

Ionotropic glutamate-like receptor $\delta 2$ binds D-serine and glycine

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The orphan glutamate-like receptor GluR $\delta 2$ is predominantly expressed in Purkinje cells of the central nervous system. The classification of GluR $\delta 2$ to the ionotropic glutamate receptor family is based on sequence similarities, because GluR $\delta 2$ does not form functional homomeric glutamate-gated ion channels in transfected cells. Studies in GluR $\delta 2^{-/-}$ knockout mice as well as in mice with naturally occurring mutations in the GluR $\delta 2$ gene have demonstrated an essential role of GluR $\delta 2$ in cerebellar long-term depression, motor learning, motor coordination, and synaptogenesis. However, the lack of a known agonist has hampered investigations on the function of GluR $\delta 2$. In this study, the ligand-binding core of GluR $\delta 2$ (GluR $\delta 2$ -S1S2) was found to bind neutral amino acids such as D-serine and glycine, as demonstrated by isothermal titration calorimetry. Direct evidence for binding of D-serine and structural rearrangements in the binding cleft of GluR $\delta 2$ -S1S2 is provided by x-ray structures of GluR $\delta 2$ -S1S2 in its *apo* form and in complex with D-serine. Functionally, D-serine and glycine were shown to inactivate spontaneous ion-channel conductance in GluR $\delta 2$ containing the *lurcher* mutation (EC_{50} values, 182 and 507 μ M, respectively). These data demonstrate that the GluR $\delta 2$ ligand-binding core is capable of binding ligands and that cleft closure of the ligand-binding core can induce conformational changes that alter ion permeation.

crystal structure | electrophysiology | isothermal titration calorimetry | ligand-binding core

GluR $\delta 2$ receptors are homologous to ionotropic glutamate receptors (iGluRs) but are not gated by L-glutamate (1, 2). The GluR $\delta 2$ receptors are localized in dendritic spines of the Purkinje cells (3), and the specific postsynaptic localization suggests that the receptor is involved in synaptic transmission (4). The majority of experimental data on the functional role of GluR $\delta 2$ in Purkinje cells originate from studies on GluR $\delta 2$ mutant mice, such as the *hotfoot* and *lurcher* strains, with naturally occurring mutations in the GluR $\delta 2$ gene, and genetically engineered GluR $\delta 2^{-/-}$ knockouts (5–9). *Hotfoot* mice express truncated GluR $\delta 2$ proteins incapable of translocation to the cell surface of Purkinje cells (7). Similar to GluR $\delta 2^{-/-}$ mice, *hotfoot* animals display an ataxic phenotype and have several deficits in cerebellar development and function, including impaired Purkinje cell synaptogenesis, motor learning and coordination, and activity-dependent α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor endocytosis during cerebellar long-term depression (6, 7, 10, 11), indicating that the presence of GluR $\delta 2$ is crucial in the Purkinje synapse.

It is still unknown whether GluR $\delta 2$ controls these functions by acting as an ionotropic ligand-gated receptor analogous to other iGluRs because no endogenous or exogenous agonist has yet been identified that can activate WT GluR $\delta 2$ or GluR $\delta 2$ -containing receptors. The most direct evidence for a role of GluR $\delta 2$ signaling has been provided by a study in which injection of an antibody specific for the putative ligand-binding core of

GluR $\delta 2$ into the mouse cerebellum causes transient cerebellar dysfunction that is manifest in the same ataxic gait observed in *hotfoot* mice (12). In addition, antibody application to Purkinje cells induced endocytosis of postsynaptic AMPA receptors, suggesting that GluR $\delta 2$ signaling may be involved in Purkinje excitatory transmission through active control of synaptic AMPA receptor content (10, 12).

In the absence of known GluR $\delta 2$ agonists, the channel function of GluR $\delta 2$ has been studied by using the properties of the mouse *lurcher* mutation (GluR $\delta 2^{Lc}$; A654T in transmembrane region-2) that generates GluR $\delta 2$, in which the ion channel is spontaneously activated in the absence of an apparent agonist (9, 13). These studies also demonstrate that GluR $\delta 2^{Lc}$ possesses channel properties that are similar to those of other iGluRs.

