Characterization of the 1H-Cyclopentapyrimidine-2,4(1H,3H)-dione Derivative (S)-CPW399, as a Novel, Potent and Subtype-Selective AMPA Receptor Full Agonist with Partial Desensitization Properties.
Experimental Section.

Melting points were determined using an Electrothermal 8103 apparatus and are uncorrected. IR spectra were taken with Perkin-Elmer 398 and FT 1600 spectrophotometers. $^1$H-NMR spectra were recorded on a Bruker 200 MHz spectrometer with TMS as internal standard; the values of the chemical shifts ($\delta$) are given in ppm and coupling constants ($J$) in Hz. All chemicals used were of reagent grade, and all reactions were carried out under argon atmosphere. Progress of the reaction was monitored by TLC on silica gel plates (Riedel-de-Haen, Art. 37341). Merck silica gel (Kieselgel 60) was used for flash chromatography (230-400 mesh) columns, and a Dowex 50WX8-400 resin was used to purify the final products. Extracts were dried over MgSO$_4$, and solvents were removed under reduced pressure. Elemental analyses were performed on a Perkin-Elmer 240C elemental analyzer, and the results are within ±0.4% of the theoretical values. Yields refer to purified products and are not optimized.

6,7-Dihydro-1H-cyclopenta-2-thioxo-4(1H)-pyrimidinone (4). A mixture of ethyl 2-oxocyclopentanecarboxilate 5 (10 g, 64.0 mmol) and thiourea (5.75 g, 75.5 mmol) was heated at 180° C for 2.5h. After cooling, methanol (26 ml) was added to the mixture, and the insoluble material was collected by filtration to give 4 (2.2 g, 20% yield) as a brownish amorphous solid, which was recrystallized from methanol: mp: >300° C. $^1$H-NMR (DMSO-d$_6$) $\delta$ 1.96 (m, 2H), 2.47 (m, 2H), 2.62 (m, 2H), 12.17 (br s, 1H), 12.56 (br s, 1H). Anal. (C$_7$H$_8$N$_2$O$_2$S) C, H, N.

6,7-Dihydro-1H-cyclopentapyrimidine-2,4(1H,3H)-dione (5). A mixture of 4 (1.0 g, 5.95 mmol) and chloroacetic acid (2.0 g, 21.0 mmol) in 40% MeOH (76 ml) was refluxed for 16h. After the reaction mixture was concentrated, the resulting precipitate was collected by filtration and recrystallized from methanol to give 5 (0.74 g, 82% yield) as colorless prisms: mp >300° C. $^1$H-NMR (DMSO-d$_6$) $\delta$ 1.94 (m, 2H), 2.43 (m, 2H), 2.64 (m, 2H), 10.72 (br s, 1H), 11.03 (br s, 1H). Anal. (C$_7$H$_8$N$_2$O$_2$) C, H, N.
(S)-1-(2-Amino-2-carboxyethyl)-6,7-dihydro-1H-cyclopenta-2-thioxo-4(1H)-pyrimidinone (2a, (S)-CPW405). To a vigorously stirred solution of 4 (0.5 g, 3.0 mmol) in dry N,N-dimethylformamide (DMF) (10 ml) was added a 60% suspension of sodium hydride in mineral oil (0.11 g, 3.0 mmol). The mixture was stirred at room temperature overnight. Then (S)-3-[(tert-butoxycarbonyl)amino]oxetan-2-one (0.185 g, 0.99 mmol) was added portion wise over 1 h. Stirring was continued overnight. The next day, the mixture was evaporated under reduced pressure (0.5 mmHg), and TFA (2.5 ml) was added to the ice-cooled residue, and the mixture was stirred at room temperature for 12 h. Excess of TFA was removed under reduced pressure, and the residue was applied to a column of Dowex 50WX8-400 resin (0.5 mmol of cation/ml of resin). The column was eluted with 50% aqueous ethanol to remove the excess unbound starting material and then with 1 M aqueous pyridine. The ninhydrin-positive fractions of the 1 M aqueous pyridine eluate were combined and evaporated to dryness under reduced pressure. Recrystallization of the residue from methanol yielded 2a (0.42 g, 56% yield) as a white solid: mp 225° C dec. \( ^1H\)-NMR (DCl/D\( _2 \)O, pH 1) \( \delta \) 2.18 (m, 2H), 2.73 (m, 2H), 3.08 (m, 2H), 4.62-5.06 (m, 3H). Anal. (C\(_{10}\)H\(_{13}\)N\(_3\)O\(_3\)S) C, H, N. The compound was then converted to the corresponding hydrochloride salt and recrystallized from methanol/ethyl acetate as colorless prisms.

