REVIEW

New insights into the structure and function of glutamate receptors: the orphan receptor δ2 reveals its family's secrets

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Abstract. The ionotropic glutamate receptors (iGluRs) form ligand-gated ion channels that mediate the vast majority of excitatory neurotransmission in the mammalian brain. These receptors play central roles not only in normal neurodevelopmental and neurophysiological processes but also in certain neuropathological processes. Molecular cloning of genes for iGluRs in the past decade has advanced our understanding of the basic properties of iGluRs, such as ion selectivity, ligand binding, and anchoring at synapses. Although the gene for the δ2 glutamate receptor (GluRδ2) was cloned on the basis of homology screening, GluRδ2 has been referred to as an "orphan" receptor because it does not form functional glutamate-gated ion channels. However, ataxia in many types of mice is caused by spontaneous mutation of GluRδ2. Analysis of two such mutants, *lurcher* and *hotfoot*, has provided key insights into the GluRδ2 signaling in neurons. Furthermore, characterization of mutant GluRδ2 has revealed unexpected clues to two fundamental features regarding the structure and function of iGluRs – gating and assembly. Studies have recently shown that the transmembrane region where the *lurcher* mutation is located probably plays a crucial role in channel gating. The mechanism that controls iGluR subunit assembly seems to involve the extracellular N-terminal domain where the *hotfoot* mutation is located. An understanding of mechanisms responsible for gating and assembly is essential for the comprehension of neuronal function and dysfunction. Although reverse genetics is useful in deciphering glutamate signaling, these findings demonstrate the power of classic approaches to forward genetics on mutant mice. (Keio J Med 52 (2): 92–99, June 2003)

Key words: assembly, gating, Purkinje cell, *lurcher*, *hotfoot*

Introduction

Fast excitatory neurotransmission in the mammalian central nervous system is mediated by ionotropic glutamate-gated receptors (iGluRs): α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA) receptors.[1] Glutamate signaling is essential for normal neurodevelopment and synaptic plasticity associated with learning and memory. However, when iGluRs are overstimulated, glutamate signaling causes "excitotoxicity," which plays a role in neuronal cell death in many neuropathological processes, such as ischemia, Alzheimer's disease, Parkinson's disease, and Huntington's disease.[2,3] Therefore, glutamate signaling is a "Jekyll-and-Hyde" type of signaling – it is crucial for normal synaptic functions but has the potential to cause severe neuronal destruction. In other words, neurons are equipped with mechanisms to precisely control glutamate signaling; when these mechanisms are disrupted, then the neurons undergo destruction. An understanding of such mechanisms is essential for an appreciation of neuronal function and dysfunction.

In the past decade, molecular cloning efforts have identified genes for four AMPA receptor subunits (GluR1–GluR4), five kainate receptor subunits (GluR5–GluR7, KA1, and KA2), and seven NMDA receptor subunits (NR1, NR2A–NR2D, NR3A, and NR3B).[1,4–6] These findings have greatly advanced our understanding of the basic properties of iGluRs, such

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as ion selectivity, ligand binding, and anchoring at synapses. However, two fundamental issues remain unclear. One is the mechanism of ''gating,'' *i.e.*, the conformational changes associated with the opening and closing of ion channels induced by external signals. The importance of the precise control of gating of ion channels is illustrated by several hereditary diseases attributable to a point mutation that causes sub-millisecond to millisecond delays in the activation or inactivation of gating.[7,8] Another unclear issue is the mechanism that controls subunit assembly; functional iGluRs are heteromers *in vivo*, and various combinations of the subunits are responsible for the functional heterogeneity of iGluRs.[9,10]