Despite the hints at the function of GluR $\delta 2$ in mature cerebellum, the role of GluR $\delta 2$ remains unclear, largely because an endogenous ligand remains unidentified. Among the glutamate receptor family, GluR $\delta 2$ and the closely related GluR $\delta 1$ are the only receptor subunits without a known ligand, because the putative NMDA receptor subunits NR3A and NR3B recently were shown to bind glycine (14, 15).

Results

X-Ray Structure of GluR $\delta 2$ -S1S2 in Its *Apo* Form. To investigate the ligand-binding properties of GluR $\delta 2$, we generated a soluble construct representing the putative ligand-binding core of GluR $\delta 2$ (referred to as GluR $\delta 2$ -S1S2; Fig. 1A) by using a strategy similar to previous studies of iGluRs (16).

As an important first step toward understanding the function of GluR $\delta 2$ at the molecular level, we determined the x-ray structure of soluble GluR $\delta 2$ -S1S2 protein (Fig. 1B). The structure of GluR $\delta 2$ -S1S2 *apo* reveals a clamshell-like structure comprising two domains (D1 and D2) that adopts an open conformation similar to the *apo* structure of the AMPA receptor GluR2-S1S2 (16). GluR $\delta 2$ -S1S2 *apo* crystallized as a twofold symmetric dimer (Fig. 1B). This dimer is similar to that of GluR2-S1S2 (16) and the heteromeric NMDA receptor dimer of the ligand-binding cores of NR1 and NR2A (17). The interface-

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Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR $\delta 2^{Lc}$, mouse *lurcher* mutation; GluR $\delta 2$ -S1S2, ligand-binding core construct of GluR $\delta 2$; iGluR, ionotropic glutamate receptor; ITC, isothermal titration calorimetry; NASP, 1-naphthyl acetyl spermine.

The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2V3T and 2V3U).

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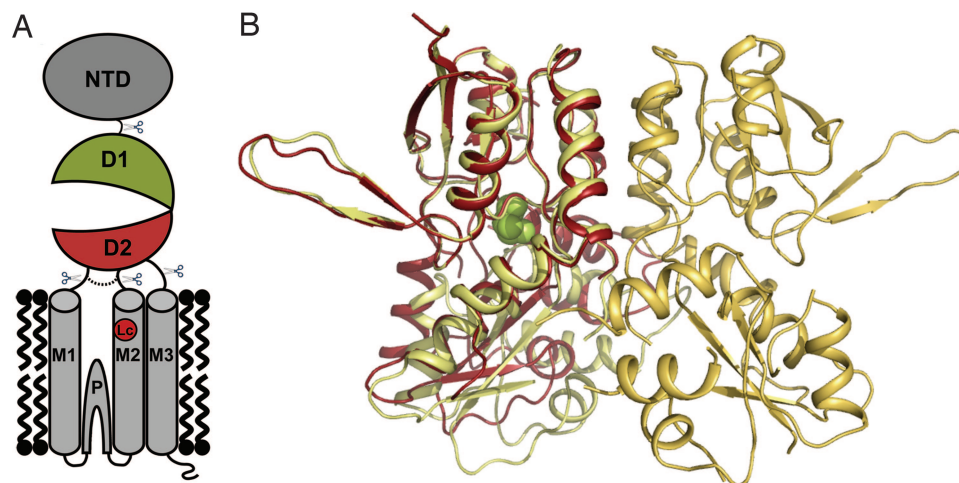


Fig. 1. The structure of the ligand-binding core of GluR δ 2. (A) Domain organization of the GluR δ 2 receptor subunit. The architecture is similar to other glutamate receptors (AMPA, kainate, and NMDA receptors), with an extracellular N terminus, three transmembrane segments (M1, M2, and M3), a reentrant membrane loop (P), and an intracellular C terminus. The extracellular regions harbor the N-terminal domain (NTD) and the ligand-binding core (D1 and D2). The red dot shows the approximate position of the lurcher mutation (A654T). The boundaries of the GluR δ 2-S1S2 construct are indicated by scissors, and the dotted line represents the Gly-Thr linker. (B) Representation of the twofold symmetric dimer of GluR δ 2-S1S2 *apo* (in yellow). The structure of GluR δ 2-S1S2 in complex with D-serine has been superimposed onto GluR δ 2-S1S2 *apo* and is shown in red. D-serine is displayed as green spheres.

accessible surface area is $\approx 1,085 \text{ \AA}^2$ per protomer and primarily made up of D1 residues located in helices D and J and the first interdomain β strand. In addition, two calcium ions are bound at this interface. For additional information on the dimer interface, see [supporting information \(SI\) Fig. 5](#).

