Coordination chemogenetics for activation of GPCR-type glutamate receptors in brain tissue

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Abstract

Direct activation of cell-surface receptors is highly desirable for elucidating the physiological roles of receptors. However, subtype-selective ligands are very limited because of the high homology among receptor subtypes. A potential approach for selective activation of a receptor subtype is chemogenetics, in which both point mutagenesis of the receptors and designed ligands are used. However, ligand-binding properties are affected in most current methods. Here, we developed a chemogenetic method for direct activation of metabotropic glutamate receptor 1 (mGlu1), which plays essential roles in cerebellar functions in the brain. Our screening identified a mGlu1 mutant, mGlu1(N264H), that was directly activated by palladium complexes. Notably, a palladium complex showing low cytotoxicity successfully activated mGlu1 in mGlu1(N264H) knock-in mice, revealing that activation of endogenous mGlu1 is sufficient to evoke the critical cellular mechanism of synaptic plasticity, a basis of motor learning in the cerebellum.

Introduction

Cell-surface receptors have indispensable roles in transmitting extracellular information into cells. Most receptors belong to protein families composed of highly homologous subtypes, each with unique physiological functions. In elucidating the roles of each receptor subtype, it is highly desirable to develop activators that selectively target a particular subtype. However, the high homology of the orthosteric ligand-binding site hampers the development of subtype-selective agonists^{1,2}. Instead, researchers have focused on positive allosteric modulators (PAMs)³, which increase the efficacy of endogenous ligands by binding to an allosteric site as subtype-selective ligands. Although PAMs represent potentially powerful therapeutic agents to increase the efficacy of receptors in situations where the concentration of endogenous ligands is reduced, researchers should be careful when using PAMs to clarify the physiological functions of receptors. Given the non-uniform concentration of endogenous ligands such as neurotransmitters in tissues⁴, the effects of a PAM may reflect the concentration of the ligands rather than the receptor function. Thus, direct activators with high subtype-selectivity are ideal for understanding the physiological roles of the target receptor.

A potential method for direct activation of target receptors is chemogenetics, in which proteins are genetically engineered to interact with the designed ligand selectively⁵. As pioneering research, a κ -opioid receptor mutant, which abrogated affinity to the natural ligand yet preserved stimulation by an artificial ligand, has been reported⁶. As improved approaches, designer receptors exclusively activated by designer drugs (DREADDs), which have low constitutive activity and high selectivity to an artificial ligand, clozapine-*N*-oxide, have been developed by directed molecular evolution of muscarinic acetylcholine receptors⁷. In association with improving the designed ligands, DREADDs are powerful tools to control cellular signaling of target cells in tissues or living animals^{8,9}. For ligand-gated ion channels, engineered receptors (PSAMs) and designed agonists (PSEMs) pairs have been reported using the bump-and-hole

strategy^{10,11}. Moreover, functional switching of cell-surface receptors has been achieved by tethering photo-switchable ligands to target receptors (see Discussion for details)¹²⁻¹⁴. Although powerful for manipulating cellular signals, these methods are not suitable for understanding the physiological roles of each receptor subtype because engineered receptors lacking affinity to endogenous ligands need to be ectopically expressed.

Thus, developing new chemogenetic methods to directly activate receptor subtypes with synthetic ligands without affecting the original receptor function is highly desirable. As a potential approach, we have recently reported a chemogenetic method using coordination chemistry for the AMPA receptor (AMPA) and metabotropic glutamate receptor 1 (mGlu1), which belong to ion-channel-type (ionotropic) and G protein-coupled-type (metabotropic) glutamate receptors, respectively^{15,16}. In this method, structure-based incorporation of coordinating amino acid residues as allosteric sites into glutamate receptors allowed metal-complex-associated activation of glutamate receptors in HEK293T cells or cultured neurons. Although useful for chemogenetic activation of these receptors, the chemogenetic coordination acted as a PAM, which increased the affinity of glutamate to the receptors.

In this report, we developed a direct activation method of mGlu1 by chemogenetics, termed dA-CBC (direct-activation via coordination-based chemogenetics). In the engineered receptor, a metal-complex [Pd(bpy), Pd(bpy)(NO₃)₂; bpy, 2,2'-bipyridine] directly activated the mGlu1 mutant without potentiating the affinity of glutamate. We also designed a derivative of Pd(bpy) with low toxicity to neurons. In mice with the mGlu1 mutation, the newly developed palladium complex successfully induced downstream signals of mGlu1 in acute cerebellar slices of the mice. Notably, the palladium complex evoked synaptic plasticity such as long-term depression (LTD), a putative cellular model of information storage for motor learning in the cerebellum of mammals. Thus, dA-CBC revealed that activation of mGlu1 is sufficient to evoke a critical cellular mechanism of memory in the cerebellum.

Results

Screening the direct-activator-type mGlu1 mutant by dA-CBC.

mGlu1, with a large extracellular and a 7-transmembrane (7TM) domain, belongs to the class C G-protein coupled receptor (GPCR) family. The extracellular domain is composed of a Venus Flytrap (VFT) domain and a cysteine-rich domain (CRD) for glutamate binding and as a semi-rigid linker, respectively (**Fig. 1a**)¹⁷. Glutamate binding to the VFT domain induces closure of the domain in the mGlu1 homodimer, which is transmitted to the 7TM domain via CRD for receptor activation (**Fig. 1a**). We reported chemogenetic methods previously for allosteric activation of glutamate receptors by regulating the open and closed form of the VFT domain using metal coordination, termed on-cell coordination chemistry (OcCC). We identified a mGlu1 mutant, mGlu1(P58H/N264H), whose glutamate-induced responses were allosterically activated by Pd(bpy) (**Fig. 1b**, top). In the mutant, 1 μ M of glutamate, which is insufficient for receptor activation, successfully induced intracellular signals in the presence of Pd(bpy). Importantly, the mGlu1 mutant was activated by Pd(bpy) even in the absence of glutamate, which guided our hypothesis that the introduction of histidine mutations in appropriate positions should induce direct activation of the mGlu1 by metal coordination without potentiating the affinity of glutamate (**Fig. 1b**, bottom). This strategy is termed dA-CBC, and our previous OcCC is renamed A-CBC (activation via coordination-based chemogenetics).

Here, we prepared seven mGlu1 mutants with two histidine mutations on both upper and lower lobes at the entrance of the VFT domain (**Fig. 1c**). The mutation sites, which are not involved in glutamate binding, were selected to minimize the influence on receptor function and based on structural information available for open (*i.e.*, apo) and closed (*i.e.*, glutamate-binding) conformations^{18,19}. We hypothesized that domain closure occurs by metal coordination instead of glutamate binding when the mutated histidines work as a bidentate chelator for metal ions or complexes. mGlu1 is a Gq-coupled GPCR,

which elevates the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) via phospholipase C (PLC) activation. Receptor activation was evaluated by monitoring $[\text{Ca}^{2+}]_i$ changes using a fluorescent Ca^{2+} indicator, Fura-2, after treatment with 10 μM metal ions (Ni^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} or Pd^{2+}) or a metal complex ($\text{Pd}(\text{bpy})$) in HEK293 cells transfected with each mGlu1 mutant (**Supplementary Fig. 1a**). The 1st screening identified five mutants (P57H/N264H , P58H/N264H , A59H/N264H , E60H/N264H , K61H/N264H) that were prominently activated by $\text{Pd}(\text{bpy})$ (**Fig. 1d, e** and **Supplementary Fig. 1b**). Although Pd^{2+} also activated three mutants (P58H/N264H , E60H/N264H , K61H/N264H), the obtained Δratio values were not that high when compared with those by $\text{Pd}(\text{bpy})$. Other metal ions (Ni^{2+} , Cu^{2+} , Zn^{2+} or Cd^{2+}) failed to activate these mGlu1 mutants. We selected three mutants (P58H/N264H , A59H/N264H , K61H/N264H) as hit mutants from the 1st screening because these three mutants showed intact glutamate-induced responses and prominent $\text{Pd}(\text{bpy})$ -induced responses (**Fig. 1e**). The calculated half-maximal effective concentration (EC_{50}) values of $\text{Pd}(\text{bpy})$ were 1.2–3.8 μM for the three mutants (**Fig. 1f**). The dose-dependency of glutamate-evoked responses was not affected significantly in these mutants when compared with that of wild-type (WT) mGlu1 (**Supplementary Fig. 2**), indicating that introduction of these histidine mutations did not affect the original receptor function of mGlu1.

