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Modulation of agonist binding to AMPA receptors by CX546: Differential effects across brain regions and GluA1-4/TARP combinations

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Running Title: Ampakine effects across brain areas and GluA/TARP variants

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Abbreviations: TARPs: transmembrane AMPA receptor regulatory proteins.

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ABSTRACT

Ampakines are cognitive enhancers that potentiate AMPA receptor currents and synaptic responses by slowing receptor deactivation. Their efficacy varies greatly between classes of neurons and brain regions, but the factor responsible for this remains unclear. Ampakines also increase agonist affinity in binding tests in ways that are related to their physiological action. We therefore examined (i) if ampakine effects on agonist binding vary across brain regions and (ii) if they differ across receptor subunits expressed alone and together with TARPs (transmembrane AMPA receptor regulatory proteins) which associate with AMPA receptors in the brain. We found that the maximum increase in agonist binding (E_{max}) caused by the prototypical ampakine CX546 differs significantly between brain regions, with effects in hippocampus and cerebellum being nearly three times larger than in thalamus, brainstem and striatum, and cortex being intermediate. These differences can be explained at least in part by regional variations in receptor subunit and TARP expression because combinations prevalent in hippocampus (GluA2 with TARPs γ3 and γ8) exhibited E_{max} values nearly twice those of combinations abundant in thalamus (GluA4 with γ2 or γ4). TARPs seem to be critical because GluA2 and GluA4 alone had comparable E_{max} and also because hippocampal and thalamic receptors had similar E_{max} after solubilization with Triton X-100 which likely removes associated proteins. Taken together, our data suggest that variations in physiological drug efficacy such as the three-fold difference previously seen in recordings from hippocampus versus thalamus may be explained by region-specific expression of GluA1-4 as well as TARPs.

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INTRODUCTION

Ampakines are benzamide compounds that allosterically potentiate AMPA receptor currents, prolong synaptic responses (Arai et al., 1996, 2002; Arai and Kessler, 2007), facilitate long term potentiation (Arai et al., 2004), and enhance memory encoding in animals and humans (Staubli et al., 1994; Lynch et al., 1997; Hampson et al., 1998). They also have shown therapeutic potential for various pathological conditions such as Alzheimer's disease, schizophrenia and depression (Lynch, 2006).

In two earlier studies we found that ampakine effects vary greatly between neurons. In hippocampus, CX546 [1-(1,4-benzodioxan-6-ylcarbonyl)piperidine] was nearly 10-fold more effective in prolonging EPSCs in pyramidal cells than in interneurons or in stratum radiatum giant cells, an ectopic version of pyramidal cells (Xia and Arai, 2005). Major differences were likewise seen between hippocampal and thalamic neurons, i.e., prolongation of EPSC duration by CX546 was about 3-fold larger in hippocampal CA1 pyramidal cells than in two subdivisions of the thalamus (Xia et al., 2005). Understanding the causes for these differences is important for any attempt to interpret the effect of these drugs on behavior and to assess their potential for clinical applications, but the factors responsible for the differential effects are not yet clear and it is therefore difficult to predict how effective ampakines will be in other neuronal systems.

Several factors can potentially contribute to the observed variations in ampakine efficacy. The AMPA receptor subunits GluA1-4, in both of their two splice variants called flip and flop, are differentially expressed in the brain (Keinanen et al., 1990; Sommer et al., 1990). Moreover, AMPA receptors are tightly associated with and modulated by proteins called TARPs (Transmembrane AMPA receptor Regulatory Proteins). These proteins again exhibit distinct distributions in the brain (Tomita et al., 2003, Moss et al., 2003) and they differ from each other in the way they alter receptor kinetics (Tomita et al., 2005; Kott et al., 2007; Suzuki et al., 2008). It should be noted that AMPA receptor currents have recently been found to be controlled by yet another family of transmembrane proteins (Schwenk et al., 2009) and additional modulatory proteins cannot be ruled out.

Evidence that ampakine effects may differ between subunits and splice variants has been provided in several earlier studies, but overall the differences seemed modest. One of the most frequent findings has been a slight preference for flop over flip variants (Arai et al., 2000; Xia et al., 2005). However, cyclothiazide, which has a particularly strong flip preference (Partin et al., 1994), was much less discriminating than ampakines in the comparative physiological studies mentioned above (Xia and Arai, 2005; Xia et al., 2005), suggesting that differences in splice variants were of minor importance. Subunit composition also does not seem to explain the observed variations in drug effect, in part because most neurons express multiple subunits that are usually assembled into heteromeric receptors (Petralia and Wenthold, 1992; Lu et al., 2009). One of the most important factors in this regard is the presence of GluA2 because receptors containing this subunit do not pass calcium and because it often

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distinguishes interneurons from pyramidal cells (Geiger et al., 1995). However, in Xia and Arai (2005), receptors in both pyramidal neurons and radiatum giant cells were found to contain GluA2, yet ampakine effects differed drastically between these cells. It therefore seems likely that factors other than subunit and flip-flop type contribute to ampakine efficacy. Of particular interest are the TARPs of which 6 variants (γ2-γ5, γ7, γ8) have now been shown to associate with AMPA receptors (Nicoll et al., 2006; Kato et al., 2008) and for which regional expression appears to be more distinctive than for receptor subunits. In the adult brain, for example, the TARPs γ3 and γ8 are strongly dominant in hippocampal neurons while γ4 is largely absent, and an inverse pattern is found in the thalamus (Tomita et al., 2003; Moss et al., 2003). For the present study, we have used binding assays to examine the impact of some of these factors on drug efficacy. The design of the experiments is based on earlier observations that ampakines increase the affinity for agonists in binding tests and that the increase in agonist binding correlates positively with the efficacy to slow response deactivation in physiological recordings (Kessler et al., 1996; Arai et al., 2002; Kessler and Arai, 2006). In one set of experiments we have expressed GluA1-4 alone and in combination with the four Type I TARPs that are most widely distributed in the forebrain (γ2, γ3, γ4, γ8) and examined if there are differences in ampakine potency and efficacy. In parallel, we have examined if there are variations in drug effects between brain regions, whether they mirror those seen in physiology, and whether they can be explained by those seen with recombinant receptors.

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METHODS

Plasmids. Dr. K. Partin (Colorado State University, Fort Collins, CO) generously provided the cDNA for GluA1i and GluA2i in the mammalian expression vector pRK5 and GluA4i in mammalian expression vector pRK7. The cDNAs for TARP subunits γ2, γ3, γ4, and γ8 in mammalian expression vector pcDNA3 were generously supplied by Dr. D. S. Bredt (University of California, San Francisco, CA).

Transient transfection of HEK293 cells. HEK293 cells were grown in a 10 cm culture dish until they were 70-90% confluent. Cells were then transfected with 10 µg total plasmid DNA using Lipofectamine 2000 (DNA:Lipofectamine = 1:2) in Opti-MEM serum-free medium (Invitrogen). These co-transfections contained 5 µg of a plasmid with AMPA receptor cDNA and 5 µg of a plasmid with TARP cDNA. When AMPA receptors were expressed alone, 5 µg of a non-coding vector was used in place of the TARP plasmid. The transfection mixture was replaced with normal culture medium containing 100 µM DNQX after 16-18 hours. Cells were harvested for binding tests 40-50 hours after starting the transfection.

Brain dissections. Sprague-Dawley rats were anesthetized with halothane and decapitated according to an institutionally approved protocol and in observation of the guidelines of the National Institutes of Health. Adults were 3-5 months old and pups were of postnatal day 14-16. The brain was extracted from the skull, immediately placed in ice-cold HBS (Hepes-buffered saline; 150 mM NaCl, 20 mM HEPES, pH 7.4), and dissected into eight general regions: frontal cortex, parietal cortex, striatum, hippocampus, thalamus, hypothalamus, cerebellum, and brain stem. The sections were frozen in 320 mM sucrose plus 0.2 mM EGTA (pH 7.0).

Membrane preparation and binding assays. P_2 fractions were prepared from frozen brain sections using a conventional protocol involving homogenization in isotonic sucrose, differential centrifugation, osmotic lysis, and repeated washing (Kessler and Arai, 2006). On the day of the experiment, membranes were thawed, tip-sonicated, washed again, and suspended in HT (Hepes/Tris buffer; 100 mM HEPES, 100µM EGTA, pH 7.4). Protein was measured according to the method of Bradford, using bovine serum albumin as standard. Binding was measured at 25°C so that the basic kinetic properties of the receptors would be similar to those of receptors in excised-patch recordings. For binding tests, membrane aliquots (5-50 µg protein) were mixed with 20 nM [^{3}H]fluorowillardiine and appropriate additions in HT to a final volume of 50 µl. CX546 was added from 166.7-fold concentrated stock solutions in DMSO; control samples contained DMSO at the equivalent final concentration of 0.6%. Samples were incubated at room temperature for 30 min and then centrifuged at 22,000 *x g* for 10 min. The supernatant was removed and pellets were washed with 1.5 ml ice-cold wash buffer (100 mM NaCl, 50 mM KSCN, 5 mM Tris/HCl, pH 7.4). Pellets were dissolved with Beckman Tissue Solubilizer (BTS-450) and scintillation fluid (containing 13 mM acetic acid) was added to determine radioactivity.