A structural alignment was performed to locate the closest structural relatives of other iGluRs, showing that the overall structure of GluR δ 2-S1S2 resembles that of GluR2-S1S2, with a larger loop inserted between helices F and G ([SI Fig. 6](#)). Despite the overall similarity to GluR2, the structural comparisons clearly demonstrated that the amino acid composition of the ligand-binding cavity of GluR δ 2 is most similar to that of NR1. The α amino acid-binding motif seen in other iGluRs (16–19) is essentially conserved in GluR δ 2. Of importance, GluR δ 2 contains a tryptophan (Trp-741) and a valine (Val-687) within the ligand-binding pocket at positions similar to those in NR1 (Trp-731 and Val-689). The tryptophan and valine in NR1 have been suggested to create a local environment that prevents binding of L-glutamate (19).

Based on structural superimpositions of the two domains D1 and D2 of GluR δ 2 individually onto the corresponding domains in fully closed structures of GluR2, GluR5, GluR6, and NR1, we hypothesized that the binding site of GluR δ 2 in the fully closed state mostly resembles that of NR1. Because GluR δ 2 and NR1 appear to have a number of features thought to exclude L-glutamate analogs from the ligand-binding pocket (19), it seemed plausible to us that potential GluR δ 2 ligands are relatively small and similar to known NR1 ligands. We therefore conducted isothermal titration calorimetry (ITC) studies on a series of small amino acids to examine their ability to bind to purified GluR δ 2-S1S2. Among these amino acids, D-serine and glycine were shown to bind to GluR δ 2-S1S2, with K_d values in the low millimolar range (Fig. 2). Because heat is absorbed, the ligand binding is endothermic and therefore entropy-driven.

X-Ray Structure of GluR δ 2-S1S2 in Complex with D-Serine. To gain information on the interactions between D-serine and GluR δ 2-S1S2, we determined the complex structure at a resolution of 1.7 $\text{\AA}$ (Fig. 1B). The electron density for D-serine in the binding site of GluR δ 2 is well defined, thereby enabling unambiguous positioning of the ligand and identification of ligand-receptor interactions (Fig. 3A). The binding of the α -amino group of

D-serine involves contacts with the backbone carbonyl of Ala-523, the side-chain hydroxyl group of Thr-525, and the carboxylate of Asp-742, whereas the side-chain guanidinium group of Arg-530 is a primary anchor for the α carboxylate group of D-serine (Fig. 3A). Furthermore, hydrogen bonds are provided to the α -carboxylate group of D-serine by backbone amides of Thr-525 and Ala-686. The overall binding mode of D-serine in GluR δ 2 is similar to the binding mode of D-serine in NR1 (19), however, with one marked difference. The side-chain hydroxyl group of Ser-688 in NR1 serves as the anchor for the β -hydroxy group of D-serine, whereas in GluR δ 2 the corresponding residue is an alanine (Ala-686); hence, this interaction has been eliminated in GluR δ 2. Instead, the Tyr-543 located in the interdomain linker region of GluR δ 2 substitutes for the role of Ser-688 in NR1 by providing a hydrogen bond from the side-chain hydroxyl group to the β -hydroxy group of D-serine. Of note, D-serine completely fills out the ligand-binding cavity in this closed conformation of GluR δ 2-S1S2, and no water molecules are within hydrogen-bonding distances of D-serine (Fig. 3B, [SI Fig. 7](#), and [SI Table 1](#)). Two thiocyanate ions are positioned in the

