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

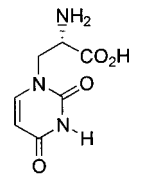
Giuseppe Campiani,*,[||] Elena Morelli,[§] Vito Nacci,[||] Caterina Fattorusso,[#] Anna Ramunno,[||] Ettore Novellino,[§] Jeremy Greenwood,[⊥] Tommy Liljefors,[⊥] Roger Griffiths,[†] Colin Sinclair,[†] Helen Reavy,[†] Anders S. Kristensen,[‡] Darryl S. Pickering,[‡] Arne Schousboe,[‡] Alfredo Cagnotto,[◇] Elena Fumagalli,[◇] and Tiziana Mennini[◇]

Dipartimento Farmaco Chimico Tecnologico (DFCT), Universita' degli Studi di Siena, via Aldo Moro, 53100 Siena, Italy, Dipartimento di Chimica Farmaceutica and Tossicologica (DCFT) and Dipartimento di Chimica delle Sostanze Naturali (DCSN), Universita' di Napoli Federico II, via D. Montesano 49, 80131 Napoli, Italy, Department of Medicinal Chemistry (DMC) and Department of Pharmacology (DP), NeuroScience PharmaBiotech Research Center, The Royal Danish School of Pharmacy, DK-2100 Copenhagen, Denmark, School of Biology, University of St. Andrews, Fife KY16 9ST, Scotland, U.K., and Istituto "Mario Negri", via Eritrea 62, 20157 Milano, Italy

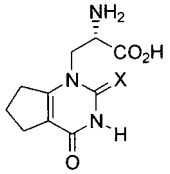
Received July 13, 2001

Abstract: (S)-CPW399 (**2b**) is a novel, potent, and subtype-selective AMPA receptor full agonist that, unlike (*S*)-willardiine and related compounds, in mouse cerebellar granule cells, stimulated an increase in $[Ca^{2+}]_i$, and induced neuronal cell death in a time- and concentration-dependent manner. Compound **2b** appears to be a weakly desensitizing, full agonist at AMPA receptors and therefore represents a new pharmacological tool to investigate the role of AMPA receptors in excitotoxicity and their molecular mechanisms of desensitization.

Introduction. Glutamic acid (Glu) effects are mediated by activation of a range of excitatory amino acid (EAA) receptors, namely (i) ionotropic receptors (iGluRs: NMDA, AMPA, and KA receptors) and (ii) the metabotropic glutamate receptors. AMPA receptors (AMPA-R) are homo- or hetero-oligomeric assemblies of four different receptor protein subunits, GluR1−4.[1] In addition, each of the four AMPA-R proteins have been characterized in flip and flop splice variant forms.[2] Although Glu and its receptors are involved in learning, memory, and other plastic changes in the CNS, paradoxically, excessive stimulation of glutamate receptors appears to underlie a number of human neurological disorders and neurodegenerative diseases. Desensitization of iGluRs shapes synaptic responses and provides a critical mechanism of neuroprotection at central synapses. Although several factors are known to modulate the kinetics of desensitization of AMPA-R, the molecular basis of the desensitization process and its physiological relevance are still poorly understood. Several lines of evidence suggested that amino acids in the S2 region were critical for the desensitization properties of GluR1, a subunit that, although homologous at the amino acid level with the GluR3 subunit, desensitizes with a slower rate constant. The molecular reason for this difference in desensitization rate constant and differences in binding affinity of subtype-selective agonists at GluR1 and GluR3 were recently reported, and a model of the AMPA-R binding site for desensitizing agonists was proposed.[3] Consequently, there is great interest in the development of novel compounds that, interacting selectively with specific AMPA-R subunits, may be useful in investigating the molecular mechanisms of desensitization and the role of AMPA-R in excitotoxicity. Here, we describe the synthesis and the pharmacological characterization of the new AMPA-R agonist **2b**. Though structurally related to the naturally occurring EAA agonist willardiine, the unique structural feature of **2b** is the presence of a bicyclic skeleton that makes it a novel weakly desensitizing AMPA-R full agonist which is a potent neurotoxin. Furthermore, molecular modeling of the possible binding mode of **2b** at AMPA-Rs is also discussed with regard to its subtype selectivity.