Although hit mutants and $\text{Pd}(\text{bpy})$ pairs were obtained, it was unclear whether $\text{Pd}(\text{bpy})$ acts as a direct activator or an activator coupled with PAM activity (*i.e.*, ago-PAM)² for the mGlu1 mutants. Then, in the next step (*i.e.*, 2nd screening), we aimed to find mGlu1 mutants activated by $\text{Pd}(\text{bpy})$ directly without potentiating the affinity of glutamate. To evaluate the PAM effect selectively by excluding the direct activation effect of $\text{Pd}(\text{bpy})$, we used a mGlu1-selective orthosteric antagonist, LY367385 (ref. 20), which competitively binds with glutamate to the VFT domain (**Supplementary Fig. 3a**). As shown in **Supplementary Figures 3b and 3c**, pretreatment with LY367385 suppressed glutamate-induced responses. Importantly, 10 μM LY367385 also inhibited

Pd(bpy)-induced Ca^{2+} responses in the mGlu1(P58H/N264H) mutant (**Supplementary Fig. 3d and 3e**), which showed that direct activation of Pd(bpy) can be suppressed by the orthosteric antagonist. Then, we directly evaluated the positive allosteric effect of Pd(bpy) for the glutamate-induced response by pretreatment with LY367385 (**Supplementary Fig. 3f**). The dose-dependent glutamate-induced responses of the hit mutants were obtained even in the presence of LY367385, and the positive allosteric effect of Pd(bpy) was largely different among these mutants (**Fig. 1g and Supplementary Fig. 3g, h**). The EC_{50} values of glutamate were shifted to the left for the P58H/N264H or K61H/N264H mutant (**Fig. 1g**). In contrast, a prominent shift of the EC_{50} value was not observed for the A59H/N264H mutant, indicating that the A59H/N264H mutant was activated by Pd(bpy) without potentiating glutamate affinity. In contrast, Pd(bpy) acted as ago-PAM for P58H/N264H and K61H/N264H mutants. Thus, the screening results identified the A59H/N264H mutant and Pd(bpy) as a hit pair for dA-CBC of mGlu1.

Direct activation mechanism of the mGlu1 mutant by dA-CBC. We next investigated the activation mechanism of the mGlu1 A59H/N264H mutant. The corresponding single mutants (A59H and N264H mutants) were prepared to examine the bidentate coordination of Pd(bpy) to both histidine residues. As shown in **Figure 2a**, Pd(bpy) failed to activate the A59H mutant. In contrast, the N264H mutant was unexpectedly activated by Pd(bpy). In both mutants, glutamate responses were intact, suggesting that receptor function was unaffected by the mutations (**Supplementary Fig. 4**). Regarding other Pd(bpy)-responsive mutants (P58H/N264H and K61H/N264H), the corresponding single mutants (P58H and K61H) failed to be activated by Pd(bpy) (**Fig. 2a**). These results indicate that the single N264H mutation is sufficient for the chemogenetic activation of mGlu1.

Considering the putative activation mechanism of dA-CBC (**Fig. 1b**, bottom), a coordination partner of the N264H mutation for Pd(bpy) binding is required on the upper

lobe of the VFT domain. We found four coordinating amino acid residues (H54, H55, E60, H111) within 15 Å from the N264 residue in the crystal structure of the closed-form of the VFT domain (**Fig. 2b** and **Supplementary Table 1**). These candidate amino acids were exchanged to phenylalanine or alanine in the N264H mutant, and Pd(bpy)-induced responses were examined for these mutants. As shown in **Figure 2c**, the H54F/N264H and H55F/N264H mutant failed to be activated by Pd(bpy). However, glutamate-induced responses were also lost in the H54F/N264H mutant but not the H55F/N264H mutant. Given that H54 interacts with D107 in the crystal structure¹⁸, substituting this residue is likely to be lethal for receptor activity in the H54F/N264H mutant. Collectively, although the involvement of H54 cannot be excluded, endogenous H55 is probably the coordination partner of the N264H mutation for Pd(bpy)-induced chemogenetic activation. The EC₅₀ value of Pd(bpy) for the N264H mutant was 1.6 μM, and glutamate-induced responses were unaffected by the N264H mutation (**Fig. 3a,b**). Importantly, the dose-dependency of the glutamate-induced responses in the presence of LY367385 was unaffected in the presence of Pd(bpy), indicating that the affinity of glutamate was not potentiated by Pd(bpy) in the mGlu1(N264H) mutant (**Fig. 3c**). Thus, the single point mutation (N264H) is sufficient for the direct activation of mGlu1 by Pd(bpy).

The chemogenetic activation of the mGlu1(N264H) mutant in HEK293 cells was examined in more detail. Western blotting indicated that the total expression level of the N264H mutant was comparable to that of WT mGlu1 (**Fig. 3d**). A newly designed mGlu1-selective fluorescent probe, FITM-Cy3, was designed to quantify the expression level of mGlu1 on the cell surface under live-cell conditions (**Fig. 3e**). FITM, a negative allosteric modulator (NAM) of mGlu1²¹, which binds to the 7TM domain²², was conjugated with an anionic fluorescent dye, sulfo-Cy3, to obtain FITM-Cy3. Confocal live imaging clearly showed that FITM-Cy3 visualizes surface mGlu1 with a dissociation constant (*K_d*) of 6.8 nM in HEK293 cells (**Supplementary Fig. 5**), and FITM-Cy3 clarified that surface expression of the N264H mutant was comparable to that of WT

mGlu1 (**Fig. 3f**). We also examined reversibility and selectivity of the Pd(bpy)-induced activation of mGlu1. As shown in **Figure 3g**, repeated $[Ca^{2+}]_i$ increases were observed after washing out Pd(bpy) in the culture medium. The Pd(bpy)-induced mGlu1 responses were inhibited by LY367375 or FITM, which binds to the VFT or 7TM domain of mGlu1, respectively (**Supplementary Fig. 6a, b**). Moreover, U73122, a PLC inhibitor, suppressed the Pd(bpy) induced response (**Supplementary Fig. 6c**), which indicates that Pd(bpy) activates the conventional Gq-pathway of mGlu1. Thus, we can directly and reversibly activate mGlu1 and its downstream signal pathways with minimal disturbance of the receptor function by using a pair of Pd(bpy) and single-point mutagenesis (N264H) of mGlu1 in the dA-CBC method.

Design of low cytotoxic Pd(bpy) derivatives for neurons. Palladium complexes have attracted much attention as anti-cancer drugs because their geometry and complex forming processes are similar to those of platinum-based drugs such as cisplatin^{23,24}. The putative molecular mechanism of the anti-tumor activity underlies DNA binding of these metal complexes. However, neurotoxicity has been reported for the clinical use of platinum drugs²⁵. Although previous reports show palladium complexes having favorable neurotoxicity when compared with that of platinum drugs, the neurotoxicity of palladium complexes needs to be examined when used in neurons or brain tissues.