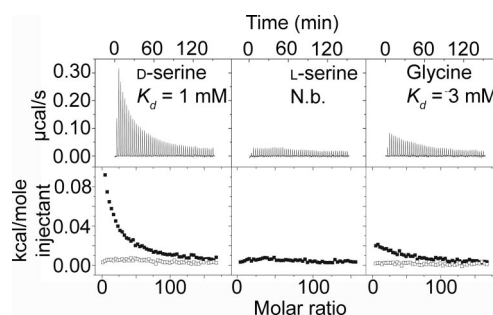
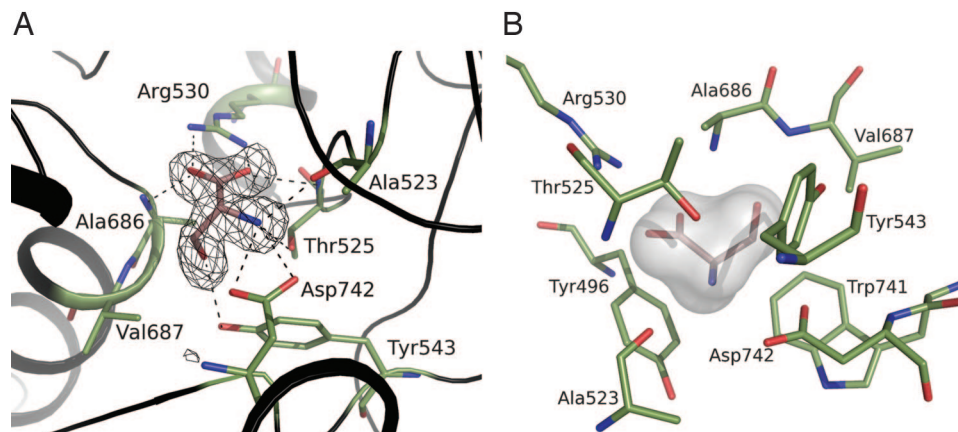


Fig. 2. ITC studies on GluR δ 2-S1S2. Raw data (*Upper*) and isotherms (*Lower*) of D-serine, L-serine, and glycine are presented. The filled squares represent titrations of GluR δ 2-S1S2 with ligands, and the open squares represent titrations with ligands in buffer only. The graphs show that heat is absorbed after each injection of the ligand and that the heat signal diminishes as the protein becomes saturated with the ligand. The K_d values of D-serine and glycine binding correspond to fitted $1/K_d$ values of $1.11 \pm 0.04 (10^3 \text{ M}^{-1})$ and $0.36 \pm 0.03 (10^3 \text{ M}^{-1})$, respectively. N.b., no binding. Molar ratio is the ratio of ligand relative to GluR δ 2-S1S2.



vicinity of the D-serine-binding site, with closest distances of 4.4 Å (D-serine hydroxyl group to sulfur atom of one thiocyanate ion) and 5.8 Å (D-serine carboxylate oxygen atom to sulfur atom of second thiocyanate ion).

Electrophysiological Evidence for Binding of D-Serine and Glycine to GluRδ2. To examine the functional significance of binding of D-serine and glycine to GluRδ2, WT GluRδ2 was expressed in *Xenopus* oocytes and studied by using voltage-clamp electrophysiology. No detectable current was observed after application of D-serine (1 mM) or glycine (1 mM) to oocytes injected with cRNA encoding WT GluRδ2 ($n > 20$). Because GluRδ2 has been proposed to form heteromeric receptors with other glutamate receptor subunits (20), we examined coexpression of GluRδ2 in combination with a number of other subunits. No D-serine or glycine current response either with or without L-glutamate could be detected from oocytes coinjected with cRNA encoding GluRδ1/GluRδ2 ($n = 6$), GluRδ2/GluR1 ($n = 4$), GluRδ2/GluR2 ($n = 4$), GluRδ2/KA1/KA2 ($n = 6$), GluRδ2/GluR6 ($n = 4$), GluRδ2/NR1 ($n = 8$), GluRδ2/NR2A ($n = 4$), GluRδ2/NR2B ($n = 4$), GluRδ2/NR2C ($n = 6$), GluRδ2/NR2D ($n = 4$), or GluRδ2/NR3B ($n = 4$).