Although the δ2 glutamate receptor (GluRδ2) is classified as an iGluR, GluRδ2 has been referred to as an ''orphan'' receptor because it does not form functional glutamate-gated ion channels when expressed, either alone or with other iGluRs, in heterologous cells, nor does it bind to glutamate analogs.[11,12] Nevertheless, GluRδ2, which is predominantly expressed in cerebellar Purkinje cells, plays a crucial role in cerebellar functions: mice that lack the gene encoding GluRδ2 display ataxia and impaired synaptic plasticity.[13] The mechanisms by which GluRδ2 is involved in cerebellar functions have not been fully elucidated, because of the lack of specific pharmacological tools with which to manipulate GluRδ2. It is unlikely that GluRδ2 directly participates in normal parallel fiber – Purkinje cell synaptic transmission because the transmission was completely blocked by specific antagonists to conventional AMPA receptors.[14] Recently, studies of several mutant mice, such as *lurcher*, *hotfoot*, and GluRδ2 knock-out mice, have provided the key to understanding GluRδ2 signaling in neurons.[14] GluRδ2 has a channel pore similar to that of other glutamate receptors; the channel is functional at least when the *lurcher* mutation is present. GluRδ2 must be transported to the Purkinje cell surface to function; the absence of surface GluRδ2 causes the ataxic phenotype of *hotfoot* mice. Furthermore, the characterization of GluRδ2 has also revealed unexpected clues to the two fundamental yet unclear issues regarding the structure and function of iGluRs – gating and assembly. These two issues are addressed in this review, which is based on part of my presentation at the 1280th Meeting of the Keio Medical Society. Here I will focus on findings from studies of *lurcher* and *hotfoot* mice performed in my laboratory.

The *Lurcher* Mutation and Gating Mechanisms of iGluRs

General design of iGluRs

Recent studies have established that iGluRs have a modular structure (Fig. 1A): an amino-terminal leucine/isoleucine/valine-binding protein (LIVBP)-like domain and a ligand-binding bipartite lysine/arginine/ornithine-binding protein (LAOBP)-like domain on the extracellular side of the plasma membrane, three transmembrane domains (TM1, TM3, and TM4), an ion channel-forming re-entrant loop segment (P-loop or TM2), and a cytoplasmic carboxyl-terminal region.[3] Ligand binding is achieved by the LAOBP-like domain, which consists of S1 and S2 regions.[15] Two lobes formed by these regions close upon ligand binding, which leads to opening of the ion channel gate (Fig. 1A). The re-entrant loop formed by TM2 determines the ion selectivity of the channel pore.[16] The cytoplasmic tails of iGluRs are important in synaptic clustering and in regulating receptor activity that involves intracellular signaling via interaction with anchoring proteins.[17]

The lurcher mutation

The ataxia of *lurcher* mice results from a point mutation at position 654 (Ala → Thr) at the end of TM3 of GluRδ2 (Fig. 1B); this particular mutation causes constitutive activation of mutant channels ($GluR\delta2^{Lc}$) in the absence of ligand binding[18] and eventually leads to the death of Purkinje cells. Because the channel properties of $GluR\delta2^{Lc}$ are similar to those of AMPA or kainate receptors, it has been concluded that GluRδ2 has a channel pore that can be functional at least when this mutation is present at TM3.[19]

Interestingly, the *lurcher* mutation in GluRδ2 is in a highly conserved motif (SYTANLAAF [the site of mutation is underlined]), which is at the end of TM3 in all iGluRs (Fig. 1B). Moreover, a similar yet rudimentary motif is found in TM2 of the bacterial glutamate-gated channel GluR0[20] and the bacterial pH-gated K^+ channel KcsA (Fig. 1B). Thus, this motif may play a crucial role in the function of iGluRs and may have evolved with K^+ channels from a common ancestor.[21] Recently, the structure of TM2 in KcsA K^+ channels during gating-associated movement was characterized: the TM2 helix rotates and tilts away from the permeation pathway[22,23] to open the channel gate. Therefore, coworkers in my laboratory and I hypothesize that the gating of iGluRs is achieved by a similar mechanism associated with the movement of this motif in TM3. Indeed, this motif is ideally located, between the agonist-binding pocket and the channel pore, for participation in the transduction of agonist binding to channel gating (Fig. 1A).

