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Probe-dependence in the allosteric modulation of a G protein-coupled receptor: Implications for detection and validation of allosteric ligand effects

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Running title: Allosteric probe-dependence at a GPCR

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Abbreviations: ACh, acetylcholine; C_7/3-phth, heptane-1,7-bis-(dimethyl-3'-phthalimidopropyl) ammonium bromide ; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle medium; E2L, second extracellular loop; ERK1/2, extracellular signal-regulated kinases 1/2; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; LY2033298, 3-amino-5-chloro-6-methoxy-4-methyl-thieno[2,3-b]pyridine-2-carboxylic acid cyclopropylamide; mAChR, muscarinic acetylcholine receptor ; McN-A-343, 4-*I*-[3-chlorophenyl]carbamoyloxy)-2-butynyltrimethylammnonium chloride; [^{3}H]NMS, [^{3}H]-N-methylscopolamine; [^{3}H]QNB, [^{3}H]-quinuclidinyl benzilate; TM, transmembrane domain

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ABSTRACT

We recently described 3-amino-5-chloro-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxylic acid cyclopropylamide (LY2033298) as a novel allosteric modulator of M_4 muscarinic acetylcholine (ACh) receptors (mAChRs) on the basis of its ability to preferentially potentiate the actions of ACh at the M_4 mAChR subtype. In the current study, we show that LY2033298 can also bind to the M_2 mAChR and mediate robust positive or negative allosteric effects depending on the orthosteric ligand used as a probe of receptor activity. This finding of striking "probe-dependence" indicates that the previously-described selectivity of the modulator does not arise as a consequence of selective affinity for a poorly conserved allosteric site, but rather due to subtype-selective cooperativity with ACh upon interaction with a common allosteric binding site. Moreover, a comparison of the effects of the modulator on orthosteric ligand affinity relative to signaling through a [^{35}S]GTPγS or extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation assay at the M_2 mAChR revealed that, although the effects on binding were positive in all instances, the effects on signaling were either positive or strongly negative depending on the agonist and the pathway. Mutational analysis identified residues Y177 and W99$^{3.28\ 1}$ as contributing to the binding of LY2033298, whereas the orthosteric site residues, Y104$^{3.33}$ and Y403$^{6.51}$ contributed to the ability of the ligand to impose pathway-biased modulation. Collectively, these findings have important implications for the detection and validation of allosteric modulators of G protein-coupled receptors (GPCRs), because they highlight the potential for ligand misclassification or lack of appreciation of off-target allosteric activities.

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INTRODUCTION

The mAChRs are prototypical Family A GPCRs. Five mAChR subtypes have been identified and classified functionally on the basis of G protein signal transduction preferences; M_1, M_3 and M_5 mAChR subtypes preferentially activate $G_{q/11}$ proteins, whereas M_2 and M_4 mAChRs signal preferentially through $G_{i/o}$ proteins (Caulfield, 1993). However, all five mAChRs are promiscuous in their coupling preferences and exhibit signaling behavior that extends beyond the simple G protein-second messenger paradigm, including the recruitment of convergent pathways such as the MAP kinases (Lanzafame *et al.*, 2003). Because these receptors are widely expressed throughout the CNS and the periphery, they have emerged as therapeutic targets for treatment of a diverse range of pathologies, including schizophrenia, Alzheimer's disease, pain, respiratory disorders, bladder dysfunction, and irritable bowel syndrome (Eglen *et al.*, 2001; Wess *et al.*, 2007). However, a major drawback to the selective targeting of the mAChRs has been the high degree of homology conservation within the orthosteric (ACh-binding) pocket.

One approach showing promise in overcoming some of the difficulties associated with selective mAChR drug targeting is to focus on allosteric binding sites. Indeed, the mAChRs are one of the best-characterized model systems for understanding the actions of small molecule allosteric modulators of GPCRs, and a relatively rich allosteric pharmacology has emerged in recent years for various mAChR subtypes (Birdsall *et al.*, 2005; Gregory *et al.*, 2007; Conn *et al.*, 2009). Commonly-proposed advantages of targeting receptor allosteric sites include the possibility of greater selectivity due to lower sequence conservation within allosteric pockets across subtypes of a given GPCR, as well as the potential to fine-tune physiological signaling in a more spatial and temporally-selective manner (Christopoulos, 2002; May *et al.*, 2007c). However, it is often underappreciated that selectivity in the actions

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of allosteric modulators can also be achieved through the expression of selective cooperativity (positive or negative) between allosteric and orthosteric ligands at one receptor subtype while retaining neutral cooperativity at all other subtypes of the same receptor family (Lazareno *et al.*, 1996). That is, it is possible for an allosteric site to also be conserved across subtypes of a given GPCR, but the magnitude and direction of the allosteric effect can vary dramatically between these subtypes.