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Transiently transfected HEK293 cells were rinsed in their culture dish with cold HB (Harvest Buffer; 150 mM NaCl, 10 mM Tris/HCl, 100 µM EGTA, pH 7.4) followed by incubation in this buffer for 2 min at 0°C. In all steps preceding binding, cells and buffers were kept at 0-4°C. Cells were harvested with a transfer pipette and homogenized with a PowerGen 125 tissue homogenizer (Fisher Scientific). Homogenates were washed twice by centrifugation at 37,000 x *g* for 15 min and resuspension in cold HB. Homogenates were then subjected to at least one freeze/thaw cycle. To measure binding, cell membranes were thawed, washed, and resuspended in HT. Aliquots (10-30 µg protein) were mixed with [^{3}H]fluorowillardiine and appropriate additions in HT to a final volume of 50 µl and incubated for 15 min at room temperature. Incubations were terminated by addition of ice-cold wash buffer (5 ml) and immediate filtration through GF/C glass fiber filters (Whatman). Filters were quickly rinsed with an additional 15 ml of ice-cold wash buffer and placed in scintillation fluid to determine the radioactivity content. For recombinant receptors without and with co-expression of TARPs, CX546 dose responses relations were measured with [^{3}H]fluorowillardiine at a concentration equal to one fifth of the K_D determined for individual subunits by Kessler and Arai (2006). For all assays, non-specific binding was determined by inclusion of 10 mM glutamate and subtracted from total binding. The binding data were normalized to binding without drug and fitted with a sigmoidal function (bottom asymptote =100, n_{Hill}=1) to determine the EC_{50} for the drug (apparent drug affinity) and the maximum change in binding, called E_{max}. The latter is calculated as the % increase over baseline binding in the absence of drug. Prism (GraphPad) was used for data analysis.

SDS-PAGE and Western blots. P_2 brain membrane fractions (40-100 µg protein) were combined with SDS-PAGE sample buffer and boiled for 2-5 minutes. Proteins were separated in 4-12%, Bis-Tris, precast gels (Criterion XT gels; Bio-Rad) for 1 hr at 190 V and transferred to PVDF membrane at 50 V for 1 hr. The membranes were blocked for 1 hr with 5% dry milk and then incubated with primary antibody in TPBS (10 mM phosphate buffered saline, 0.05% Tween) for 12-18 hrs at 4°C. After 3 washes in TPBS, blots were incubated in IRDye680 (926-32221; from LI-CORE) or IRDye800 (926-32220) goat anti-rabbit antibody (1:10,000) for 1 hr at room temperature and rinsed 3 times in TPBS and then twice in PBS. Blots were visualized and quantified using the Odyssey infrared imaging system (LI-CORE). The quality of this system was tested against NIH-imaging software (Image J) with Western blots of serial dilutions of recombinant GluA4. Infrared signals were also compared to detection with chemiluminescence (LAS-3000, Fujifilm). The Odyssey software gave similar results as Image J and superior linearity compared to chemiluminescence, consistent with findings reported by Wang et al. (2007). Primary antibodies were: anti-GluA1 (AB1504; 1 µg/ml), anti-GluA2 (AB1768; 0.5 µg/ml), anti-GluA3 (MAB5416; 5 µg/ml), and anti-GluA4 (AB1508; 1.5 µg/ml) from Chemicon International. Anti-actin (A2066; 1:200) was from Sigma.

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Statistical analysis. Binding data of recombinant receptors (E_{max} and EC_{50}) were analyzed by one-way ANOVA (SPSS 12.0). Some curve fittings were directly compared by F-test (Graph Pad, Prism). Data from Western blots were analyzed by t-test.

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RESULTS

Determination of ampakine EC_{50} and efficacy (E_{max})**.** Interactions between CX546 and the AMPA receptor were assessed from changes in the binding of the agonist [^{3}H]fluorowillardiine (FW). We used [^{3}H]FW because it exhibits high affinity binding without inclusion of the chaotropic ion thiocyanate which drastically diminishes the ampakine effect but is necessary to achieve reliable binding of the more conventional agonist [^{3}H]AMPA (Kessler and Arai, 2006). In most assays, membranes were incubated with a fixed concentration of [^{3}H]FW and varying concentrations of CX546 up to the solubility limit of 6 mM. As illustrated in figure 1A, [^{3}H]FW binding became progressively larger as drug concentration increased. A sigmoidal curve with a Hill coefficient of 1 has previously been shown to provide an adequate fit for all ampakines (Kessler and Arai, 2006). The fitted curve provides an upper asymptote which defines the maximal drug effect and an EC_{50} value which represents the potency of the drug under the test conditions. The maximal percent increase in [^{3}H]FW binding over baseline will be called E_{max} or 'efficacy' and it is obtained by subtracting 100% from the upper asymptote value (see figure 1A). As shown earlier (Kessler and Arai, 2006) and illustrated in figure 1B, the increase in [^{3}H]FW binding results from a decrease in the dissociation constant K_D for this agonist while B_{max} remains unchanged. The experimentally determined E_{max} value evidently depends on the agonist concentration. If b(a) represents binding without drug at the agonist concentration [a] and b'(a) represents binding in the presence of drug, then the ratio $R(a) = b'(a)/b(a) = (E_{max}/100 +1)$ is given by the equation $R(a) = (a + K_D) \bullet (a + K_D')^{-1}$, where K_D and K_D' represent the agonist dissociation constants without and with ampakine. With $a >> K_D$ this ratio approaches 1. On the other hand, at infinitesimally small agonist concentrations, R is equal to K_D/K_D' and thus equal to the factor by which agonist affinity is increased by the drug. For practical purposes, the [^{3}H]FW concentration in our tests was chosen to be as small as possible while still giving reliable binding data. For recombinant receptors this was selected to be about one tenth or less of the K_D constant. It will be shown below that under these conditions E_{max} is minimally altered by small perturbations in agonist K_D.

CX546 exhibits specificity for different combinations of receptor subunits and TARP variants. Figure 2A shows the effect of CX546 on the homomeric receptors GluA1, GluA2 and GluA4. For the latter two subunits, the drug effect was nearly identical with an E_{max} of 99 ± 7 % for GluA2 (n=6) and 101 ± 13 % for GluA4 (n=4). The EC_{50} was likewise similar with values of 652 ± 105 µM and 501 ± 106 µM, respectively. For GluA1 receptors, on the other hand, the drug effect was much smaller with an E_{max} of about 15%; the EC_{50} estimate of 2.4 mM accordingly has a high error margin. Tests with GluA3 are not shown because they were hampered by low receptor expression and inconsistencies in drug effects not seen with any other subunit.

A central goal of this study has been to examine if TARPs influence the effect of CX546. To test this, HEK293 cells were transfected in parallel with AMPA receptor subunits alone and in combination with each of the TARPs γ2, γ3, γ4, and γ8, and binding assays were done side by side for the various combinations. The main observation has been

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that E_{max} values can be greatly influenced by TARPs, as illustrated by two examples in figures 2B and C. Co-expression of GluA2 with the TARP γ3 significantly increased the E_{max} from 99 ± 7 % to 128 ± 7 % (n=6), or by about 30% ($p<0.001$; figure 2B). A very different effect was observed, however, when GluA4 was expressed without and with γ4 (figure 2C). In this case, the E_{max} was significantly *lower* in the presence of the TARP (74 ± 4 %) than with GluA4 alone (101 ± 13 %, n=4). Additional tests were conducted to verify that E_{max} changes are not secondary to changes in FW affinity. We have previously shown that γ2 may change agonist affinity up to 1.5-fold (Kessler et al., 2008) and a similar size shift has been observed for the GluA-TARP combinations tested here (see figure 2B inset). The effect of CX546 was therefore measured again for GluA2 + γ3 at a 1.5-fold increased [^{3}H]FW concentration of 6 nM to maintain equal distance to the agonist K_D. As shown in figure 2B, the E_{max} was essentially the same as when measured at 4 nM. This confirms that minor changes in agonist K_D do not significantly alter E_{max} at the low [^{3}H]FW concentration selected for our tests. It should also be noted that a reduction in E_{max} as seen with GluA4 + γ4 would be contrary to expectation if FW affinity is reduced. An additional influence on E_{max} could be that TARP co-expression changes the ratio of incompletely versus fully assembled receptors and that this in turn altered the apparent efficacy of CX546 (see Discussion in Supplemental Material). We therefore used Blue Native PAGE to evaluate the oligomeric state of recombinant receptors without and with TARPs. Most recombinant receptors were found to be present as fully assembled tetramers. No receptor monomers were detected in either preparation and the low level of receptor dimer did not change with co-expression of TARPs (see Supplementary Figure S1-A,B). This indicates that the changes in E_{max} caused by TARPs result from changes in receptor pharmacology rather than from different levels in receptor processing.