(S)-1-(2-Amino-2-carboxyethyl)-6,7-dihydro-1H-cyclopentapyrimidine-2,4(1H,3H)dione (2b, (S)-CPW 399). Similarly to the procedure as described for 2a, the title compound was prepared starting from 5. After recrystallization from methanol, (S)-CPW 399 was obtained as a white solid: mp 210° C dec.. \( ^1H\)-NMR (DCl/D\( _2 \)O, pH 1) \( \delta \) 2.14 (m, 2H), 2.71 (m, 2H), 3.03 (m, 2H), 4.41-4.62 (m, 3H). Anal. (C\(_{10}\)H\(_{13}\)N\(_3\)O\(_4\)) C, H, N. The compound was then converted to the corresponding hydrochloride salt and recrystallized from methanol/ethyl acetate as colorless prisms.

Molecular Modeling. Conformational analysis was performed on the anionic tri-ionized form of (S)-CPW-399 in solution, by Montecarlo searching using the MMFFs forcefield in Macromodel with the GB-
SA solvation model.\textsuperscript{1} A local minimum within 1 kJ/mol of the global minimum was found, resembling the bound conformations of AMPA and glutamate, according to the GluR2-S1S2J crystal structures of Armstrong and Gouaux.\textsuperscript{2} The ligand geometry was checked by optimization with AM1-SM2\textsuperscript{3} and B3LYP/6-311+G(d,p)/PB-SCRF quantum mechanical theories\textsuperscript{4}, giving similar results. The ligand was docked into the binding sites of available agonist-bound crystal structures of GluR2-S1S2J, whereby the most likely binding mode and approximate degree of domain closure were readily apparent given the size and character of the bicyclic system; the binding mode resembles more that of glutamate and particularly kainate than that of AMPA. A homology model of GluR3 was built as previously described\textsuperscript{5}, including water molecules, the location of which were confirmed using the GRID program.\textsuperscript{5-7} Protons were added to the models of the GluR2 and GluR3 binding sites in complex with (S)-CPW399, and the hydrogen bonding networks completed unequivocally by manually adjusting rotatable bonds and water orientations to maximize favorable interactions. In both models, a spherical zone was then defined, including all residues and waters partly or wholly within 13Å of the ligand. A series of partial optimizations were performed on the zones, again using the MMFFs forcefield\textsuperscript{1}, specifying a dielectric constant of 4\textsuperscript{1}: first the proton positions were allowed to relax, and then the ligand, waters, and all side chain atoms out to a radius of 11Å from the ligand. Relatively minor changes in position and conformation were noted, with the side chains adjusting their conformations to fit the ligand. Hydrogen bond distances were measured, and are presented in Figure 3.

Male CRL:CD(SD)BR-COBS rats (Charles River) were killed by decapitation, cortices were rapidly dissected from other brain regions and used immediately for membrane preparation.