We have recently described the *in vitro* and *in vivo* pharmacological characteristics of a novel allosteric modulator, LY2033298, which is an allosteric agonist and a selective potentiator of the actions of ACh at the human M_4 mAChR but exhibits minimal interaction with the endogenous agonist at other mAChR subtypes (Chan *et al.*, 2008; Nawaratne *et al.*, 2008; 2010; Leach *et al.*, 2010; 2011). Interestingly, when examining the potential for species variability in the actions of this modulator between human and rodent M_4 mAChRs, we found that the variability could be attributed to differences in the cooperativity between the modulator and ACh between species, and not due to differences in the allosteric binding pocket between the human and rodent receptors (Suratman *et al.*, 2011). Moreover, we also noted striking differences in the magnitude of the allosteric effect depending on the nature of the orthosteric ligand used. For instance, LY2033298 caused robust potentiation of the actions of the agonists, ACh and oxotremorine, at M_4 mAChRs, but was only weakly positive when combined with the agonist, xanomeline, neutral when tested against the antagonist, [^{3}H]quinuclidinyl benzylate ([^{3}H]QNB), and weakly negative when combined with the antagonist, *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) (Leach *et al.*, 2010; Suratman *et al.*, 2011). A change in the nature and extent of allosteric modulation at a given receptor depending on the type of orthosteric ligand used is referred to as "probe dependence" (Kenakin, 2005;

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Keov *et al.*, 2011) and has substantial implications for the detection, validation and potential therapeutic utility of allosteric ligands.

Given these findings, the aim of the current study was to re-evaluate the notion of allosteric modulator selectivity in the context of a receptor that is closely related to the M_4 mAChR, but which shows less potential for allosteric interaction between LY2033298 and the endogenous agonist; a finding that reflects either variability in the allosteric binding pocket or else a manifestation of differential cooperativity between subtypes. For this purpose, we chose the M_2 mAChR. By utilizing different agonists and antagonists, as well as a series of mutant M_2 mAChR, we provide evidence for a "common" allosteric binding pocket utilized by LY2033298 across mAChR subtypes, and also reveal striking examples of probe dependence that have wider implications for allosteric ligand drug discovery.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, penicillin/streptomycin, and hygromycin-B were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from ThermoTrace (Melbourne, Victoria, Australia). [^{3}H]NMS (70.0 Ci/mmol) and [^{3}H]QNB (120 Ci/mmol) were purchased from PerkinElmer Life Sciences. [^{35}S] guanosine 5'-O-[gamma-thio]triphosphate) ([^{35}S]GTPγS; >1000 Ci/mmol) was purchased from PerkinElmer Life Sciences and Amersham Biosciences. The *Sure- Fire*^{TM} cellular ERK1/2 assay kits were a generous gift from TGR BioSciences (Adelaide, Australia). AlphaScreen^{TM} reagents were from PerkinElmer Life Sciences. LY2033298, and xanomeline tartrate were synthesized in-house at Eli Lilly (Indianapolis, IN). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Receptor mutagenesis, cell culture and membrane preparation

FlpIn Chinese hamster ovary (CHO) cells stably expressing muscarinic M_2 mAChRs (M_2 mAChR FlpIn-CHO) wild type (WT) or mutant were generated and cultured as described previously (Stewart *et al.*, 2009). Cell membranes were prepared as described previously (Valant *et al.*, 2008).