TM3 of iGluRs: to gate or not to gate

To test our hypothesis, we introduced the Ala → Thr mutation, *i.e.*, the *lurcher* mutation, at the corresponding position in an AMPA receptor GluR1 ($GluR1^{Lc}$). Like $GluR\delta2^{Lc}$, $GluR1^{Lc}$ formed channels that were

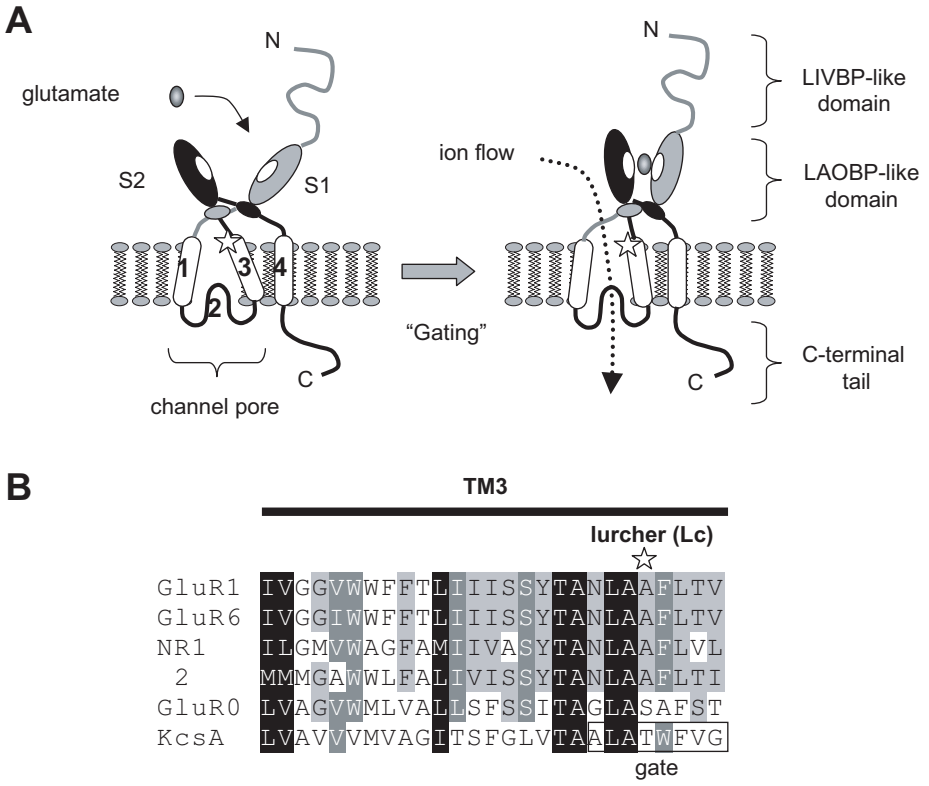


Fig. 1 Schematic illustration of the structure of ionotropic glutamate receptors and glutamate-induced conformational changes associated with channel gating. (A) The LIVBP-like domain is at the N terminus. Two extracellular segments of the LAOBP-like domain (S1 and S2) form the ligand-binding pocket of ionotropic glutamate receptors (left). Two lobes formed by S1 and S2 segments close upon ligand binding, which leads to opening of the ion channel gate (right). Three short segments connect the structure to the transmembrane regions (numbered 1–4). The site of the mutation in GluRδ2 of *lurcher* mice is in the conserved motif SYTANLAAF in transmembrane domain 3 (TM3) and is indicated by ☆. (B) Comparison of the amino acid sequence of GluRδ2 with those of representative glutamate receptors and K^+ channels (AMPA receptor GluR1, kainate receptor GluR6, NMDA receptor NR1, prokaryotic glutamate-gated channel GluR0, and bacterial K^+ channel KcsA). TM3 of glutamate receptors and of GluR0 and TM2 of KcsA are shown. Letters are shaded according to the percentage of conservation of similar amino acids (100%, 80%, and 60%) in each position. A box indicates the residues in KcsA that rotate during channel opening[24,25]. The location of a mutation in GluRδ2 of *lurcher* mice is also indicated by ☆.

