Supporting Information

Role of Stoichiometry in the Dimer-Stabilizing Effect of AMPA Receptor Allosteric Modulators

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The main article explores the differences in dimerization between 1 and 2 modulator/dimer models in the context of experimental data for thiazide modulators of the GluA2 ligand-binding domain. In the supporting information, we describe the detailed methodologies used in this work (Figures S1 and S2) and include a discussion of models of dimerization (Equations S1 to S27 and Figures S3 to S6) to support our conclusions.

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Visualization of the Modulator-Binding Cavity

The modulator-binding pocket was generated to depict the volume located at the dimer interface that is known to be accessible to positive allosteric modulators. Fifteen GluA2 structures that include bound modulators were aligned for the residues sequence selections, KPMFS and SKGYG, on both chains of the dimer using UCSF Chimera’s MatchMaker Tool. The alignment was repeated for all chain arrangements (i.e., A:B dimer aligned with B:A dimer for the same modulator). The fifteen modulators include thiazide-type modulators: 1) cyclothiazide (CYTZ) [PDB: 1LBC], 2) hydroflumethiazide (HFMZ) [PDB: 3ILU], 3) trichlormethiazide (TCMZ) [PDB: 3ILT], 4) IDRA-21 [PDB: 3IL1], 5) chlorothiazide [PDB: 3IK6], 6) hydrochlorothiazide [PDB: 3IJX], 7) althiazide [PDB: 3IJO], 8) NS1493 [PDB: 3H6U], 9) NS5206 [PDB: 3H6V], 10) NS5217 [PDB: 3H6W], A-subsite binding modulators: 11) aniracetam [PDB: 2AL5], 12) CX614 [PDB: 2AL4], and full-spanning sulfonamide modulators: 13) dimeric biarylsulfonamide [PDB: 3BBR], 14) PEPA [PDB: 3M3L], 15) LY404187 [PDB: 3KGC]. The composite of modulators was combined into a single file and surfaced in UCSF Chimera.

Fit of BioSAXS data

Figure S1. Experimental SAXS data for 0.1 mM LBD in the presence of 0.15 mM CYTZ. The Oligomer program from the ATSAS suite was used to fit the data to a mixture of theoretical CRYSOL-derived scattering curves. Oligomer generated a best fit curve (red) by optimizing the mixture of theoretical scattering curves for the monomer (based on chain A from the GluA2 LBD PDB: 1FTJ) and the dimer (based on chain A and C from the GluA2 LBD PDB: 1FTJ). To visually illustrate the difference between the curves, Oligomer fits were generated using the following pairs of theoretical curves: monomer-dimer.
(red), monomer-monomer (green), and dimer-dimer (blue). A mixture of 50.4% dimer and 49.6% monomer produced the minimum discrepancy between the experimental and calculated scattering curves.

Figure S2. Experimental SAXS data for a LBD dimerization series that includes 0 mM, 0.2 mM, and 2 mM HFMZ concentrations. The data are shown for (A) 0.03 mM, (B) 0.06 mM, and (C) 1.0 mM LBD concentrations. The experimental data (open circles), monomer fit (green), dimer fit (blue), and monomer-dimer mixture fit (red) are displayed in each HFMZ-LBD concentration plot. The dimerization data are summarized in Figure 4A.
Fit of data to models

The data sets were collected over two synchrotron runs, one consisting of modulator concentration-dimerization curves at three ligand-binding domain (LBD) concentrations and the other consisting of a more extended modulator concentration-dimerization curve at a single LBD concentration and a LBD concentration-dimerization curve at a single modulator concentration. All five data sets were fit simultaneously to Equations S6 and S14 using a SIMPLEX algorithm to minimize the sum of squares. The equations are all in terms of the free modulator concentration (L), but only the total modulator concentration is known from the data. For each iteration in the minimization process, a full dimerization curve (1000 points) was generated, and the total modulator concentration (free plus bound) was determined for each point. A linear interpolation was then used to determine a calculated fraction of dimer for each total modulator value in the data set, which in turn allowed the calculation of the sum of squares. The data were well fit with a single $K_4$ for all three modulators, and $K_3$ values specific to each modulator. The $K_3$ values were well determined by the data, but fitting using fixed values of $K_4$, suggested that the value was not well constrained by the data, in that reasonable fits could be obtained for values of $K_4$ between 20 and 200 mM, with a shallow minimum at approximately 40 mM.

