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Transmembrane AMPA Receptor Regulatory Proteins and Cornichon-2 Allosterically Regulate AMPA Receptor Antagonists and Potentiators*

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AMPA receptors mediate fast excitatory transmission in the brain. Neuronal AMPA receptors comprise GluA pore-forming principal subunits and can associate with multiple modulatory components, including transmembrane AMPA receptor regulatory proteins (TARPs) and CNIHs (cornichons). AMPA receptor potentiators and non-competitive antagonists represent potential targets for a variety of neuropsychiatric disorders. Previous studies showed that the AMPA receptor antagonist GYKI-53655 displaces binding of a potentiator from brain receptors but not from recombinant GluA subunits. Here, we asked whether AMPA receptor modulatory subunits might resolve this discrepancy. We find that the cerebellar TARP, stargazin (γ-2), enhances the binding affinity of the AMPA receptor potentiator [^{3}H]-LY450295 and confers sensitivity to displacement by non-competitive antagonists. In cerebellar membranes from stargazer mice, [^{3}H]-LY450295 binding is reduced and relatively resistant to displacement by non-competitive antagonists. Coexpression of AMPA receptors with CNIH-2, which is expressed in the hippocampus and at low levels in the cerebellar Purkinje neurons, confers partial sensitivity of [^{3}H]-LY450295 potentiator binding to displacement by non-competitive antagonists. Autoradiography of [^{3}H]-LY450295 binding to stargazer and γ-8-deficient mouse brain sections, demonstrates that TARPs regulate the pharmacology of allosteric AMPA potentiators and antagonists in the cerebellum and hippocampus, respectively. These studies demonstrate that accessory proteins define AMPA receptor pharmacology by functionally linking allosteric AMPA receptor potentiator and antagonist sites.

The AMPA type glutamate receptor mediates most fast excitatory synaptic transmission in brain. AMPA receptor comprise heterotetramers of the glutamate binding and pore-forming subunits, GluA1–4, which are each alternatively spliced to yield flip and flop isoforms (1–3). In addition to the principal GluA subunits, neuronal AMPA receptors typically contain auxiliary transmembrane AMPA receptor regulatory protein (TARPs)[2] subunits (4, 5), which enhance receptor trafficking (6) and modulate channel gating (7–10). Neuronal AMPA receptors may also associate with other transmembrane proteins, including CNIH (cornichon) proteins and CKAMP44, which can further modulate receptor trafficking and channel function (11–14).

AMPA receptors play fundamental roles in controlling behavior, learning, and memory; dysfunction of these receptors likely underlies a variety of neuropsychiatric disorders (15–21). Accordingly, numerous pharmacological efforts have sought either to promote AMPA receptor function with potentiators or to block channel function with antagonists. AMPA receptor potentiators enhance channel permeation by either slowing deactivation (channel closure following glutamate removal) or blunting desensitization (channel closure in the continued presence of glutamate) (22). AMPA potentiators can enhance synaptic transmission and thereby promote growth factor release and neurogenesis (23–25). Preclinically, AMPA potentiators have shown promising activity in models of depression (26), cognitive impairment (27), and Parkinson disease (28). AMPA receptor antagonists that blunt synaptic transmission have been pursued as therapeutics for epilepsy, neurodegeneration, and neuropathic pain. Multiple classes of AMPA receptor antagonists have been identified. Antagonists, such as 6-cyano-7-nitroquinoxaline-2,3-dione, compete with glutamate at the agonist binding site and occlude channel opening (29). Non-competitive antagonists, such as GYKI-53655, reportedly interact at an alternative site to allosteric potentiators and inhibit channel gating downstream of glutamate binding (30, 31).

TARPs have complex interactions with AMPA receptor modulator drugs. TARPs convert certain competitive antagonists, including 6-cyano-7-nitroquinoxaline-2,3-dione, into partial agonists (32). By increasing glutamate affinity, TARPs also blunt effects of competitive antagonists (33). By contrast, TARP γ-2 increases the affinity of certain non-competitive antagonists, such as GYKI-53655 (33). The molecular mechanism for channel block by non-competitive antagonists remains unclear (31), and resolving this may provide insights for mechanisms that underlie channel gating.

Interactions between AMPA receptors potentiators and antagonists have also yielded valuable clues regarding their mechanisms of action. The IC_{50} for GKYI-53655 is shifted 10-fold to right by cyclothiazide (34). Although initially interpreted as evidence for direct interaction between these sites, subsequent studies showed that cyclothiazide cannot displace a

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[2] The abbreviations used are: TARP, transmembrane AMPA receptor regulatory protein; LBD, ligand binding domain.

radiotracer from the GYKI-53655 binding site (9). Additional insight was provided by binding studies with [^{3}H]-LY395153, which potently labels the potentiator site (35). Interestingly, GYKI-53655 potently blocks binding of [^{3}H]-LY395153 to AMPA receptors in brain membranes but does not affect binding to recombinant receptors (35). Here, we addressed this discrepancy and discovered an unexpected role for AMPA receptor accessory proteins in functionally linking AMPA receptor potentiator and non-competitive antagonist binding sites. These results provide valuable insights for understanding and refining the neuropharmacology of AMPA receptors.

EXPERIMENTAL PROCEDURES

Buffers, Reagents, Plasmids, and Cell Culture—All buffers and reagents were purchased from either Sigma-Aldrich or Thermo Fisher Scientific (Pittsburgh, PA). All cDNAs were human, except for rat iGluA2R, and were cloned into pcDNA 3.1 mammalian expression plasmids (Invitrogen). Compounds used in binding assays were synthesized at Lilly Research Laboratories (Indianapolis, IN). For electrophysiology experiments, HEK293T cells were maintained at 37 °C in 5% CO_2 high glucose DMEM medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin and split bi- or tri-weekly. HEK293T cells were plated onto 25-mm coverslips and were transiently transfected using FuGENE 6 according to the manufacturer's protocols (11814443001; Roche Applied Science). Experiments were conducted 48–72 h post-transfection.