constitutively activated in the absence of ligand binding.[19] When glutamate was applied to 293 cells expressing GluR1 (Fig. 2A), rapidly desensitizing currents were recorded (Fig. 2B, left). Glutamate also activated currents through $GluR1^{Lc}$ despite constitutive channel activation (Fig. 2B, right), a finding suggesting that the constitutively activated channels were not locked in the open configuration. However, the mutation severely slowed the kinetics of ligand-gated channels. We also observed similar effects of the *lurcher* mutation on kainate receptor GluR6 and NMDA receptors NR1 and NR2A.[19] Furthermore, in the SYTANLAAF motif of GluR1, we have recently identified additional residues that cause *lurcher* mutation-like effects (Kamiya *et al*., *submitted*) and whose periodicity is characteristic of amino acids in α-helices (Fig. 3A). These results are consistent with the view that the gating of iGluRs is mediated by the structure at the end of TM3, including the SYTANLAAF motif. According to this model, the end of TM3 forms α-helices that cross one another, and ligand binding at the LAOBP-like domain (Fig. 1A) induces tilting and rotation of the SYTANLAAF motif in TM3 to open the ion permeation pathway (Fig. 3B). When the *lurcher* mutation is introduced into this motif, the larger side-chain volume of the substituted amino acid may inhibit the complete closure of the gate even in the absence of the ligand and may slow the closure of the gate after the ligand is released (Fig. 3B).

An alternative to our hypothesis about the channel-gating mechanisms of iGluRs is that the gating of iGluRs is achieved by the conformational change in the channel pore at TM2. These two hypotheses are not mutually exclusive, because the rotation at the end of TM3 can be allosterically coupled with the conformational change at the channel pore. For example, in the family of cyclic nucleotide-gated K^+ channels, ligand binding-induced rotation of the S6 helices, which correspond to TM3 of iGluRs, is thought to be associated

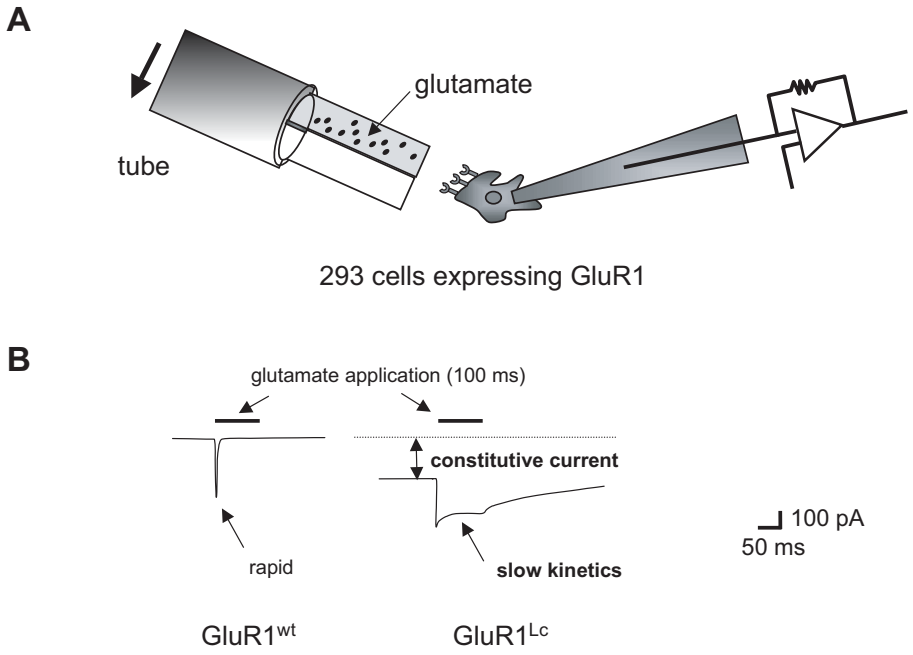


Fig. 2 Patch-clamp recordings of constitutive and glutamate-induced currents in 293 cells expressing glutamate receptors. (A) Schematic illustration of the patch-clamp recording. Currents passing through the glutamate receptor under a fixed voltage are monitored by a recording electrode placed in the 293 cells expressing glutamate receptors. Control solution was gravity-fed into one of the two lumens of the θ-shaped glass tubing; glutamate-containing solution was fed into the other. The cell or a patch of cell membranes was positioned near the interface formed between continuously flowing control and drug solutions. Solution exchange was made by rapidly moving the θ-shaped glass with a Piezo translator. With this system, all of the solution can be exchanged in 200 μs. (B) Typical current responses in 293 cells expressing wild-type GluR1 ($GluR1^{wt}$) or *lurcher* GluR1 ($GluR1^{Lc}$). Application of 1 mM glutamate for 100 ms to 293 cells expressing $GluR1^{wt}$ induced rapidly inactivated current. In contrast, constitutive current was observed in $GluR1^{Lc}$-expressing cells in the absence of ligands. Moreover, the application of glutamate induced further currents with very slow desensitization and deactivation kinetics.

with conformational changes in the channel pore[24,25]; these conformational changes open the channel gate.