A more systematic comparison across different GluA-TARP combinations is shown in figure 3 and summarized in table 1. In combination with GluA2 receptors, γ8 increased E_{max} to 136 ± 8 % and thus was even more effective than γ3. On the other hand, γ2 and γ4 produced at best a marginal increase that was not statistically significant (103 and 112%, respectively). In contrast, all four TARPs decreased E_{max} when co-expressed with GluA4. This reduction was significant for all TARPs, but it was most prominent with γ2 (71 ± 8 %) and γ4 (74 ± 4 %) and least with γ3 (84 ± 4 %) and γ8 (81 ± 10 %). Thus, in overall comparisons TARPs introduced a nearly two-fold disparity among some receptor-TARP combinations (136% for GluA2 + γ8, versus 71% for GluA4 + γ2). Seen from a different angle, however, both receptor subunits exhibited the largest E_{max} values when co-expressed with γ3 or γ8 and the smallest values when expressed with γ2 or γ4 (figure 3D), suggesting that the nature of the TARP is an important determinant for drug efficacy. On the other hand, the potency of CX546 was not significantly changed by the TARPs with either receptor subunit. Likewise, no clear changes were seen when GluA1 was co-expressed with TARPs, but this may

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largely be due to the smallness of the drug effect and the resultant difficulty in detecting differences between dose-response curves.

CX546 binding varies in different regions of rat brain. The effect of CX546 was also examined in membranes prepared from eight brain regions. Concentration-response curves are shown in figure 4 and values for E_{max} and EC_{50} are summarized in table 2. It is apparent that E_{max} varied considerably between brain regions. The most striking difference is seen between hippocampus and thalamus from adult animals in which E_{max} values were 189 ± 5% and 68 ± 6%, respectively (n=3; $p<0.001$). Adult cerebellum exhibited a high E_{max} like hippocampus, but striatum, brainstem, and hypothalamus had an E_{max} closer to that of the thalamus (80-91%), and cortical membranes were intermediate with an E_{max} around 110%. In PND14 pups, E_{max} values for most brain regions were smaller than in adults (figure 4B) and regional differences were less pronounced, but hippocampus and thalamus still exhibited the largest disparity. One notable case is the cerebellum in which E_{max} increased more than two-fold from pups to adults and thus to a much larger degree than in other brain regions. The EC_{50} for CX546 also varied between brain regions, being highest in the hippocampus (4.2 mM) and lowest in thalamus and striatum (1.4 mM). However, adult cerebellum despite its high E_{max} exhibited an EC_{50} in the lower range (2 mM) and thus there is no clear correlation between EC_{50} and E_{max} values.

It was further examined if solubilization of brain AMPA receptors influences the effects of CX546. Solubilized AMPA receptors have higher affinity for agonists (Hall et al., 1992), but we have previously shown that ampakines are still effective in increasing agonist binding in the solubilized state (Kessler and Arai, 2006). For the present study, we used stringent solubilization with 5% Triton X-100 in order to effectively remove TARPs from the receptor (see Supplementary Figure S1-C,D). As shown in figure 4C, this greatly reduced the disparity in the CX546 effect between hippocampus and thalamus. The E_{max} values which had differed almost three fold in native membranes were 85% and 75% upon solubilization and were intermediate between those for native receptors. Likewise, after solubilization the EC_{50}s differed less than two-fold (302 and 596 μM). Interestingly, they were in a similar range as those measured with homomeric receptor, but lower than those obtained before solubilization. These results suggest that the AMPA receptors *per se* exhibit very similar ampakine profiles in these two brain regions and they support the notion that factors external to the receptors contribute in important ways to drug efficacy and potency.

AMPA-R subunit expression in hippocampus and thalamus. Since drug effects differed most prominently between hippocampus and thalamus, we further examined on Western blots the relative content of GluA1-4 in the membrane fractions used for the binding studies. As shown in figure 5, the relative abundance of the four subunits differed significantly between the two brain regions. In particular, GluA1 expression was negligible in thalamus and GluA4 was much more abundant relative to the other three subunits. If we tentatively assume that signal strength on blots is not very different for the various antibodies, then we can estimate that GluA4 accounts for about half of the AMPA

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receptor subunits in thalamus membranes but only about 7% in hippocampal membranes. Similar proportions have been reported for AMPA receptor transcript expression (Keinanen et al., 1990; Beneyto and Meador-Woodruff, 2004).

Relationship between binding and physiological properties according to AMPA receptor models. The present study was prompted in part by our earlier observations that drugs that are most effective in prolonging deactivation also cause very large increases in agonist binding. Receptor simulations were therefore used to examine if these basic relationships can be explained on theoretical grounds. Changes in the binding constant K_D and deactivation time constant tau_{deact} were calculated for several receptor models after systematically changing specific sets of rate constants. The findings are shown in figure 6 for a conventional single-ligand model (Kessler et al., 2008) and for the 2-ligand model employed by Sekiguchi et al. (2002). The latter was used because it may reproduce in good approximation the mode of operation of the presumed functional unit of the receptor, i.e., the subunit dimer (Sun et al., 2002), in particular because it takes into account the steep cooperativity in desensitization which structural information suggests to be a basic aspect of dimer operation (Sun et al., 2002). As shown, a consistent and large increase in binding affinity was seen with both receptor models if a reduction in the rate of agonist dissociation was combined with a reduction in the rate of desensitization. Slowing channel closing produced much smaller changes, especially in more complex models (figure 6B), and changes in desensitization rate constants alone caused a reduction in binding affinity (not shown, but see Hall et al., 1993). Condition 'c' in Figure 6C is reminiscent of the effects seen with CX546 in that a ten-fold slowing in response deactivation is accompanied by a significant increase in binding affinity and responses to prolonged agonist application become non-desensitizing. Similar results were obtained in simulations with other receptor models (not shown), including those proposed by Zhang et al. (2006) and Robert and Howe (2003). The latter was examined because it models the binding of four ligands, but its validity remains to be studied further because its predictions for basic binding properties seem to be at variance with actual binding data (see Kessler et al., 2008, for details). In any case, it must be emphasized that all simulation results are qualitative because actual receptor operation is probably more complex than described by any of these models. No attempts were made to simulate the interactions between ampakines and TARPs because the impact of the latter on kinetic rate constants is not yet understood in sufficient detail.

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DISCUSSION

Ampakine effects on recombinant receptors. A major conclusion from these studies has been that ampakine effects differ across receptor subunits and that they depend further on the TARP associated with the receptor. In comparisons between subunits, E_{max} was almost the same for GluA2 and GluA4 (~100%), but much lower for GluA1. For unknown reasons, data for transiently expressed GluA3 varied, but experiments with stably expressed GluA3 exhibited E_{max} values around 70% or higher (not shown). This suggests that low E_{max} is probably specific for GluA1. The second and perhaps more important observation has been that these efficacies are modulated by TARPs. Interestingly, the shifts in E_{max} were in opposite directions for GluA2 and GluA4 and resulted in overall differences as large as two-fold. Moreover there was a consistent pattern in comparisons between TARPs in that combinations with γ3 and γ8 always exhibited higher E_{max} than combinations with γ2 and γ4. TARPs have recently been shown to differ also in modulating various physiological response parameters (Milstein et al., 2007; Cho et al., 2007; Suzuki et al., 2008), but these effects tended to be similar for γ2 and γ3 on the one hand versus γ4 and γ8 on the other. Thus the pairwise grouping was different from the one seen here where E_{max} was largest for γ3 and γ8. This suggests that the influence on E_{max} is not secondary to a change in a previously characterized physiological parameter and that it represents a novel aspect of GluA-TARP interactions.

Ampakine effects on brain regions. Another finding of this study has been that there are major differences in the CX546 dose response relations between brain regions. Most notably, the E_{max} was almost three times larger in the hippocampus than in most subcortical regions, while cortex exhibited low to intermediate E_{max}. It should be noted that broad comparisons of this kind serve to characterize the dominant receptor populations and that it does not imply that all neurons in a region have identical properties. In fact, in physiological recordings the largest contrast in drug effects was seen between neurons within hippocampal area CA1. However, the interneurons and radiatum giant cells which exhibited minimal drug effects constitute only a minute percentage of the neurons in this area and our binding data reflect mainly the properties of receptors on pyramidal cells and dentate gyrus granule cells. Interestingly, the nearly three-fold difference in E_{max} between hippocampus and thalamus is reminiscent of our earlier observation that CX546 is about three times more effective in prolonging synaptic responses in hippocampal pyramidal cells compared to thalamic neurons (Xia et al., 2005). Although the numerical likeness may be coincidental, the finding that hippocampal pyramidal cell receptors consistently exhibited the highest drug efficacy in physiology and binding seems to point to a fundamental property of these receptors.

The differences between brain regions may be at least partially explained by our observations with recombinant GluA-TARP combinations. AMPA receptors on pyramidal neurons are usually calcium impermeable and hence contain GluA2, whereas receptors on interneurons and in subcortical regions are often enriched in GluA1 and GluA4 (Geiger et al, 1995; Beneyto and Meador-Woodruff, 2004). In agreement with this, hippocampal membranes

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contained a large proportion of GluA2 while GluA4 was dominant in thalamus membranes. With regard to TARPs, mRNA analysis and immunostaining have shown that γ3 and γ8 are preferentially expressed in hippocampus while γ2 and γ4 are abundant in the thalamus (Klugbauer et al., 2000; Tomita et al., 2003; Moss et al., 2003; Fukaya et al., 2006). Thus, receptors in hippocampal pyramidal cells can be assumed to contain GluA2 in association with γ3 and γ8, whereas receptors combining GluA4 with γ2 or γ4 are more representative of the thalamus. The fact that the E_{max} of the former combinations was almost twice that of the 'thalamic' combinations - and that GluA1 is too low in thalamus to make a significant contribution - is therefore likely to be at least partially responsible for the difference seen between hippocampal and thalamic membranes. Of course, a more comprehensive analysis would have to take into account contributions from GluA3 and from the flop counterparts of GluA1-4, as well as changes that may occur in heterotetrameric combinations of receptors which are more typical in brain (Lu et al., 2009). Nonetheless, our data suggest that the prevalence of the TARP subtype is an important factor for ampakine efficacy.