Pd(bpy) toxicity was examined in PC12 cells, a neuron-like cell-line, or in primary cortical neurons (**Fig. 4**). The effect of Pd(bpy) on neuronal outgrowth, a characteristic feature of neurons, was evaluated in PC12 cells after differentiation of the cells by nerve growth factor (NGF). As shown in **Figure 4b**, neurite outgrowth was not affected even at a high concentration of Pd(bpy) ($[Pd(bpy)] = 100 \mu M$). However, 30 μM Pd(bpy) clearly suppressed cell growth in undifferentiated PC12 cells (**Fig. 4c**). In primary cortical neurons, gradual $[Ca^{2+}]_i$ elevation was observed by treatment with 10 μM Pd(bpy), which became more prominent by 30 μM Pd(bpy) (**Fig. 4d**). Importantly,

this $[Ca^{2+}]_i$ elevation was suppressed by neither the mGlu1 inhibitors (LY367385 and FITM) nor the PLC inhibitor (U73122) (**Supplementary Fig. 7**), which indicated a side effect of Pd(bpy) at the concentration used with the neurons. Considering the action of palladium complexes as anti-cancer drugs in the intracellular area, these unfavorable effects of Pd(bpy) are likely caused by cell permeability of the complex.

We then newly designed palladium complexes bearing hydrophilic or anionic ligands to suppress permeation of these compounds into cells (**Fig. 4a**). As shown in **Figure 4e**, 3 μ M Pd(OH-bpy) and Pd(sulfo-bpy) successfully activated mGlu1(N264H). However, Pd(EG-bpy) showed weakened responses, and Pd(di-sulfo-bpy) failed to activate mGlu1(N264H) at the same concentration (**Fig. 4e**). The EC_{50} values of Pd(OH-bpy) and Pd(sulfo-bpy) for mGlu1(N264H) activation were 1.1 and 0.62 μ M, respectively (**Fig. 4f**). We next examined the effect of Pd(OH-bpy) or Pd(sulfo-bpy) on cell growth of PC12 cells. As with the case of Pd(bpy), cell growth was suppressed by 30 μ M Pd(OH-bpy). In contrast, for Pd(sulfo-bpy), cell growth was unaffected even at the high concentration ($[Pd(sulfo-bpy)] = 100 \mu$ M) (**Fig. 4c**). More critically, in cortical neurons, the influence on $[Ca^{2+}]_i$ elevation was not observed following treatment with 30 μ M Pd(sulfo-bpy) (**Fig. 4d**), suggesting that the side effects were successfully suppressed using Pd(sulfo-bpy) bearing the anionic ligand. Consistently, the solubility of Pd(sulfo-bpy) was clearly improved when compared with that of Pd(bpy) (**Fig. 4g**). In the case of Pd(bpy), a linear increase of the absorbance at 314 nm was lost over 300 μ M Pd(bpy) in artificial cerebrospinal fluid (ACSF), a buffer solution used for brain tissue experiments in the next section. In contrast, linearity of the absorbance was observed over 1 mM for Pd(sulfo-bpy), indicating that Pd(sulfo-bpy) stock solutions can be prepared in ACSF, which is another improvement when applying the palladium complex to brain slices.

Knock-in of the N264H mutation does not alter mGlu1 function in mice. With Pd(sulfo-bpy) showing low cytotoxicity in hand, we aimed to evaluate chemogenetic

activation of endogenous mGlu1 to understand the physiological roles in the mouse brain. The mGlu family is composed of eight subtypes (mGlu1–8) divided into three groups (group I–III) according to their pharmacological properties¹⁷. mGlu1 belongs to the group I family with the cognate mGlu5 and is highly expressed in the dendritic regions of Purkinje cells in the cerebellum²⁶ (**Fig. 5a**). The mGlu1 agonist 3,5-dihydroxyphenylglycine (DHPG) is typically used to examine the physiological role of mGlu1; however, DHPG cannot discriminate between mGlu1 and mGlu5. Moreover, mGlu5 is expressed in neurons and non-neuronal (glial) cells such as astrocytes²⁷. Thus, the contribution of DHPG in brain tissues is complicated. Alternatively, mGlu1 selective PAM reagents would be useful; however, the effect of PAM reflects the difference in the extracellular glutamate concentration, as described in the Introduction. Therefore, a direct activation method of mGlu1, such as our dA-CBC strategy, should be ideal to understand the physiological roles of mGlu1 in brain tissues.

We generated knock-in (KI) mice harboring the point mutation (N264H) in the mouse gene using the CRISPR/Cas9 system²⁸ to chemogenetically control endogenous mGlu1 function (**Fig. 5b** and **Supplementary Fig. 8a**). The KI mouse was termed the coordination-based chemogenetics (CBC) ready mouse (*mGlu1^{CBC/CBC}* and *mGlu1^{CBC/+}* for homozygous and heterozygous, respectively). Bodyweight and gross anatomy of the cerebellum were unaffected in *mGlu1^{CBC/CBC}* mice (**Supplementary Fig. 8b, c**). However, given the severe ataxic phenotypes in *mGlu1* knock-out (*mGlu1^{-/-}*) mice^{29,30} and autosomal-recessive congenital cerebellar ataxia by familial mutations in the *mGlu1* gene in humans^{31–33}, we analyzed *mGlu1^{CBC/CBC}* mice in detail.

Western blotting of mGlu1 indicated that the expression level of mGlu1 was unchanged in the cerebellum of *mGlu1^{CBC/CBC}* mice (**Fig. 5c**). In addition, the expression of excitatory postsynaptic glutamate receptors [AMPA and δ 2-type glutamate receptor (GluD2)] were also unaffected in the mutant mice. The cell-surface expression level of mGlu1 in the acutely prepared cerebellar slices was examined using FITM-Cy3.

Prominent fluorescence was observed from the molecular layer of the cerebellum, where mGlu1 is highly expressed (**Fig. 5a, d**). Given the membrane impermeability of FITM-Cy3 and disappearance of fluorescence in the co-presence of excess FITM, the fluorescent signals correspond to cell-surface mGlu1 and surface mGlu1 levels were the same between WT and *mGlu1^{CBC/CBC}* mice (**Fig. 5d**).

Next, we evaluated the distribution of the mGlu1(N264H) mutant in the cerebellum. Immunohistochemistry using an anti-mGlu1 antibody clearly indicated that mGlu1 localized in the dendritic spines of Purkinje cells (**Fig. 5e, f** and **Supplementary Fig. 9a, b**). Notably, super-resolution microscopy showed that immune-positive signals for mGlu1 were predominantly localized at the edges of synaptic contacts, which were represented by signals for a parallel fiber (PF)–Purkinje cell synapse specific-postsynaptic protein (GluD2) and a presynaptic protein (bassoon), indicating perisynaptic localization of mGlu1 in both WT and mutant *mGlu1^{CBC/CBC}* mice (**Fig. 5g** and **Supplementary Fig. 9c**). This result is consistent with the similar subcellular localization of mGlu1 revealed by immunoelectron microscopy^{33,34}.

Subsequently, the function of the mGlu1(N264H) mutant was evaluated by whole-cell patch-clamp recordings of Purkinje cells in the cerebellar slices. We first confirmed that excitatory postsynaptic currents (EPSCs) evoked by PF and climbing fiber (CF) stimuli (PF-EPSCs and CF-EPSCs, respectively) were normal in amplitude and the paired-pulse ratio, reflecting presynaptic functions³⁵, of *mGlu1^{CBC/CBC}* mice (**Fig. 5h** and **Supplementary Fig. 10**). mGlu1 is required for synapse elimination of surplus CF inputs to Purkinje cells during development to make a one-to-one relationship in adults, and the relationship is impaired in *mGlu1*-deficient mice. In this context, 90% of Purkinje cells had a single step of CF-EPSC amplitude by increasing CF stimuli in adult *mGlu1^{CBC/CBC}* mice, as with the case of WT (91%, $P > 0.05$ by two-way repeated ANOVA; **Fig. 5i**), suggesting that mGlu1(N264H) is functioning normally for CF synapse elimination during development. Furthermore, burst PF stimulation (2–10 times of PF stimuli at 100

Hz) induced robust TRPC-mediated slow currents (designated as slowEPSCs) by activation of the perisynaptic mGlu1 in *mGlu1^{CBC/CBC}* Purkinje cells and WT cells (**Fig. 5j**). Because these slowEPSCs are often used to evaluate the degree of mGlu1 activation³⁶, these results indicate that the mGlu1(N264H) mutant functions in a neuronal activity-dependent manner.