Injection of GluR $\delta 2^{Lc}$ mutant cRNA in oocytes resulted in receptors with spontaneous channel activation, producing inward currents sensitive to the open-channel blocker 1-naphthyl acetyl spermine (NASP) (21) (SI Fig. 8). Although Klein and Howe (22) have suggested that a similar mutation in homomeric GluR1 AMPA receptors increases the affinity of L-glutamate such that the channel can be activated by low nanomolar concentrations of contaminant L-glutamate, no known activators have been described for WT GluR $\delta 2$ or GluR $\delta 2^{Lc}$. For this reason, we assume that channels formed by GluR $\delta 2^{Lc}$ are spontaneously active. Surprisingly, application of D-serine to GluR $\delta 2^{Lc}$ resulted in a concentration-dependent reduction in the current by up to $85 \pm 2\%$ relative to inhibition by NASP (EC_{50} value of $182 \pm 11 \mu M$; $n = 9$; Fig. 4A). Similarly, glycine also reduced the current in a concentration-dependent manner by up to $81 \pm 3\%$ (EC_{50} value of $507 \pm 40 \mu M$; $n = 7$; Fig. 4A), whereas application of L-serine, D-aspartate, D-glutamate, and L-glutamate (all at 1 mM) did not cause marked changes in the GluR $\delta 2^{Lc}$ current (Fig. 4B). L-aspartate (1 mM) had a modest effect on GluR $\delta 2^{Lc}$ currents, inhibiting currents by $22 \pm 2\%$ ($n = 16$) relative to NASP (Fig. 4B). However, L-aspartate was only able to maximally reduce the current by $26 \pm 1\%$ relative to NASP with a corresponding EC_{50} value of $268 \pm 53 \mu M$ ($n = 11$).

Because D-serine completely fills out the ligand-binding cavity in the closed conformation of GluRδ2-S1S2 (Fig. 3B), it seems unlikely that L-aspartate could bind in this fully closed receptor conformation.

We also tested 31 additional D- and L-amino acids for activity at oocytes expressing WT GluR82 or GluR82^{Lc}. None of these amino acids induced detectable responses at WT GluR82, and none displayed higher potency at GluR82^{Lc} than D-serine (SI Table 2).

Characterization of Ligand Binding to GluR δ 2^{Lc} by Rapid Application. It has previously been shown that agonists induce a large D1–D2 domain closure (20–26°) in the isolated ligand-binding core of other iGluRs (16, 18, 19) and that antagonists stabilize the open conformation of the ligand-binding core (19, 23). At GluR2, the degree of domain closure correlates with the extent of receptor activation and desensitization (24). A domain closure of up to 30° is introduced upon binding of D-serine to GluR δ 2–S1S2 relative to the *apo* structure (molA, 30°; molB, 26°; Fig. 1*B*). Our observation of a near-complete reduction of GluR δ 2^{Lc} current upon D-serine application, combined with the extensive domain closure in the structure of GluR δ 2–S1S2 in complex with D-serine, suggests that ligand binding may lead to desensitization of the GluR δ 2^{Lc} receptor.

To assess whether WT GluR82 elicited a rapidly desensitizing current upon ligand binding, we expressed GluR82 in HEK-293 cells and used a rapid perfusion system to apply D-serine or glycine to the receptors during voltage-clamp electrophysiological recordings. GFP was fused to the C terminus of GluR82 to enable identification of cells with high levels of expression. No detectable current was observed when excised membrane patches ($n = 10$) or intact whole cells expressing either WT GluR82 ($n = 11$) or GluR82-GFP ($n = 16$) were rapidly switched into 10 mM D-serine or 10 mM glycine (Fig. 4C). Because extracellular Ca^{2+} has previously been shown to modulate GluR82^{Lc} function (13), we similarly switched intact whole cells expressing WT GluR82 into 10 mM D-serine in recording solution with reduced (0 mM, $n = 12$) or enhanced (10 mM, $n = 12$) extracellular Ca^{2+} , but no detectable current response was observed.

