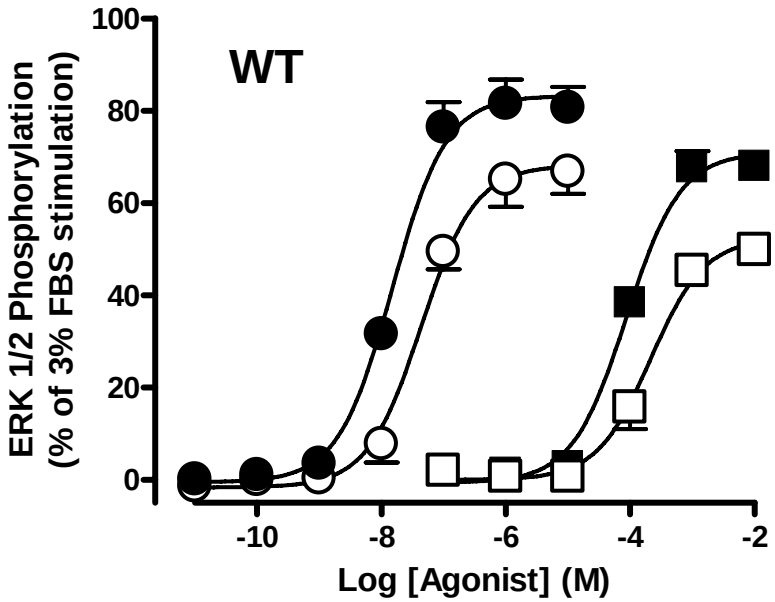
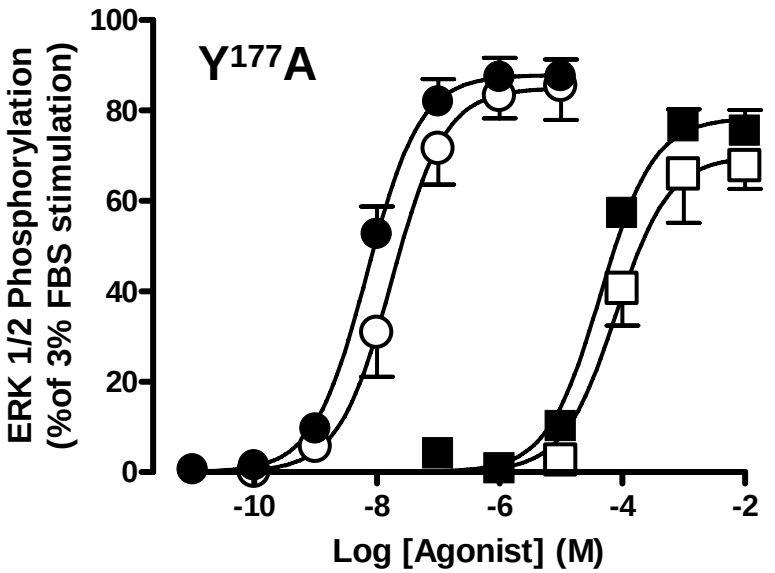
SUPPLEMENTARY FIGURES

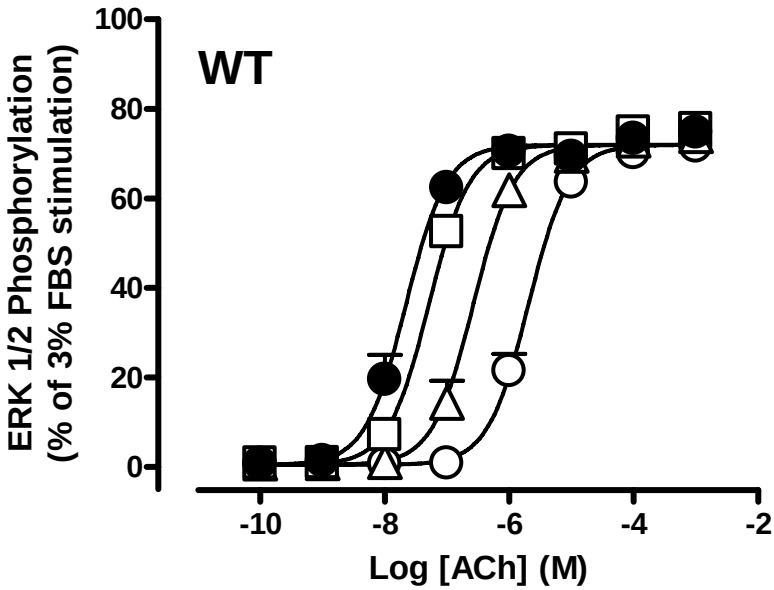
Supplementary Figure 1 Mutation of Tyr^{177}Ala in the allosteric pocket reduces the potency of DDBL-4 to act as a negative allosteric modulator of orthosteric agonist efficacy at the human M_2 mAChR. ACh (circles) or TMA (squares)-mediated ERK1/2 phosphorylation in the absence (solid symbols) or presence (open symbols) of 100μM DDBL-5 for 5 min at 37°C in CHO FlpIn cells stably expressing either the wild type (WT) or Tyr^{177}Ala (Y^{177}A) mutant M_2 mAChR. Data represent the mean + standard error of the mean obtained from 3-4 experiments conducted in duplicate.