Radioligand Equilibrium Binding Assays

M_2 mAChR FlpIn-CHO membrane homogenates (5-20 µg) were incubated in a 500 µl total volume of assay buffer containing either [^{3}H]NMS (0.5 nM) or [^{3}H]QNB (0.5 nM) and a range of concentrations of LY2033298 (10 nM to 100 µM) at 30 °C for 90 min. Radioligand saturation binding and affinity parameters in these cells have been reported previously

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(Gregory *et al.*, 2010). Interaction binding studies were also performed between [^{3}H]NMS, an IC_{20} concentration of various mAChR agonists (ACh, oxotremorine, oxotremorine-M, tetramethylammonium, pilocarpine, xanomeline or McN-A-343), and increasing concentrations of LY2033298 by co-incubating 20 μg of M_2 mAChR FlpIn-CHO membrane in the presence of guanosine-5′-(βγ-imino)triphosphate (Gpp(NH)p), as described previously (Nawaratne *et al.*, 2010). For all experiments, nonspecific binding was defined by 10 μM atropine, and the effects of vehicle were also determined. Incubation was terminated by rapid filtration through Whatman GF/B filters using a Brandell cell harvester (Gaithersburg, MD). Filters were washed three times with 3 ml aliquots of ice-cold 0.9% NaCl buffer and dried before the addition of 4 ml of scintillation mixture (Ultima Gold, PerkinElmer Life Sciences). Vials were then left to stand until the filters became uniformly translucent before radioactivity was determined in dpm using scintillation counting.

[^{3}H]NMS Dissociation Kinetic Assays

M_2 mAChR FlpIn-CHO cell membranes (20 μg for wild type or 5 μg for Y177A) were equilibrated with [^{3}H]NMS (0.5 nM) in a 1 ml total volume of assay buffer for 90 min at 30°C. Atropine (10 μM) alone or in the presence of LY2033298 was then added at various time points to prevent the reassociation of [^{3}H]NMS with the receptor. In subsequent experiments designed to investigate the effect of a range of modulator concentrations on [^{3}H]NMS dissociation rate, a "two-point kinetic" experimental paradigm was used where the effect of increasing concentrations of LY2033298 on [^{3}H]NMS dissociation was determined at 4 and 40 min. This approach is valid to determine [^{3}H]NMS dissociation rate constants if the full time course of radioligand dissociation is monophasic both in the absence and presence of modulator (Kostenis *et al.*, 1996) (Lazareno *et al.*, 1995); this was the case in our current study. All other details of these assays are as described previously (Valant *et al.*,

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2008). Termination of the reaction and determination of radioactivity were performed as described above.

[^{3}H]NMS Association Kinetic Assay

A two-point kinetic experimental paradigm was used where the effect of increasing concentrations of allosteric modulator on [^{3}H]NMS association was determined at 3 and 30 min. M_2 mAChR FlpIn-CHO cell membranes (20 µg) were added into a 1 ml total volume of assay buffer at 30 °C containing [^{3}H]NMS (0.5 nM) alone or in the presence of a range of concentrations of LY2033298 at 3 and 30 min. Termination of the reaction and determination of radioactivity were performed as described above.

[^{35}S]GTPγS Binding Assay

M_2 mAChR FlpIn-CHO cell membranes (5-25 µg) were equilibrated in a 500 µl total volume of assay buffer containing 10 µM guanosine 5'-diphosphate and a range of concentrations of ligands (ACh, oxotremorine, oxotremorine-M, tetramethylammonium, pilocarpine, xanomeline or McN-A-343) in the absence or presence of LY2033298 (0.1 to 10 µM) at 30 °C for 60 min. After this time, 50 µl of [^{35}S]GTPγS (1 nM) was added, and incubation continued for 30 min at 30 °C. Termination of the reaction and determination of radioactivity were performed as described above.

ERK1/2 Phosphorylation Assay

These assays were performed using the AlphaScreen-based *SureFire* kit as described in detail previously (May *et al.*, 2007a) (Avlani *et al.*, 2007). For each agonist, the pERK1/2 response was determined to peak between 5 and 8 min (data not shown), and subsequent concentration-response curves were constructed at the peak time point. All data were

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expressed as a percentage of ERK1/2 phosphorylation mediated after a 6 min exposure to Dulbecco's modified Eagle's medium containing 3% FBS or normalized to the maximal response of the endogenous agonist, ACh, when specified.