For radioligand binding experiments, HEK293E cells were maintained in suspension using a plate shaker (160 rpm) and incubated at 37 °C, 5–8% CO_2, humidified with DMEM/F-12 medium (Hybritech, San Diego, CA) supplemented with HEPES (20 mM), Pluronic F-68 (0.075% w/v), tropolone (0.4 μg/ml), and nucellin/humulin (5 μg/ml). On the day of transfection, cells were centrifuged at 1000 × *g* and resuspended to 2×10^6 cells/ml. Using prewarmed media containing no supplements, the DNA-transfection reagent complex was made by adding 5 μg cDNA per ml with 10 μl of X-tremeGENE 1539 (Roche Applied Science), gently mixed for 60–90 min at room temperature, and then added to the cells. Cells were harvested 48 h post-transfection.

Tissue and Recombinant Cell Membrane Preparation—Stargazer and wild-type mice were euthanized with CO_2 and decapitated. Brains were dissected rapidly and homogenized using a Polytron in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Cells were pelleted and homogenized using a Polytron in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4). Homogenates were centrifuged at 1000 g to remove nuclei and unbroken cells. Both tissue and cell homogenates were centrifuged again at 4 °C at 38,000 × *g* for 20 min. To remove endogenous glutamate, pellets were resuspended, washed with buffer, and centrifuged for 20 min. This process was repeated a total of four times. After the final wash, pellets were frozen on solid CO_2 and stored at −80 °C.

Radioligand Binding—Membranes were incubated with 50 nM [^{3}H]-LY450295 (ViTrax Radiochemicals, Placentia, CA) and other pharmaceutical agents as indicated for 2 h at 4 °C. Assay buffer comprised 50 mM Tris-HCl (pH 7.4) and 500 μM L-glutamate (Tocris Bioscience, Ellisville, MO). Nonspecific binding was determined by including 10 μM LY450108, a related AMPA receptor potentiator (36). All binding was terminated by rapid filtration using a TOMTEC 96-well cell harvester (Hamden, CT) through GF/A filters presoaked with 0.3% polyethyleneimine. The filters were washed with 5 ml of ice-cold 50 mM Tris buffer (pH 7.4) and air-dried overnight. The dried filters were placed on PerkinElmer Life Sciences MeltiLex A melt-on scintillator sheets, and the radioactivity was counted using a PerkinElmer Life Sciences Wallac 1205 Betaplate counter (Perkin Elmer Life Sciences). For binding studies, homomeric GluA transfections were used to ensure a uniform receptor composition. GluA2 was selected for binding studies due to its inclusion in most hippocampal (GluA1/GluA2 heteromeric) and cerebellar neuronal (GluA2/GluA3 and GluA2/GluA4 heteromeric) AMPA receptors (37). In some experiments, experimental variability caused binding to exceed 100% of control.

Electrophysiology—Agonist-evoked currents were recorded from transfected HEK293T cells as described (38). Recordings were made using thick-walled borosilicate glass electrodes (Sutter Instruments, Novato, CA) pulled and fire-polished to a resistance of 2–5 megohms. All cells were voltage-clamped at −80 mV; data were low pass-filtered at 5 kHz and digitized at 10 kHz using Axoclamp 200B and Axopatch software and hardware (Molecular Devices, Sunnyvale, CA). For each experiment, the transfected HEK293T cells were maintained in external solution containing the following: 117 mM TEA, 13 mM NaCl, 5 mM $BaCl_2$, 1 mM $MgCl_2$, 20 mM CsCl, 5 mM glucose, and 10 mM Na-HEPES (pH 7.4). The intracellular electrode solution contained the following: 160 mM *N*-methyl-D-glucamine, 4 mM $MgCl_2$, 40.0 mM Na-HEPES (pH 7.4), 12 mM phosphocreatine, 2.0 mM Na_2-ATP (pH 7.2) adjusted with H_2SO_4. Osmolarity was adjusted to 290 ± 5 mOsM.

Transfected HEK293T cells were lifted and perfused with a 16-barrel glass capillary pipette placed 100–200 μm from the cell (VitroCom, Mountain Lakes, NJ). Solutions were switched by sliding the pipette array with a solution exchange rate of <20 ms. Glutamate (1 mM), LY450295 (10 μM), and GYKI-53655 (10 μM) were applied where indicated. Because of the low conductance from homomeric GluA2 receptors used in binding studies (39, 40), we transfected HEK293T cells with GluA1 + GluA2 in the presence or absence of γ-2. GluA2 incorporation into heteromeric complexes with GluA1 was confirmed via a linear *I-V* curve from −80 to + 80 mV. Preincubation of the potentiator, empirically determined to maximize intercell potentiation reliability, was for a period of 1 min followed by 30-s pulses of agonist in the presence of compounds.

Autoradiography—Sagittal brain sections were cut at 12 μm, thaw mounted onto gelatin-coated slides, and stored at −80 °C. Sections were incubated for 2 h in 50 mM Tris-HCl containing 50 nM [^{3}H]-LY450295, 500 μM L-glutamate, and other agents as indicated. Sections were rinsed with 50 mM ice-cold Tris-HCl for 10 min, dried, and exposed to a Fujifilm Imaging Plate for 15 days.

Data Analysis and Statistics—Radioligand binding studies were analyzed using a Microsoft Excel™ workbook and were graphed using GraphPad Prism software (La Jolla, CA). The electrophysiology data are represented as mean ± S.E. and were the result of at least three independent experiments. Analyses

involving three or more data sets were performed with a one-way analysis of variance with a Tukey Kramer post hoc analysis using GraphPad Prism software. Analyses involving two data sets were performed with an uncorrected Student's *t* test or with a Student's *t* test with a Welsh correction, only if the variances were statistically different. Percent inhibition was calculated as,

$$\text{Inhibition (\%)} = 100 - [(I_{\text{Glu-SS in LY450295+GYKI-53655}}/I_{\text{Glu-SS in LY450295}}) \times 100] \quad \text{(Eq. 1)}$$

where $I_{\text{Glu-SS}}$ is the glutamate-evoked steady state current in LY450295 and GYKI-53655 or just LY450295. Significance was set as a p value of <0.05. B_0 refers to the specific potentiator binding, which occurs in the absence of any added antagonist.

