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Induction of excitatory and inhibitory presynaptic differentiation by GluD1

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ABSTRACT

The δ subfamily of ionotropic glutamate receptor subunits consists of GluD1 and GluD2. GluD2, which is selectively expressed in cerebellar Purkinje neurons, has been shown to contribute to the formation of synapses between granule neurons and Purkinje neurons through interaction with Cbln1 (cerebellin precursor protein1) and presynaptic Neurexin. On the other hand, the synaptogenic activity of GluD1, which is expressed not in the cerebellum but in the hippocampus, remains to be characterized. Here, we report that GluD1 expressed in non-neuronal HEK cells, induced presynaptic differentiation of granule neurons through its N-terminal domain in co-cultures with cerebellar neurons, similarly to GluD2. We also show that GluD1 rescued the defect of synapse formation in GluD2-knockout Purkinje neurons, indicating the functional similarity of GluD1 and GluD2. In contrast, GluD1 expression alone did not induce presynaptic differentiation in co-cultures of HEK cells with hippocampal neurons. However, when Cbln1 was exogenously added to the culture medium, GluD1 induced presynaptic differentiation of not only glutamatergic presynaptic terminals but also GABAergic ones. Cbln1 is not expressed in hippocampal neurons but is expressed in entorhinal cortical neurons projecting to the hippocampus. In co-cultures of HEK cells expressing GluD1 and entorhinal cortical neurons, both glutamatergic and GABAergic presynaptic terminals were formed on the HEK cells without exogenous application of Cbln1. These results suggest that GluD1 might contribute to the formation of specific synapses in the hippocampus such as those formed by the projecting neurons of the entorhinal cortex.

1. Introduction

The ionotropic glutamate receptor subunits (iGluRs) are classified into four subfamilies: AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type, NMDA (*N*-methyl-D-aspartate)-type, kainate-type and δ-type, according to their agonists and sequence homology. The former three constitute cation channels gated by binding to glutamate, and contribute to excitatory synaptic transmission and modulation [1,2]. On the other hand, the δ subfamily, consisting of GluD1 and GluD2, do not bind to glutamate [3,4]. GluD2 is selectively expressed on the postsynaptic membrane at synapses between cerebellar granule neurons and Purkinje neurons. In GluD2 knockout mice, the number of synapses between granule and Purkinje neurons is decreased [5,6]. In addition, GluD2 expression in non-neuronal HEK cells was shown to induce the formation of functional glutamatergic presynaptic terminals of granule neurons through the N-terminal domain (NTD) [7,8]. Furthermore, subsequent studies revealed that postsynaptic GluD2 binds to presynaptic Neurexin through Cbln1 secreted from granule neurons [9–11].

In contrast, functional analyses of GluD1 have been limited. GluD1 is expressed relatively abundantly in many areas of the central nervous system in the early postnatal stage. As development proceeds, GluD1 expression becomes restricted to the hippocampus, cochlear and vestibular hair cells, and spiral ganglion cells [2,12–14]. In GluD1 knockout mice, a relatively mild deficiency in hearing ability was reported [15]. Interestingly, mutations in the GluD1 gene have been detected in some autism patients [16]. It was also shown that GluD1 expressed in HEK cells induces presynaptic differentiation of cerebellar granule neurons, similarly to GluD2 [7]. However, how similar functions of GluD1 and GluD2 are, and whether GluD1 contributes to synapse formation in the hippocampus, where endogenous GluD1 is expressed, remain unclear. To address these issues, we have performed analyses using co-culture preparations of non-neuronal HEK cells expressing GluD1 or its mutants, and neurons prepared from the cerebellum, hippocampus or entorhinal cortex.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; Cbln, cerebellin precursor protein; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Div, day in vitro; EGFP, enhanced green fluorescent protein; EPSC, excitatory postsynaptic current; GABA, γ-aminobutyric acid; HEK, human embryonic kidney; IPSC, inhibitory postsynaptic current; NMDA, *N*-methyl-D-aspartate; NTD, N-terminal domain; Vgat, vesicular GABA transporter; Vglut1, vesicular glutamate transporter 1.