Data analysis

Computerized nonlinear regression was performed using Prism 5.03 (GraphPad Software, San Diego, CA). Radioligand binding curves for interaction with LY2033298 were fitted to an allosteric ternary complex model (Eq. 1) to derive estimates of allosteric modulator affinity (K_B), cooperativity between the modulator and the radioligand (α') or the agonist (α); values of α or $\alpha' > 1$ denote positive cooperativity, whereas values of $0 < \alpha$ or $\alpha' < 1$ denote negative cooperativity (Christopoulos *et al.*, 2002; May *et al.*, 2007b):

$$E = \frac{B_{max}[A]}{[A] + \left(\frac{K_A K_B}{\alpha'[B] + K_B}\right)\left(1 + \frac{[I]}{K_I} + \frac{[B]}{K_B} + \frac{\alpha[I][B]}{K_I K_B}\right)} \quad (1)$$

where K_A, K_B and K_I represent the equilibrium dissociation constants of the radioligand, allosteric ligand and second (unlabeled) orthosteric ligand, respectively, and [A], [B] and [I] denote their respective concentrations. Interaction studies between radioligand and the modulator in absence of inhibitor, i.e. [I] = 0, allowed determination of α'.

Dissociation and association kinetic data were fitted to monoexponential functions to derive observed rate constants of either dissociation or association. Finally for whole cell functional studies, the interaction between the orthosteric agonists and LY2033298 was fitted to the following two forms of an operational model of allosterism and agonism (Aurelio *et al.*, 2009; Leach *et al.*, 2007) to derive functional estimates of modulator affinity, cooperativity and efficacy:

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$$E = \frac{E_m\left(\tau_A[A]\left(K_B + \alpha\beta[B]\right) + \tau_B[B]K_A\right)^n}{\left([A]K_B + K_AK_B + [B]K_A + \alpha[A][B]\right)^n + \left(\tau_A[A]\left(K_B + \alpha\beta[B]\right) + \tau_B[B]K_A\right)^n} \quad (2)$$

$$E = \frac{E_m\left(\tau_A[A]\left(K_B + \alpha\beta[B]\right) + \tau_B[B]EC_{50}\right)^n}{EC_{50}{}^n\left(K_B + [B]\right)^n + \left(\tau_A[A]\left(K_B + \alpha\beta[B]\right) + \tau_B[B]EC_{50}\right)^n} \quad (3)$$

where E_m is the maximum attainable system response for the pathway under investigation, [A] and [B] are the concentrations of orthosteric agonist and allosteric modulator/agonist, respectively, K_B is the dissociation constant of the allosteric modulator, EC_{50} is the concentration of orthosteric (full) agonist yielding 50% of the response between minimal and maximal receptor activation in the absence of allosteric ligand, n is a transducer slope factor linking occupancy to response, α is the binding cooperativity factor (as described above), β is an empirical scaling factor describing the allosteric effect of the modulator on orthosteric agonist signaling efficacy, respectively, and τ_A and τ_B are operational measure of the ligands' respective signaling efficacies that incorporate receptor expression levels and efficiency of stimulus-response coupling (Aurelio *et al.*, 2009; Leach *et al.*, 2007). For this analysis, the entire family of curves for a given agonist-modulator combination at each receptor were fitted by constraining the dissociation constant parameter, pK_B, to the value determined in the radioligand binding assays. Equation 2 was used in interaction studies performed between LY2033298 with partial agonists, while equation 3 was used when the modulator was interacted with full agonists, depending on the pathway investigated. This is so because equation 3 is only valid in cases where the orthosteric agonist has high efficacy ($\tau >> 1$) such that K_A is >> [A].

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All affinities, potencies, efficacies, and cooperativity parameters were estimated as logarithms (Christopoulos, 1998). Results are expressed as means ± S.E.M. unless otherwise stated. Statistical analyses were by Student's *t*-test, or one-way ANOVA followed by Bonferroni's post-test, as appropriate. Values of $p < 0.05$ were considered statistically significant.