Consistent with the intact subcellular localization and function of the mGlu1(N264H) mutant endogenously expressed in mice, the ataxic phenotype observed in *mGlu1^{-/-}* mice was never observed in the *mGlu1^{CBC/CBC}* mice (**Supplementary Movie 1**). In addition, *mGlu1^{CBC/CBC}* mice showed high performance in the rota-rod test (**Supplementary Fig. 11**), which contrasts to *mGlu1^{-/-}* mice whose behaviors were severely impaired in motor coordination, as reported previously^{29,30}. Taken together, the introduction of the N264H mutation in mGlu1 does not affect original receptor activity *in vivo*.

Induction of synaptic plasticity by mGlu1 dA-CBC in the cerebellum. In brains, synaptic plasticity such as long-term potentiation (LTP) and LTD of excitatory transmission has been proposed as a cellular substrate for learning and memory^{37,38}. In particular, cerebellar LTD, which occurs at PF–Purkinje cell synapses by the simultaneous activation of PF and CF inputs, generally underlies a certain form of motor-related learning. mGlu1 is highly expressed at PF–Purkinje cell synapses as described (**Fig. 5a, d**) and absolutely required for the induction of cerebellar LTD. We first examined whether cerebellar LTD occurs in acute cerebellar slices from *mGlu1^{CBC/CBC}* mice following electric stimulation (**Fig. 6a**). When PF stimuli were applied repetitively and simultaneously with Purkinje cell depolarization, mimicking a CF input, at 1 Hz (LTD-stim), a robust LTD occurred in *mGlu1^{CBC/CBC}* mice ($68 \pm 4\%$ at $t = 26\text{--}30$ min) and WT mice ($72 \pm 5\%$ at $t = 26\text{--}30$ min, $P > 0.05$ by Mann-Whitney U test; **Fig. 6b**). Furthermore, application of the mGlu1/mGlu5 activator, DHPG (50 μM for 10 min under

a current-clamp mode) also caused a chemical-induced LTD (chemLTD) in cerebellar slices from WT ($55 \pm 7\%$ at $t = 31-35$ min) and *mGlu1^{CBC/CBC}* ($56 \pm 6\%$ at $t = 31-35$ min, $P > 0.05$ by Mann-Whitney U test; **Fig. 6c**) mice. These results also emphasize that the mGlu1(N264H) mutant works functionally.

We then examined the direct activation of mGlu1 using dA-CBC in brain slices to clarify the contribution of mGlu1 activation in LTD. For this purpose, 1 μ M Pd(sulfo-bpy) was used as the treatment instead of 50 μ M DHPG. As shown in Figure 6d, after washing out Pd(sulfo-bpy), sustained decreases of the PF-EPSC were observed in *mGlu1^{CBC/CBC}* mice ($71 \pm 5\%$ at $t = 31-35$ min; **Fig. 6d**). In contrast, the decrease was not observed by treating WT mice with Pd(sulfo-bpy) ($99 \pm 4\%$ at $t = 31-35$ min, $P < 0.01$ vs *mGlu1^{CBC/CBC}* by Kruskal-Wallis test followed by the Scheffe *post hoc* test), indicating successful activation of endogenous mGlu1 and induction of synaptic plasticity using Pd(sulfo-bpy). Interestingly, Pd(sulfo-bpy)-induced chemLTD was also observed in heterozygous *mGlu1^{CBC/+}* mice ($68 \pm 5\%$ at $t = 31-35$ min; **Fig. 6d**) and similar to homozygous *mGlu1^{CBC/+}* mice ($P > 0.05$ vs *mGlu1^{CBC/CBC}* by Kruskal-Wallis test followed by the Scheffe *post hoc* test; **Fig. 6d**), suggesting that a half amount of mGlu1 in WT mice is sufficient to induce chemLTD. In summary, dA-CBC was successfully applied for chemogenetic activation of mGlu1 in mice, which clarified the essential roles of mGlu1 activation for LTD, the putative mechanism of motor learning in the cerebellum.

Discussion

We developed a direct-activation method of mGlu1 without affecting glutamate affinity using dA-CBC, in which a single point mutation (N264H) was sufficient for chemogenetics activation by Pd(bpy). To demonstrate the chemogenetic activation of mGlu1 endogenously expressed in neurons, we generated mGlu1(N264H)-KI (*mGlu1^{CBC/CBC}*) mice using the CRISPR-Cas9 system and obtained *mGlu1^{CBC/CBC}* mice that exhibited no obvious abnormal phenotypes. Pd(sulfo-bpy), which showed low

toxicity to neurons, successfully activated the mGlu1 mutant in the cerebellar slice prepared from *mGlu1^{CBC/CBC}* mice. Notably, Pd(sulfo-bpy) induced chemLTD in cerebellar slices, which indicated that activation of endogenous mGlu1 is sufficient to evoke the cellular basis of motor learning.

Genetic studies indicate that mGlu1 has essential roles in motor learning. Global mGlu1 knock-out (KO) mice show an ataxic phenotype and lack cerebellar LTD^{29,30}. Introduction of a mGlu1 transgene with a Purkinje cell-specific L7 promoter into the mGlu1 KO mice restored the cerebellar LTD, suggesting essential roles of mGlu1 in cerebellar function³⁹. In addition, mGlu1 conditional knock-out (cKO) using the tetracycline-controlled gene regulation system revealed mGlu1 function in adult mice⁴⁰. These genetic approaches are undoubtedly powerful in understanding the physiological roles of mGlu1. However, the expression level and transcription timing are different when compared with those of the native system. Moreover, in the gene knock-out or transgene approaches, adaptive compensation of related genes or fluctuation of the expression level of related proteins are issues. In fact, the expression level of mGlu5 in Purkinje cells is significantly increased in both mGlu1 cKO mice and spinocerebellar ataxia type 1 mice showing decreased expression of mGlu1^{41,42}. In contrast, in the mGlu1 dA-CBC strategy, introducing a single point mutation (N264H) was sufficient for chemogenetic modulation, allowing preparation of knock-in (*mGlu1^{CBC/CBC}*) mice, in which the expression and function of the receptors were not affected. Indeed, we successfully controlled endogenous mGlu1 function using low cytotoxic Pd(sulfo-bpy) in acute cerebellar slices. Although *in vivo* application of this technique has not been examined, this approach has the potential for *in vivo* regulation of endogenous mGlu1 if the palladium complex can be delivered efficiently to brains. As another direction of this study, mGlu1(N264H) can be expressed selectively in target neurons, which contributes to our understanding of mGlu1-dependent neuronal circuits in various brain regions.

In addition to well-used chemogenetic methods such as DREADD⁷⁻⁹ and PSAM/PSEM^{10,11}, functional regulation of cell-surface proteins has been achieved by chemical biology manipulations. A powerful approach is tethering photo-switchable ligands to the target protein via cysteine modification^{12,14} or a protein tag^{13,14}. These methods are used successfully for photo-switching of cell-surface receptors or ion channels in neurons. Genetic code expansion enables the introduction of photocaged and photo-switchable groups as non-canonical amino acids to control protein function by photo-irradiation⁴³. Metal coordination has also been used for functional switching of cell-surface receptors, in which the ligand-binding region of GPCRs has been mutated into metal coordination sites for metal-induced activation^{44,45}. Although all these methods are powerful for the functional regulation of target receptors, the original receptor function is often affected. Consequently, chemogenetic control of endogenous receptors is quite challenging, and only a few successful examples have been reported. In this context, the current dA-CBC method, which enables control of endogenous mGlu1 function in cerebellar slices prepared from mGlu1(N264H)-knock-in mice, is a notable chemogenetic strategy for controlling endogenous receptor function.