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2. Materials and methods

2.1. Cell cultures

Cerebella were dissected out from newborn ICR mouse pups and incubated in Ca^{2+}- and Mg^{2+}-free HBSS containing 0.1% trypsin and 0.05% DNase for 15 min at 37 °C [17]. GluD2 knockout mice were provided by M. Mishina and backcrossed with ICR mice [5,7]. Neurons were dissociated by trituration and seeded on poly-D-lysine-coated cover glass in DMEM/F12-based medium containing 2% fetal bovine serum. The next day, 75% of the medium was replaced with serum-free medium. Half of the medium was replaced with serum-free medium 4 and 10 days after dissociation. To inhibit glial proliferation, cytosine β-D-arabinofuranoside (5 μM) was added to the medium from 4 days after dissociation. Hippocampal and entorhinal cortical neuronal cultures were prepared from newborn ICR mouse and Wistar rat pups, respectively. They were grown on poly-D-lysine coated glass in Neurobasal medium (Gibco). Half of the medium was replaced with fresh medium 4 and 10 days after dissociation.

2.2. Vectors and expression

The expression vectors of GluD1 and GluA1 (flop) were constructed previously [7]. GluD1 and all mutants were tagged with an HA-epitope sequence at their N-terminal ends. The expression vector of myc-Cbln1 was constructed previously [18], and those of rat $GABA_A$ receptor subunits α1, β2 and γ2 were provided by S. Kawaguchi. HEK cells were transfected with expression vectors for EGFP (pEGFP-N1; Clontech), with or without HA-GluD1 or its mutants, and with or without GluA1 or $GABA_A$ receptor subunits using Lipofectamine 2000 (Invitrogen). To transfect expression vectors into Purkinje neurons, direct microinjection into the nuclei using a glass micropipette was performed.

2.3. Co-culture assay

The co-culture assay was performed as described previously [7]. The day after transfection, HEK cells were seeded into neuronal cultures on day in vitro (div) 12 (2×10^4 cells/cm^2). For preparation of culture medium containing Cbln1, HEK cells were transfected with myc-Cbln1 and incubated in Neurobasal medium for 2 days [18]. Then, the Cbln containing medium (250 μl) was added to the co-culture medium (250 μl). Experiments were performed on div 14 or 15.

2.4. Immunocytochemistry

Cultured cells were fixed in phosphate buffered saline containing 4% paraformaldehyde for 10 min at room temperature. After permeabilization in phosphate buffered saline containing 0.5% Tween 20, samples were processed for immunofluorescent staining. After washing, the cover glass was mounted with glycerol-based medium AntiFade (Invitrogen). The primary and secondary antibodies used were guinea pig anti-vglut1 antibody (Chemicon; 1:20,000), rabbit anti-vgat antibody (Synaptic Systems; 1:1000), monoclonal anti-bassoon antibody (Stressgen; 1:1000), and Alexa 568-conjugated anti-rabbit or anti-guinea pig IgG antibody (Invitrogen; 1:400). Images were captured with an FV1000 confocal laser scanning microscope (Olympus). The conditions for capturing images and the threshold were kept constant throughout a series of experiments.

2.5. Image analysis

For quantification of the vglut1, vgat or bassoon signal on HEK or Purkinje cells, the cells' edges were defined using the EGFP signal by thresholding and smoothing. Then, fluorescent intensity on the edges of HEK cells was measured. The vglut1 signals on Purkinje cells were segregated using the watershed algorithm, and the number of vglut1-positive puncta (0.05–1 μm^2) on the edges was counted. Images were analyzed with ImageJ (National Institutes of Health; rsb.info.nih.gov/ij/).

2.6. Electrophysiology

Whole-cell patch-clamp recording was performed using the external solution consisting of (in mM): 145 NaCl, 5 KOH, 2 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.3, at room temperature (20–24 °C). 100 μM cyclothiazide (CTZ, Tocris) was added to prevent desensitization of AMPA-type glutamate receptors in excitatory postsynaptic current (EPSC) recording. In some experiments, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris) or 20 μM bicuculline (Tocris) was added to the external solution to suppress GluA1-mediated or $GABA_A$ receptor-mediated currents, respectively. In EPSC recording glutamate was electrophoretically applied to neurons around the HEK cell to increase the activity. Patch pipettes were filled with the internal solution consisting of (in mM): 147 CsCl, 10 CsOH, 5 EGTA and 10 HEPES, pH7.3. The electrode resistance was 3–5 MΩ. The membrane potential was held at −60 mV. Only recordings with an input resistance of >100 MΩ and series resistance of <25 MΩ were accepted. Recording was performed with an EPC-9 amplifier (HEKA), and the recorded current was low-pass filtered at 1.5 kHz.