1, (S)-willardiine



2a, X=S, (S)-CPW405
2b, X=O, (S)-CPW399

Chemistry. Synthesis of **2b** ((S)-CPW399) and its thioanalogue **2a** ((S)-CPW405) is outlined in Scheme 1. (*S*)-3-[(*tert*-butoxycarbonyl)amino]oxetan-2-one, in turn prepared from (*S*)-serine, can act as a chiral electrophilic alanine cation equivalent which reacts with nucleophiles to provide optically pure alanine derivatives.[4] This synthetic strategy was applied to the synthesis of the two willardiine analogues. The synthesis of the key intermediates **4** and **5** and the alkylation of the uracil ring were performed as previously described.[5,6]

In Vitro Pharmacology. 1. Radioligand Binding Assays. Native iGluRs from rat brain membranes and recombinant homomeric AMPA-Rs (GluR1−4) expressed in Sf9 cells (Table 1, and Figure 1 of Supporting Information) were used as testing systems. Excitotoxic effects were evaluated on mouse cerebellar granule cells (Figure 1). In ligand binding studies, both **2b** and **2a** displaced [^{3}H]AMPA from native AMPA-R binding sites, exhibiting K_i values of 747 nM and 1370 nM, respectively. Both compounds also exhibited a lower affinity for the ligand binding site of native KA receptors while no affinity was found for the NMDA receptor nor its

* To whom correspondence should be addressed. Tel: 0039-0577-234172. Fax: 0039-0577-234333. E-mail: campiani@unisi.it.
[||] DFCT-Universita' degli Studi di Siena.
[§] DCFT-Universita' di Napoli Federico II.
[#] DCSN-Universita' di Napoli Federico II.
[⊥] DMC-The Royal Danish School of Pharmacy.
[†] University of St. Andrews.
[‡] DP-The Royal Danish School of Pharmacy.
[◇] Istituto di Ricerche Farmacologiche "Mario Negri".

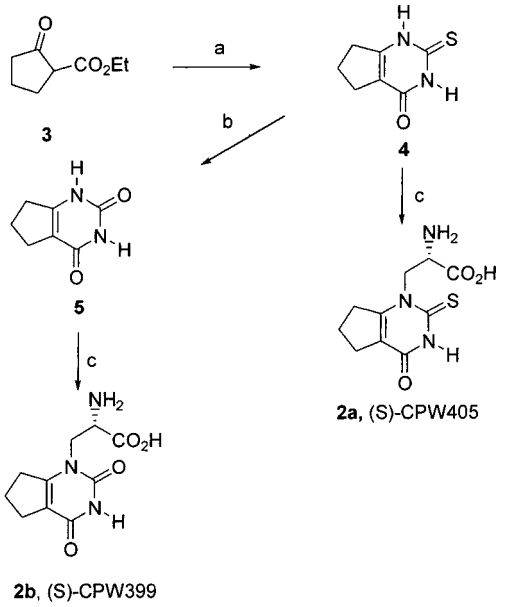
10.1021/jm015552m CCC: $20.00
Published on Web 11/17/2001

Table 1. Affinity of **2b** ((S)-CPW399) for Native iGluRs (from Rat Brain) and Affinity and Potency at Recombinant Homomeric AMPA Receptors