**[^3H]AMPA, [^3H]kainic acid and [^3H]CGP 39653 binding.** The tissue was homogenized using an Ultra Turrax TP-1810 in the following ice cold buffers: for [^3H]AMPA binding - Tris/HCl, 30 mM, pH 7.1 containing 2.5 mM CaCl2; for [^3H]kainic acid binding - Tris/acetate, 50 mM, pH 7.1 containing 2.5 mM CaCl2 and for [^3H]CGP 39653 binding - Tris/HCl 5 mM, pH 8. The homogenate was centrifuged at 48000xg for 10 min at 4°C (three times for [^3H]AMPA and [^3H]kainic binding; once for [^3H]CGP 39653 binding). After the final centrifugation the remaining pellet was resuspended in the same fresh buffer (with 10 mM EDTA for [^3H]CGP 39653 binding) and incubated at 37°C for 30 min (or 10 min for [^3H]CGP 39653 binding). The suspension was then centrifuged at 48000xg for 10 min at 4°C, the supernatant discarded and the pellet homogenized in the same fresh buffer (without EDTA for [^3H]CGP 39653 binding) and frozen at -20°C (for [^3H]CGP 39653 binding one additional centrifugation was undertaken and the remaining pellet then frozen at -20°C).

**[^3H]MDL 105,519 binding.** Rat cerebral cortex was homogenized in a Potter-Elvejeim homogenizer with a teflon pestle in 10 vol of ice-cold 0.32 M sucrose, pH 7.4 and centrifuged at 1000xg for 10 min. The supernatant was collected, centrifuged at 20000xg for 20 min and the resulting pellet (P2) resuspended in 20 vol of fresh water, maintained in ice for 15 min and then centrifuged at 8000xg for 20 min. The supernatant and the “buffy-coat” were carefully collected and centrifuged at 48000xg for 20 min. The pellet obtained was stored at -20°C until the day of the binding assay.

**[^3H]AMPA binding.** On the day of assay, the frozen tissue suspension was thawed and centrifuged at 48000xg for 10 min at 4°C. The pellet obtained was resuspended in fresh Tris/HCl, 30 mM, pH 7.1 containing 2.5 mM CaCl2 and re-centrifuged. The pellet obtained was finally resuspended in Tris/HCl, 30
mM, pH 7.1 containing 2.5 mM CaCl$_2$ and 100 mM KSCN. $[^3]$H]AMPA binding$^8$ was undertaken in a final incubation volume of 0.5 ml comprising 0.25 ml of membrane suspension (10 mg of tissue/sample), 0.25 ml $[^3]$H]AMPA (sp.act. 40 Ci/mmol, NEN; final concentration 5 nM) and 10 µl of displacing agents or solvent. Non-specific binding was obtained in presence of 10 µM AMPA. Incubation (30 min at 4°C) was stopped by rapid filtration under vacuum (Brandell MR 48R) through GF/C filters which were then washed with 12 ml of ice-cold buffer. The filters were placed in vials containing 4 ml of liquid scintillation (Ultima Gold MV, Packard) and counted in a LKB 1214 rackbeta scintillation counter.

$[^3]$H]Kainic acid binding. On the day of assay, the frozen tissue suspension was thawed and centrifuged at 48000xg for 10 min at 4°C. The pellet obtained was resuspended in 6.5 vol of 5 mM EGTA, incubated for 10 min at 4°C and re-centrifuged as above. The pellet obtained was resuspended in Tris/acetate, 50 mM, pH 7.1 and washed twice by centrifugation. The pellet obtained was finally resuspended in Tris/acetate, 50 mM, pH 7.1. $[^3]$H]Kainic acid binding$^9$ was undertaken in a final incubation volume of 1ml comprising 0.5 ml of membrane suspension (8 mg of tissue/sample), 0.5 ml $[^3]$H]kainic acid (sp.act. 58 Ci/mmol, NEN; final concentration 2 nM) and 20 µl of displacing agents or solvent. Non-specific binding was obtained in presence of 1 µM kainic acid. Incubation (60 min at 4°C) was stopped by rapid filtration under vacuum (Brandell MR 48R) through GF/C filters which were then washed with 12 ml of ice-cold buffer and counted as above.

$[^3]$H]CGP 39653 binding. On the day of assay, the frozen pellet was thawed and washed twice by resuspension in Tris/HCl, 5 mM, pH 7.7 with centrifugation at 48000xg for 10 min at 4°C. The pellet obtained was finally resuspended in the same fresh buffer. $[^3]$H]CGP 39653 binding$^{10}$ was done in a final incubation volume of 1 ml consisting of 0.5 ml of membrane suspension (10 mg of tissue/sample), 0.5 ml $[^3]$H]CGP 39653 (s.a. 48.9 Ci/mmol, NEN; final concentration 2 nM) and 20 µl of displacing agents or solvent, non specific binding was obtained in presence of 100 µM L-glutamic acid. Incubation (60 min at
4°C) was stopped by rapid filtration under vacuum (Brandell MR 48R) through GF/B filters which were then washed with 12 ml of ice-cold buffer and counted as above.