The dA-CBC strategy for mGlu1 is based on structural changes to the VFT domain. Initial X-ray structural analyses of the VFT domain of mGlu1 with glutamate showed a conformational change from a resting open- to an active closed state^{18,19}. However, both agonist-bound open and antagonist-bound closed states have also been reported in structural analyses. Regarding these controversial observations, FRET-based analyses⁴⁶ on live cells and recent structural analyses of full-length mGlu receptors using cryo-electron microscopy⁴⁷⁻⁴⁹ strongly suggest that closing of the VFT domain correlates with receptor activation. As described, mGlu1 belongs to the class C GPCR family. The class C GPCRs include mGlu receptors, γ -aminobutyric acid B receptors, Ca²⁺-sensing receptors and sweet and amino acid taste receptors, which share the large extracellular ligand-binding domain as a unique structural feature. Notably, structural analyses of the

ligand-binding domain or full-length of these receptors have been reported recently⁴⁷⁻⁵². These studies indicate that structural changes to the ligand-binding domain, known as the Venus Flytrap mechanism, are essential for activating class C GPCRs. Thus, our dA-CBC, a structure-based chemogenetic approach, can be potentially used to understand the physiological roles of other class C GPCR subtypes.

1 **Methods**

2 All synthesis procedures and compound characterizations, gross cerebellar anatomy and
3 rota-rod test are described in **Supplementary Methods**.

5 **Construction of expression vector of mGlu1 mutants**

6 Site directed mutagenesis was performed using the QuikChange II XL site-directed
7 mutagenesis kits (Stratagene) or the Q5® Site-Directed Mutagenesis kit (NEB) with
8 pBluescript II SK(+) encoding rat mGlu1¹⁵, according to the manufacturer's instruction.
9 After sequencing the cDNA region of mGlu1 mutants, the cDNA was subcloned into
10 pCAGGS vector⁵³ to obtain the expression vectors as previously reported¹⁵.

12 **Cell culture and transfection**

13 HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)
14 supplemented with 100 unit ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin and 10% FBS
15 (Sigma) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. HEK293 cells were
16 transfected with plasmids (WT mGlu1, the mGlu1 mutants, or the control vector) using
17 SuperFect transfection reagent (Qiagen) or Viafect (promega) according to the
18 manufacturer's instruction. The cells were co-transfected with pEGFP-F (Clontech),
19 pDsRed monomer-F (Clontech), or iRFP670 (kindly gifted from Prof. Verkhusa) as the
20 transfection marker. After transfection, the cells were cultured in DMEM supplemented
21 with 10% dialyzed FBS (Gibco) instead of 10% FBS to decrease cytotoxicity. After 36–
22 48 h incubation, the transfected HEK293 cells were utilized for experiments.

23 PC12 cells were maintained in DMEM supplemented with 100 unit ml⁻¹ penicillin and
24 100 µg ml⁻¹ streptomycin and 10% horse serum (Gibco) at 37 °C in a humidified
25 atmosphere of 95% air and 5% CO₂.

27 **Fluorescent Ca²⁺ imaging**

The transfected HEK293 cells were seeded on glass coverslips (Matsunami) coated with poly-L-lysine solution (Sigma-Aldrich) then incubated for 4–10 hours. HEK293 cells or cultured neurons were loaded with 5 μ M Fura-2 AM (Dojindo) for 15–20 minutes in the growth medium. Fura-2 fluorescence was measured in HBS (107 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 2 mM CaCl₂, 11.5 mM glucose and 20 mM HEPES at pH 7.4). Fluorescence images were obtained using a fluorescence microscope (IX71, Olympus) equipped with a complementary metal-oxide semiconductor (CMOS) camera (ORCA-flash 4.0, Hamamatsu Photonics) under xenon-lamp illumination, and analysed with a video imaging system (AQUACOSMOS, Hamamatsu Photonics) according to the manufacturer's protocol. The ratio of 340:380 nm fluorescence was determined from the images, on a pixel-by-pixel basis. To facilitate the screening assay in HEK293 cells, three different cell lines that expressed one of the constructs were co-cultured on a glass coverslip. Each mutant can be distinguished by co-transfected fluorescent proteins that have distinct colours as a marker, and the glutamate responses of three different mutants were assayed simultaneously. The Δ ratio was defined as the difference between the maximum and the initial ratio values. The Δ ratio was fitted with KaleidaGraph using following equation (1): $a + (b-a)/(1+(x/c)^d)$, and the EC₅₀ value was calculated.

Western blotting analyses of HEK293 cells expressing mGlu1

The transfected HEK293 cells were washed with PBS, and then lysed with RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.4 supplemented with 1% NP-40, 0.25% sodium deoxycholate, and 0.1% SDS) containing 1% Protease Inhibitor Cocktail (Nacalai tesque) at 4 °C for 30 min. The lysate was mixed with 5 $\times$ Laemmli buffer (325 mM Tris-HCl, 15% SDS, 20% sucrose, 0.06% bromophenol blue, and 250 mM DTT at pH 6.8) and incubated at room temperature for 1 h. The sample was subject to SDS-polyacrylamide gel electrophoresis SDS-PAGE (7.5%) and then transferred to a polyvinylidene fluoride membrane (Bio-rad). After blocking in TBS-T (TBS with 0.05% Tween-20)

supplemented with 5% skim milk powder (Wako) at room temperature for 1 h, the membranes were incubated overnight with primary antibodies in TBS-T supplemented with 1% skim milk powder at 4 °C. The membranes were washed three times with TBS-T, incubated with secondary antibodies in TBS-T with 1% skim milk powder at room temperature for 1 h, and washed three times with TBS-T. Used primary antibodies were as follows: anti-mGluR1 antibody (BD biosciences, 610964, 1:2000) and anti- β actin antibody (MBL, M177-3, 1:3000). Used secondary antibodies were as follows: anti-mouse-IgG-HRP antibody (MBL, 330, 1:3000). The signal was developed by ECL prime (cytiva) and detected with a Fusion Solo S imaging system (Vilber Lourmat). The target bands were manually selected, and the intensity was calculated with subtraction of background.

Confocal live cell imaging of cell-surface mGlu1 in HEK293 cells

HEK293 cells were co-transfected with mGlu1 plasmid (WT mGlu1, the mGlu1 mutant, or the control vector) and iRFP-670 as a transfection marker. The cells were added with 1 μ M FITM-Cy3, and incubated for 30 min at 16 °C to suppress endocytosis. Confocal live imaging was performed with a confocal microscope (LSM900, Carl Zeiss) equipped with a 63 $\times$, numerical aperture (NA) = 1.4 oil-immersion objective. Fluorescence images were acquired by excitation at 561 or 640 nm derived from diode lasers. To quantify the fluorescence intensity of Cy3 at the cell surface, iRFP 670 positive cells were selected and the maximum signal intensity of the cell surface was calculated by ZEN blue software (Carl Zeiss). The membrane intensity was fitted with KaleidaGraph (Synergy Software), and the K_d value of FITM-Cy3 was calculated.

Evaluation of Neurite outgrowth in differentiated PC12 cells

PC12 cells were seeded on the 24-well plate at 1.0×10^4 cells. Twelve h after seeding, the PC12 cells were differentiated by treating with 100 ng/mL NGF- β (Sigma-Aldrich), and

each concentration of Pd(bpy) was added simultaneously in DMEM supplemented with 100 unit ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin and 2% FBS. After incubation for 3 days, PC12 cells bearing neurites were randomly selected, and the length of the longest neurite in each cell was measured using ImageJ package Fiji.

Cell proliferation assay of PC12 cells

PC12 cells were seeded on the 24-well plate at 1×10^4 cells. One to two days after seeding, the cells were treated with Pd(bpy) or Pd(sulfo-bpy) for 3-4 days. To visualize the live cells, the cells were loaded with 2 µM calcein-AM (dojindo) for 15 min in HBS, and then washed with HBS. The fluorescent images of the cells were obtained with fluorescent microscopy (Axio Observer7, Carl Zeiss) equipped with CMOS camera (Axiocam712mono, Carl Zeiss), and the cell numbers were automatically counted with ZEN blue software (Carl Zeiss).