RESULTS

Non-competitive Antagonists Displace [^{3}H]-LY450295 Binding from Brain but Not from Recombinant AMPA Receptors—Previous studies characterized binding of AMPA receptor potentiator [^{3}H]-LY395153 both to brain membranes and to recombinantly expressed GluA subunits (35). Curiously, the non-competitive antagonist, GYKI-53655 potently displaced [^{3}H]-LY395153 binding to cerebrocortical membranes but did not displace binding from transfected GluA4 membranes (35). We first asked whether this discrepancy was particular to a specific brain region or GluA subunit. Using GluA2 because of its predominant incorporation into cerebellar neuronal AMPA receptors (37), we observed that GYKI-53655 readily displaced the AMPA potentiator, [^{3}H]-LY450295, from cerebellar membranes but did not displace [^{3}H]-LY450295 binding from transfected GluA2 membranes (Fig. 1*A*). Furthermore, a structurally distinct non-competitive AMPA receptor antagonist, CP-465,022, showed a similar selectivity for displacement of potentiator only from native tissues (Fig. 1*B*). Because GluA2 is incorporated into AMPA receptors across multiple brain regions, we focused on this GluA subunit (37).

Stargazin Increases [^{3}H]-LY450295 Potentiator Binding Affinity—Numerous studies show that TARPs control neuronal AMPA receptor gating and pharmacology (4, 41–45). In particular, stargazin (γ-2) slows AMPA receptor desensitization, augments steady state currents and increases the affinity for both the full agonist glutamate and the partial agonist kainate (7–10). We asked whether γ-2 might also modulate [^{3}H]-LY450295 binding. GluA2 alone produced receptors that bound [^{3}H]-LY450295 with a K_d of 81 ± 13 nM and a $B_{\max}$ of 1434 ± 74 fmol/mg protein. Nonlinear regression analysis of the specific binding revealed a single saturable, high affinity site for LY450295. Co-expression of γ-2 increased both [^{3}H]-LY450295 binding affinity (K_d = 19 ± 2.5 nM) and total binding ($B_{\max}$ = 2101 ± 67 fmol/mg protein, Fig. 2*A*). Immunoblotting showed that the increase in total binding with γ-2 occurred despite a decrease in the amount of GluA2 expressed (Fig. 2*B*), which suggests that γ-2 may promote folding or stability of functional GluA2 to increase potentiator binding (46). Displacement of [^{3}H]-LY450295 with unlabeled LY450295 revealed a similar γ-2-mediated increase in LY450295 binding affinity (GluA2 alone IC_{50} = 109.5 nM *versus* GluA2 + γ-2 IC_{50} = 29.5 nM, Fig. 2*C*). The stargazin-mediated enhancement in potentiator affinity is paralleled by the increase seen previously in native cortical tissue *versus* recombinantly expressed AMPA receptors (35).

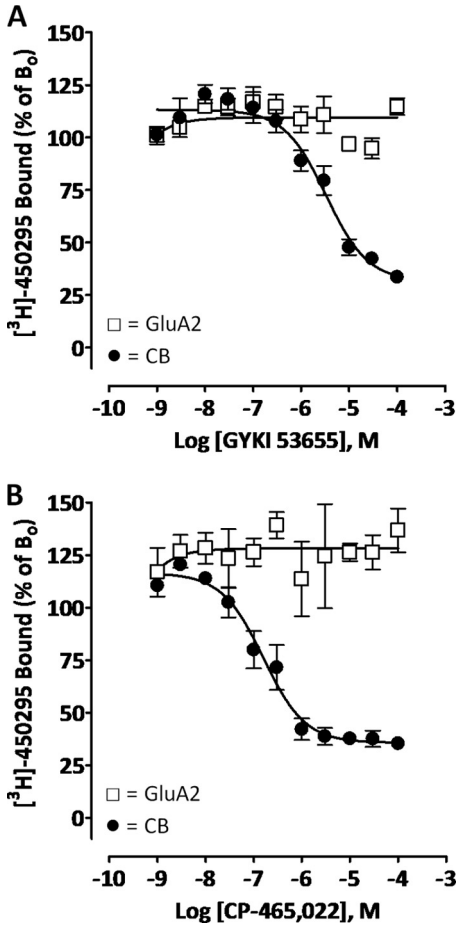

FIGURE 1. **Non-competitive antagonists displace [^{3}H]-LY450295 binding to brain but not to recombinant AMPA receptors.** *A* and *B*, non-competitive antagonists GYKI-53655 (*A*) and CP-465,022 (*B*) displace [^{3}H]-LY450295 binding from cerebellar (*CB*) membranes (*filled circles*) but not from membranes containing recombinantly expressed GluA2 (*open squares*). Data are presented as mean ± S.E.

Stargazin Enables Non-competitive Antagonists to Displace [^{3}H]-LY450295 Binding from AMPA Receptors—We next asked whether γ-2 co-transfection might also confer sensitivity of [^{3}H]-LY450295 binding to GYKI-53655. Strikingly, GYKI-53655 readily displaced [^{3}H]-LY450295 from GluA2 + γ-2 membranes similar to wild-type cerebellar membranes (Fig. 3). Also, the structurally distinct AMPA receptor antagonist, CP-465,022, showed a similar selectivity for displacement of radiolabeled potentiator binding from GluA2 + γ-2-containing membranes (Fig. 3).