The expression patterns of receptor subunits and TARPs may also explain binding results in other regions. Many subcortical areas have a high density of GluA1 or GluA4 in combination with γ4 and should therefore exhibit low drug efficacy. This is the case for instance for striatum (high in GluA1 and γ4; Klugbauer et al., 2000; Lilliu et al., 2001; Tomita et al., 2003; Beneyto and Meador-Woodruff, 2004) and many brainstem areas (GluA4 plus γ2 and γ4; Condorelli et al., 1993; Petralia and Wenthold, 1992; Moss et al., 2003). Also, the general increase in E_{max} between PND14 and adulthood may reflect the decrease in γ4 occurring in most regions (Tomita et al., 2003; Fukaya et al., 2006). Lastly, the low ampakine efficacy seen in recordings from interneurons (Xia et al., 2005) could again be due to an abundant expression of GluA1 and GluA4 (Geiger et al., 1995; Catania et al., 1995; Leranth et al., 1996) and perhaps a higher expression of γ2 than in pyramidal cells (Fukaya et al., 2006).

Physiological significance of binding E_{max}. Monitoring drug effects on agonist binding was initially used as a practical way to assess drug potencies and to test for competitive interactions among subclasses of modulators (Kessler et al., 1996), but it subsequently became apparent that changes in agonist binding are also related to effects on fast responses. This seemed at first counterintuitive because binding is determined by the equilibrium distribution of receptor states. However, the dissociation constant K_D for agonists is a function of *all* rate constants and hence is modulated in principle by any factor that influences receptor operation (Ambros-Ingerson and Lynch, 1993). In practical terms, the K_D is determined mainly by the ratio of the rate constants for ligand unbinding and binding, multiplied by the ratio of resensitization and desensitization rates. In accord with this, we have shown that cyclothiazide, whose main action is to attenuate desensitization, lowers the affinity for agonists (Hall et al., 1993; Kessler and Arai, 2006). However, most other AMPA receptor modulators were found to *increase* the affinity for agonists (Kessler and Arai, 2006) and they have in common that they are more effective in slowing response deactivation. It has previously been suggested that these drugs stabilize the non-desensitized/agonist-bound receptor states by making them energetically more favorable

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(Nagarajan et al. 2001; Arai et al. 2002), and findings from recent structural analyses support this notion (Jin et al., 2005). This would cause a slowing in agonist dissociation, and hence response decay, and at the same time make transition to desensitized states less attractive. Our receptor simulations have shown that the parallel increase in binding affinity and deactivation time constants can be understood as an immediate consequence of this stabilization of the non-desensitized states. It should also be noted, though, that the change in agonist K_D caused by a modulator is expected to be smaller if a receptor has a stronger inherent desensitization, and this may potentially explain the smaller effect of CX546 on GluA1 whose resensitization rate is the slowest among subunits (Kessler et al., 2008). Whether this also entails a smaller drug effect on deactivation rates of GluA1 remains to be determined experimentally. In any case, however, TARPs are thought to influence mainly non-desensitized receptor states (Tomita et al., 2005) and thus variations in E_{max} across TARPs are not likely to be secondary to changes in desensitization.

The extent to which modulation of E_{max} by TARPs reflects differences in efficacy to slow response deactivation remains to be examined in physiological experiments. Stargazin (γ2) alters the influence of cyclothiazide on GluA1 receptors (Tomita et al., 2006) and thus it is likely that TARPs also modify the effects of ampakines, not least because they target similar aspects of receptor kinetics. Also, the correspondences we observed between hippocampus and thalamus support the notion that binding and physiological efficacies are related. Of course, it will be imperative to examine this proposition further by testing CX546 effects in other brain regions like cerebellum and striatum. One potential complication is that drug effects in some regions, like the cerebellum, may be less homogeneous between classes of neurons and may contain contributions from glia. Nonetheless, confirming our prediction that drug efficacy in striatum and brain stem regions is low would substantiate the notion that drug effects are especially large in hippocampal pyramidal cells and it would provide an explanatory framework for the observation that ampakines have been particularly successful in many hippocampus-dependent tasks (Hampson et al., 1998; Lynch, 2006).

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Determination of drug efficacy (E_{max}) in binding assays. **A**. In most binding assays of this study, the concentration of [^{3}H]FW (fluorowillardiine) was kept constant and the concentration of CX546 was varied between 0 and 6 mM. The binding data were then normalized to binding without drug and fitted with a sigmoidal function (bottom asymptote =100, n_{Hill}=1) to determine the EC_{50} for the drug (apparent drug affinity) and the maximum efficacy called E_{max}. The latter is calculated as the % increase over baseline binding in the absence of drug. **B**. Typical saturation assay for [^{3}H]FW in the presence and absence of 6 mM CX546 (GluA2). The data points were fitted with a hyperbolic equation. As illustrated by this example, CX546 increased the apparent affinity for the agonist but maximum binding remains unchanged (B_{max} = asymptote). It is also apparent that the ratio 'binding in the presence of drug' versus 'binding without drug' becomes smaller as the agonist concentration increases.

Figure 2. Effect of CX546 on AMPA receptor subunits without and with TARPs. **A**. HEK293 cells were transiently transfected with the flip variants of GluA1, GluA2 and GluA4. Binding to membranes prepared from these cells was measured using a filtration assay (see Methods). Binding was normalized to that without drug; the data are the mean and sem of 3 (A1), 6 (A2) and 4 (A4) experiments. The data were fitted as described in figure 1 and the binding parameters EC_{50} and E_{max} are summarized in table 1. [^{3}H]FW concentrations were 1 nM (A1), 4 nM (A2) and 51 nM (A4) and they were selected to be one fifth of the published K_D value for each subunit (determined at 0°C; Kessler and Arai, 2006); this concentration represents about one tenth of the K_D at the temperature used in this study (~24°C). Average binding without drug was (in pmol/mg protein): 0.137 ± 0.03 (A1), 0.275 ± 0.04 (A2) and 0.328 ± 0.03 (A4). **B,C**. GluA2 or GluA4 was transiently transfected into HEK293 cells alone or in combination with γ3 (A2) or γ4 (A4). The data at each drug concentration are averages of 6 (A2) and 4 (A4) experiments (mean and sem). Concentration-effect curves with and without TARP differed in each case significantly at $p < 0.001$ ($F(2,10) > 72$). The inset shows that the apparent affinity for FW was reduced slightly by γ3 (K_D = 79 nM for A2 plus γ3, versus 50 nM for A2 alone); the reduction was on average 1.5 ± 0.1 fold (n=3). Therefore an additional CX546 assay was done with GluA2 plus γ3 at a 1.5-fold increased FW concentration of 6 nM in order to maintain equidistance from the agonist K_D (dashed line, average of 2 experiments). The E_{max} value at this increased FW concentration was not significantly different from that at 4 nM (225 ± 11 % vs 228 ± 9 %)(t-test, p=0.9).

Figure 3. Systematic comparison of CX546 effects. The AMPA receptor subunits GluA (A1, A2, or A4) were transiently transfected into HEK293 cells alone or with each of four TARP variants and CX546 dose response curves were measured as described in figure 2. Values for E_{max} and EC_{50} are summarized in table 1. **A.** A1 and A1/TARPs at

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1 nM [^{3}H]fluorowillardiine. The CX546 effect was small and not significantly altered by the TARPs. **B.** A2 and A2/TARPs at 4 nM [^{3}H]fluorowillardiine. γ3 and γ8 significantly increased the E_{max} for CX546 by about 30% ($p \leq 0.001$). In contrast, γ2 and γ4 caused only a slight increase in efficacy and the difference from A2 alone was not significant. EC_{50} values were not significantly altered. **C**. A4 and A4/TARPs at 51 nM [^{3}H]fluorowillardiine. All four TARPs significantly decreased the E_{max} of CX546, but the changes relative to A4 alone were largest with γ2 and γ4. **D.** Comparison of E_{max} values for GluA2 and 4. Co-expression with γ2 and γ4 consistently produced a smaller E_{max} in both subunits compared to co-expression with γ3 and γ8. Statistical significance was determined by ANOVA (see table 1.)