		2b		**2b**	
receptor	K_i[a] nM ± SEM	K_i[b] nM ± SEM	Hill slope n_H ± SEM	EC_{50}[c] μM ± SEM	Hill slope n_H ± SEM
NMDA	NA				
AMPA	747 ± 119 (1370 ± 201)				
KA	7940 ± 3200 (>10000)				
$GluR1_o$		109 ± 3[d]	0.95 ± 0.02	24.9 ± 3.6	1.06 ± 0.10
$GluR2(Q/R)_o$[e]		218 ± 16[d]	0.92 ± 0.02	13.9 ± 1.4	1.16 ± 0.21
$GluR3_o$		2137 ± 218[d]	0.98 ± 0.05	224 ± 20[d]	0.81 ± 0.04
$GluR4c_o$		1756 ± 69[d]	0.92 ± 0.07	34.3 ± 2.1	0.66 ± 0.04

[a] Binding affinity for **2a** in parentheses. NA = not active at 10^{-4} M concentration. [b] Shown are means ± SEM of three to four competition experiments, performed in triplicate. [c] Shown are means ± SEM of concentration−response experiments performed on three to five oocytes from two to three separate injection batches. [d] Statistically significantly different ($P < 0.05$) using one-way ANOVA followed by Bonferroni *t*-test. [e] $GluR2_o$(Q) for EC_{50}; $GluR2_o$(R) for K_i.

Scheme 1. Synthesis of the Title Compounds[a]



[a] (a) Thiourea, 180 °C; (b) chloroacetic acid, 40% MeOH, reflux; (c) (*S*)-3-[(*tert*-butoxycarbonyl)amino]oxetan-2-one, NaH, DMF, rt.

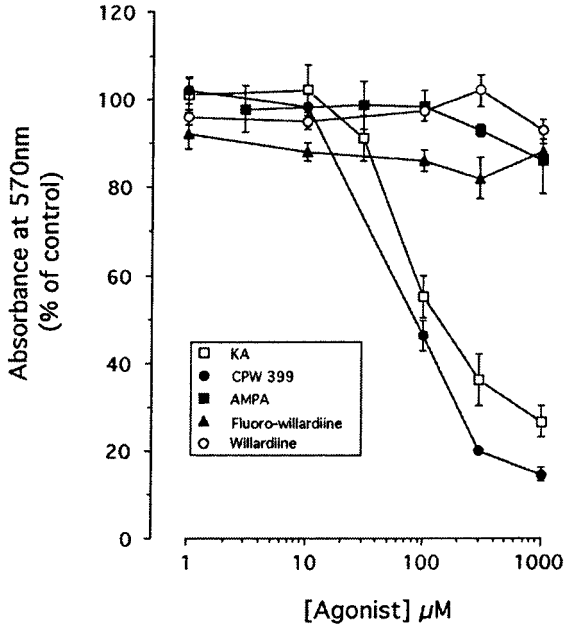


Figure 1. Neurotoxicity profile of **2b**. Concentration-dependent effect of **2b** and various AMPA/KA receptor agonists on cell viability in cultured cerebellar granule cells. Primary cultures of cerebellar granule cells, prepared from suspensions of dissociated cells from 7-day-old postnatal mouse cerebella, were maintained in culture for 7 days. Cells were then exposed for 24 h in culture-conditioned medium to increasing concentrations of agonists 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. The absorbance values for control cells were 0.238 ± 0.008. Data represent mean ± SEM values ($n = 8-16$).