GYKI-53655 Inhibits γ-2-Lacking AMPA Receptors in Presence of LY450295—Differential displacement by GYKI-53655 of [^{3}H]-LY450295 in GluA2 (no displacement, Figs. 1*A* and 3) and GluA2 co-transfected with γ-2 membranes (~70% displacement, Figs. 1*A* and 3) could indicate that LY450295 makes γ-2-lacking AMPA receptors insensitive to functional antagonism by GYKI-53655. To assess this, we measured GYKI-53655-mediated inhibition of glutamate-evoked currents. As reported previously, co-transfection of γ-2 dramatically

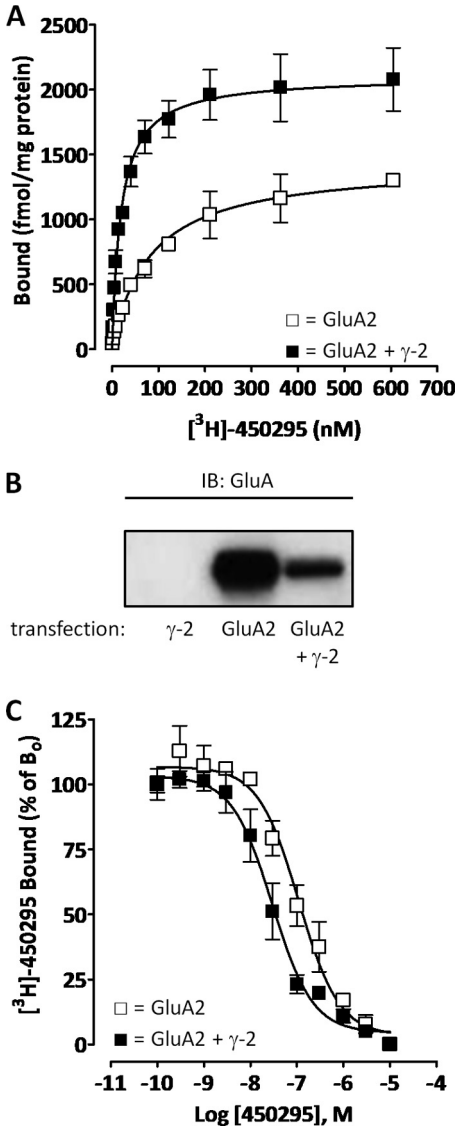

FIGURE 2. **γ-2 allosterically modulates [^{3}H]-LY450295 binding.** *A*, γ-2 co-expression (*filled squares*) increases the total binding (B_{max} ~ 2100 *versus* ~ 1430 fmol/mg protein, respectively) for [^{3}H]-LY450295 relative to GluA2 expression alone (*open squares*). *B*, this increase in B_{max} occurs despite a lower expression of GluA2 in the γ-2-containing membranes. *C*, γ-2 co-expression increases the potency of [^{3}H]-LY450295 binding (K_d ~ 30 nM for GluA2 + γ-2 *versus* ~ 110 nM for GluA2 alone). Data are presented as mean ± S.E. *IB*, immunoblot.

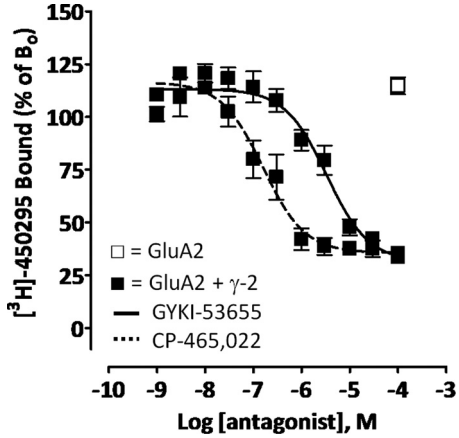

FIGURE 3. **γ-2 renders [^{3}H]-LY450295 binding to GluA2 sensitive to displacement by non-competitive antagonists.** GYKI-53655 (*solid line*) and CP-465,022 (*dotted line*) displace [^{3}H]-LY450295 from recombinant GluA2 co-expressing γ-2 (*filled squares*) (compare with GluA2 alone at 100 μM GYKI-53655 (*open square*) from Fig. 1). Data are presented as mean ± S.E.

increased glutamate-evoked currents (Fig. 4, *A–C*). LY450295 potentiated the glutamate-evoked currents from GluA1/GluA2 and GluA1/GluA2 + γ-2 heteromeric receptors (Fig. 4, *A–C*). Importantly, GYKI-53655 inhibited the LY450295-potentiated glutamate-evoked steady state currents from both γ-2-lacking and γ-2-containing AMPA receptors (Fig. 4, *A*, *B*, and *D*). Even though GYKI-53655 does not affect LY450295 binding to GluA2 receptors, these data show that GYKI-53655 regulates γ-2-lacking receptors and implies that the binding sites for GYKI-53655 and LY450295 are distinct.

[^{3}H]-LY450295 Binding to Stargazer Cerebellum Shows Reduced Sensitivity to GYKI-53655—Stargazer mice, which lack functional γ-2, display absence epilepsy and cerebellar ataxia and exhibit dramatically reduced synaptic AMPA receptor-mediated responses in cerebellar granule cells (5, 6, 47). To further assess the importance of γ-2, we characterized binding of [^{3}H]-LY450295 and found that cerebellar membranes from stargazer mice bound less [^{3}H]-LY450295 than did wild-type membranes (wild-type B_{max} ~ 1960 fmol/mg protein *versus* stargazer B_{max} ~ 1470 fmol/mg protein; Fig. 5*A*). Furthermore, [^{3}H]-LY450295 had decreased binding affinity in stargazer as compared with wild-type cerebellar membranes (~32 nM in wild-type *versus* ~60 nM in stargazer, Fig. 5*A*). Importantly, the non-competitive antagonists, GYKI-53655 and CP-465,022, only weakly displaced [^{3}H]-LY450295 binding from stargazer cerebellar membranes (Fig. 5, *B* and *C*). These data resolve the discrepancy between native and recombinant AMPA receptors for potentiator binding by establishing that γ-2 allosterically regulates AMPA receptor allosteric modulator pharmacology (35).

CNIH-2 Promotes Partial Displacement of [^{3}H]-LY450295 Binding by Non-competitive Antagonists—Recent proteomic analyses have identified several additional AMPA receptor accessory proteins that modulate channel kinetics and/or pharmacology (11, 14). In particular, CNIH (cornichon) proteins modulate surface trafficking of AMPA receptors and channel gating (11–13). CNIH-2 is present in multiple brain regions with high levels of expression occurring in the hippocampus and low levels of expression in the cerebellum, particularly within the Purkinje neuron population (12, 13). We assessed the effects of CNIH-2 on [^{3}H]-LY450295 binding. We observed a single saturable, high affinity site in HEK293E membranes transiently expressing GluA2 + CNIH-2. Similar to γ-2, we found that CNIH-2-increased total binding (Fig. 6*A*). However, unlike γ-2, CNIH-2 did not affect affinity for [^{3}H]-LY450295 (Fig. 6*B*). Interestingly, GluA2 + CNIH-2 recombinant membranes exhibited partial displacement of [^{3}H]-LY450295 binding by the non-competitive antagonists, GYKI-53655 and CP-465,022 (Fig. 6*C*). Thus, CNIH-2 can regulate AMPA receptor pharmacology in a manner similar to but not identical to TARPs.