Figure 4. Effects of CX546 on AMPA receptors in different brain regions. **A.** Effect of CX546 on [^{3}H]FW binding to membranes from eight brain regions of adult rats (3-5 months). Binding was measured at room temperature using a centrifugation assay. [^{3}H]FW was kept constant at 20 nM and the concentration of CX546 was varied between 0 and 6 mM. The data are averages from three separate experiments with two membrane preparations. Binding for each region was normalized to that in the absence of CX546. The percent values from three experiments were then averaged and are presented as mean and sem. The E_{max} and EC_{50} values and binding densities are summarized in table 2. **B.** Comparison of the same eight brain regions from rat pups (PND14). **C.** Drug effect on receptors from adult hippocampus and thalamus before and after solubilization with 5% Triton X-100 (30 min at 4°C; insoluble material removed by centrifugation). Binding to solubilized receptors was measured by a filtration assay and is the average of two separate preparations of hippocampus and thalamus. E_{max} and EC_{50} values are given in the text. Solubilization reduced the disparity in E_{max} between the brain regions and generally lowered the EC_{50}.

Figure 5. Expression of GluA1-4 in hippocampus versus thalamus. P2 brain membrane fractions were separated in 4-12% Bis-Tris Criterion XT Precast gels (Bio-Rad) and transferred to PVDF membranes. The blots were incubated with AMPA-R subunit specific antibodies followed by treatment with IRDye conjugated secondary antibodies (LI-CORE). Blots were visualized and quantified using the Odyssey imaging system (LI-CORE). Two separate membrane preparations were analyzed. The AMPA-R signal was normalized to actin and, for each subunit, all samples were quantified on the same blot. Statistical significance was determined by t-test. The normalized values for individual subunits from hippocampus were compared to the that of the corresponding receptor from thalamus (A1, p=0.015; A2, p=0.038; A3, p=0.004; A4, p=0.049). **A.** Representative Western blot of AMPA-R subunits in hippocampus and thalamus of adult rats. **B.** Average relative expression of AMPA-R subunits in thalamus compared to hippocampus. Bars indicate the standard error of the mean.

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Figure 6. Correlation between changes in binding and physiological parameters in receptor models. Binding constants K_D and deactivation time constants tau_{deact} were examined in a conventional single-ligand model (Kessler et al., 1996, 2008) (**A**) and in a 2-ligand model similar to that used used by Sekiguchi et al. (2002) **(B).** Simplified schemes of the models are shown at the top, with R indicating closed receptor states, O open states, and D desensitized states. The number following these letters indicates the number of agonists bound. Lines indicate transitions between states assumed to be significant. These transitions are governed by forward and reverse rate constants shown next to the line (left to right = above the line; right to left = below; downward = right side; upward = left side). Rate constants involving agonist association are expressed in $\mu Mol^{-1} * sec^{-1}$ and must be multiplied with the glutamate concentration in µM. All other rate constants are in sec^{-1}. To calculate binding, receptor state distributions after adding ligand were calculated iteratively until a stable steady-state was reached; binding at twelve agonist concentrations was then used to determine the dissociation constant K_D. In parallel, responses to 1 ms pulses of agonist were computed and fitted with exponential decay functions to obtain the deactivation time constant tau_{deact}. These calculations were then repeated after introducing progressively larger changes in certain rate constants in order to assess the impact on K_D and tau_{deact}. Two sets of changes in rate constants were examined in particular: (i) a reduction in the channel closing rate k_{close}, and (ii) a reduction in the agonist dissociation rate constant k_{dissoc} combined with a reduction in the desensitization rate constant k_{desens}. The factor by which each rate constant was reduced is indicated in the figure next to the symbol. In B, if two numbers are shown then the upper one indicates the slowing in all the rates of dissociation, the lower one the slowing in all the rates of desensitization. The impact on binding and physiology is shown as the factor by which the deactivation time constant (tau_{deact}) is increased (X-axis) and the factor by which the K_D constant is decreased (i.e., the factor by which binding affinity is increased; Y-axis). The changes produced in the 2-ligand model are illustrated further in **(C)** where 'a' indicates responses of and binding to the unmodified receptor and 'b' and 'c' represent receptors in which dissociation and desensitization rate constants are assumed to be slowed 10/100 times (b) or 20/400 times (c). The traces on the left show responses to a 1 ms pulse of glutamate, those at the middle responses to a 100 ms pulse of glutamate. Binding is shown in Scatchard format where 'bound' indicates the number of agonists bound per 100 receptors and the K_D is given by -1/slope of the regression line.

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Table 1. E_{max} and EC_{50} values for GluA / TARP combinations.

				Significance for E_{max} (p =)	
GluA	EC_{50} (μM)	E_{max} (%)	N	vs GluA alone	vs other TARPs
A1	2455 ± 1684	15 ± 4	3		
A1/γ2	838 ± 237	16 ± 3	3	ns	
A1/γ3	781 ± 208	23 ± 1	2	ns	
A1/γ4	164 ± 11	16 ± 4	3	ns	
A1/γ8	332 ± 124	14 ± 3	3	ns	
A2	652 ± 105	99 (± 7)	6		
A2/γ2	563 ± 109	103 ± 8	4	ns	
A2/γ3	603 ± 43	128 ± 7	6	0.001	0.011 vs γ2
A2/γ4	719 ± 135	112 ± 7	5	ns	
A2/γ8	724 ± 111	136 ± 8	4	<0.001	0.003 vs γ2; 0.017 vs γ4
A4	501 ± 106	101 (± 13)	4		
A4/γ2	508 ± 72	71 ± 8	4	0.002	
A4/γ3	582 ± 108	84 ± 4	4	0.04	
A4/γ4	655 ± 177	74 ± 4	4	0.003	
A4/γ8	430 ± 98	81 ± 10	4	0.02	

Summary of the binding data from recombinant receptors expressed in HEK293 cells (see figure 3). Binding to membranes from these cells was analyzed as described in Methods. Statistical significance was calculated by one-way ANOVA with LSD post hoc analysis. The E_{max} values of each recombinant receptor were compared to those of the corresponding co-transfections with TARPs. To account for variance between assays (GluA2 and 4 sets), data were normalized to the mean of the control (A2 or A4 without TARP). For individual tests, the control E_{max} value was subtracted from the mean control E_{max} value. The difference was then applied to the E_{max} values of the co-transfections that were assayed in parallel with the control. Analysis of variance of these corrected values indicates that the differences are statistically highly significant (A2i/TARPs, $F(4,20)=6.6$, $p=0.001$); A4i/TARPs, $F(4,15)=4.5$, $p=0.013$); the results of the post hoc analysis are shown in the table. Without normalization, some of the comparisons had p values above 0.05: A2/γ2 vs. A2/γ3 ($p=0.066$), A4 vs. A4/γ3 ($p=0.17$), and A4 vs. A4/γ8 ($p=0.11$). For GluA2 alone and GluA4 alone, the standard error shown in parenthesis was calculated before normalization.

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Table 2. Binding constants for CX546 in different regions of rat brain.

	fr-ctx	par-ctx	striatum	hippocamp.	thalamus	hypothal.	cerebellum	brainstem
E_{max} (%)								
adults	114 ± 5	104 ± 9	87 ± 4	189 ± 5	68 ± 6	80 ± 12	189 ± 1	91 ± 8
pups	71 ± 2	69 ± 7	81 ± 5	146 ± 9	59 ± 2	69 ± 7	84 ± 6	86 ± 3
ratio	*0.62*	*0.66*	*0.93*	*0.77*	*0.87*	*0.86*	*0.44*	*0.95*
EC_{50} (mM)								
adults	1.64 ± 0.1	1.54 ± 0.4	1.36 ± 0.2	4.21 ± 0.5	1.39 ± 0.3	1.71 ± 0.8	1.98 ± 0.1	2.58 ± 1.1
pups	1.58 ± 0.1	1.59 ± 0.2	1.97 ± 0.5	3.16 ± 0.5	0.95 ± 0.3	1.69 ± 0.1	1.33 ± 0.2	1.32 ± 0.3
ratio	*0.96*	*1.03*	*1.45*	*0.75*	*0.68*	*0.99*	*0.67*	*0.51*

Binding to brain membranes was measured at room temperature for the eight indicated brain regions (n=3); fr-ctx = frontal cortex, par-ctx = parietal cortex. A constant concentration of [^{3}H]fluorowillardiine (20 nM) was used with addition of 0-6 mM CX546, as described in figure 4. The data were transformed and fitted with a sigmoidal curve to determine E_{max} (expressed as percent increase from control) and the potency of CX546 (EC_{50}). 'ratio' indicates the ratio 'pup' / 'adult'.