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RESULTS

Rationale behind the choice of M_2 mAChR agonists and mutants

A range of mAChR agonists were selected for the study (Fig. 1A), based on their ability to fully or partially activate the M_2 mAChR. As high efficacy agonists, we selected acetylcholine (ACh), oxotremorine and oxotremorine M. As lower efficacy agonists, we selected tetramethylammonium (TMA), pilocarpine and xanomeline. We also included the bitopic agonist, McN-A-343, which we recently identified as being able to bridge both the orthosteric site and the allosteric site of the M_2 mAChR, (Valant *et al.*, 2008). Fig. 1B shows a snake diagram of the secondary structure of the wild type (WT) M_2 mAChR, indicating the residues mutated in the current study. Based on previous work (Gregory *et al.*, 2010), we focused on four key residues, two in TM3, $W99^{3.28}$ and $Y104^{3.33}$, one in the second extracellular loop (E2L), Y177, and one in TM6, $Y403^{6.51}$. Both tyrosine residues in the TM3 and TM6, $Y^{3.33}$ and $Y^{6.51}$ respectively, are involved in the binding of the endogenous ligand, ACh, via π-cation interactions, and were mutated to alanine residues (Heitz *et al.*, 1999; Spalding *et al.*, 2006). Alanine mutation of the $W^{3.28}$ of the mAChRs is known to reduce the affinity of allosteric modulators such as gallamine at the M_1 subtype, or LY2033298 at the M_4 subtype (Matsui *et al.*, 1995; Leach *et al.*, 2011). Finally, mutation of an aromatic residue in the equivalent position in the E2L has been previously reported to drastically reduce prototypical allosteric modulator affinity, such as gallamine or C_7/3-phth at the M_2 and M_4 subtypes (Valant *et al.*, 2008); this residue is Y177 in the M_2 mAChR.

LY2033298 binds to the M_2 mAChR and displays probe dependence with antagonists

LY2033298 was initially characterized in equilibrium binding assays against the orthosteric antagonist [^{3}H]QNB at the M_2 mAChR WT as well as the four mutant receptors (Fig. 2A).

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For these experiments, we utilized concentrations of the modulator ranging from 10 nM to 100 μM; we could not go higher due to solubility limitations. Increasing concentrations of LY2033298 caused a significant inhibition of the specific binding of the radioligand, suggesting high negative cooperativity. Application of an allosteric ternary complex model (Eq. 1) to the data provided an estimate of the affinity of LY2033298 for the allosteric site of the unoccupied receptor as well as an estimate of the cooperativity between LY2033298 and the radioligand whose logarithm that was not significantly different from -3 (Table 1), indicative of a negative allosteric interaction that is indistinguishable form a competitive interaction (i.e., $\alpha' \rightarrow 0$). Interestingly, when $W99^{3.28}$ was mutated to alanine, the affinity of the modulator was significantly reduced, whereas mutation of Y177A resulted in no change in the specific radioligand binding. This suggested that mutation of this tyrosine in the E2L of the receptor either abrogated the cooperativity between LY2033298 with [^{3}H]QNB (α' = 0), or substantially reduced the binding affinity of the allosteric ligand. To further probe this latter possibility, experiments were repeated at the WT and Y177A M_2 mAChRs using [^{3}H]NMS as the orthosteric radioligand. As shown in Fig. 2B, increasing concentrations of LY2033298 caused a modest but consistent enhancement in the equilibrium binding of [^{3}H]NMS at the WT receptor (log α' = 0.50 ± 0.04; i.e., α' = 3) , in contrast to the negative allosteric effect observed with [^{3}H]QNB. This finding of probe dependence is consistent with the fact that the two radioligands, despite recognizing a similar binding pocket, represent chemically distinct series and thus adopt different orientations within the orthosteric pocket such that they are differentially affected by the conformational change induced by LY2033298. Despite the differences in cooperativity factors for the two antagonists, the estimated dissociation constant for LY2033298 at the unoccupied M_2 mAChR (4.43 ± 0.13; n = 3) was not significantly different ($p < 0.05$) from that determined against [^{3}H]QNB, as expected. Notably, when the Y177 residue was mutated to alanine, the effect of LY2033298

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on [^{3}H]NMS binding was abolished, suggesting that this residue is indeed vital for the binding of the modulator. Interestingly, the tyrosine mutations within the orthosteric binding site, Y104A$^{3.33}$ and Y403A$^{6.51}$, drastically modified the allosteric properties of LY2033298 as determined against [^{3}H]QNB. Mutation of Y104A$^{3.33}$, which is known to reduce orthosteric agonist and antagonist affinity, also significantly reduced the binding of LY2033298 while switching the cooperativity between LY2033298 and [^{3}H]QNB from negative to positive. Mutation of Y403A$^{6.51}$, did not affect the affinity of the modulator for the receptor, but did convert the cooperativity from negative to positive.