Mechanisms of Allosteric Modulator Binding

Allosteric modulators can bind to the symmetrical dimer interface on GluA2 at either one binding site or two, depending upon how the modulator is oriented in the subsites of the binding surface. For the purposes of this discussion, the signal will be the dimer of two LBD either in solution or in the intact receptor. Fitting data to a full model including binding to both monomer and dimer did not produce significantly better fits than fitting to a model assuming binding to the dimer alone, so the subsequent discussion will assume binding to the dimer. A more detailed treatment of the differences between models is given at the end of this section. The presence of agonist will be assumed in all cases. This is relevant mainly to the intact receptor in that, in presence of agonists and in the absence of modulator at equilibrium, the nondimer form is favored. For the LBD in the absence of modulator, equilibrium strongly favors the monomer.

In the case of the intact receptor, the formation of the dimer interface can be viewed as an equilibrium between two forms of the receptor: $R'$ is the desensitized form where the dimer interface is disrupted and $R$ is the form with the intact dimer interface. The importance of this is that the binding of a modulator is independent of receptor concentration. In the case of the LBD, the dimer is formed by the binding of two proteins ($R + R$), so that the dimerization would be dependent upon the concentration of the LBD. Each of the four cases will be presented followed by a comparison of the predicted results.

One binding site per dimer interface, intact receptor: The binding mechanism, assuming no binding to dissociated dimer ($R_2'$), is given by:

$$R_2' \rightleftharpoons_{K_4} R_2 + L \rightleftharpoons_{K_3} R_2L$$  \hspace{1cm} \text{(S1)}$$

Solving for the fraction of receptors with intact interface ($R_2 + R_2L$):
Where, \( \tilde{R} \) is \((R_2 + R_2L)/(R_2 + R_2L + R_2')\) and \(L\) is the free concentration of modulator.

**One binding site per dimer interface, LBD:** The binding mechanism, assuming no binding to the monomer \((R)\), is given by:

\[
R + R \xrightleftharpoons{k_4} R_2 + L \xrightleftharpoons{k_3} R_2L
\]

Solving for the fraction of dimer:

\[
\tilde{R} = \frac{1}{K_4 \left(1 + \frac{L}{K_3}\right)} \left(1 + \frac{1}{K_4 \left(1 + \frac{L}{K_3}\right)}\right)
\]

or

\[
\tilde{R} = \frac{2}{K_4 \left(1 + \frac{L}{K_3}\right)} \left(1 + \frac{2}{K_4 \left(1 + \frac{L}{K_3}\right)}\right)
\]

Where, \( \tilde{R} \) is the fraction of dimeric receptors \((2R_2 + 2R_2L)/(2R_2 + 2R_2L + R)\), \(R\) is the free concentration of the monomeric form of the LBD, and \(L\) is the free concentration of modulator. The factor of two arises from the fact that two individual monomers make up the dimer. Because the concentration of \(R\) is dependent upon \(L\), this form of the equation is not very useful. Using the quadratic equation, however, it can be expressed in terms of the total receptor concentration expressed as a monomer \((R_t)\):

\[
R_t = R + 2R_2 + 2R_2L
\]

Solving for the fraction of associated dimer \((R_2 + R_2L + R_2L^2)\):

\[
\frac{1}{R} = \frac{1 + \sqrt{1 + \frac{8R_t \left(1 + \frac{L}{K_3}\right)}{K_4 \left(1 + \frac{L}{K_3}\right)}}}{1 + \sqrt{1 + \frac{8R_t \left(1 + \frac{L}{K_3}\right)}{K_4 \left(1 + \frac{L}{K_3}\right)}}}
\]