associated glycine binding site (Table 1). (*S*)-Willardiine displayed a similar affinity and degree of selectivity among the iGluRs as **2b** for binding to native AMPA-Rs (IC_{50} = 800 nM) and native KA receptor (IC_{50} = 27 ± 7 μM).[6] The thio analogue **2a** allows AMPA-R selectivity to be retained although reducing the affinity by some 2-fold. Further neurochemical studies were conducted using only the more potent analogue, **2b**. AMPA-R subtype selectivity was investigated by examining the binding affinity of **2b** at homomeric rat GluR1−4. Binding data (Table 1) reveal that, in general, **2b** showed high affinity for recombinant AMPA-Rs (especially for $GluR1_o$ and $GluR2_o$(*R*)). The superior affinity of **2b** compared to (*S*)-willardiine[6] is probably due to extra hydrophobic interactions given by the cyclopentane-fused ring placed at positions 5 and 6 of the uracil ring of **2b**. The pharmacological profile of **2b** at recombinant rat GluR1−4 revealed an approximately 20-fold selectivity for $GluR1_o$ over $GluR3_o$ or $GluR4c_o$. Compound **2b** shows a similar, high affinity for GluR1/GluR2 and lower affinity for GluR3/GluR4, suggesting a pharmacological subdivision of AMPA-R into two groups, 1/2 versus 3/4. Similar selectivity profiles have been reported for (*S*)-willardiine and (*S*)-5-fluoro-willardiine at GluR1 versus GluR4.[6] The AMPA-R agonist (*S*)-4-bromohomoibotenic acid also exhibits a GluR1/2 versus GluR3/4 selectivity[3], suggesting that a similar mechanism may be involved for **2b** selectivity. Yonetani−Theorell analysis[7] of the displacement of (*R,S*)-[^{3}H]AMPA binding to $GluR1_o$ by **2b** (Figure 2 of Supporting Information) indicated a competitive interaction, implying that it binds to the same site as AMPA.

2. Electrophysiological Assay. The potency (EC_{50}) of **2b** at recombinant rat AMPA-R was measured on receptors expressed in *Xenopus lævis* oocytes (Table 1, Figure 2). Interestingly, at $GluR1_o$ and $GluR2_o$(Q) **2b** had high potency and at $GluR3_o$ a lower potency, while the potency at $GluR4c_o$ was not different from that at $GluR1_o$. This discrepancy between the affinity and potency of **2b** at $GluR4c_o$ could signify that the efficacy

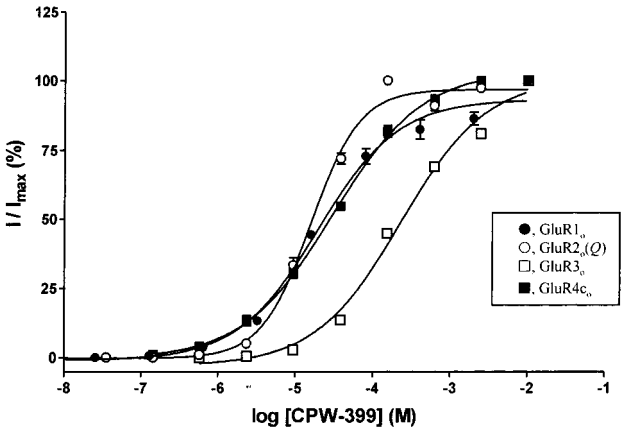


Figure 2. Potency of **2b** at recombinant AMPA-R. Receptors were expressed in *X. lævis* ooxytes, and responses to **2b** were measured as described in Materials and Methods. Shown are typical concentration−response curves from individual oocytes for each of the four homomeric AMPA receptors tested (repeated in 2−4 oocytes). EC_{50} and n_H were determined by nonlinear iterative fitting to a logistic equation. ●, $GluR1_o$ (EC_{50} = 19.3 μM, n_H = 0.85); ○, $GluR2_o$(Q) (EC_{50} = 15.6 μM, n_H = 1.39); □, $GluR3_o$ (EC_{50} = 230 μM, n_H = 0.79); ■, $GluR4c_o$ (EC_{50} = 29.7 μM, n_H = 0.76).

and/or desensitization properties of **2b** at this subunit are different from the other subunits. In the presence of cyclothiazide, which inhibits AMPA-R desensitization, saturating concentrations of **2b** gave responses which were of a magnitude similar to that of the responses produced by saturating concentrations of L-glutamate, indicating that **2b** is a full agonist. However, the maximal steady-state, desensitized responses of **2b** were larger than those of L-glutamate but less than those of kainate. This suggests that **2b** desensitizes AMPA-R less than other full agonists such as L-glutamate or AMPA, but more than the partial agonist kainate.