Clinical implications

Studies of the *lurcher* mutation provided the first direct evidence that a mutation that affects the gating of a neurotransmitter-gated channel causes the degeneration of the neurons that express it. Because the therapeutic potential of voltage-gated channel blockers is often related to their effects on gating,[26,27] a drug targeted against the gate-related structure at the end of TM3 of iGluRs may hold therapeutic potential for diseases associated with excitotoxicity.

The *Hotfoot* Mutations and Assembly of iGluRs

The hotfoot mutations

Ataxia in the *hotfoot* mouse *ho4J* is caused by a deletion that occurs in the GluRδ2 gene and results in the loss of 170 amino acids from the LIVBP-like domain (approximately 400 amino acids) (Fig. 4A).[28] Unlike the dominantly inherited *lurcher* allele, the *hotfoot* alleles carry recessive loss-of-function mutations that cause ataxia in the absence of obvious Purkinje-cell death. We found that the mutation in *ho4J* mice inhibited the function of GluRδ2 by impairing its exit from the endoplasmic reticulum[29] (ER), which was mainly localized in the Purkinje cell soma (Fig. 4B). In addition to *ho4J*, at least 16 independent *hotfoot* alleles have been identified.[30] Interestingly, many of these *hotfoot* alleles also have deletions of nucleotides that code for 40 to 95 amino acids in the LIVBP-like domain of GluRδ2; these deletions also affect the transport of GluRδ2 to the cell surface.[31] Coimmunoprecipitation assays indicated that all these deletions reduced the intermolecular interaction between individual GluRδ2 molecules. Thus, the LIVBP-like domain seems to be crucial for oligomerization of GluRδ2. These results indicate that GluRδ2 must be transported to the surface of Purkinje cells to function and that the ataxic phenotype of *hotfoot* mutants is caused by the loss of GluRδ2 from the cell surface.

LIVBP-like domain of iGluRs: some assembly required

Unlike the functions of other domains, the function of the LIVBP-like domain in iGluRs has not been