To more directly probe the ability of LY2033298 to allosterically modulate orthosteric antagonist binding properties, the effect of the modulator on the rate of association and dissociation of an antagonist was monitored at the M_2 mAChR WT (Fig. 2C and Supp. Figs. 1A and B). We chose [^{3}H]NMS rather than [^{3}H]QNB because of the faster rate of dissociation of the former radioligand at the M_2 mAChR. As shown in Fig. 2C, LY2033298 retarded both the association and the dissociation kinetics of [^{3}H]NMS, however over slightly different concentration ranges such that LY2033298 was more potent at slowing the dissociation (pEC_{50} = 5.03 ± 0.06) than the association (pEC_{50} = 4.70 ± 0.08) of [^{3}H]NMS, thus explaining why LY2033298 was a modest potentiator of [^{3}H]NMS equilibrium binding. In theory, the potency of an allosteric modulator that follows the simple allosteric ternary complex model in affecting the rate of orthosteric radioligand dissociation should be equal to the quotient of the dissociation constant of the modulator for the free receptor and the cooperativity factor, i.e. K_B/α; in contrast, the potency of the modulator for slowing orthosteric radioligand association rate should equal the K_B only, as it is reflecting the pre-equilibration of the modulator with the free receptor prior to addition of radioligand (Lazareno and Birdsall, 1995; Kostenis and Mohr, 1996). Accordingly, the experimentally-

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determined potency of LY2033298 for slowing the association rate of [^{3}H]NMS (pEC_{50} = 4.70) is in good agreement with the pK_B value determined for the modulator from equilibrium binding assays (4.43 - 4.75, depending on which radioligand was used). Similarly, the slightly higher potency for the modulator in slowing [^{3}H]NMS dissociation (5.03) reflects the slight positive cooperativity between the radioligand and the modulator, as the dissociation kinetic experiments quantify the affinity of the modulator for the radioligand-occupied receptor (theoretical value of pK_B/α' predicted from the individual K_B and α' values from the [^{3}H]NMS equilibrium binding experiments is 4.93).

In contrast, when dissociation kinetic experiments were performed at the M_2 mAChR Y177A, no appreciable change of the dissociation rate of [^{3}H]NMS could be observed, supporting the hypothesis that mutation of the tyrosine in the E2L of the M_2 mAChR substantially reduces the binding of LY2033298. This latter finding is consistent with similar observations at the M_4 mAChR, whereby mutation of F186A (at the equivalent position in the E2L to Y177 at the M_2 mAChR) was identified as a key contributor to the binding affinity of LY2033298 for the allosteric site on that receptor (Nawaratne *et al.*, 2010). Taken together, these radioligand binding experiments conclusively show that LY2033298 recognizes an allosteric site on the M_2 mAChR, in addition to its well-characterized interaction with the M_4 mAChR.

LY2033298 exhibits binding probe dependence with agonists at the WT M_2 mAChR

To determine the effect of LY2033298 on agonist affinity and cooperativity, interaction studies were performed using [^{3}H]NMS (0.5 nM), LY2033298 and an IC_{20} concentration of agonist at the WT M_2 mAChR (Fig. 3; Table 2). Increasing concentrations of LY2033298 positively modulated the affinity of all the mAChR agonists studied, but to markedly

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different extents, again indicating the occurrence of probe dependence. The effect of the modulator on ACh affinity was reasonable (α = 16), but not as pronounced as we have previously noted at the M_4 mAChR (α values ranging up to 60) (Chan *et al.*, 2008; Leach *et al.*, 2011; Leach *et al.*, 2010; Nawaratne *et al.*, 2010; Suratman *et al.*, 2011). Even lower degrees of positive cooperativity were observed with pilocarpine (2.8) and McN-A-343 (8.9), whereas TMA (37) and xanomeline (36) were modulated to a greater extent than ACh. The most dramatic increases in cooperativity were observed when LY2033298 was combined with oxotremorine (390) or oxotremorine M (190).