We also performed rapid application of ligand onto membrane patches from HEK-293 cells transfected with GluR2^{Lc}. In agreement with earlier studies (13, 21), GluR2^{Lc} transfected cells displayed large spontaneous membrane conductance with an inwardly rectifying current-voltage relationship when the

regulation of function of WT GluR δ 2-containing receptors, it also remains possible that GluR δ 2 carries out additional roles independent of ligand binding. Our findings thus suggest a path forward toward evaluating GluR δ 2 in the cerebellum, in addition to suggesting previously unrecognized features of the ligand-binding pocket within the glutamate receptor family).

Materials and Methods

Expression and Purification. Determination of the boundaries of GluR δ 2–S1S2 was based on sequence alignments of previously crystallized S1S2 constructs from other iGluRs (16, 18). The expression construct was made as follows: the S1 and S2 segments constituting the ligand-binding core of GluR δ 2 were PCR-amplified from full-length rat GluR δ 2 cDNA (GenBank accession no. NM.024379) by using the primers S1 forward, ATTA-GAATTCCGTGGAGGTGTGGTTCTACGC; S1 reverse, TTATGGTACCCCTTCGCAGTAGGACCCC; S2 forward, ATATGGTACCTCCATCCAGTCTCTTCAGG; and S2 reverse, AATTCTCGAGTTACAGGTCACACTGGCCATTC. The PCR products were digested with restriction enzymes and simultaneously ligated into pET-28a(+) (Novagen, Madison, WI).

The expression construct was transformed into the *Escherichia coli* cell line Origami 2 (Novagen). An overnight culture was used to inoculate three liters of Hyper Broth (AthenaES, Baltimore, MD) containing 30 μ g/ml kanamycin and 12.5 μ g/ml tetracycline and grown to an OD₆₀₀ of $\approx$ 1.0 at 37°C with 120 rpm of shaking before expression was induced by adding isopropyl- β -D-thiogalactopyranoside to 250 μ M. The protein was expressed overnight at 25°C with 120 rpm of shaking. The expressed protein contains an N-terminal His tag, a trypsin cleavage site, and the GluR δ 2–S1S2 sequence [440-GVVLRLV . . . GVLLRR-551], [664-SIQSLQ . . . NGQCDL-813] separated by a Gly–Thr linker. After cell harvesting and lysis, the protein was purified by Ni²⁺ affinity chromatography. The His tag was cleaved by trypsin digestion, and the protein was further purified by ion-exchange chromatography and gel filtration. An extra N-terminal glycine in the crystallized GluR δ 2–S1S2 construct was a remnant of the trypsin cleavage site.

A selenomethionine derivative was prepared by inoculating an overnight culture into 1 liter of minimal media, and the culture was grown at 37°C to an OD₆₀₀ of 0.6. The temperature was set to 30°C, and the following amino acids were supplemented as solids: 50 mg of selenomethionine, 100 mg of lysine, 100 mg of threonine, 100 mg of phenylalanine, 50 mg of leucine, 50 mg of isoleucine, and 50 mg of valine. After 15 min, expression was induced by adding isopropyl- β -D-thiogalactopyranoside to 1 mM. The protein was expressed overnight at 30°C.

Crystallization. Experiments were set up by using the hanging-drop method at 20°C with drops of 1 plus 1 μ l of protein [7–10 mg/ml in 10 mM Hepes (pH 7.0)/20 mM NaCl/1 mM EDTA] and reservoir solution consisting of 8–12% polyethylene glycol 4000/0.1 M Tris-HCl (pH 8.5)/50 mM calcium chloride for GluR δ 2 *apo* and of 15–25% polyethylene glycol 4000/0.1 M cacodylate buffer (pH 6.5)/0.2 M sodium thiocyanate for the complex between GluR δ 2–S1S2 and D-serine (20 mM). Crystals were transferred through a cryoprotectant with 20–30% glycerol in reservoir solution before flash-cooling.