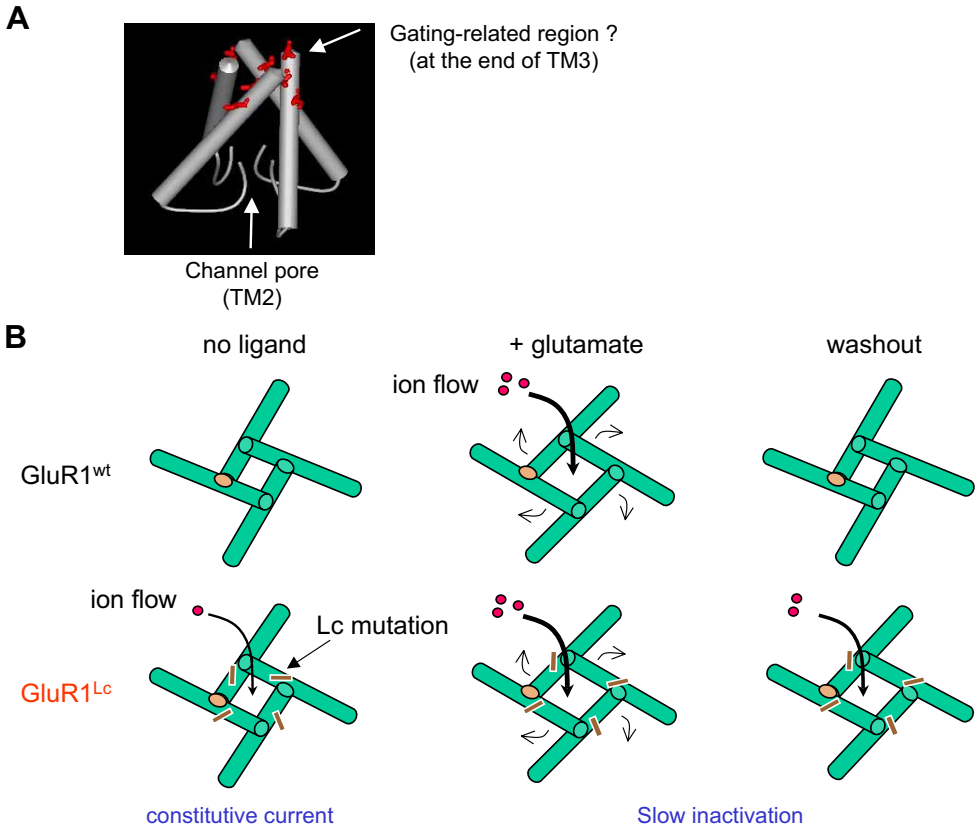
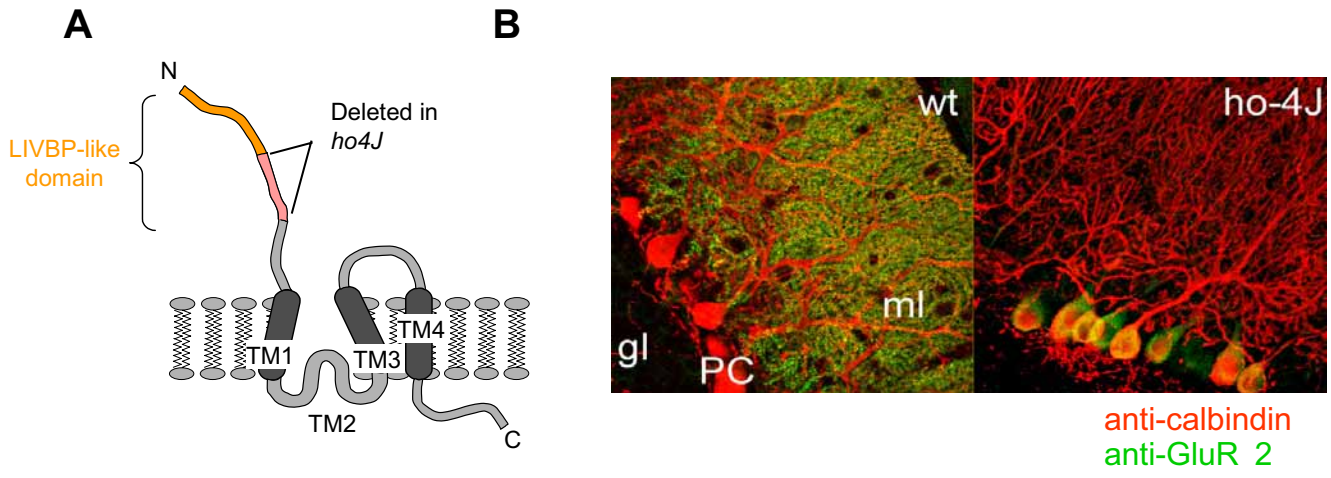


Fig. 3 A model of glutamate-receptor gating. (A) An oblique view of TM3 of GluR1. The position of TM3 is based on coordinates for KcsA K^+ channels. In the region possessing the *lurcher* mutation, the red areas are the sites of substituted amino acids with larger side chains than the original residues. A channel pore made by TM2 is also shown. (B) A potential arrangement of the TM3 helices in the closed and open conformation. This model is based on the KcsA K^+ gating model. Shown are top views of TM3 helices of glutamate receptors in the closed state without ligand binding (left panels), the open state during glutamate application (middle panels), and the deactivation state after the ligand is washed away (right panels). According to this model, the end of TM3 helices form bundles, the ends of the bundles cross one another, and ligand binding at the LAOBP-like domain (Fig. 1A) induces tilting and rotation of the TM3 to open the ion permeation pathway. When the *lurcher* mutation is present ($GluR1^{Lc}$), the larger side-chain volume of the substituted amino acid residue may inhibit the complete closure of the gate, even in the absence of the ligand; thus, constitutive currents are produced. Although ligand binding can further rotate the TM3 to allow more ion flow, TM3 cannot rapidly return to its original position because of the large side-chain volume; thus, current inactivation in $GluR1^{Lc}$ channels is relatively slow.