**Two binding sites per dimer interface, intact receptor:** The binding mechanism, assuming no binding to dissociated dimer \((R_2')\), is given by:

\[
R_2' \xrightleftharpoons{k_4} R_2 + L \xrightleftharpoons{k_3} R_2L + L \xrightleftharpoons{k_2} R_2L_2
\]

Assuming no cooperativity of modulator binding:

\[
R_2' \xrightleftharpoons{k_1} R_2 + L \xrightleftharpoons{0.5k_3} R_2L + L \xrightleftharpoons{2k_3} R_2L_2
\]

Solving for the fraction of associated dimer \((R_2 + R_2L + R_2L_2)\):
Where, \( \bar{R} \) is the fraction of dimer and \( L \) is the free concentration of modulator.

**Two binding sites per dimer interface, LBD:** The binding mechanism, assuming no binding to dissociated dimer, is given by:

\[
R + R \xleftarrow{K_4} \rightarrow R_2 + L \xleftarrow{K_3} \rightarrow R_2L + L \xleftarrow{K_2} \rightarrow R_2L_2
\]  
\( S10 \)

Assuming no cooperativity of modulator binding:

\[
R + R \xleftarrow{K_4} \rightarrow R_2 + L \xleftarrow{0.5K_4} \rightarrow R_2L + L \xleftarrow{2K_4} \rightarrow R_2L_2
\]  
\( S11 \)

Solving for the fraction of dimer:

\[
\bar{R} = \frac{2R}{K_4 \left( 1 + 2L + \frac{L^2}{K_3} \frac{K_4}{K_3} \right)} \quad \text{or} \quad \bar{R} = \frac{2R}{K_4 \left( 1 + 2L + \frac{L^2}{K_3} \frac{K_4}{K_3} \right)}
\]  
\( S12 \)

Where, \( \bar{R} \) is the fraction of dimer, \( R \) is the monomeric form of the LBD, and \( L \) is the free concentration of modulator. The factor of two arises from the fact that two individual monomers make up the dimer. Again the concentration of \( R \) is dependent upon \( L \); but the quadratic equation, however, it can be expressed in terms of the total receptor concentration expressed as a monomer (\( R_t \)):

\[
R_t = R + 2R_2 + 2R_2L + 2R_2L_2
\]  
\( S13 \)

\[
\bar{R} = \frac{-1 + \sqrt{1 + \left( \frac{8R_t}{K_4} \left( 1 + 2L + \frac{L^2}{K_3} \frac{K_4}{K_3} \right) \right)}}{1 + \left( \frac{8R_t}{K_4} \left( 1 + 2L + \frac{L^2}{K_3} \frac{K_4}{K_3} \right) \right)}
\]  
\( S14 \)