Autoradiography Demonstrates TARP-mediated Sensitivity of [^{3}H]-LY450295 Binding—γ-2 is the predominant TARP subunit in the cerebellum, whereas γ-8 predominates in hip-

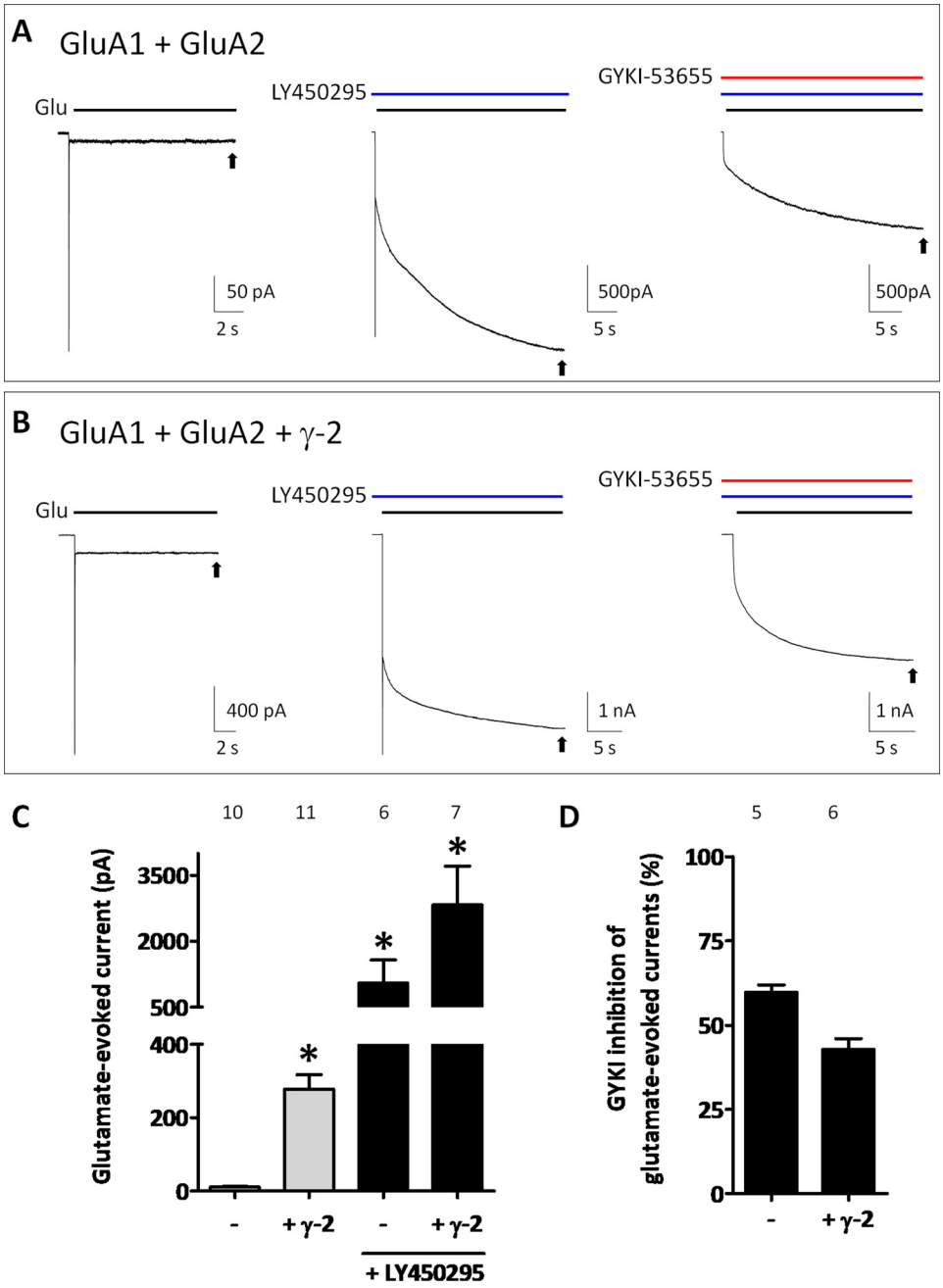

FIGURE 4. **GYKI-53655 inhibits LY450295 potentiated currents from AMPA receptors in the presence or absence of γ-2.** Glutamate-evoked currents from GluA1/GluA2 heteromers in the absence (*A*) or presence (*B*) of γ-2 are enhanced by LY450295 (*C*, *black bars*), and these potentiated currents are blocked by GYKI-53655 (*D*). Note that in the presence of 10 μM LY450295, GYKI-53655 inhibits GluA2-containing AMPA receptors expressed alone or co-expressed with γ-2. *Arrows* indicate the time point at which the steady state current was measured. * denotes $p < 0.05$ when compared to GluA1/GluA2 expressed alone. Data are presented as mean ± S.E.

pocampus (48). We used autoradiography to visualize [^{3}H]-LY450295 binding throughout the brain. Sagittal sections were incubated with ~50 nM [^{3}H]-LY450295, and co-application of unlabeled LY450108 served as a measure of non-specific binding (Fig. 7, *A* and *D*). In wild-type mice, [^{3}H]-LY450295 binding was present in diverse brain regions with the highest levels occurring in the hippocampus and cerebellum (Fig. 7*A*). The non-competitive antagonist CP-465,022 reduced [^{3}H]-LY450295 binding in both brain regions (Fig. 7, *A* and *E*).