Animal experiments

Pregnant ICR mice maintained under specific pathogen-free conditions and C57BL6/J mice were purchased from Japan SLC, Inc (Shizuoka, Japan). The animals were housed in a controlled environment (25 °C, 12 h light/dark cycle) and had free access to food and water, according to the regulations of the Guidance for Proper Conduct of Animal Experiments by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Use Committees of Nagoya University, Kyoto University, and Keio University.

Preparation of primary cortical neuronal culture

Glass coverslips (Matsunami) in 24-well plates (BD Falcon) were coated with poly-D-lysine (Sigma-Aldrich) and washed with sterile water three times. Cerebral cortices from 16-day-old ICR mouse embryos were aseptically dissected and digested with 0.25 w/v% trypsin (Nacalai tesque) for 20 min at 37 °C. The cells were re-suspended in Neurobasal Plus medium (Invitrogen) supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml) and filtered by Cell Strainer (100 µm, Falcon) and centrifuged at 1,000 rpm for 5 min. The cells were resuspended in Neurobasal Plus medium supplemented with 2% of B-27 Plus Supplement (Invitrogen), 1.25 mM GlutaMAX I (Invitrogen), penicillin (100 units/ml), and streptomycin (100 µg/ml) and plated at a density of 2×10^5 cells on the 24-well plate. The cultures were maintained at 37 °C in a 95% air and 5% CO₂ humidified incubator. The neurons were used for fluorescent Ca²⁺ imaging experiment at 3 DIV (day in vitro).

Evaluation of the solubility of Pd complexes in ACSF

Pd complexes were dissolved in ACSF (125 mM NaCl, 2.5 mM KCl, 2 mM Ca₂Cl, 1 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 10 mM Glucose) gassed with 5% CO₂ and 95% O₂, and incubated for 1 h at 4 °C. The solution was centrifuged for 15 min at 15,000 rpm, and the supernatant was collected. The UV-vis absorption spectra of the samples were measured in the range of 250–450 nm with 1 nm resolution using a UV-vis spectrophotometer UV-2600 (SHIMADZU) at room temperature. Maximal absorption in aqueous solution at 312 nm was used to quantification.

Generation of *mGlu1^{CBC/CBC}* and *mGlu1^{-/-}* by CRISPR/Cas9 system

Mutant mice carrying mGlu1 N264H mutation were generated using the CRISPR-Cas9 system according to our previously report²⁸ with some modification. For generation of the mutant mice, *pX330* plasmid DNA vector⁵⁴, donor single-stranded oligodeoxynucleotide (ssODN), and fertilized eggs of C57BL/6J mice were used. The

sequence (5'-GACAAAATCTACAGCAATGC-3') in exon 3 was selected as the gRNA target, and it was inserted into entry site of the *pX330*. The donor ssODN was designed to induce a point mutation of N264H (c.790A>C) as shown in Figure 5b, and the 113 mer ssDNA was synthesized (Integrated DNA Technologies). Female C57BL/6J mice were injected with pregnant mare serum gonadotropin and human chorionic gonadotropin with a 48-h interval, and mated with male C57BL/6J mice. We then collected zygotes from oviducts in mated female and mixture of the *pX330* (circular, 5 ng/μL, each) and the ssODN (10 ng/μL) was microinjected into zygotes. Subsequently, survived injected zygotes were transferred into oviducts in pseudopregnant ICR female and newborns were obtained. Genotypes of the F0 mice were determined by PCR with the following primer sets; *forward* for WT and N264H (5'-GCAATACCACCCTCCTCTGA-3'), *reverse* for WT (5'-CGCATGGCACTCAGTAACC-3'), *reverse* for N264H (5'-AGCTCTTCTCGCCAGCATG-3') to give 321 bp or 218 bp product for WT or N264H, respectively. The candidate F0 mice were mated with the C57BL/6J mice to obtain F1 offspring. Genotypes of F1 mice were determined by PCR with the following primer sets; *forward* (5'-GCAATACCACCCTCCTCTGA-3') and *reverse* (5'-CGCATGGCACTCAGTAACC-3'), and the PCR products were sequenced by Sanger sequencing. The F1 mice having mGlu1 N264H mutation were crossed with the C57BL/6J mice to obtain *mGlu1^{CBC/+}* mice. The *mGlu1^{CBC/+}* mice were mated to obtain *mGlu1^{CBC/CBC}* mice.

The genotype analysis of the F1 mice also gave a mouse showing 5 bp deletion (c.790_794del) in the same exon. This deletion caused frameshift mutation and introduction of a stop codon in the same exon as shown in **Supplementary Figure 12**. Thus, the indel mice were crossed with the C57BL/6J mice to obtain *mGlu1^{-/+}* mice. The *mGlu1^{-/+}* mice were mated to obtain *mGlu1^{-/-}* mice.

Preparation of acute cerebellar slices

C57BL/6J mice (aged 3 weeks) were anesthetized with isoflurane, and their brains were transferred to ice-cold cutting solution buffer (120 mM Choline Chloride, 3 mM KCl, 8 mM MgCl₂, 1.25 mM NaH₂PO₄, 28 mM NaHCO₃, 22 mM Glucose, 0.5 mM Na ascorbate) gassed with 5% CO₂ and 95% O₂ and incubated for 5 min. Sagittal cerebellar slices were cut with a microslicer (Linear slicer Pro7; DOSAKA EM) in cutting solution buffer and then incubated in ACSF gassed with 5% CO₂ and 95% O₂ at room temperature.

Western blotting analysis of the cerebellar slices

The cerebellar slices prepared from *mGlu1*^{+/+} mice or *mGlu1*^{CBC/CBC} mice, were homogenized in homogenization buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA [pH 8.0]) and sonicated. Samples were solubilized by 1.0 % SDS and denatured at 70 °C in Laemmli sample buffer. Proteins were then resolved on SDS-PAGE and analyzed using western blotting with primary antibodies (polyclonal antibodies obtained from Frontier institute; anti-mGluR1a [Frontier Institute, rabbit, 1:5,000], anti-GluD2 [Frontier Institute, guinea pig, 1:1,000] and anti-pan AMPAR [Frontier Institute, guinea pig, 1:1,000] and secondary antibodies (horseradish peroxidase-conjugated anti-rabbit antibody [Cytiva, 1:2,500], Alexa Fluor 488-conjugated anti-rabbit antibody [ThermoFisher, 1:2,500], and Alexa Fluor 488-conjugated anti-guinea pig antibody [ThermoFisher, 1:2,500]). Chemiluminescent or fluorescent signals were detected with iBright FL1000 (ThermoFisher) and quantified using ImageJ (NIH).

Confocal live cell imaging of cell-surface mGlu1 in the cerebellar slices

The cerebellar slices prepared from *mGlu1*^{+/+} mice or *mGlu1*^{CBC/CBC} mice, were incubated with 10 nM FITM-Cy3 and 10 µg/mL Hoechst33342 for 30 min at room temperature in a humidified atmosphere of 5% CO₂ and 95% O₂. As a negative control, cerebellar slices prepared from *mGlu1*^{+/+} mice were incubated with 10 nM FITM-Cy3, 10 µg/mL Hoechst33342, and 500 nM FITM. Then, the slices were subjected to confocal live

imaging using a confocal microscope (LSM900, Carl Zeiss). The fluorescence intensity of Cy3 at the molecular layer were quantified by ZEN blue software (Carl Zeiss).