**Comparison of one site vs. two sites, intact receptor:** The important point to note is that the dimerization, rather than the binding of modulator, is measured, so that the signal arises both from the modulator-free dimerized state as well as the modulator-bound dimerized state. Considering first the one site model for the intact receptor (equation \( S1 \)), the level of dimerization in the absence of modulator is given by \( 1/(K_4+1) \), which is \( \bar{R} = A \) in Figure S1; and 50% dimerization is reached at a free modulator concentration equal to \( K_3(K_4-1) \). This formulation only makes sense for \( K_4 \) greater than 1, since when \( K_4 \) is equal to 1, the receptor is already 50% dimerized in the absence of modulator. What we really want to know is the half-maximal effect of the modulator, that is, the modulator concentration that gives a degree of dimerization halfway between the dimerization in the absence of modulator and complete
dimerization (\( R = A + 0.5*B \) in Figure S1). We can call this the EC\(_{50}\) and it is reached at a free modulator concentration equal to \( K_3(K_4+1) \). The Hill slope is one in all cases. With the possibility of binding to two sites (equation S8), only one of which is required to stabilize the dimer (i.e., the dimer cannot dissociate when either one or two modulators are bound), the dimerization curve as a function of modulator concentration exhibits an apparent positive cooperativity (binding is not cooperative) and 50% dimerization is reached at a modulator concentration of \( K_3\left(\sqrt{K_4} - 1\right) \) for \( K_4 \geq 1 \), and the EC\(_{50}\) is \( K_3\left(\sqrt{K_4 + 2} - 1\right) \). Note that apparent positive cooperativity arises from the stabilization of the dimer by occupation of one or two of the binding sites and that binding of two modulators rather than one increases the apparent affinity from \( K_3(K_4+1) \) to \( K_3\left(\sqrt{K_4 + 2} - 1\right) \). In these two mechanisms, the constant \( K_4 \) is unitless, so that the units of EC\(_{50}\) remain molar.

**Comparison of the concentration dependence of the intact receptor and LBD:** From equations S6 and S14, the EC\(_{50}\) for dimerization of the LBD can be determined in terms of the equilibrium constants. The difference between the LBD and the intact receptor is that the EC\(_{50}\) and the dimerization in the absence of modulator for the LBD is also dependent upon the total receptor concentration. The dimerization in the absence of modulator, is given by:

\[
\bar{R} = \frac{-1 + \sqrt{1 + \frac{8R_t}{K_4}}}{1 + \sqrt{1 + \frac{8R_t}{K_4}}} \tag{S15}
\]

This is the same whether one or two binding sites are present. Considering first one binding site model, the point of 50% dimerization is the modulator concentration equal to:

\[
K_3\left(\frac{K_4}{R_t} - 1\right) \tag{S16}
\]

for \( K_4/R_t > 1 \). The EC\(_{50}\) is somewhat more complex:

\[
K_3\left(4 + \frac{K_4}{2R_t} \left(1 + \sqrt{1 + \frac{8R_t}{K_4}}\right)\right) - 1 \tag{S17}
\]

Unlike the case of the intact receptor, the EC\(_{50}\) is dependent upon the total concentration of the LBD (\( R_t \)), with a decreasing EC\(_{50}\) as the concentration of LBD increases. Note that the 4 and -1 in S17 can be simplified to 3, but it is left in this form for comparison to equation S19.
For the two binding site model ($K_4/R_t > 1$), the point of 50% dimerization is the modulator concentration equal to:

$$K_3 \left( \frac{K_4}{R_t} - 1 \right)$$

The EC$_{50}$ for the two-site model is:

$$K_3 \left( \sqrt{\frac{4 + \frac{K_4}{2R_t}}{1 + \frac{8R_t}{K_4}}} - 1 \right)$$

Clearly, the EC$_{50}$ for the two-site model will always be lower than that of the one-site model.

**Apparent cooperativity of modulator-dependent dimerization**: A measure of cooperativity is the modulator concentration required to produce 80% dimerization (EC$_{80}$) divided by EC$_{20}$. EC$_{80}$ and EC$_{20}$ are measured between dimerization in the absence of modulator and full dimerization (see Figure S1). If one considers a simple Hill equation, this ratio would be 16 for a Hill slope of one. A value higher than this would be consistent with apparent negative cooperativity and a value less than this, with apparent positive cooperativity. As expected for the one binding site model for the intact receptor, the ratio is 16:

$$\frac{EC_{80}}{EC_{20}} = \frac{4K_4(K_4 + 1)}{K_3(K_4 + 1)} = 16$$

For the two-site model with the intact receptor, apparent cooperativity is always positive (ratio is between 10.472 and 4, for $K_4 = 0$ to $K_4$ approaches infinity) and dependent upon $K_4$ (i.e., the ratio decreases and apparent positive cooperativity increases with increasing $K_4$):

$$\frac{EC_{80}}{EC_{20}} = \frac{\sqrt{1 + 4(K_4 + 1)} - 1}{\sqrt{1 + \frac{1}{4}(K_4 + 1)} - 1}$$