2.7. Statistics

All data were expressed as mean ± SEM. Steel–Dwass's multiple comparison test was used to determine the significance of differences unless otherwise stated.

3. Results and discussion

3.1. NTD of GluD1 is essential for induction of presynaptic differentiation

There are two extracellular domains in GluD1, as in other ionotropic glutamate receptor subunits. They are the N-terminal domain (NTD, also called a leucine/isoleucine/valine binding protein (LIVBP) domain), and a ligand-binding domain. The latter is involved in glutamate binding in other ionotropic glutamate receptors [19,20]. The NTD is indispensable for the synaptogenic activity of GluD2 in the cerebellum [7]. We first examined whether the NTD of GluD1 is also critical for the synaptogenic activity, or not. A deletion mutant lacking the NTD (GluD1ΔN) and a chimeric protein in which the transmembrane segment of platelet derived growth factor receptor was fused to the NTD (GluD1N-TM), were constructed. The HEK cells expressing GluD1 or GluD1N-TM accumulated vglut1 and bassoon signals, but not vgat signal in co-cultures with cerebellar neurons (Fig. 1). Vglut1 is a marker for glutamatergic presynaptic terminals, vgat for GABAergic ones, and bassoon for all presynaptic terminals. The HEK cells expressing GluD1ΔN failed to accumulate any signals. We confirmed that GluD1, GluD1ΔN and GluD1N-TM were expressed on the plasma membrane of the HEK cells by staining the HA-tag (data not shown). These results indicate that the NTD of GluD1 is critical for the synaptogenic activity, and that GluD1 can induce the pre-

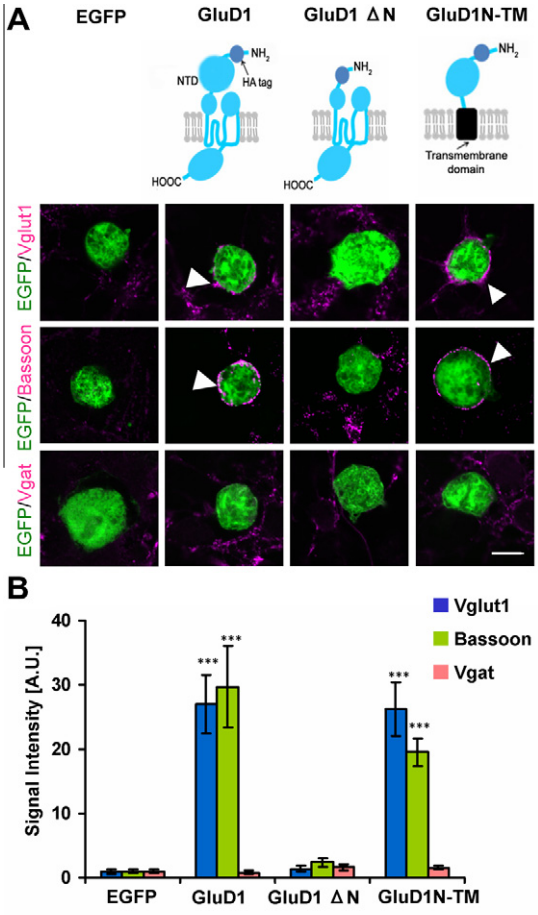

Fig. 1. GluD1's NTD is critical for the induction of formation of glutamatergic presynaptic terminals. (A) HEK cells transfected with EGFP alone (green), or with EGFP plus GluD1, GluD1ΔN or GluD1N-TM, were co-cultured with cerebellar neurons. Structures of the transfected GluD1, GluD1ΔN and GluD1N-TM are shown on the top. Vglut1, bassoon, or vgat were stained (magenta, arrowheads), respectively. Scale bar indicates 10 μm. Vglut1 and bassoon signals were detected on the HEK cells expressing GluD1 or GluD1N-TM. (B) Quantification of signal intensities of vglut1, bassoon, and vgat on the HEK cells. The number of cells was 30 for each. Statistically significant differences compared to the EGFP control are indicated by asterisks (***$P < 0.001$).

synaptic differentiation of cerebellar granule neurons, similarly to GluD2.