**[^3H]MDL 105,519 binding**. On the day of assay, the frozen pellet was thawed and washed 4 times by resuspension in Tris acetate, 50 mM, pH 7.4 and centrifugation at 48000xg for 10 min at 4°C. The pellet obtained was finally resuspended in the same fresh buffer.[^3H]MDL 105,519 binding[^1] was undertaken in a final incubation volume of 0.5 ml consisting of 0.25 ml of membrane suspension (5 mg of tissue/sample), 0.25 ml[^3H]MDL 105,519 (sp.act. 69 Ci/mmol, Amersham; final concentration 4 nM) and 10 µl of displacing agents or solvent. Non-specific binding was obtained in presence of 1 mM glycine. Incubation (30 min at room temperature) was stopped by rapid filtration under vacuum (Brandell MR 48R) through GF/B filters which were then washed with 12 ml of ice-cold buffer and counted as above.

**Molecular Biology.** The rat AMPA receptor clones GluR1, GluR2(R), GluR2(Q) and GluR3, in pBluescript (pBSK) vector (Stratagene, La Jolla, CA) were supplied by Dr. Stephen Heinemann (The Salk Institute, La Jolla, CA) while GluR4c was a gift from Dr. A. Buonanno (NIH, Bethesda, MD). For preparation of high-expression cRNA transcripts, GluR1, GluR3 and GluR4c were inserted into the vector pGEMHE[^12] as previously described.[^5][^13] GluR2(Q)pBSK(-) was digested with Xho I plus Xba I, treated with Klenow enzyme to produce blunt ends and ligated into the (5')-BamH I and (3')-Hind III sites of pGEMHE, which were also blunt-ended using the Klenow enzyme, giving 350 bp 5'- and 525 bp 3'-untranslated sequence. cDNAs were grown in XL1 Blues bacteria (Stratagene) and prepared using column purification (Qiagen, Chatsworth, CA). cRNA was synthesized from these cDNAs using the mMessage mMachine T7 mRNA-capping transcription kit (Ambion Inc., Austin, TX).

**Recombinant Baculovirus Construction and Sf9 Cell Culture.** The baculovirus-Sf9 cell system was employed to express recombinant rat AMPA receptor homomeric complexes used for radioligand binding assays. All manipulations of virus and insect cells, including maintenance of cell culture, transfection,
plaque purification, amplification and expression of receptor, were according to standard protocols in ‘Guide to Baculovirus Expression Vector Systems and Insect Cell Culture Techniques’, (Life Technologies, Paisley, UK) and ‘Baculovirus Expression Vector System: Procedures and Methods Manual’, 2nd ed. (PharMingen, San Diego, CA). The creation and expression of recombinant GluR1o, GluR2o(R) and GluR3o baculoviruses and Sf9 cell culture have been described previously.15,16 Recombinant GluR4cO baculovirus was made by digesting GluR4cOpGEMHE with BamH I plus Eag I and subcloning the GluR4c insert into the (5’)-BamH I and (3’)-Eag I sites of the PharMingen baculovirus transfer vector pVL1393 followed by use of the PharMingen BaculoGold transfection kit.