Considering now the LBD with one binding site, the cooperativity depends upon both $R_t$ and $K_4$:

$$\frac{EC_{80}}{EC_{20}} = \frac{K_4}{8R_t} \left( 5 \sqrt{1 + \frac{8R_t}{K_4}} + 5 \right) \left( 5 \sqrt{1 + \frac{8R_t}{K_4}} + 3 \right) - 1$$

If $R_t > K_4$, the ratio approaches 42.6667, and if $K_4 > R_t$, the ratio approaches 64 (apparent negative cooperativity in all cases). If $R_t > K_4$, the LBD is essentially dimeric in the absence of allosteric modulator.

Considering now the LBD with two binding sites, the cooperativity also depends upon both $R_t$ and $K_4$.
If $R \gg K_4$, the ratio approaches 16 (no cooperativity), and if $K_4 \gg R$, the ratio approaches 8 (apparent positive cooperativity).

**Rationale for the use of the model assuming binding to the dimer:** Assuming a one-binding site cyclical model, the modulator-bound dimer can be reached either by dimerization of the LBD (i.e., $R$) followed by binding to the dimer (Path A) or binding to the monomer followed by dimerization with an modulator-free LBD (Path B). Both Path B and the cyclical model would predict a decrease in dimerization at high modulator concentrations because the concentration of free $R$ would become depleted and not be available to bind to RL.

The dimerization functions for the three models are:

**Cyclical model:**

$$\bar{R} = \frac{-\left(1 + \frac{L}{K_1}\right) + \left(1 + \frac{L}{K_3}\right) \sqrt{1 + \frac{8R_1}{K_4}} \left(1 + \frac{L}{K_1}\right)}{1 + \frac{8R_1}{K_4} \left(1 + \frac{L}{K_3}\right)}$$

**Path A:**

$$\bar{R} = \frac{-1 + \left(1 + \frac{L}{K_1}\right)}{1 + \sqrt{1 + \frac{8R_1}{K_4} \left(1 + \frac{L}{K_3}\right)}}$$

**Path B:**

$$\bar{R} = \frac{-\left(1 + \frac{L}{K_1}\right) + \left(1 + \frac{L}{K_3}\right) \sqrt{1 + \frac{8R_1}{K_4} \left(1 + \frac{L}{K_1}\right)}}{1 + \frac{8R_1}{K_4} \left(1 + \frac{L}{K_3}\right)}$$

For Path B, no dimerization would be expected in the absence of modulator, but for Path A and the full model, the dimerization in the absence of modulator is:

$$\bar{R} = \frac{-1 + \sqrt{1 + \frac{8R_1}{K_4}}}{1 + \sqrt{1 + \frac{8R_1}{K_4}}}$$