well characterized. As the name suggests, LIVBP is a bacterial periplasmic amino acid-binding protein, like LAOBP. Indeed, in the glutamate receptors of the metabotropic family, the LIVBP-like domain serves as a ligand-binding domain.[32] In contrast, the LIVBP-like domain is important in the subtype-specific assembly of the GluR1.[33] This domain is also important in the association between NR1 and NR2A.[34] Interestingly, LIVBP-like domains of various receptors, including those of GluR4[35] and metabotropic glutamate receptors,[32] tend to dimerize with each other. These findings and the reduced intermolecular interaction between *hotfoot* GluRδ2 suggest that a major function of this domain in iGluRs is oligomerization of subunits. We hypothesize that deletions in the LIVBP-like domain of GluRδ2 in *hotfoot* mice correspond to the interface for dimerization or the structure that underlies the interface.

To examine whether this hypothesis is applicable to other iGluRs, we introduced a 170-amino acid deletion, *i.e.*, the *ho4J* mutation, at the corresponding position in the AMPA receptor GluR1 ($GluR1^{ho4J}$). Glutamate activated no current in 293 cells expressing $GluR1^{ho4J}$ (Fig. 5A). Like GluRδ2 with a *hotfoot* mutation, this mutation inhibited the function of GluR1 by impairing its exit from the ER. Coimmunoprecipitation assays further indicated that these deletions reduced the intermolecular interaction between individual GluR1 molecules. These results suggested that the *hotfoot* region in the LIVBP-like domain of other iGluRs is involved in subunit oligomerization and subsequent exit from the ER.

The mechanisms by which $GluR\delta2^{ho4J}$ and $GluR1^{ho4J}$ are retained in the ER are unclear. Although N-glycosylation of the N-terminal region of glycine receptors and nicotinic acetylcholine receptors is critical for their oligomerization and their suubsequent exit from the ER,[36,37] it is not the case with iGluRs.[29] It is possible that a quality control mechanism detects the conformation of the N-terminal region of the oligomer. If the assembled subunits are unstable because of an incompatible LIVBP-like domain, the oligomer may be retained in the ER (Fig. 5B). Alternatively, the LIVBP-like domain of iGluRs may possess a specific signal