**Radioligand Binding.** The affinities of compounds at wild-type and mutant receptors were determined from competition experiments with (R,S)-[5-methyl-3H]-AMPA (40.87 Ci/mmol; NEN, Boston, MA) as previously detailed.13,14 Competition data were fit to a logistic equation (Eqn. 1) to determine the Hill coefficient (nH) or to Eqn. 2 for calculation of the drug affinity (Ki). The competitive nature of CPW-399 binding versus (R,S)-AMPA was confirmed using Yonetani-Theorell analysis of the binding data.15

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\text{Eqn. 1 } \quad \frac{TB_{\text{max}}}{1 + ([I]/IC_{50})^{n_H}} + \text{NSB}
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\[
\text{Eqn. 2 } \quad \frac{(R\cdot L_d/K_d)}{(1 + L_d/K_d + [I]/K_i)} + \text{NSB}
\]

Kd = radioligand dissociation constant, Ki = inhibitor dissociation constant, Ld = total radioligand concentration, TBmax = total radioligand bound at zero competitor concentration, IC50 = inhibitor concentration producing 50% specific binding, nH = Hill coefficient, NSB = nonspecific binding, [I] = total competitive inhibitor concentration.

**Oocyte Preparation.** Mature female *Xenopus laevis* (African Reptile Park, Tokai, South Africa) were anesthetized using 0.1% ethyl 3-aminobenzoate and ovaries were surgically removed. The ovarian tissue was dissected and treated with 2 mg/mL collagenase in Ca2+-free Barth’s medium for 2 hr at room temperature and subsequently defolliculated using fine forceps. On the second day, oocytes were injected
with 50 nL of (~1 µg/µL) cRNA and incubated in Barth’s medium (in mM: 88 NaCl, 1 KCl, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 2.4 NaHCO₃, 10 HEPES, pH 7.4) with gentamicin (0.10 mg/mL) at 17°C. Oocytes were used for recordings from 6–10 days post-injection.

**Two-Electrode Voltage Clamp.** The oocytes were voltage-clamped with the use of a two-electrode voltage clamp (Dagan Corporation, Minneapolis, MN) with both microelectrodes filled with 3 M KCl. Recordings were made while the oocytes were continuously superfused with frog Ringer’s solution (in mM): 115 NaCl, 2 KCl, 1.8 CaCl₂, 5 HEPES, pH 7.0). Drugs were dissolved in Ringer’s solution and were added by bath application. Recordings were made at room temperature at holding potentials in the range of -80 to -30 mV. Agonist concentration-response curves were constructed by measuring the maximal current induced by increasing concentrations of agonist. Data from individual oocytes were fit to a logistic equation: 

\[ I = \frac{I_{\text{max}}}{[1 + 10^{(\log EC_{50} - \log[\text{agonist}]) \cdot n_H}]} \]

where I is the response observed at a given agonist concentration. The parameters \( I_{\text{max}} \) (maximal current observed at infinite agonist concentration), \( n_H \) (Hill coefficient), and the \( EC_{50} \) (concentration of agonist producing 50% of \( I_{\text{max}} \)) were determined by an iterative least squares fitting routine. Concentration-response curves were determined in triplicate in each oocyte. EC₅₀ and \( n_H \) values were calculated for each receptor from 3–5 separate oocytes.

**Data Analysis Software.** One-way ANOVA (followed by the Bonferroni t-test) was utilized for comparison of receptors’ parameters using SigmaStat for Windows v2.0 (SPSS Science, Chicago, IL). Values were considered statistically significantly different if \( P < 0.05 \). Electrophysiological data were analyzed using Prism v3.00 (GraphPad Software Inc., San Diego, CA) while binding data were analyzed using Grafit v3.00 (Erithacus Software Ltd., Horley, UK). For binding experiments in rat cortices, inhibition curves were analyzed using the “Allfit” program running on a IBM AT-PC. The Ki values were derived from the IC₅₀ values using the Cheng and Prusoff equation.
**Materials.** Restriction and other molecular biological enzymes were obtained from New England BioLabs (Beverly, MA). Sf900-II culture medium, gentamicin, antibiotics and plaque assay reagents were from Life Technologies (Paisley, UK). Collagenase and additional chemicals and reagents were obtained from Sigma (St. Louis, MO) or similar local suppliers.