For Path A, the fraction of dimerized protein should approach 1 monotonically as the modulator concentration increases (Figure S3A). For Path B and the cyclical model, the dimerization is nonmonotonic, increasing up to a point ($L = K_1$) and then decreasing to zero at infinite modulator concentration.
Depending upon the values of $K_1$, $K_3$ and $L$, the cyclical model follows Path A, Path B, or a combination of the two (Figure S3C). The data obtained with SAXS are monotonic, with the caveat that the concentration of allosteric modulator is limited by solubility. The question then is whether the modulator can bind to the monomer (Path B), dimer (Path A) or both (Cyclical Model). A strict adherence to Path B is unlikely because some dimer is present in the absence of allosteric modulator and micromolar binding of modulator has been measured to a constitutive dimer. For the HFMZ data, the maximum for Path B would have to be greater than 10 mM (on the order of 50 mM or greater). Since the maximum is dependent upon $K_1$, this restrains the value of $K_1$ for this model to 50 mM or greater. In order to place the maximum near one, the $K_2$ equilibrium would have to favor the dimer (i.e., $K_2$ would have to be small relative to $R_i$). If we then allow binding to both monomer and dimer, the relationship between $K_3$ and $K_4$ is fixed because $K_1K_2 = K_3K_4$. Since the degree of dimerization in the absence of modulator is small, $K_4$ must be large relative to $R_i$, at least on the order of several mM or greater (Sun et al. estimated the value to be 6 mM using analytical ultracentrifugation). Assuming that a small fraction of dimer exists in the absence of allosteric modulator, then the value of $K_3$ would have to be low relative to $K_1$. Thus, binding to the preformed dimer would be of higher affinity than binding to the monomer. In this regime, the predictions of the three models are very similar, with Path A being monotonic and Path B and the cyclical model peaking at 50 mM or greater (Figure S3D). The data can be effectively and most simply modeled with the equations for Path A, realizing that low affinity binding to the monomer remains a possibility.
Figure S5. (A) Theoretical curve for Path A showing the relationship between the constants in the dimerization equation and important points on the dimerization curve. (B) Theoretical curve for Path B showing the relationships between the constants in the dimerization equation and the important points on the dimerization curve. The free modulator concentration for the peak of the curve was determined from the derivative of the dimerization curve with respect to free modulator. (C) and (D) Predicted curves for the three models using different equilibrium constants. Because of detailed balance, only three of the four equilibrium constants are unique.

**Extension to two binding site model:** The analysis above is based on the one binding site model. Equations of the same form can be extended to the two binding site model. For this, at least two possibilities exist when considering binding to the monomer (Figures S6A & B). The first possibility would be to assume that the modulator bound only to one of the two halves of the monomer. For example, we might assume that the modulator could only bind to one half of the two symmetrical sites on the dimer interface (Site 1 or 2 in Figure S6C). In this case, the model is relatively simple (Figure S6B), with three unique equilibrium constants (assuming no cooperativity in binding); however, the curves have the same form for Path A, Path B and the full model. This would not be diagnostic for determining if binding can occur to the monomer.
The more general possibility would be that the modulator could bind to either site on the monomer with different affinities. This would result in a model with twelve equilibrium constants (Figure S6A), which could be reduced to eleven with the assumption of no cooperativity of binding. With consideration of detailed balance, the number of unique constants can be reduced to five. Using this model, it is possible to generate curves that are nonmonotonic, but the SAXS data were fit well to a model with two adjustable constants and would not be well constrained with a model using five adjustable constants. The main difference between the one binding site model and the two binding site model is that the possibility of autoinhibition for the one binding site model arises simply from the possibility that the modulator can bind to the monomeric form. For the two binding site model, autoinhibition would not occur in the absence of binding to the monomer or if binding to the monomer were exclusively to one of the two sites (Figure S6B). Only by assuming that both of the sites (sites 1 and 2 in Figure S6C) can be occupied in the monomer, can autoinhibition be generated.

Figure S6. Models for modulators binding to two sites per dimer interface. (A) A full cyclical model which allows binding to either half of a monomer. The full model requires 12 equilibrium constants. The states are described structurally in part C. The portion of the model in black is the same as part B. (B) Model assuming that the modulator can only bind to one of two sites on the monomer. (C) Structural representation of each state in the model. The binding surface for modulators has two symmetrical binding sites. A monomer has two halves of each site. The possibility exists that the modulator could bind to either half of the site (RL or RL'), and if it binds to both halves in the monomer, two modulators could be bound to a monomer (RL\textsubscript{2}). If RL or RL' can exist, then only RL can bind to RL to form R\textsubscript{2}L\textsubscript{2} and likewise only RL' and RL' can bind, which would also from R\textsubscript{2}L\textsubscript{2}. Also, RL\textsubscript{2} can bind to a free R to form R\textsubscript{3}L\textsubscript{2}.
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