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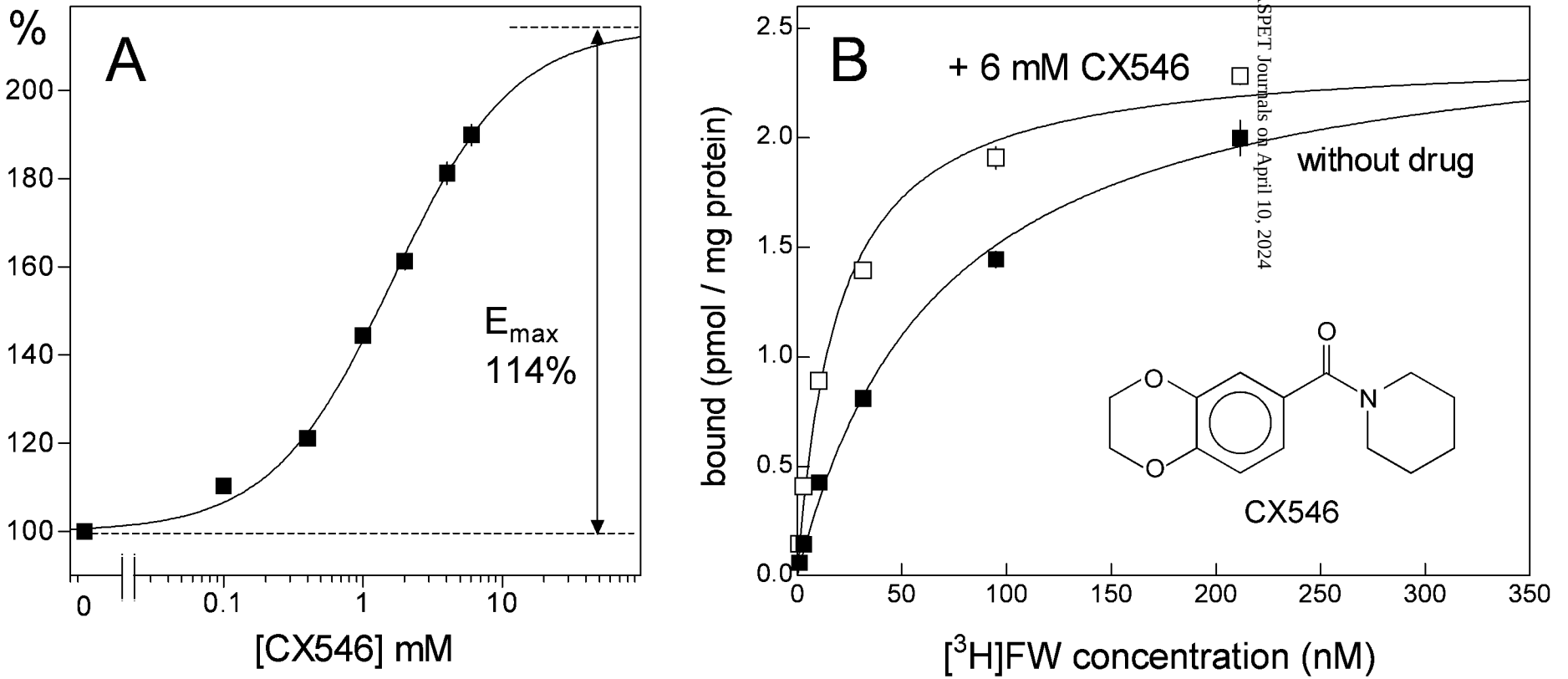

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Figure 1

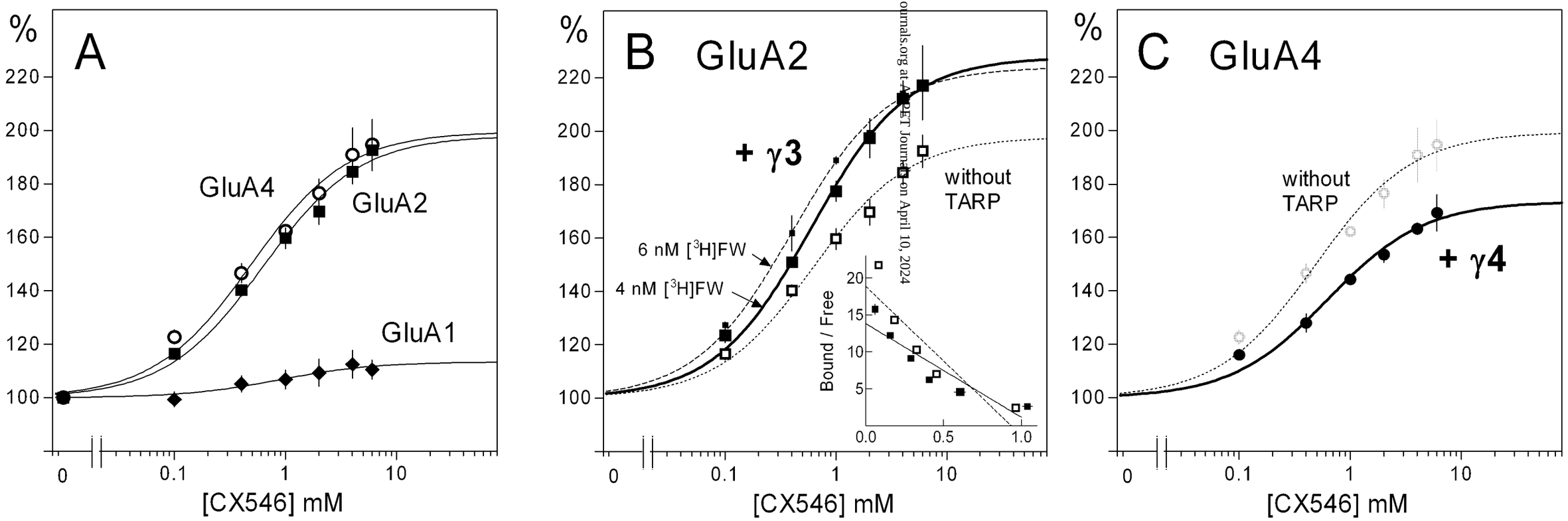

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Figure 2

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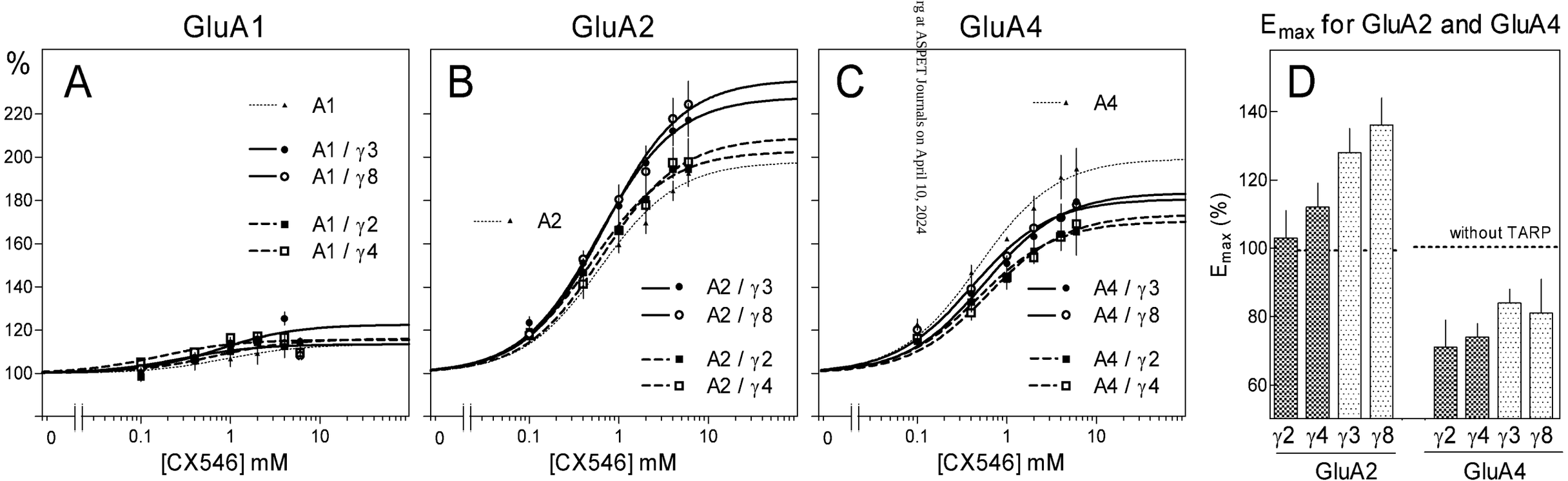