Structure Determination. GluR δ 2–S1S2 *apo* SAD-data of the selenomethionine derivative were collected to 2.75 Å resolution at cryogenic temperature at beam-line X12 (Deutsches Elektronen-Synchrotron, Hamburg, Germany). Native data of GluR δ 2–S1S2 in complex with D-serine were collected to a resolution of 1.74 Å at beam-line BL1 (BESSY, Berlin, Germany). The 14 selenium sites within the asymmetric unit (corresponding to two GluR δ 2–S1S2 *apo* molecules) were located, and the positions were refined. A model was built into the

experimental electron density by using the structure of GluR2–S1S2:(S)-ATPO [Protein Data Bank ID code 1NOT (23)] as a template. The GluR δ 2–S1S2 structure in complex with D-serine was solved by molecular replacement by using the separate domains D1 and D2 of GluR δ 2–S1S2 *apo* as search models. Residues in the allowed regions of the Ramachandran plot correspond to 92.3% (*apo*) and 98.2% (D-serine complex). For details and statistics on structure determinations and refinements, see SI Table 3. The atomic coordinates of the two structures of GluR δ 2–S1S2 have been deposited in the Protein Data Bank with accession numbers 2V3T (*apo*) and 2V3U (D-serine complex).

ITC Experiments. ITC experiments were carried out by using a VP-ITC calorimeter (MicroCal, Northampton, MA). The calorimeter cell (1.40 ml) was filled with 28 μ M GluR δ 2–S1S2 in ITC buffer [20 mM Hepes (pH 7.5)/100 mM NaCl/2 mM KCl] at 20°C. The protein concentration was determined by quantitative amino acid analysis. For titrations, 50 injections of 20 mM ligand solutions in ITC buffer were carried out at 3-min intervals, with 3 μ l in the first and 6 μ l in the subsequent injections. The heat of dilution obtained from injecting a ligand into buffer was subtracted before the fitting process. The data analysis was performed by using Origin 7.0 (MicroCal) for ITC by using a single binding-site model. The first data points in the analysis were discarded. The stoichiometry *N* was fixed to 1 in the fitting process because the experiments were conducted with a low protein concentration for a low-affinity system. This is also the reason why dissociation constants (*K_d*) are given instead of absolute values of ΔH and ΔS , because the latter parameters may be less accurately estimated (30).

Electrophysiology. For expression in *Xenopus* oocytes and HEK-293 cells, WT rat GluR δ 2 (GenBank accession no. U08256) and rat GluR δ 2^{lc} cDNAs were subcloned into a pCI-IRES-bla vector containing a T7 site upstream from the 5' UTR. The GluR δ 2–GFP fusion construct was created by insertion of a PCR-generated DNA fragment coding for a GFP tag into the N-terminal end of WT GluR δ 2 in the pCI-IRES-bla vector. The lurcher mutation was introduced into GluR δ 2 by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the protocol of the manufacturer, and verified by DNA sequencing. Constructs used for expression in *Xenopus* oocytes were linearized by restriction enzymes to produce cRNAs by using mMessage mMachine kits (Ambion, Huntingdon, U.K.). *Xenopus* oocyte preparation and maintenance, HEK-293 culturing, and electrophysiological recordings were performed essentially as described previously (24, 31). The receptor expression in HEK-293 cells was achieved by transfection of cells with vector DNA by using the lipid-based transfection reagent FuGENE 6 (Roche, Indianapolis, IN) according to the instructions of the manufacturer. During recordings, oocytes were continuously perfused with Ca²⁺- and Mg²⁺-free Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 1.9 mM BaCl₂, and 10 mM Hepes (pH 7.6). A patch-clamp recording in the whole-cell configuration was made with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Recording electrodes (5–8 M Ω) were filled with 140 cesium gluconate/5 mM Hepes/4 mM NaCl/2 mM MgCl₂/0.5 mM CaCl₂/1 mM ATP/0.3 mM GTP/5 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (pH 7.4; 23°C). Unless otherwise noted, the recording chamber was continually perfused with recording solution composed of 150 mM NaCl, 10 mM Hepes, 1 mM CaCl₂, 3 mM KCl, and 10–20 mM mannitol (pH 7.4). For outside-out patches of HEK-293 cells, rapid solution exchange was performed with a two-barrel theta glass pipette controlled by a piezoelectric translator (Burleigh Instruments, Fishers, NY); junction currents were used to estimate the speed of solution exchange after