3.2. GluD1 can rescue the defect of synapse formation in GluD2 knockout Purkinje neurons

As described above, the synaptogenic function of GluD1 was similar to that of GluD2 in co-cultures of HEK cells and cerebellar neurons. Thus, we thought that GluD1 might be able to substitute for GluD2 in the synapse formation between granule and Purkinje neurons. Microinjection of vectors encoding EGFP and GluD1 into cultured GluD2 knockout Purkinje neurons, increased the number of vglut1 signals around the Purkinje neuron compared with microinjection of a vector encoding only EGFP (Fig. 2). These results suggest that the synaptogenic activity of GluD2 could be substituted by GluD1 in the cerebellum.

3.3. GluD1 contributes to excitatory and inhibitory synapse formation only in the presence of Cbln1 in the hippocampus

In the mature animal, GluD1 is expressed not in the cerebellum, but in the hippocampus. Thus, we examined the synaptogenic

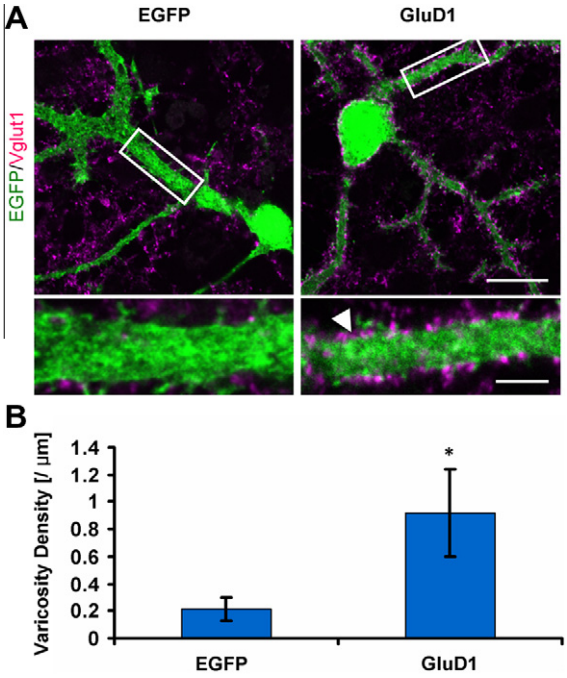

Fig. 2. Rescue of synapse formation by GluD1 in GluD2 knockout Purkinje neurons. (A) Cultured GluD2 knockout Purkinje neurons were transfected with EGFP (green), or EGFP plus GluD1, and vglut1 was stained (magenta, arrowhead). Scale bars indicate 20 μm (upper) or 5 μm (lower). (B) Densities of vglut1-positive varicosities on the Purkinje cells. The numbers of cells examined were 3 (EGFP) and 5 (EGFP plus GluD1). A statistically significant difference is indicated by an asterisk (*$P < 0.05$, one-tailed Student's *t*-test).

activity of GluD1 in co-cultures of HEK cells expressing GluD1 and hippocampal neurons. Neither vglut1 nor vgat signal was accumulated around the HEK cells (Fig. 3), suggesting that GluD1 is insufficient for synapse formation in the hippocampus. Previous studies reported that GluD2 binds to Cbln, which in turn interacts with presynaptic Neurexin, thus contributing to synapse formation [9,10]. The Cbln family consists of Cbln1-4. An essential role of Cbln1 in the synapse formation between cerebellar granule and Purkinje neurons was reported [19]. Cerebellar granule neurons express Cbln1 and Cbln3 abundantly. On the other hand, Cbln expression in the hippocampus is limited [20]. Only weak expression of Cbln1, 2 and 4 in interneurons was reported. Recent studies showed that Cbln1 and Cbln2 but not Cbln4 bind strongly to Neurexin containing splice segment 4, and induce presynaptic differentiation [21,22]. The lack or small amount of Cbln in the hippocampal cultures might be the cause of failure of synaptogenesis in the co-cultures.

When Cbln1 protein was exogenously added to the co-culture medium, both vglut1 and vgat signals were detected around the HEK cells expressing EGFP and GluD1 (Fig. 3). These results indicate that GluD1 could induce differentiation of both glutamatergic and GABAergic presynaptic terminals in the presence of Cbln1, and suggest the intriguing possibility that GluD1 might contribute to the specific synapse formation between input fibers secreting Cbln and hippocampal neurons.