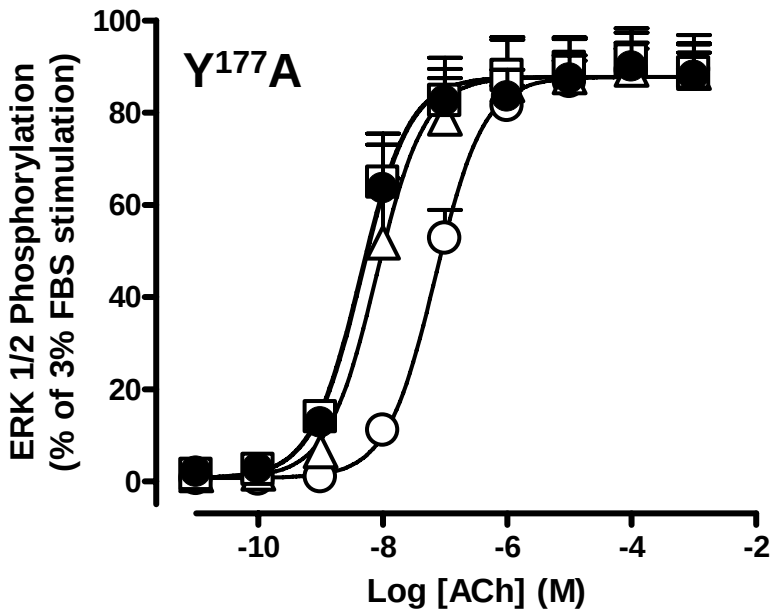
Supplementary Figure 2 Mutation of Tyr^{177}Ala in the allosteric pocket reduces the potency of the prototypical allosteric modulator, gallamine, to inhibit the actions of ACh at the human M_2 mAChR. ACh -mediated ERK1/2 phosphorylation in the absence (●) or presence of 1 μM (□), 10 μM (△) or 100 μM (○) of gallamine at either the wild type (WT) or Tyr^{177}Ala (Y^{177}A) mutant M_2 mAChR. Data represent the mean + standard error of the mean obtained from 3-4 experiments conducted in duplicate.





Supplementary Figure 1

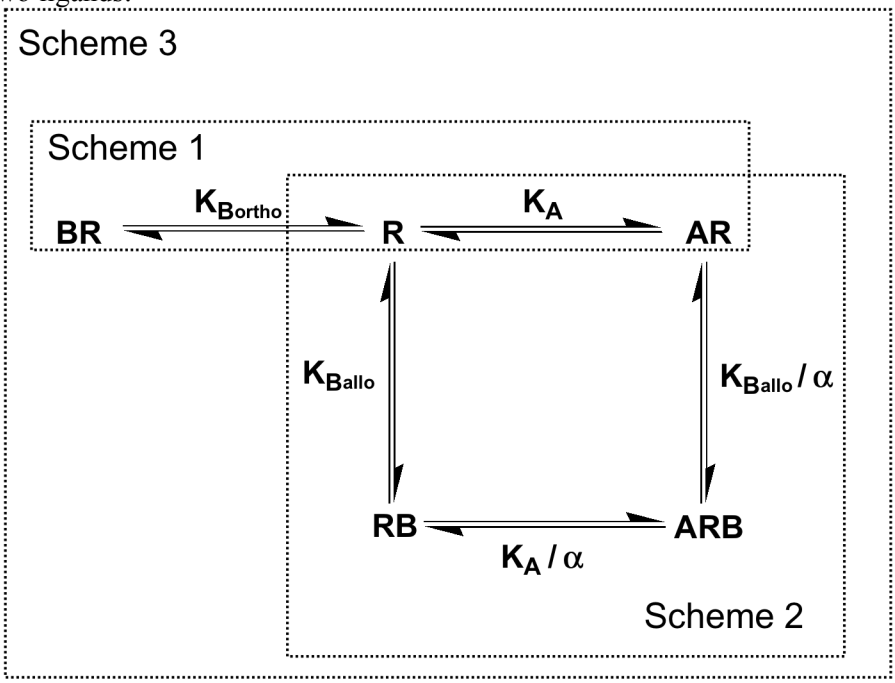




Supplementary Figure 2

SUPPLEMENTARY INFORMATION

The interaction between an orthosteric ligand, A, and a second ligand, B, that can occupy either an allosteric site or the orthosteric site (but not both simultaneously) is shown in the Figure below. K_A denotes the equilibrium dissociation constant of A for binding to the orthosteric site, whereas K_{Bortho} and K_{Ballo} denote the equilibrium dissociation constants of B for binding to the orthosteric and allosteric sites, respectively. The parameter, α, is the cooperativity factor for the allosteric interaction between the two ligands:



The complete bitopic mechanism (Scheme 3) is thus a hybrid of the standard model of competitive interaction (Scheme 1) and the allosteric ternary complex model, ATCM (Scheme 2). For a simple competitive mechanism (Scheme 1), the fractional occupancy of A in the presence of B was first derived by Gaddum (1936) (1). From that relationship, it is also possible to derive the midpoint location (potency) parameter, $[B]_{50}$, for titration of ligand B in the presence of a fixed concentration of A:

$$[B]_{50} = K_{Bortho}\left(1+\frac{[A]}{K_A}\right) \quad \text{(A1)}$$

This relationship underlies the Cheng and Prusoff equation that is routinely used to derive antagonist dissociation constants (i.e., K_{Bortho} or K_I values) from IC_{50} (i.e., $[B]_{50}$) values obtained in radioligand competition binding studies. A similar relationship can also be derived for an allosteric interaction that follows the ATCM, but in this instance the value of $[B]_{50}$ for an allosteric modulator does not change linearly with orthosteric ligand occupancy, instead reaching a limiting shift governed by the value of the cooperativity factor, α (2):

$$[B]_{50} = \frac{K_{Ballo}\left(1+\frac{[A]}{K_A}\right)}{\left(1+\frac{\alpha[A]}{K_A}\right)} \quad \text{(A2)}$$

With respect to the combined bitopic model (Scheme 3), the fractional occupancy of A in the presence of B has been derived previously (3), and is shown below:

$$\rho_A = \frac{[A]}{[A]+K_A \frac{\left(1+[B]\left(\frac{1}{K_{Bortho}}+\frac{1}{K_{Ballo}}\right)\right)}{\left(1+\frac{\alpha[B]}{K_{Ballo}}\right)}} \quad \text{(A3)}$$

The midpoint potency parameter for the titration curve of ligand B in this model is as follows:

$$[B]_{50} = \frac{K_{Ballo}\left(1+\frac{[A]}{K_A}\right)}{\left(1+\frac{\alpha[A]}{K_A}\right)+\frac{K_{Ballo}}{K_{Bortho}}} \quad \text{(A4)}$$

For the purposes of the current study, we were interested in the situation where the cooperativity of the allosteric interaction is highly negative, i.e., when the value of α approaches 0. Under this condition, the midpoint potency parameter of the titration curve of ligand B in the presence of a fixed concentration of A according to the ATCM (Scheme 2; equation A2) becomes:

$$[B]_{50\,(\alpha\to 0)} = K_{Ballo}\left(1+\frac{[A]}{K_A}\right) \quad \text{(A5)}$$

whereas in the bitopic orthosteric/allosteric model (Scheme 3; equation A4), it becomes:

$$[B]_{50\,(\alpha\to 0)} = \frac{K_{Ballo}\left(1+\frac{[A]}{K_A}\right)}{1+\frac{K_{Ballo}}{K_{Bortho}}} \quad \text{(A6)}$$

A comparison between equations A1 and A5 illustrates the fact that allosteric interactions characterized by very high degrees of negative cooperativity become indistinguishable from simple orthosteric competitive interactions with respect to antagonist potency estimates, and the application of a simple (orthosteric) competitive model to the data will thus yield a good estimate of antagonist binding affinity irrespective of whether the binding is to an allosteric site or the orthosteric site. For antagonist potency estimates using the bitopic model described by Scheme 3 (equation A6), which is characterized by two different dissociation constants for ligand B, there are three situations that need to be considered when α approaches 0:

a) $K_{Bortho} >> K_{Ballo}$, i.e., ligand B has a much lower affinity for the orthosteric site than for the allosteric site. Antagonist potency is then described by the following:

$$[B]_{50\,(K_{Bortho}\to\infty)} = K_{Ballo}\left(1+\frac{[A]}{K_A}\right) \quad \text{(A7)}$$

b) $K_{Ballo} >> K_{Bortho}$. i.e., ligand B has a much lower affinity for the allosteric site than for the orthosteric site:

$$[B]_{50\,(K_{Ballo}\to\infty)} = K_{Bortho}\left(1+\frac{[A]}{K_A}\right) \quad \text{(A8)}$$

c) $K_{Ballo} = K_{Bortho}$ i.e., ligand B has identical affinities for the orthosteric and allosteric sites.

$$[B]_{50\,(K_{Bortho}=K_{Ballo})} = \frac{K_{Ballo}\left(1+\frac{[A]}{K_A}\right)}{2} = \frac{K_{Bortho}\left(1+\frac{[A]}{K_A}\right)}{2} \quad \text{(A9)}$$

A comparison between equations A1, A5, A7 and A8 shows that they are all of the same form and will thus yield the same estimate of antagonist affinity from the $[B]_{50}$ once corrected for orthosteric ligand occupancy. Equation A9 is a special limiting case, but even there the resulting antagonist affinity estimate will only be discrepant from its true value by a factor of 2.

SUPPLEMENTARY REFERENCES

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3. May, L. T., Leach, K., Sexton, P. M., and Christopoulos, A. (2007) *Annu Rev Pharmacol Toxicol* **47**, 1-51