3. Excitotoxicity Assay and Increases in Intracellular Calcium. The effect of **2b** on neuronal viability was investigated by exposing primary cultures of mouse cerebellar granule cells at 7 days in vitro to increasing concentrations (1−1000 μM) of agonists for a continuous 24 h period. The results presented in Figure 1 show that **2b** exerts a concentration-dependent toxic effect on granule cells with an approximate EC_{50} value of 70−80 μM. The cytotoxic actions of **2b** were also time-dependent: continuous exposure for 4 h resulted in statistically significant less toxicity at concentration above 100 μM (*P* values were set at 95% confidence limits; analyzed by Tukey's test for multiple comparisons using Minitab release 8; CLE COM Ltd., Birmingham, U.K.). Kainate also exerted a concentration-dependent toxic action (EC_{50} ~100 μM) while (*S*)-AMPA, (*S*)-willardiine, and 5-fluorowillardiine exhibited no apparent excitotoxicity. Co-administration of a nontoxic dose of 200 μM cyclothiazide (at concentrations >250 μM, cyclothiazide exhibits cytotoxicity toward cerebellar granule cells) with varying concentrations of AMPA was seen to unmask an excitotoxic effect (Figure 3 of Supporting Information). 5-Fluorowillardiine and, to a lesser extent, willardiine induced an excitotoxic effect when co-administered with a nontoxic concentration of cyclothiazide (not shown). Under these conditions, the rank order of excitotoxic potency was AMPA ≈ fluorowillardiine > willardiine, with respective ~EC_{50} values being of the order 3 μM, 3 μM, and >200 μM. Of special significance was the finding that cyclothiazide had only a minimal effect in enhancing **2b**-induced excitotoxicity, which is in agreement with the electrophysiological data where cyclothiazide gave only weak potentiation of **2b**-stimluated currents. The pharmacology of **2b**-induced toxicity was subsequently investigated. Competitive and noncompetitive antagonists of the NMDA receptor, APV and TCP, respectively, along with nifedipine, offered no protection against **2b**-induced toxicity. In contrast, the AMPA/KA receptor competitive antagonists, CNQX and NBQX, both protected against **2b**-induced toxicity. To dissect more clearly the receptor involvement mediating **2b** excitotoxicity, experiments were undertaken in which granule cells were exposed to **2b** alone and in combination with the noncompetitive AMPA-R antagonist, GYKI 53655.[8] It could be demonstrated that 20 μM GYKI 53655 affords complete neuroprotection against excitotoxic concentrations of **2b** (at least up to 300 μM concentrations of the agonist) while still providing marked protection at an appreciably higher concentration (1 mM) of the excitotoxin. Excitotoxic neuronal cell death appears to be associated with a large increase in the intracellular free-calcium concentration ($[Ca^{2+}]_i$) following hyperactivation of EAA receptors. Compound **2b** stimulated an increase in $[Ca^{2+}]_i$ in a concentration-dependent fashion, attaining a value of 6-fold that of resting cells at maximum stimulation (~100 μM) (Figure 4 of Supporting Information). Furthermore, **2b**-stimulated increases in $[Ca^{2+}]_i$ were not enhanced by the presence of cyclothiazide but were abolished by either 10 μM NBQX or CNQX, markedly attenuated by 10 μM nifedipine, and unaffected by 500 μM D-APV.