Figure 3

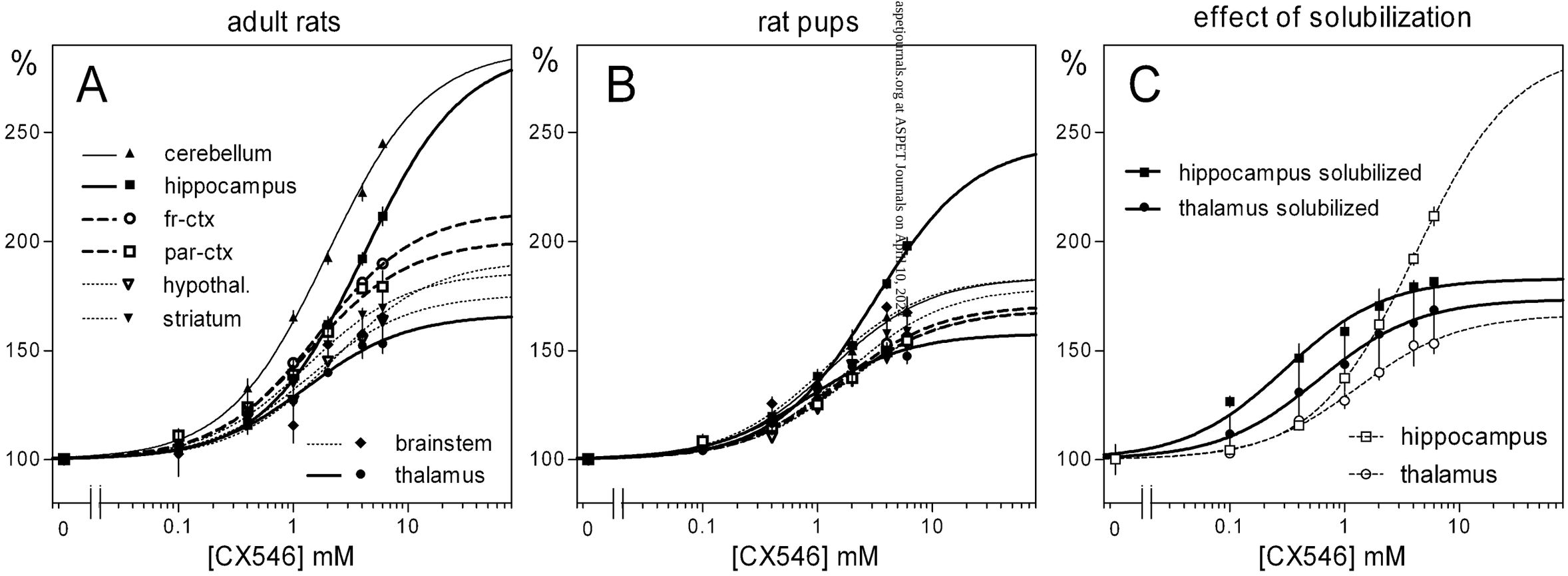

Figure 4

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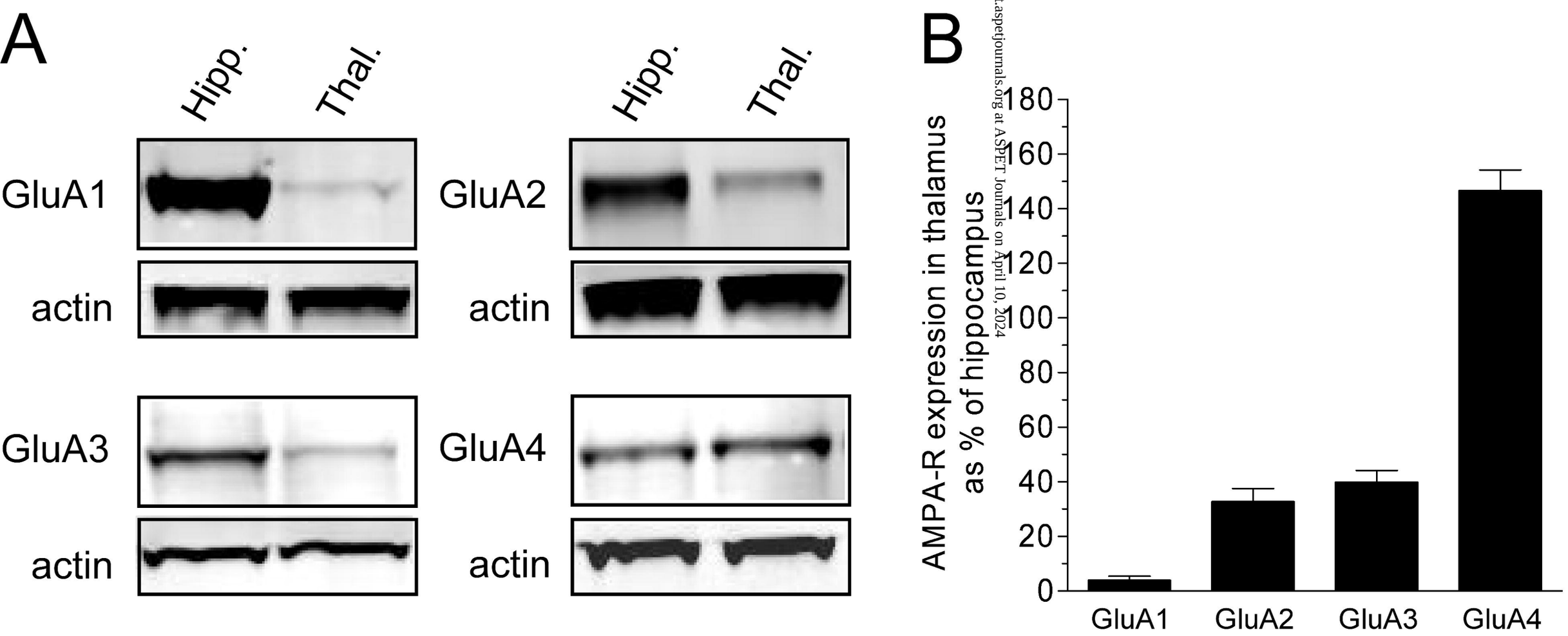

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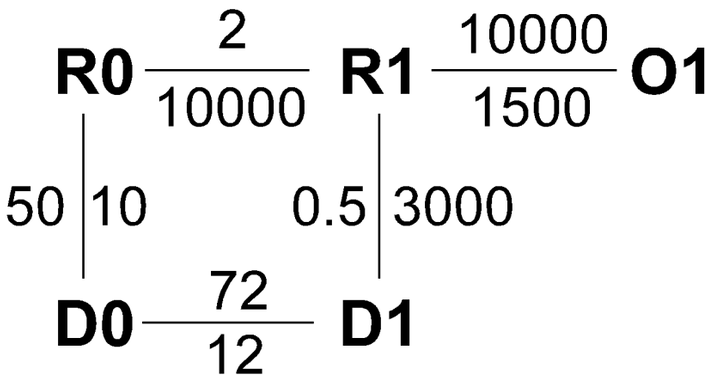
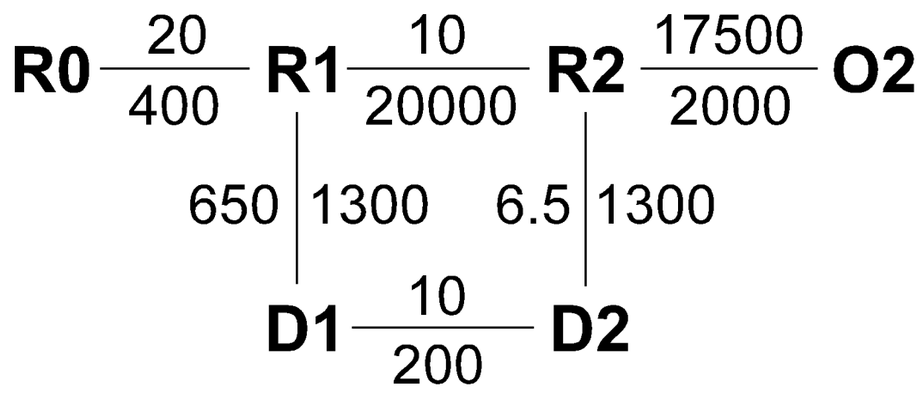
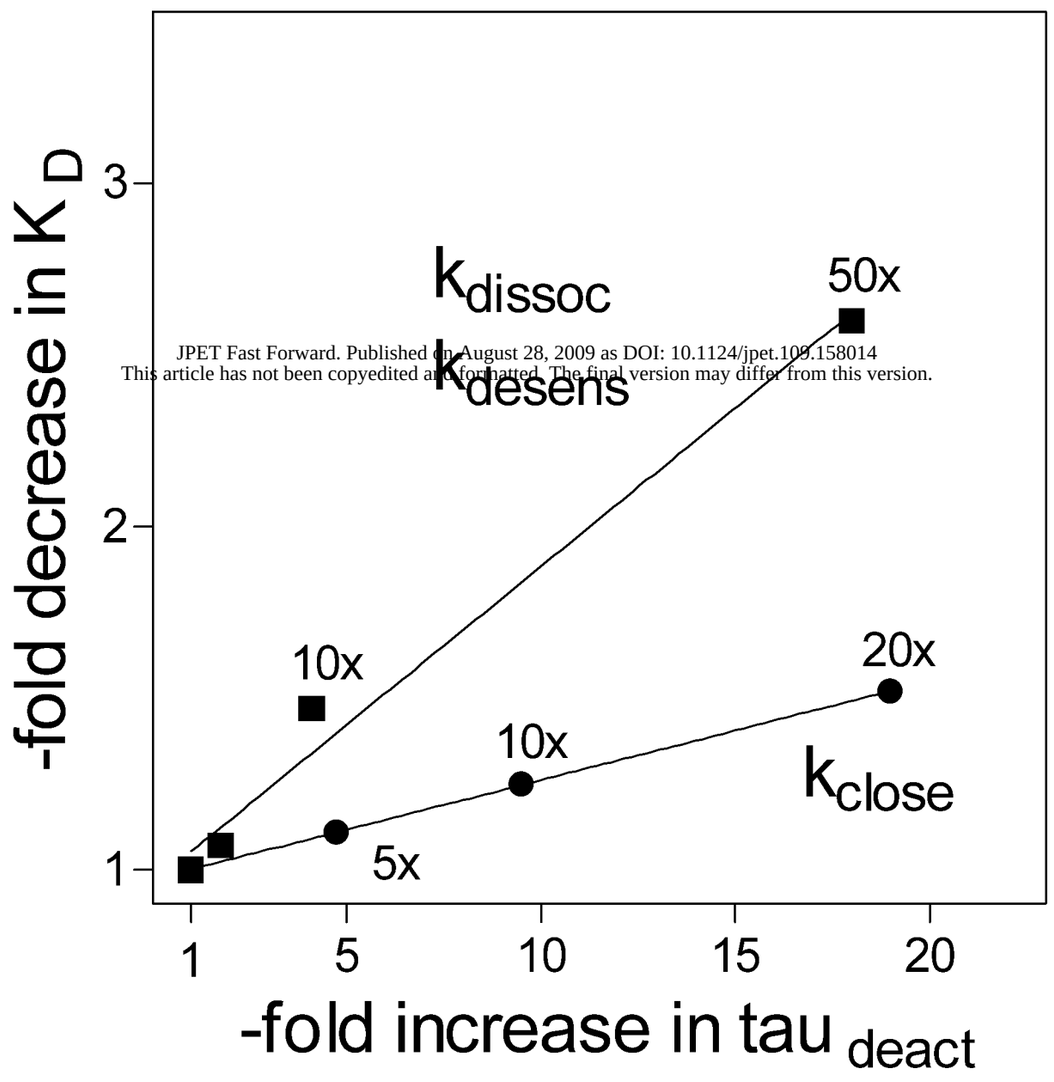
Figure 5

