI) and Purkinje cells. Cbln1-GluD2 signaling likely suppresses MLI–PC inhibitory responses by lowering protein tyrosine phosphorylation levels via PTPMEG, anchored to the C-terminus of GluD2 in wild-type PCs [40]. A *Src* inhibitor reduces increased inhibitory responses in *Grid2*-null PCs.

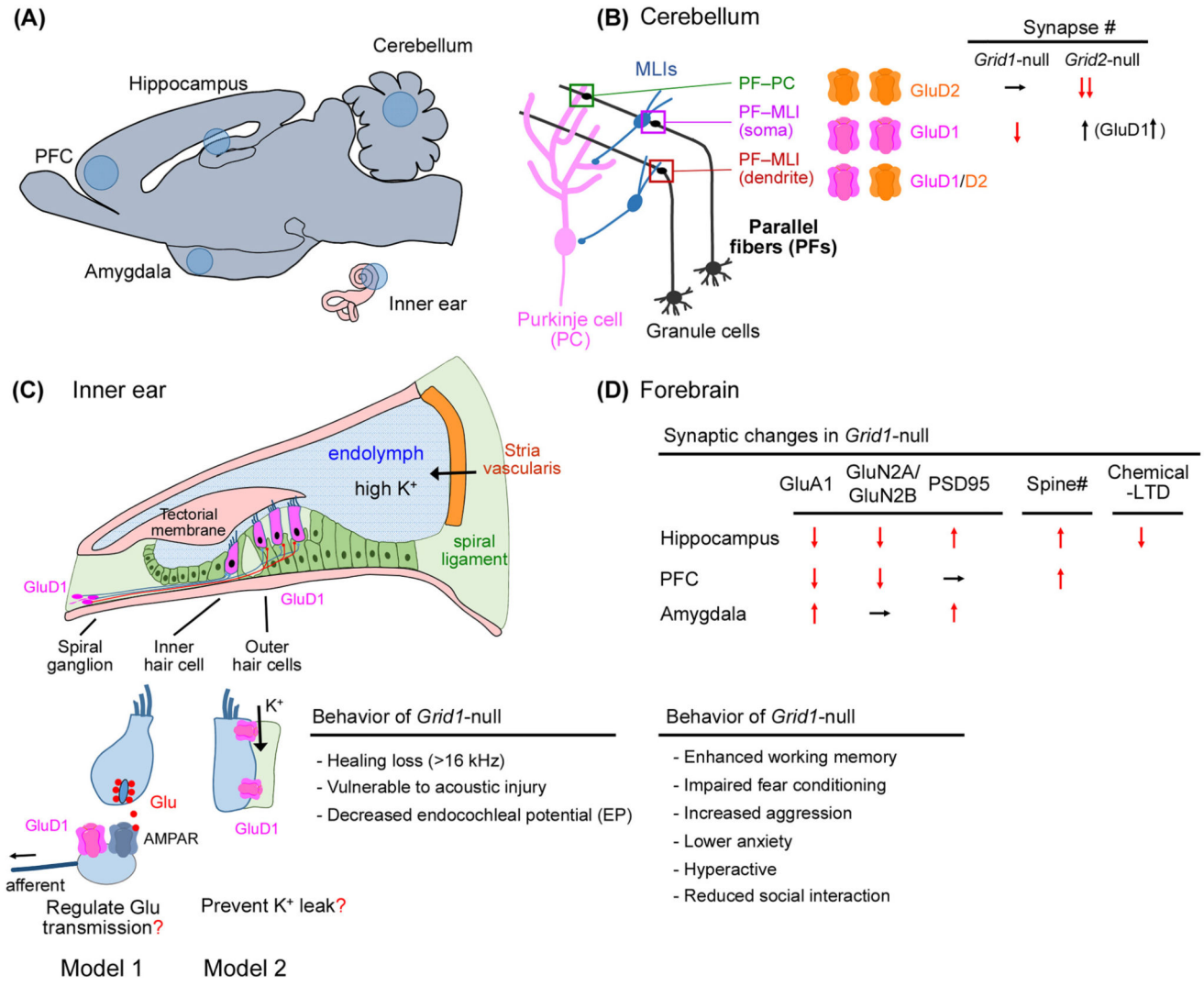

Figure 3. GluD1 signaling in various brain regions.

(A) Expression of GluD1 in the forebrain, cerebellum and inner ear in adult mice. (B) GluD1 signaling in the cerebellar cortex. GluD1 is expressed at parallel fiber (PF)–molecular interneuron (MLI) synapses, especially those forming on MLI soma. GluD2 is highly expressed at PF–Purkinje cell (PC) synapses and weakly at MLI-PC synapses forming on MLI dendrites. In *Grid1*-null mice, PF–MLI synapses decrease significantly. Conversely, somatic PF–MLI synapses increase in *Grid2*-null mice, probably because of compensatory increase in GluD1 expression [12]. (C) GluD1 signaling in the inner ear. Schematic drawing of the organ of Corti indicating GluD1 expression in the inner hair cells, outer hair cells and spiral ganglia (top) [5]. Two models illustrate the cause of hearing loss in *Grid1*-null mice (bottom). GluD1 expressed in the afferent fibers may regulate glutamatergic synaptic transmission (model 1). GluD1 may serve as a cell adhesion molecule to maintain the endocochleal potential, which helps drive receptor currents into cochlear hair cells, by restricting K^+ leakage from hair cell areas (model 2). (D) GluD1 signaling in the forebrain. Changes in the synaptic proteins, spine numbers and chemical LTD induced by a mGlu1

agonist in three forebrain regions (top) are shown, together with behavioral phenotypes (bottom) of *Grid1*-null mice. ↓, reduced; ↑, increased; →, no changes.

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