In stargazer, [^{3}H]-LY450295 binding is reduced substantially in the cerebellum and reduced modestly in the hippocampus (Fig. 7, *B* and *D*). In γ-8 knock-out, [^{3}H]-LY450295 binding was reduced significantly in hippocampus but not in cerebellum (Fig. 7, *C* and *D*). Importantly, the [^{3}H]-LY450295 binding that remained in the cerebellum of stargazer mice was not displaced by CP-465,022 (Fig. 7*E*). In the γ-8 knock-out hippocampus residual [^{3}H]-LY450295 was partially resistant to displacement by the non-competitive antagonist (Fig. 7*E*). Together, these findings establish that TARPs and accessory proteins such as CNIH-2 allosterically regulate AMPA receptor pharmacology in specific brain regions.

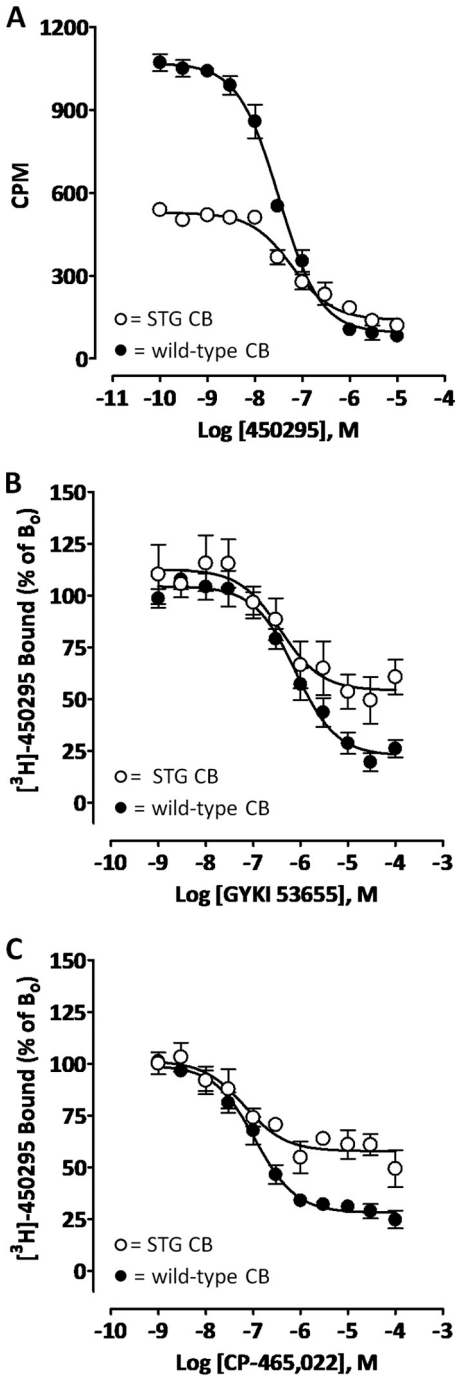

FIGURE 5. **γ-2 modulates [^{3}H]-LY450295 binding in cerebellum.** *A*, cerebellar membranes from stargazer mice (*STG CB*, *open circles*) exhibit reduced total [^{3}H]-LY450295 binding (B_{max} ~ 1960 *versus* ~ 1470 fmol/mg protein, respectively) and reduced binding affinity (K_d ~ 32 nM *versus* ~ 60 nM, respectively) relative to wild-type (wild-type CB, *filled circles*). Non-competitive antagonists GYKI-53655 (*B*) and CP-465,022 (*C*) more weakly displace [^{3}H]-LY450295 binding from stargazer cerebellar membranes as compared with wild-type cerebellar membranes (GYKI, ~46% *versus* ~77% displaced, respectively; CP, ~42% *versus* ~72% displaced, respectively). Data are presented as mean ± S.E.

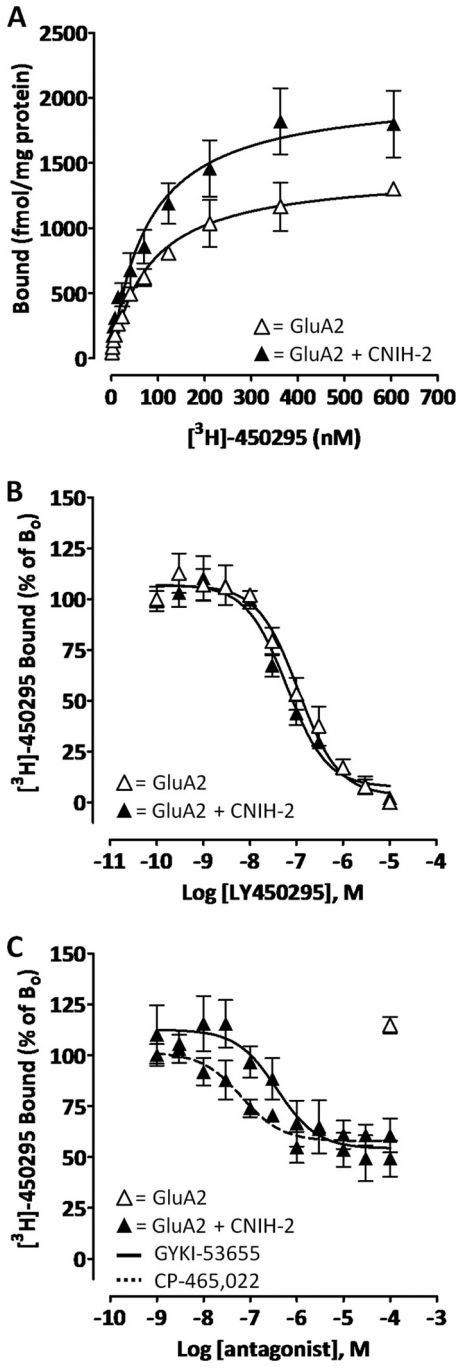

FIGURE 6. **CNIH-2 confers partial displacement of [^{3}H]-LY450295 binding by non-competitive antagonists.** *A*, CNIH-2 co-expression (*filled triangles*) increases the total binding (B_{max} ~2050 fmol/mg protein) for [^{3}H]-LY450295 relative to GluA2 expression alone (*open triangles*). *B*, CNIH-2 co-expression with GluA2 has no significant effect LY450295 potency relative to GluA2 alone (*open triangles*). *C*, non-competitive antagonists GYKI-53655 (~46% displacement, *solid line*) and CP-465,022 (~43% displacement, *dotted line*) partially displace [^{3}H]-LY450295 binding from GluA2 + CNIH-2 receptors (*filled triangles*) (compare with GluA2 alone at 100 μM GYKI-53655 (*open triangle*) from Fig. 1). Data are presented as mean ± S.E.