A 1-ligand model



B 2-ligand model





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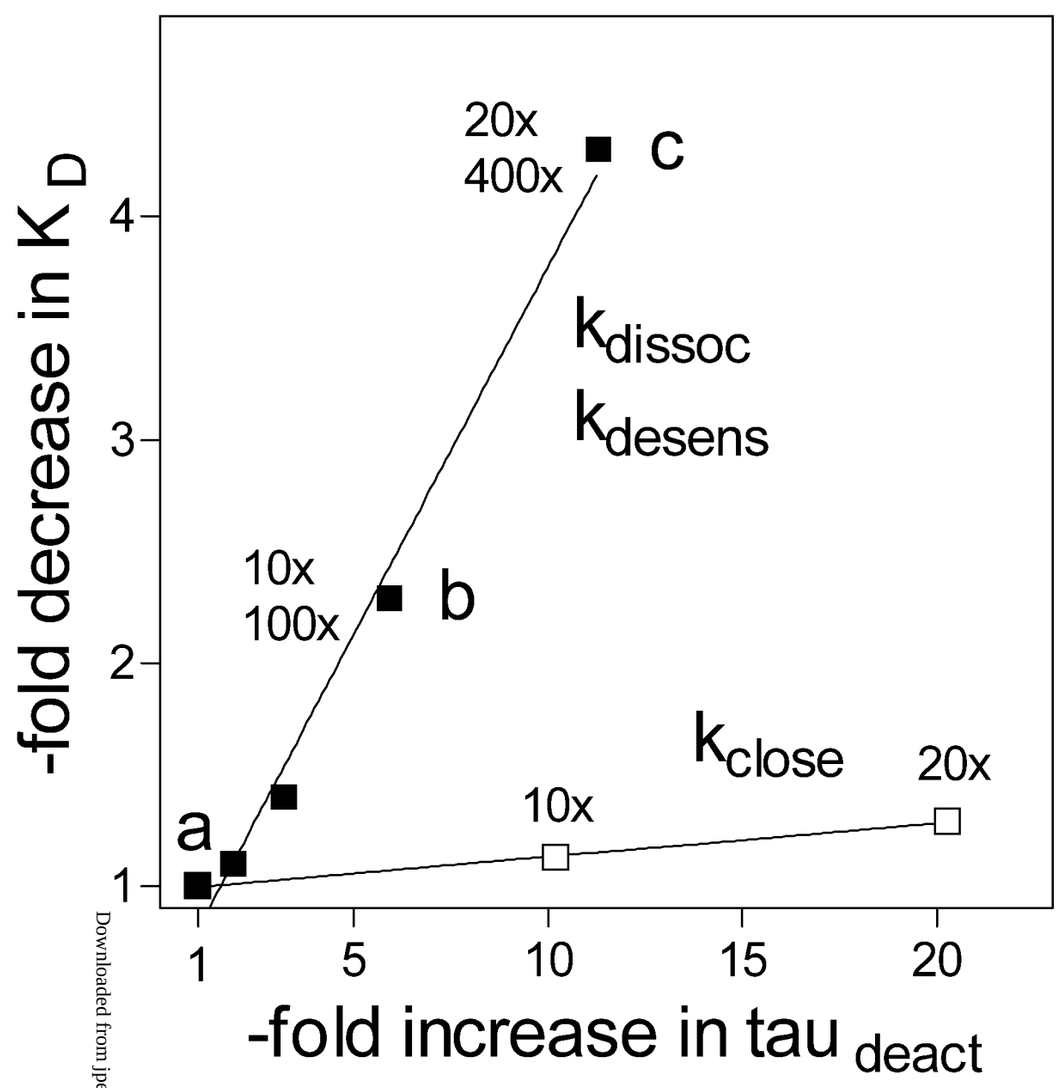

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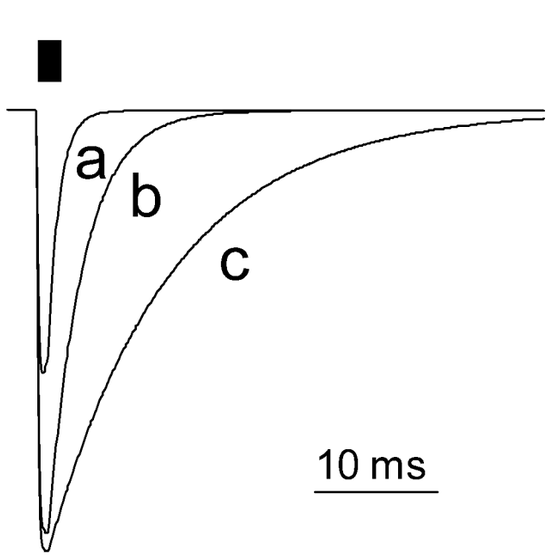

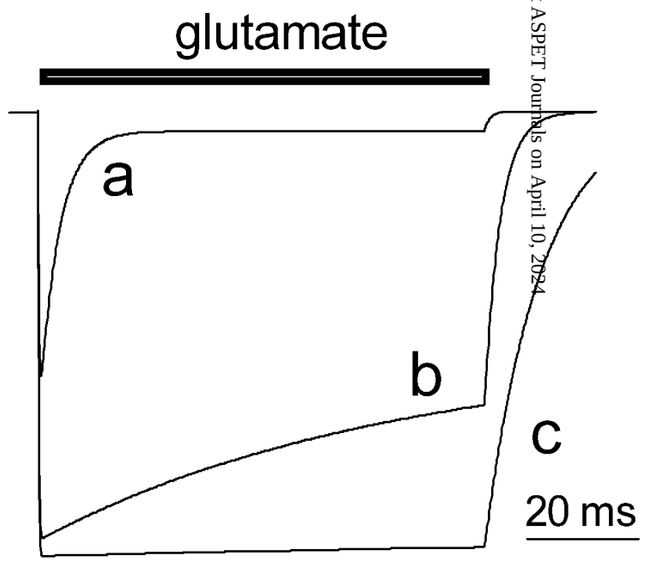

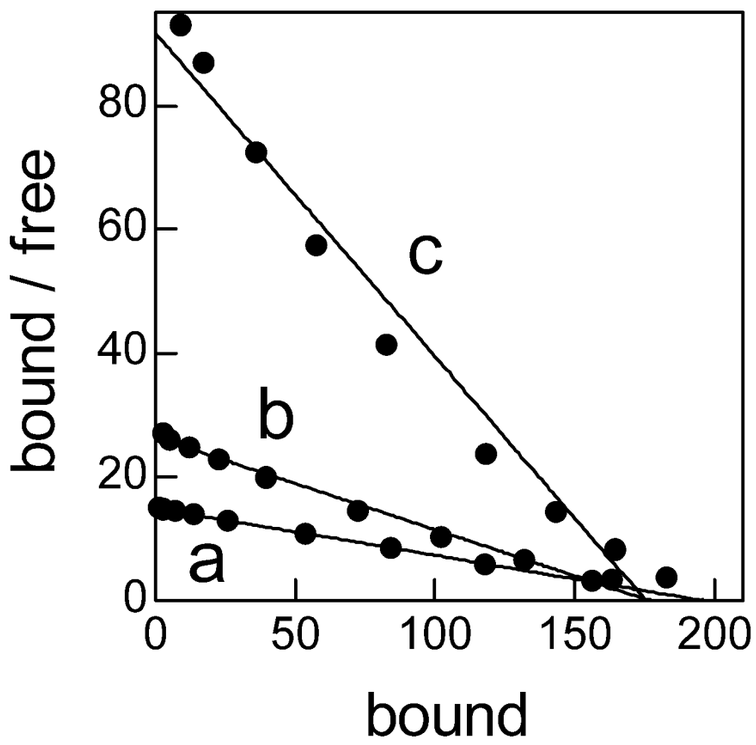

Figure 6