Immunohistochemistry of the cerebellar slices

Under deep anesthesia with a pentobarbital, mice were fixed by cardiac perfusion with 0.1 M sodium phosphate buffer (PB, pH7.4) containing 4% paraformaldehyde (4% PFA/PB); the brain was then removed and soaked in 4% PFA/PB for 2h. After rinsing the specimens with PB, parasagittal slices (50- μ m thickness) were prepared using a microslicer (DTK-2000; D.S.K.). Microslicer sections were permeabilized with 0.1% Triton X-100 (Sigma) in PB with 10% normal donkey serum or 2% normal goat serum/2% bovine serum albumin (BSA) for 20 min. Sections of freshly frozen brains were also prepared by cryostat (Leica) at 20 μ m thickness, mounted on glass slides and fixed with 3% glyoxal for 15 min, followed by blocking with 10% donkey serum for 30 min at RT and permeabilization with PBS containing 0.1% Triton X-100 (wash buffer). Immunohistochemical staining was performed using selective primary antibodies (anti-mGlu1 α (1:1,000; Frontier Institute), anti-calbindin (1:10,000; Swant), anti-GluD2 (1:500; Frontier Institute) and anti-Bassoon (1:500, Synaptic Systems) antibodies) overnight at room temperature, followed by incubation with the proper fluorescent dye-conjugate secondary antibodies (1: 1,000, Invitrogen) for 1 h. Finally, the sections were mounted with Fluoromount-G (SouthernBiotech). Photographs were taken with a confocal laser-scanning microscope (FLUOVIEW, FV1000; Olympus) and a super-resolution microscopy (OSR; Olympus).

Electrophysiology of the cerebellar slices

Whole-cell recordings were made from visually identified Purkinje cells using a 60 $\times$ water-immersion objective attached to an upright microscope (BX51WI, Olympus) at room temperature, as described previously⁵⁵. The resistance of patch pipettes was 1.5–3

MΩ when filled with an intracellular solution of the following composition: 65 mM Cs-methanesulfonate, 65 mM K-gluconate, 20 mM HEPES, 10 mM KCl, 1 mM MgCl₂, 4 mM Na₂ATP, 1 mM Na₂GTP, 5 mM sucrose, and 0.4 mM EGTA, pH 7.25 (295 mOsm/kg). The solution used for slice storage and recording consisted of the following: 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM D-glucose, bubbled continuously with a mixture of 95% O₂ and 5% CO₂. Picrotoxin (100 μM; Sigma) was always added to the saline to block inhibitory synaptic transmission. To evoke EPSCs derived from CF (CF-EPSCs) and PF (PF-EPSCs) inputs onto Purkinje cells, square pulses were applied through a stimulating electrode placed on the granular layer and the molecular layer (50 μm away from the pial surface), respectively. The selective stimulation of CFs and PFs was confirmed by the paired-pulse depression (PPD) and paired-pulse facilitation (PPF) of EPSC amplitudes at a 50-ms interstimulus interval (ISI), respectively. To search for multiple CFs innervating the recorded Purkinje cell, the stimulating electrode was moved systematically in the granular layer, and the stimulus intensity was gradually increased at each stimulation site (pulse width, 50 μs; strength, 0–200 μA). For the LTD sessions, PF-EPSCs were recorded successively at a frequency of 0.1 Hz from Purkinje cells clamped at –80 mV. After a stable PF-EPSC amplitude had been observed for at least 5 min, a conjunctive stimulation that consisted of 30 single PF stimuli together with a 200 ms depolarizing pulse from a holding potential of –60 to +20 mV was applied for LTD induction. To chemically induce LTD, 50 μM (S)-3,5-dihydroxyphenylglycine (DHPG) or 1 μM Pd(sulfo-bpy) was applied for 10 min in a current-clamp mode. Access resistances were monitored every 10 s by measuring the peak currents in response to 2 mV, 50-ms hyperpolarizing steps throughout the experiments; the measurements were discarded if the resistance changed by >20% of its original value. The normalized EPSC amplitude on the ordinate represents the EPSC amplitude for the average of six traces for 1 min divided by that of the average of six traces for 1 min immediately before the conjunctive

stimulation. The EPSCs were recorded using an Axopatch 200B amplifier (Molecular Devices), and the pClamp system (version 9.2; Molecular Devices) was used for data acquisition and analysis. The signals were filtered at 1 kHz and digitized at 4 kHz.

Statistical analysis

All data are expressed as mean $\pm$ s.e.m. We accumulated the data for each condition from at least three independent experiments. We evaluated statistical significance with the two-tailed Welch's t-test, Mann-Whitney U test, one-way ANOVA with Dunnet's test, or Kruskal-Wallis test. A value of $P < 0.05$ was considered significant.

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Author contributions

S.K. and I.H. initiated and designed the project. K.O., Y.Michibata and R.K. performed the construction of mGlu1 plasmids. K.O. and Y.Miura performed Ca²⁺ imaging in HEK293 cells. K.O. and T.D. performed synthesis and confocal imaging in HEK293 cells. K.O. performed toxicity assay and solubility assay in PC12 cells. S.M. and S.T. generated mutant mice using CRISPR/Cas9 system. K.O., W.K., H.N. and S.K. bred mutant mice. K.O., T.Y., E.M. and M.Y. performed imaging or western blot analysis in cerebellar slices. W.K., M.I. and M.Y. performed electrophysiological experiments. K.O., W.K. and S.K. wrote the manuscript. All authors discussed and commented on the manuscript.

Competing interests

The authors declare no competing financial interests.

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Figure 1 | Screening of the direct-activation-type mGlu1 mutants. (a) Schematic illustration of glutamate-induced conformational changes of mGlu1. (b) Schematic illustration of the coordination-based chemogenetics (CBC). Upper: allosteric activation of mGlu1 by A-CBC (OcCC in our previous terminology). Bottom: direct activation of mGlu1 by dA-CBC. (c) Conformational change of the VFT on the glutamate binding. Upper: open form in the apo state (Protein Data Bank (PDB) 1EWT). Lower: closed form in the glutamate-binding state (PDB 1EWK). The mutation sites at the upper and lower lobes are highlighted in red and blue. (d) Pd(bpy)-induced mGlu1 responses in HEK293 cells. Left: representative trace of Ca^{2+} response induced by 10 μM of Pd(bpy) in HEK293 cells transfected with the plasmid of mGlu1 P58H/N264H mutant (red) or WT mGlu1 (black). The ΔRatio is defined as the difference between the maximum and the initial ratio values. Right: averaged ΔRatio in the presence or absence of 10 μM of Pd(bpy). (n = 20). ***Significant

difference ($P < 0.001$, two-tailed Welch's t-test). n.s., not significant ($P > 0.05$). **(e)** The heatmap shows the averaged Δ ratio induced by 10 μ M glutamate, metal ions, or a metal complex. See **Supplementary Figure 1b** for each data. **(f)** Concentration-dependent curves for Pd(bpy) in HEK293 cells expressing mGlu1 WT (black), P58H/N264H (red), A59H/N264H (orange) or K61H/N264H (blue). ($n = 20$). EC_{50} values were 1.2, 3.8, and 2.3 μ M for P58H/N264H, A59H/N264H, and K61H/N264H, respectively. **(g)** Evaluation of the positive allosteric effect of Pd(bpy) to the mGlu1 mutants. Effects of 3 μ M Pd(bpy) on the concentration-dependency of the glutamate-responses were examined in the presence of 10 μ M LY367385 in HEK293 cells expressing the hit mutants (P58H/N264H, A59H/N264H, K61H/N264H). ($n = 20$). Data are presented as mean $\pm$ s.e.m. See **Supplementary Figures 3g and 3h** for the representative traces.

Figure 2 | Mechanism of direct activation of the mGlu1 mutant by dA-CBC. (a) Pd(bpy)-induced mGlu1 responses in HEK293 cells expressing the mGlu1 mutant. Left: representative traces of Ca^{2+} responses induced by 10 μM of Pd(bpy) in HEK293 cells expressed with the mGlu1 N264H (red), P58H (grey), A59H (blue), or K61H (green) mutants. Right: averaged Δ ratio. (n = 20). (b) Mutated coordinating amino acid residues within 15 Å from N264 in the crystal structure of the VFT domain (PDB 1EWK). (c) Averaged Δ ratio induced by 10 μM Pd(bpy) (left) and 10 μM glutamate (right). (n = 20). ***Significant difference ($P < 0.001$, One-way ANOVA with Dunnett's test). Data are presented as mean $\pm$ s.e.m.