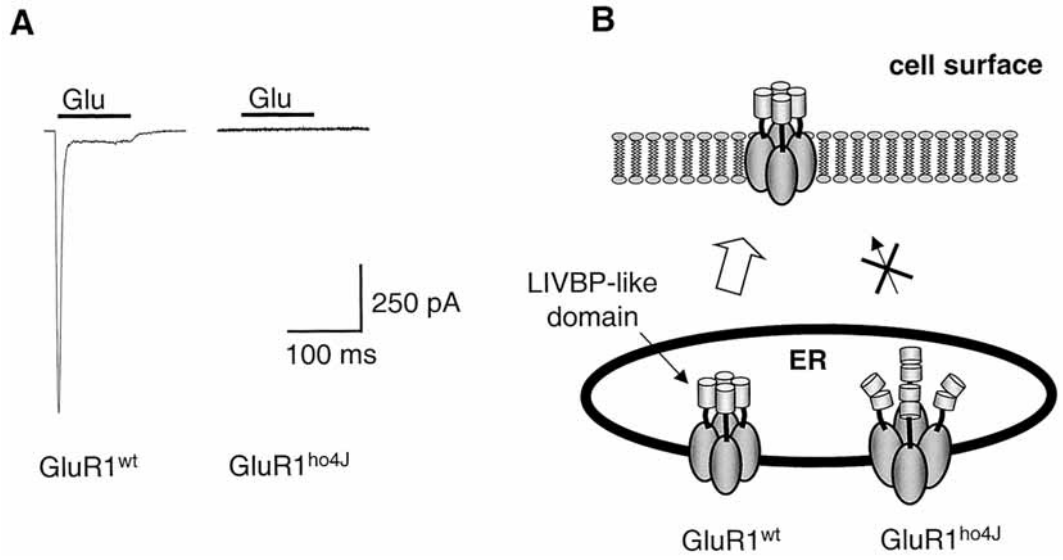


Fig. 5 The effect of the *ho4J* mutation in GluR1 and a model of the mechanism by which the *ho4J* mutant receptor is retained in the endoplasmic reticulum (ER). (A) Electrophysiological analysis of wild-type GluR1 ($GluR1^{wt}$) and *ho4J* GluR1 ($GluR1^{ho4J}$) expressed in 293 cells. Glutamate-induced currents were recorded by the method described in Fig. 2A. (B) Model of the retention of the ionotropic glutamate receptors (*e.g.*, GluR1) in the ER and the expression of these receptors on the cell surface. Ionotropic glutamate receptors oligomerize in the ER, and the oligomers are then transported to the cell surface. Oligomers that contain a *ho4J* subunit are retained in the ER, probably by the ER quality control mechanism, and are eventually degraded.

Fig. 4 Schematic drawing of the *hotfoot* mutation *ho4J* and its effect on GluRδ2 localization in Purkinje cells. (A) The *ho4J* mutation is located in the LIVBP-like domain of GluRδ2. (B) Confocal microscopic analysis of the retention of GluRδ2 in *ho4J* Purkinje cells. Cerebellar sections from wild-type (wt) and *ho4J* heterozygous mice were prepared and stained with anti-GluRδ2 antibody and anti-calbindin D28K antibody. In wild-type mice (left), GluRδ2 (green) is located predominantly in fine dendritic spines of Purkinje cells (PC; red) in the molecular layer (ml). However, in *ho4J* mice (right), GluRδ2 remains in the somas of PCs, thus causing cerebellar ataxia.

recognized by unidentified protein trafficking mechanisms, as reported recently for GluR1 and GluR2.[38] In this case, the *hotfoot* deletions may impair the proper presentation of this motif to the trafficking machinery. Both views are consistent in that iGluRs are preassembled in the ER and only the oligomers that pass the control mechanism can be transported to the cell surface.

Clinical implications

All iGluRs function as heteromers *in vivo*.[9,10] For example, AMPA receptors are heteromers that consist of GluR1 through GluR4, and each subunit has a specific function. A dynamic combination of these subunits may control the functional heterogeneity of iGluRs *in vivo*, but whether a mechanism prevents the coassembly of iGluR subunits belonging to different families is still unclear. Therefore, characterization of the LIVBP-like domain will provide key insight into the developmental and tissue-specific functions of iGluRs. As proposed for G protein-coupled receptors,[39] defining the oligomerization interface and its molecular dynamics could yield previously unidentified sites that can serve as targets for novel therapeutic agents. Compounds that modulate receptor assembly could represent a new class of noncompetitive drugs with distinct selectivity and activity profiles.

It has become increasingly clear that glutamate signaling is mainly modulated *in vivo* by membrane trafficking processes controlling the expression of receptors on the cell surface.[17] For example, AMPA receptor trafficking plays a critical role in two prominent examples of synaptic plasticity: long-term potentiation and long-term depression. Indeed, studies of the *hotfoot* mice have demonstrated the importance of the LIVBP-like domain in the function of GluRδ2 *in vivo*. Therefore, further studies are warranted to better characterize the mechanisms of the LIVBP-like domains in controlling the trafficking of iGluRs.

Concluding Remarks

It is ironic that natural mutations affecting GluRδ2, an enigmatic member of the iGluR family, have revealed very important clues to two fundamental issues regarding the structure and function of iGluRs – gating and assembly. In the era in which the whole-genome sequence of several organisms and reverse genetics are available, these fortuitous discoveries have made us realize again the power of classic genetic approaches in naturally occurring mutant mice. Perhaps the lesson we should learn from these mutant mice is that we should be humble and listen to what nature is telling us. A large number of spontaneous mutations affect GluRδ2, probably because it is encoded by a large gene (approximately 1.4 Mb) and its mutation causes a nonlethal ataxic phenotype. Therefore, we expect that forward-genetics approaches in which the alkylating agent ethylnitrosurea is used as a mutagen will result in the production of new types of ataxic mutant mice and provide further clues to the function of GluRδ2 and iGluRs.

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