**Excitotoxicity Assays. Materials.** Mice (CD1 strain) were obtained from Charles River U.K. Ltd (Kent, England). All cell culture plasticware was purchased from NUNC A/S, Denmark. Foetal calf serum was from Sera-Lab Ltd (Sussex, England), and poly-L-lysine, trypsin, soybean trypsin inhibitor, DNAse, p-aminobenzoic acid, insulin, penicillin G and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldiazonium bromide (MTT) were obtained from Sigma Chemicals (Poole, England). D,L(±)-2-amino-5-phosphonopentanoic acid (D-APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione (NBQX) were purchased from Tocris Cookson (Bristol, England). N-[1-(2-thienyl)cyclohexyl]-piperidine (TCP), GYKI 53655 and nifedipine were from RBI (Natick). The acetoxymethyl ester of Fluo-3 (Fluo-3/AM) and Pluronic F-127 were purchased from Molecular Probes Inc. (Eugene, Oregon). All other chemicals were of the purest grade available from regular commercial sources [Merck Ltd. (Dorset, UK); Sigma Chemical Co. (St. Louis, MO); Fluka Chemicals Ltd. (Dorset, UK)].

**Cerebellar granule cell cultures.** Primary cultures of cerebellar granule cells were prepared as described previously. Using this method, it has been established that this culture comprises at least 95% granule cells. Briefly, cerebella from 7-day-old CD1 mice were dissociated by mild trypsinization and subsequent trituration in a solution containing soybean trypsin inhibitor and DNase. Dissociated cells were pelleted (5 min at 1000 rpm) and resuspended in Dulbecco’s modified Eagle medium supplemented with 19.5 mM KCl, 24 mM glucose, 2 mM glutamine, 7 µM p-amino-benzoic acid, insulin (100 mU/L), penicillin G (5 x 10^5 U/L) and foetal calf serum (10% v/v; heat inactivated). Cells were seeded in 96-well plates (25x10^6 cells per plate) pre-coated with a 1% (w/v) solution of poly-L-lysine and cultured at 37 °C in a humidified
atmosphere of 5% CO₂/95% air. Experiments were performed on cells after 7 days in culture. Cytosine arabinoside (20 µM final concentration) was added 48-hr after plating to prevent glial proliferation.

**Assessment of cytotoxicity.** Cytotoxicity was assessed by a spectrophotometric method which measures the viability of cells on the basis of their ability to bioreduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Whether or not MTT reduction occurs within active mitochondria or extramitochondrially remains the subject of some debate. MTT is yellowish in color when dissolved in a balanced salt solution without phenol red. In viable cells the tetrazolium ring of MTT is selectively cleaved yielding blue/purple crystals of the formazan derivative. The crystals are dissolved in acidified isopropanol and the resulting color read spectrophotometrically. A decrease in absorbance when compared to control cells provides a quantitative assessment of cell damage.

Cells were cultured as described above in 96-well plates (NUNC) for 7 days. The growth medium was removed from the cells and they were immediately washed twice with 100 µl HBS-1 (125 mM NaCl; 5 mM KCl; 20 mM NaHCO₃; 50 mM HEPES; 5 mM D-glucose; 0.9 mM CaCl₂, pH 7.4) per well at 37 °C prior to exposure with 100µl aliquots of test solutions (made up in conditioned medium) under the same assay conditions. The concentrations of test solutions used are specified at appropriate sections of the text and figure/table legends. Following exposure for 24h the cells were assayed for cytotoxicity. To assay for cell damage, the cells were washed with 125 µl HBS-1 per well and then 50 µl of MTT solution (final concentration 0.2 mg/ml) was added to each well. The cells were incubated in the dark at 37 °C for 10 min before addition of 125 µl of Triton X-100 / 0.04M HCl in anhydrous isopropanol. The plates were then wrapped in aluminum foil and left overnight at 4 °C to ensure solubilization of the blue/purple formazan crystals. The absorbance was read at 570 nm in a Dynatech MR5000 plate reader and cell viability expressed as a percent of control (untreated, 'medium alone' cells).