Molecular Modeling Studies. To better understand the novel pharmacological properties of **2b**, molecular modeling of its binding mode at AMPA-Rs was performed. The optimized models of **2b** bound to GluR2 and GluR3 (Figure 3) indicate the likely source of the exhibited subunit selectivity since the only nonconserved residue in the vicinity of the ligand is Y716-(GluR1) [Y702 in GluR2] vs F728(GluR3) [F724 in GluR4].[3] Whereas, in the case of GluR2, O-4 of **2b** makes a H-bond of moderate strength to W2 (2.33 Å), which is in turn strongly bound to Y702 and T686, the same is not true of the GluR3−agonist complex. For GluR3, there is only one opportunity for W2 to H-bond to the receptor (T712), and the H-bond between W2 and oxygen at C-4 is weakened (2.46 Å). There is also an indirect effect whereby a putative interdomain "lock" is lengthened, i.e., the H-bond between E402(GluR2)/E426(GluR3) [S1 domain] and T686(GluR2)/T712-(GluR3) [S2 domain]: 1.99 Å (GluR3) vs 1.86 Å (GluR2). This may affect the desensitization properties of **2b** at the various AMPA-Rs. The net effect of these interactions is that **2b** has selective affinity toward those receptors containing tyrosine rather than phenylalanine at this position in the binding pocket. In this regard, **2b** probably behaves in a fashion similar to (*S*)-4-bromohomoibotenic acid (BrHIBO).[3,9] However, due to the extra heteroatom O-2 on the uracil ring, the net negative charge of the **2b** heterocycle is less concentrated on the oxygen in contact with W2 than in the case of BrHIBO, and the selectivity displayed by **2b** is correspondingly

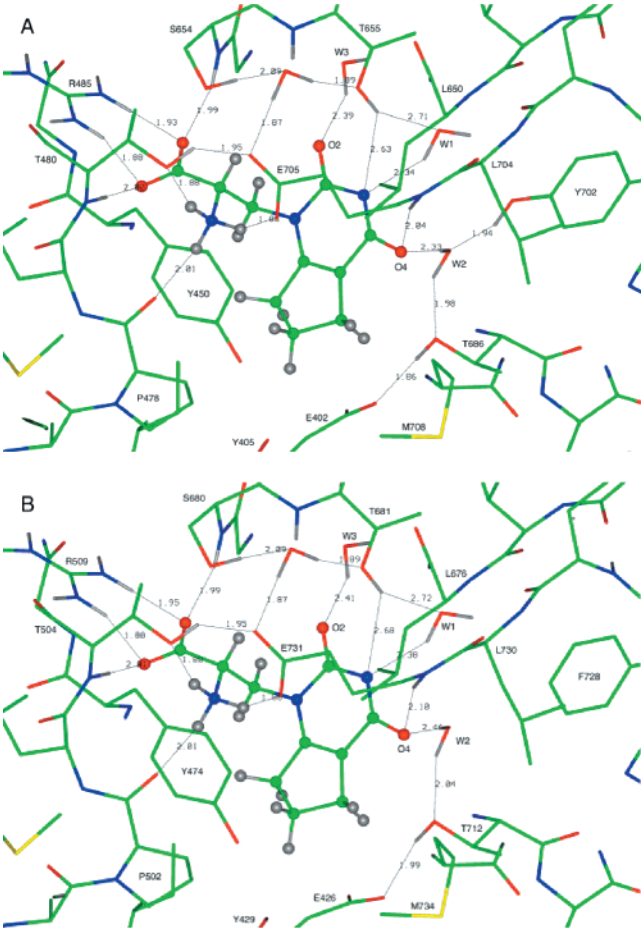


Figure 3. Molecular modeling of **2b** binding to AMPA-R. A: Compound **2b** modeled in the binding site of GluR2−S1S2J. B: Compound **2b** in the binding site of a homology-built model of GluR3. Numbering as per ref 3. W1−3 are receptor water molecules which connect the ligand to the receptor via hydrogen bonds. Most protons and some residues, including Y723 (GluR2) in front of the ligand, have been omitted for clarity.

less pronounced. Although the GluR1/2 versus GluR3/4 binding selectivity of **2b** is similar to that of willardiine itself,[6] its higher affinity is most likely due to the hydrophobic contact between the cyclopentane ring and the receptor pocket.