DISCUSSION

The principal finding of this study is that TARP subunits functionally link potentiator and antagonist binding sites within AMPA receptors. As published previously (35), we found that GYKI-53655 displaces AMPA potentiators from native AMPA receptors but not from recombinant receptors containing only a GluA subunit. We resolved this paradox by observing that TARPs γ-2/γ-8, and to a lesser extent CNIH-2, confer sensitivity of AMPA receptor potentiator binding to displacement by GYKI-53655 and another non-competitive AMPA receptor antagonist. Although GYKI-53655 did not dis-

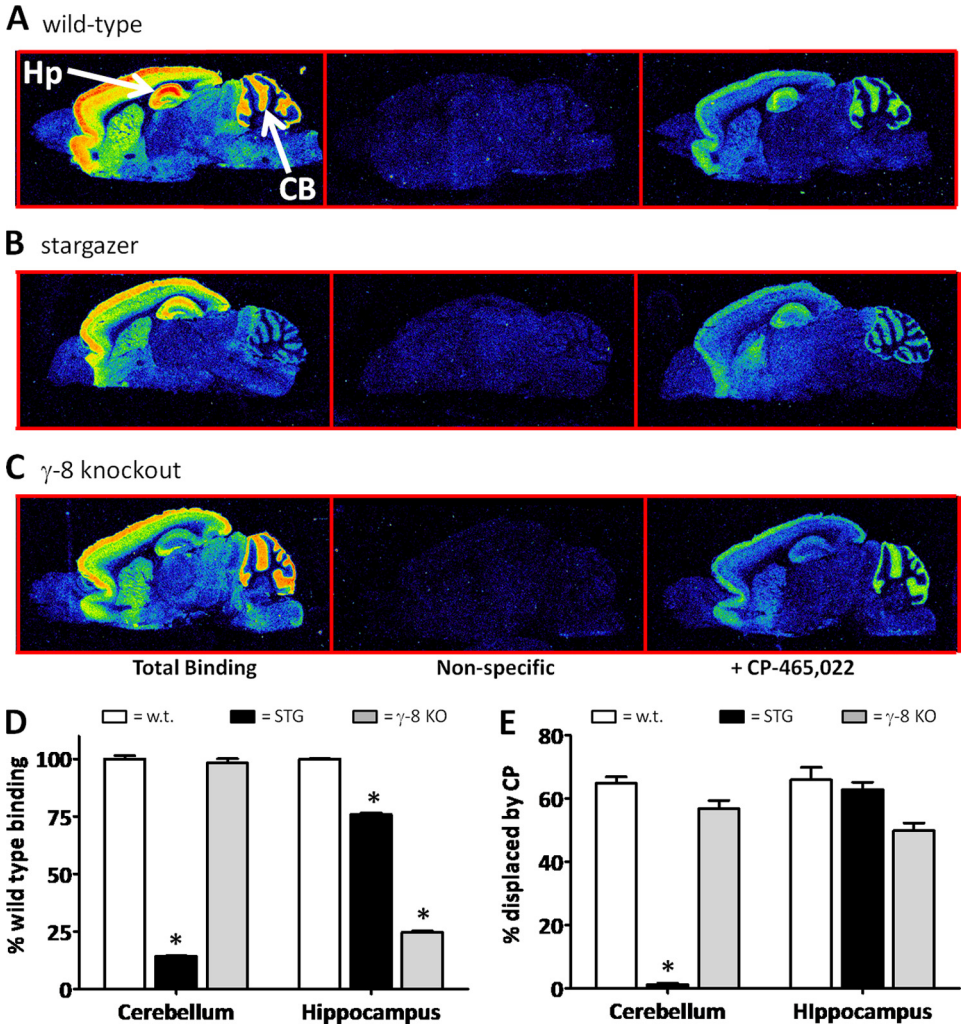

FIGURE 7. **TARPs regulate [^{3}H]-LY450295 sensitivity to displacement by non-competitive antagonists.** *A–C*, autoradiograms of [^{3}H]-LY450295 binding alone (*left panels*) in the presence of 10 μM LY450108 (nonspecific binding, *middle panels*) or in the presence of 10 μM CP-465,022 (*right panels*) for wild-type (*A*), stargazer (*B*), or γ-8 knock-out (*C*) mice. *D*, quantification shows dramatically reduced [^{3}H]-LY450295 binding in stargazer cerebellum (*CB*) and in γ-8 knock-out hippocampus (*HP*). *E*, CP-465,022 does not displace the residual [^{3}H]-LY450295 binding in stargazer cerebellum (*STG*), and CP-465,022 shows reduced efficacy in γ-8 knock-out hippocampus. An *asterisk* denotes $p < 0.05$ when compared with wild-type binding (*D*) or CP-465,022-mediated displacement (*E*). Data are presented as mean ± S.E.

place [^{3}H]-LY450295 in recombinant γ-2-lacking AMPA receptors, it nonetheless inhibited LY450295 potentiated currents from such receptors. Furthermore, γ-2 increases the affinity of both AMPA potentiators and antagonists (7–10, 33). Using receptor autoradiography and transgenic stargazer or γ-8 knock-out mice, we visualized the essential roles for these TARPs in controlling AMPA receptor pharmacology across the brain.