**Measurement of intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ).** Fluo-3/AM loading and fluorescence measurements (excitation wavelength, 490 nm; emission wavelength, 538 nm) were
performed essentially as described by Kardos et al.\textsuperscript{23} Apart from the dye-loading solutions, all other solutions contained 1 mM furosemide (an anion channel blocker) which prevented leakage of Fluo-3 into the medium. After loading with Fluo-3A/M in balanced salt solution (BSS) [125 mM NaCl, 5 mM KCl, 1.0 mM MgSO\textsubscript{4}, 0.9 mM CaCl\textsubscript{2}, 10 mM HEPES and 5 mM glucose, pH7.4], cells were washed, exposed to test solutions contained in a modified BSS (Mg\textsuperscript{2+}-free) and the fluorescence (F) readings obtained using a Fluoroskan II (Labsystems, Finland) fluorescence plate reader. On removing test solution, calibration was performed by addition of the ionophore A23187 (10 µM) in BSS, which allowed sufficient influx of Ca\textsuperscript{2+} to attain the saturation level of binding with the intracellularly trapped Fluo-3 (F\textsubscript{max}). To this medium was added 5 mM CuSO\textsubscript{4} (dissolved in 0.9% NaCl) in order to quench the fluorescence and yield the minimum value (F\textsubscript{min}). The observed relative fluorescence values for the cells were used in the following equation to calculate the free cytosolic Ca\textsuperscript{2+} concentration: [Ca\textsuperscript{2+}]\textsubscript{i} = KD(F-F\textsubscript{min})/F\textsubscript{max} - F) where the KD is 450 nM. The observed fluorescence (F) in the presence of test compounds increases on binding of Ca\textsuperscript{2+} without shifts in excitation or emission wavelengths.\textsuperscript{24}
Figure 1. Pharmacological Profile of (S)-CPW-399 at Recombinant AMPA Receptors.

Receptors were expressed in Sf9 cell membranes and drug affinity measured by (R,S)-[^3]H]AMPA competition binding assays. $K_i$ was determined by non-linear, iterative fitting of the data as described in Materials and Methods. Shown are mean ± S.D. of triplicate determinations from single experiments (repeated 2-3 times).
Figure 2. Yonetani-Theorell Analysis of (S)-CPW-399 Inhibition Mode.

Specific inhibition of (R,S)-AMPA binding by (S)-CPW399 (★, 0 nM; ✧, 50 nM; θ, 100 nM; σ, 200 nM) using 5.97 nM [3H](R,S)-AMPA as radiolabel. Nonspecific binding was determined in the presence of 1 mM L-glutamate and subtracted from total binding to give the specific binding. Shown is one experiment which was repeated once with similar results. Parallel lines indicate a competitive inhibition mode. (slopes (x 10⁻⁶): ★, 3.96; ✧, 4.23; θ, 3.66; σ, 4.21).
Primary cultures of cerebellar granule cells, prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella, were maintained in culture for 7-days. Cells were then exposed for 24-hr in culture-conditioned medium to increasing concentrations of AMPA in the absence or presence of 200 µM cyclothiazide prior to assessment of cell viability by MTT staining. The absorbance at 570 nm was expressed as a percentage of control (no added agonist) cells. Data represent mean ± SEM values (n = 8-16).
Figure 4. Concentration-dependent effect of (S)-CPW399 on stimulated increases in $[\text{Ca}^{2+}]_i$ in cultured cerebellar granule cells. Primary cultures of cerebellar granule cells, prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella, were maintained in culture for 7-days. At the start of the experiment, cells were first washed with balanced salt solution and loaded with Fluo-3/AM before exposure to increasing concentrations of (S)-CPW399 in the absence or presence of 100 µM cyclothiazide. Measurement of $[\text{Ca}^{2+}]_i$ was undertaken as described in Materials and Methods. Data are the mean ± SEM (n = 8-16). Increases in $[\text{Ca}^{2+}]_i$ are presented as fold basal (i.e. the resting level of $[\text{Ca}^{2+}]_i$) which for 7-DIV cells in this series of experiments was 93.2 ± 2.5 nM.
References and Notes.


(17) Cheng, Y.; Prusoff, W.H. Relationship Between the Inhibition Constants (Ki) and the Concentration of Inhibitor which Causes 50 per cent Inhibition (IC\(_{50}\)) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, 22, 3099-3108.