3.4. GluD1 contributes to excitatory and inhibitory synapse formation in co-cultures with entorhinal cortical neurons

The main inputs to the hippocampus come from the entorhinal cortex, which expresses a substantial amount of Cbln1 [20]. We dissociated neurons from the entorhinal cortex, and co-cultured them with HEK cells expressing EGFP and GluD1. Both vglut1

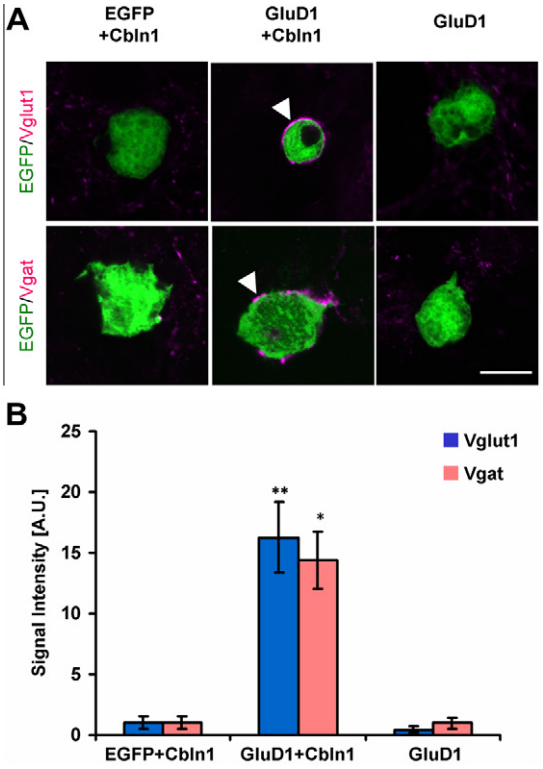

Fig. 3. Synaptogenic activity of GluD1 in co-cultures with hippocampal neurons. (A) HEK cells transfected with EGFP alone (green), or EGFP plus GluD1, were co-cultured with hippocampal neurons in the presence or absence of Cbln1, and vglut1 or vgat was stained (magenta, arrowheads). Scale bar indicates 10 μm. Both vglut1 and vgat signals were detected on the HEK cells only in the presence of Cbln1. (B) Quantification of signal intensities of vglut1 and vgat on the HEK cells. The number of cells was 15 for vglut1 and 17 for vgat. Statistically significant differences are indicated by asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$).