Structural studies provide insight regarding regulation of AMPA receptors by pharmacological agents and auxiliary subunits. GluA subunits are three-pass transmembrane proteins and the ligand binding domain (LBD) comprises amino acid residues from the extracellular N terminus and the loop between the second and third transmembrane domains. Structural studies of the LBD (49) and more recently of the GluA2 tetramer (50) have provided insight regarding molecular mechanisms for AMPA receptor gating. The tetrameric complex is assembled in a dimer-of-dimer conformation. Agonist binding induces closure of the clam-shaped LBD, which leads to separation of the attached transmembrane helices and then to channel opening (51). This is followed rapidly by rearrangement of the LBD dimer interface and channel desensitization. AMPA receptor potentiators bind at the interface of LBD dimers and prevent the conformational changes that cause desensitization (52).

Medicinal chemistry has created several classes of AMPA receptors potentiators that have differential specificity for AMPA receptor subtypes. The prototypical AMPA potentiator cyclothiazide preferentially enhances current from GluA flip isoforms (53), whereas 4–2-(phenylsulfonylamino)ethylthio-2,6-difluoro-phenoxyacetamide preferentially augments currents from flop isoforms (54). TARPs modulate the pharmacology of AMPA potentiators. Specifically, in AMPA receptors containing γ-2, cyclothiazide and 4–2-(phenylsulfonylamino)-ethylthio-2,6-difluoro-phenoxyacetamide can activate both flip and flop GluA isoforms (55). We made extensive use of the biarypropylsulphonamide type of potentiator, LY450295 (35), which potently blocks channel desensitization and deactiva-

tion. Structural studies show that this class of AMPA potentiators bridges the cleft between LBD subunits and stabilizes the glutamate-bound LBD dimer structure (56, 57). Furthermore, [^{3}H]-LY450295 potently labels AMPA potentiator binding sites in recombinant and native receptors only in the presence of agonist (35).

To test how TARPs exert their actions on AMPA potentiator binding, we measured the potentiator affinity. Our results showed that γ-2 increased potency of AMPA potentiator binding, indicating allosteric modulation. AMPA potentiators blunt receptor desensitization by binding to and stabilizing the interface between dimer pairs of the LBD (52). TARPs also blunt channel desensitization, and this is thought to occur through interactions of the first extracellular loop of TARP with the LBD (58). Our data support this model and suggest that TARPs facilitate dimerization of LBDs and therefore enhance the binding affinity of AMPA potentiators.

AMPA receptor antagonists also have been valuable tools to understand receptor gating mechanisms. Pharmacological studies have identified non-competitive antagonists including the 2,3-benzodiazepines, such as GYKI-53655 (59), and quinazolinones, such as CP-465,022 (60). Radioligand binding indicates that the 2,3-benzodiazepines and the quinazolinones interact with overlapping sites on the receptor; however, their binding site remains uncertain. Blocking receptor desensitization by cyclothiazide (30) or by mutating GluA1 leucine 497 to a tyrosine (31) decreases the affinity of GYKI-53655. Also, GYKI-53655 dissociates more rapidly from activated than from non-activated receptors suggesting that GYKI-53655 binds preferentially to a closed state of the AMPA receptor (31).

A domain-swapping and site-directed mutagenesis strategy showed that residues between LBD and the channel transmembrane domains are essential for antagonism by GYKI-53655. This region therefore was interpreted to be the binding site for non-competitive antagonists (31). This finding suggested a model whereby conformational changes in the regions that link the LBD to the transmembrane helices during channel opening distort the binding site for GYKI-53655 and reduce its affinity. This model was questioned by studies showing that co-transfection with γ-2 restored GYKI-53655 inhibition of the GYKI-53655-"insensitive" GluA1 variant (33). Indeed, γ-2 increases inhibitory potency of GYKI-53655 on non-mutated GluA1 (33). As TARPs stabilize the open non-desensitized state, the γ-2-mediated increase in GYKI-53655 affinity would not have been predicted by the previous model.

Our studies showed that displacement of AMPA potentiator [^{3}H]-LY450295 binding by either GYKI-53655 or CP-465,022 requires a TARP or, to a lesser extent, a CNIH-2 subunit within the receptor complex. These data resolved a paradox from previous studies showing that AMPA potentiator binding to native AMPA receptors but not recombinant GluA4 alone is sensitive to inhibition by GYKI-53655 (35). Our data also showed that TARPs enhance affinity of [^{3}H]-LY450295 binding. As predicted by these results, we found a reduction in [^{3}H]-LY450295 binding to cerebellar membranes from stargazer mice. The residual binding was insensitive to displacement by GYKI-53655 with the residual displacement likely resulting from other TARPs. We further characterized [^{3}H]-LY450295 pharmacology across mouse brain by autoradiography. In γ-8 knock-out mice, we found a dramatic loss of [^{3}H]-LY450295 binding in the hippocampus, which fits with the regional distribution for γ-8 (61, 62). We also found that the residual hippocampal binding of [^{3}H]-LY450295 in γ-8 knock-outs exhibited somewhat reduced displacement by a non-competitive antagonist. Robust hippocampal CNIH-2 expression and/or alternative TARP likely accounts for the residual antagonist activity.

Our studies provide key insights regarding the functional interactions of TARPs with AMPA receptors. It had been shown previously that the first extracellular loop of γ-2 controls AMPA receptor gating, whereas the C terminus is important for surface and synaptic trafficking (8, 63). The first extracellular domain of γ-2 also mediates the increase in GYKI-53655 affinity (33). Furthermore, γ-2 can change the conformation of the linker between the ligand binding core and the transmembrane domain of AMPA receptors to restore GYKI-53655 antagonism for a receptor with mutations in this linker region (33). Our findings support the hypothesis that TARPs enable non-competitive antagonists to block potentiator binding, suggesting that TARPs propagate perturbation of the juxtamembrane linker region to the LBD. Promoting conformational communication between these regions of AMPA receptors may explain how TARPs regulate AMPA receptor pharmacology to enhance channel gating. Future structural studies of AMPA receptors in complex with TARP and other auxiliary subunits will clarify the nature of these molecular interactions.

Either augmenting or antagonizing AMPA receptor function represents intriguing pharmacological approaches for a variety of neuropsychiatric disorders. However, the ubiquity of neuronal AMPA receptors suggests that subtype selectivity may be desired for modulating transmission in specific pathways. Understanding how auxiliary subunits modify AMPA receptor pharmacology may help design more selective agents for targeting AMPA receptors.

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