Figure 3 | Characterization of mGlu1 N264H mutant. (a, b) Concentration-dependent curves for Pd(bpy) (in a) and glutamate (in b) in HEK293 cells expressing mGlu1(N264H) or WT mGlu1. (n = 20). (c) Evaluation of the positive allosteric effect of Pd(bpy) to the mGlu1(N264H) mutant. Effect of 3 μM Pd(bpy) on the concentration-dependency of the glutamate-responses in the presence of 10 μM LY367385 in HEK293 cells expressing mGlu1(N264H) is shown. (n = 20). (d) Evaluation of the protein expression level of mGlu1(N264H) by western blotting. (n = 3, biologically independent experiments). ***Significant difference ($P < 0.001$, One-way ANOVA with Dunnet's test). n.s., not significant ($P > 0.05$). (e) Chemical structure of FITM-Cy3, a fluorescent probe for mGlu1. (f) Evaluation of the cell-surface expression of mGlu1(N264H) by live imaging using FITM-Cy3. Upper: Representative confocal images of the HEK293 cells transfected with WT mGlu1, mGlu1(N264H), or vector control after addition of 1 μM FITM-Cy3. Lower: quantification of fluorescent intensity on the cell surface. (n = 30). Scale bars, 20 μm. ***Significant difference ($P < 0.001$, One-way ANOVA with Dunnet's test). n.s., not significant ($P > 0.05$). (g) The Ca^{2+} response induced by the repetitive treatment of 3 μM Pd(bpy). Left: representative trace of the Ca^{2+} response induced by 3 μM Pd(bpy) in HEK293 cells expressing WT mGlu1 (black) or mGlu1(N264H) (red). Pd(bpy) was treated repetitively after washing the cells with the culture medium and measurement buffer for 10 min. Right: averaged ΔRatio induced by 3 μM Pd(bpy). (n = 20). Data are presented as mean ± s.e.m.

Figure 4 | Low toxic Pd(bpy) derivatives for neuronal studies. (a) Chemical structures of the Pd(bpy) derivatives. (b) Evaluation of the effect of Pd(bpy) for NGF-induced neurite outgrowth of PC12 cells. Left: representative fluorescent images of PC12 cells treated with or without Pd(bpy). PC12 cells were visualized using Calcein-AM. Right: concentration-dependency of Pd(bpy) for neurite outgrowth in the PC12 cells after treatment of 1 ng/mL NGF. (n = 20). Scale bars, 50 μm. (c) Concentration-dependency of Pd(bpy) derivatives on the cell growth in undifferentiated PC12 cells. (n = 3). (d) The abnormal Ca²⁺ response by Pd(bpy) (black) or Pd(sulfo-bpy) (red) in the cultured cortical neurons. Left: representative trace of the Ca²⁺ response induced by 30 μM Pd(bpy) (black) or Pd(sulfo-bpy) (red). Right: concentration-dependence curves for Pd(bpy) (black) or Pd(sulfo-bpy) (red). (n = 30). (e) Ca²⁺ responses of Pd(bpy) derivatives for mGlu1 N264H mutant in HEK293 cells. Left: averaged Δratio induced by 3 μM Pd(bpy) derivatives. (n = 15-20). Data are presented as mean ± s.e.m. (f) Concentration-dependent curves for Pd(sulfo-bpy) (red) and Pd(OH-bpy) (green) in HEK293 cells expressing mGlu1 N264H mutant (n = 20). (g) Solubility assay of the Pd(bpy) (black) and Pd(sulfo-bpy) (red) in ACSF using UV-vis spectroscopy. Linear increment of absorbance at 312 nm corresponding to the palladium complexes indicates the solubility of the complexes. The concentration dependency of the absorbance of Pd complexes in the high (left) and low (right) concentration range (n = 3). Data are presented as mean ± s.e.m.

Figure 5 | Chemogenetic regulation of endogenous mGlu1 in brain tissue. (a) The architecture of the cerebellum in a mouse. Left: schematic illustration of a sagittal section of the mouse brain and enlarged image of the cerebellum. Middle: schematic illustration of major components of the cerebellar cortical neuronal circuits. Right: PF-Purkinje cell synapses. PF, parallel fiber; CF, climbing fiber; AMPAR, AMPA receptor; IP₃R, inositol triphosphate receptor; PLC, phospholipase C; TRPC, transient receptor potential channel canonical. (b) Illustration for preparing mGlu1(N264H) KI mice (*mGlu1^{CBC/CBC}*) using CRISPR-Cas9 system. (c) Evaluation of the protein expression level of mGlu1, AMPAR and GluR2 between *mGlu1^{+/+}* and *mGlu1^{CBC/CBC}* in the cerebellum by western blotting. (d) Quantification of the surface expression level of mGlu1 between *mGlu1^{+/+}* and *mGlu1^{CBC/CBC}* in acute cerebellar slices using FITM-Cy3. Scale bars, 200 μ m. (n = 2–5, biologically independent experiments). ***Significant difference (*P* < 0.001, One-way ANOVA with

Dunnet's test). n.s., not significant ($P > 0.05$). **(e, f)** Conventional confocal (in **e**) and super-resolution (in **f**) microscopic images of immune-positive signals for mGlu1 (red) and calbindin (white) in the molecular layer of a *mGlu1^{CBC/CBC}* cerebellar slice. Scale bars, 20 μm (in **e**) and 2 μm (in **f**). **(g)** Super-resolution microscopic images of immune-positive signals for mGlu1 (red), GluD2 (green) and Bassoon (blue). Scale bars, 1 μm . An enlarged view is shown in a right panel. Scale bars, 200 nm. **(h)** Representative CF-EPSC traces from *mGlu1^{+/+}* (top) and *mGlu1^{CBC/CBC}* (bottom). **(i)** A histogram showing the percentage of the number of CFs innervating single Purkinje cells. [$n = 32$ cells (*mGlu1^{+/+}*) or 30 cells (*mGlu1^{CBC/CBC}*)]. **(j)** mGlu1-mediated slowEPSC data. By adjusting PF stimulus intensities, similar sizes of PF-evoked AMPA-EPSC (left traces) were obtained. Then, slowEPSC (right traces) were recorded by application of 2–10 times of PF stimuli at 100 Hz in the presence of AMPAR blockers. Amplitudes of slowEPSC were normalized by amplitudes of AMPA-EPSCs and plotted against the number of stimulations (right graph). [$n = 9$ cells (*mGlu1^{+/+}*) or 13 cells (*mGlu1^{CBC/CBC}*)]. Data are presented as mean $\pm$ s.e.m.

Figure 6 | Cerebellar LTD is induced by direct activation of the mGlu1 mutant by dA-CBC. (a) A schematic diagram illustrating the experimental set-up. Conjunctive stimulus [PF/ ΔV -stim, 30 $\times$ (PF stimuli plus PC depolarization) at 1 Hz] was applied to induce LTD at PF–Purkinje cell synapses (LTD-stim). **(b)** Averaged data of LTD recordings from *mGlu1*^{+/+} (black circles) and *mGlu1*^{CBC/CBC} (red circles). Insets, representative PF-EPSC traces just before (blue; *t* = -1 min) and 30 min after (black and red) LTD-stim in *mGlu1*^{+/+} and *mGlu1*^{CBC/CBC} mice, respectively. Right graph shows a cumulative probability of the degree of LTD at *t* = 30 min. [*n* = 7 cells (*mGlu1*^{+/+}) or 6 cells (*mGlu1*^{CBC/CBC})]. **(c, d)** Averaged data of DHPG-induced (in **c**) or Pd(sulfo-bpy)-induced (in **d**) chemLTD recordings from *mGlu1*^{+/+} (black circles), *mGlu1*^{CBC/CBC} (red circles) and *mGlu1*^{CBC/+} (green circles in **d**) mice. Insets, representative PF-EPSC traces just before (blue; *t* = -1 min) and 25 min after (black, red or green) drug washout in each mouse group. Right graph shows a cumulative probability of the degree of LTD at *t* = 35 min. [*n* = 7 cells (*mGlu1*^{+/+}) or 8 cells (*mGlu1*^{CBC/CBC})]. Data are presented as mean $\pm$ s.e.m.