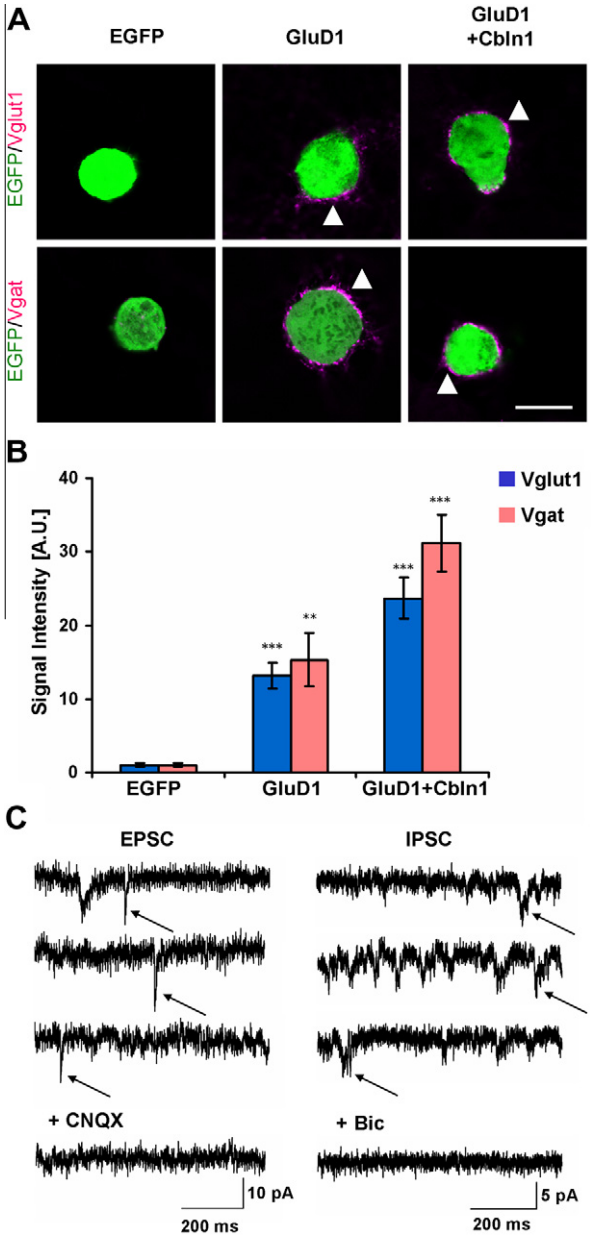

Fig. 4. Synaptogenic activity of GluD1 in co-cultures with entorhinal cortical neurons. (A) HEK cells transfected with EGFP alone (green), or with EGFP plus GluD1, were co-cultured with entorhinal cortical neurons in the presence or absence of Cbln1, and vglut1 or vgat was stained (magenta, arrowheads). Scale bar indicates 10 μm. Both vglut1 and vgat signals were detected on the HEK cells expressing GluD1. Addition of Cbln1 increased the intensity of vglut1 and vgat signals. (B) Quantification of signal intensities of vglut1 and vgat on the HEK cells. The number of cells was 13 for vglut1 and 16 for vgat. Statistically significant differences are indicated by asterisks ($^{**}P < 0.01$; $^{***}P < 0.001$). (C) Representative EPSC-or IPSC-like currents (arrows) recorded from HEK cells expressing EGFP and GluD1, together with GluA1, or $GABA_AR$ α1, β2 and γ2 subunits. The bottom traces were recorded in the presence of CNQX or bicuculline (Bic).

and vgat signals were detected around the HEK cells (Fig. 4A and B), suggesting that GluD1 might contribute to the synapse formation between entorhinal cortical neurons and hippocampal neurons. Addition of Cbln1 increased the amount of both vglut1 and vgat signals, suggesting that perhaps only a fraction of excitatory and inhibitory neurons in the entorhinal cortex secreted Cbln1.

Finally, we examined whether the presynaptic terminals formed on the HEK cells were functional or not. The HEK cells were transfected with not only EGFP and GluD1 but also GluA1 or $GABA_A$ receptor subunits (α1, β2 and γ2). GluA1 is a subunit of ionotropic AMPA type glutamate receptor [1,2], and α1, β2 and γ2 form ionotropic $GABA_A$ receptor [23]. When whole-cell voltage-clamp recording was performed from the HEK cells expressing GluD1 plus GluA1, excitatory postsynaptic current (EPSC)-like events were recorded (Fig. 4C). These currents were suppressed by CNQX, an antagonist for AMPA receptors. On the other hand, inhibitory postsynaptic current (IPSC)-like events were recorded from HEK cells that expressed GluD1 plus the α1, β2 and γ2 subunits of $GABA_A$ receptor. These currents were suppressed by bicuculline, an antagonist for $GABA_A$ receptors. These results indicate that GluD1 could contribute to the functional synapse formation at both glutamatergic and GABAergic synapses.

Entorhinal cortical neurons projecting to the hippocampus have been considered to be the layer 2, 3, 5 and 6 glutamatergic neurons [24,25]. GluD1 might be involved in the synapse formation between these neurons and hippocampal neurons. On the other hand, which neurons formed vgat-positive varicosities on the HEK cells expressing GluD1 is not known. Some GABAergic neurons in the entorhinal cortex or adjacent areas might secrete Cbln and might have formed GABAergic presynaptic terminals on the HEK cells. Whether GluD1 is involved in GABAergic synapse formation in vivo is an intriguing issue to be addressed in the future.

The present findings indicate that GluD1 contributes to synapse formation through its NTD and can substitute for GluD2 in the synaptogenic activity in the cerebellum. Moreover, GluD1 might contribute to the formation of specific synapses in the hippocampus formed by the input fibers secreting Cbln, such as the projecting fibers from the entorhinal cortex. And finally, GluD1 could be

involved in synapse formation not only at excitatory synapses but also at inhibitory synapses.

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