Conclusion. Taken together, these results support the action of **2b** as a novel, subtype-selective, partially desensitizing, full agonist of AMPA-R. The AMPA-R subtype selectivity exhibited by willardiine analogues is attributable to the dione nature of the uracil ring structure and the differential interactions of these oxygen atoms with water molecules and tyrosine/phenylalanine residues which form an H-bonding matrix in the ligand binding site. In **2b**, the cyclopentane-fused ring confers a higher affinity than willardiine itself due to additional hydrophobic interactions in the binding pocket. In addition, we propose that this cyclopentane-fused ring hinders the closure of the S1−S2 domains, which is a requirement of receptor desensitization. This gives rise to the unique pharmacological properties of **2b** which, unlike glutamate, AMPA, or (*S*)-willardiine itself (full agonists that rapidly promote receptor desensitization), is a full agonist that is weakly desensitizating. This can account for the potent neurotoxic profile of **2b** in comparison to (*S*)-willardiine (a full agonist with no excitotoxic potential). Compound **2b** produces a large calcium response and toxicity due to its partially desensitizing nature, a property shared by kainate. Consequently, this compound is a new pharmacological tool to investigate the molecular mechanisms of desensitization and neurotoxicity of AMPA-R, leading to a new class of AMPA-R agonists.

Acknowledgment. We thank the European Union (CEC BIOTECH. PROGRAMME − Dem. Contr. CT 98-0223) and MURST − Rome, Italy (PRIN 99) for financial support.

Supporting Information Available: Experimental details (chemistry, molecular modeling, and pharmacology (ref), Figures 1−4). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Dingledine, R.; Borges, K.; Bowie, D.; Traynelis, S. F. The glutamate receptor ion channels. *Pharmacol. Rev.* **1999**, *51*, 7−61.

(2) Sommer, B.; Keinanen, K.; Verdoorn, T. A.; Wisden, W.; Burnashev, N.; Herb, A.; Kohler, M.; Takagi, T.; Sakmann, B.; Seeburg, P. H. Flip and flop: a cell-specific functional switch in glutamate-operated channels in the CNS. *Science* **1990**, *249*, 1580−1585.

(3) Banke, T. G.; Greenwood, J. R.; Christensen, J. K.; Liljefors, T.; Traynelis, S. F.; Schousboe, A.; Pickering D. S. Identification of amino acid residues in GluR1 responsible for ligand binding and desensitization. *J. Neurosci.* **2001**, *21*, 3052−3062.

(4) Sun, G.; Uretsky, N. J.; Wallace, L. J.; Shams, G.; Weinstein, D. M.; Miller, D. D. Synthesis of chiral 1-(2′amino-2′-carboxyethyl)-1,4-dihydro-6,7-quinoxaline-2,3-diones: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor agonists and antagonists. *J. Med. Chem.* **1996**, *39*, 4430−4438.

(5) Watanabe, Y.; Usui, H.; Shibano, T.; Tanaka, T.; Kanao, M. Synthesis of monocyclic and bicyclic 2,4(1*H*,3*H*)-pyrimidinediones and their serotonin antagonist activities. *Chem. Pharm. Bull.* **1990**, *38*, 2726−2732.

(6) Jane, D. E.; Hoo, K.; Deverill, M.; Bleakman, D.; Mandelzys, A. Synthesis of willardiine and 6-azawillardiine analogues: pharmacological characterization on cloned homomeric human AMPA and kainate receptor subtypes. *J. Med. Chem.* **1997**, *40*, 3645−3650.

(7) Yonetani, T. The Yonetani-Theorell graphical method for examining overlapping subsites of enzyme active centers. *Methods Enzymol.* **1982**, *87*, 500−509.

(8) Jensen, J. B.; Schousboe, A.; Pickering, D. S. AMPA receptor mediated excitotoxicity in neocortical neurons is developmentally regulated and dependent upon receptor desensitization. *Neurochem. Int.* **1998**, *32*, 505−513.

(9) Greenwood, J. R.; Liljefors, T.. Subtype selectivity at AMPA receptors: the roles of water-mediated H-bonding. *Molecular Graphics and Modelling Society meeting on Structure-based Drug Design*; St. Catherine's College: Oxford, UK, 13−15 December 2000.

JM015552M