LY2033298 differentially modulates mAChR agonist intrinsic efficacy in a ligand- and pathway-dependent manner

We subsequently investigated the effect of LY2033298 on M_2 mAChR signaling at two different pathways, [^{35}S]GTPγS binding to activated Gα proteins and pERK1/2. [^{35}S]GTPγS binding was chosen as a proximal measure of receptor activation, while the pERK1/2 assay was chosen because it measures a downstream response that is also a point of convergence of multiple cellular pathways, some of them potentially G protein-independent. If the system behaved according to a simple two-state model and the two pathways are sequentially linked to one another, then we would expect to see a correlation between the direction and degree of modulation of a given agonist at both the proximal and distal pathways (Keov *et al.*, 2011). In contrast, if the allosteric modulator imposes bias on the signaling of the agonist, then a divergence between the effect on [^{35}S]GTPγS binding and pERK1/2 may be seen.

The interaction between LY2033298 and each of the agonists at the WT M_2 mAChR is shown in Fig. 4 (pERK1/2) and Fig. 5 ([^{35}S]GTPγS), while Fig. 6 and Table 3 summarize the functional cooperativity estimates ($\alpha\beta$ values) for each agonist/pathway obtained from

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application of equations 2 (for the partial agonists) and 3 (for the full agonists) to the data. From these experiments, it could be seen that LY2033298 on its own was able to mediate a degree of direct receptor activation, indicative of allosteric agonism; this was most noticeable in the pERK1/2 experiments (Fig. 4), presumably because this pathway is more downstream of receptor activation than the [^{35}S]GTPγS assay. More importantly, it was also evident that LY2033298 exhibited markedly different degrees of not only positive, but also negative, functional cooperativity depending on the agonist used; in contrast, only different degrees of positive cooperativity were noted on agonist binding properties (Table 2; Fig. 3). The most striking effects were observed with pilocarpine and xanomeline, which showed a reduction of maximal agonist response in the presence of increasing concentrations of LY2033298, despite the fact that the modulator, on its own, increased the activity of the receptor, i.e., behaved as an allosteric agonist (Figs. 4 and 5). This finding indicates that the conformation of the receptor that binds LY2033298 and either pilocarpine or xanomeline is clearly distinct from the conformation(s) engendered by any of the ligands on their own, as the ternary complexes formed yield inactive receptor states, whereas the binary ligand-receptor complexes are active.

Although the reversals in efficacy of xanomeline and pilocarpine in the presence of LY2033298 were the most obvious indicators of biased receptor states, it was also evident that more subtle differences existed in the profiles of modulation of the other agonists. For instance, ACh experienced greater potentiation at the level of the [^{35}S]GTPγS assay than pERK1/2, whereas oxotremorine, TMA and McN-A-343 showed the reverse preferences (Fig. 6A). Because the modulation of agonist affinity was positive in all instances, the variations in the functional cooperativity between agonists across the two pathways suggest that the underlying modulation of agonist efficacy may be negative for multiple agonists

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beyond the obvious case of pilocarpine and McN-A-343. This is illustrated more clearly in Figure 6B, where the cooperativity factors determined in the binding assays (α values) were subtracted from the functional $\alpha\beta$ factors to yield estimates of β for each agonist at each pathway.

Mutations in the orthosteric site of M_2 mAChR affect functional cooperativity factors.

The functional interaction studies were subsequently extended to the mutant receptors, and the estimates of cooperativity obtained are shown in Table 3 and Fig. 7; Supplementary Figs 2-7 show the complete concentration-response data for these experiments. Not surprisingly, no effect of LY2033298 was noted at the M_2 mAChR Y177A receptor, consistent with our hypothesis that the modulator does not bind appreciably to this E2L mutant. The main finding at the W99A$^{3.28}$ mutant was the selective reduction in the cooperativity between McN-A-343 and LY2033298. Interestingly, both Y104A$^{3.33}$ and Y403A$^{6.51}$ mutations caused marked reductions in agonist signaling efficacy that could be uniformly rescued by LY2033298 at the latter mutant (Fig. 7C), but only for the most efficacious agonists at the former (Fig. 7B). Moreover, at these mutant receptors the pERK1/2 response was always potentiated to a greater extent than the [^{35}S]GTPγS response; the mutant receptors essentially behave according to a classic two-state system (Keov *et al.*, 2011), with the biased modulation evident at the WT receptor being lost at these mutants.

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DISCUSSION

There are three major findings from this study. First, the recently-described "selective" M_4 mAChR modulator, LY2033298, interacts with the M_2 mAChR via a site that shares similarities with the common allosteric site in mAChRs. This highlights the fact that allosteric modulators can achieve receptor targeting not only by binding to less-conserved sites, but also via subtype-selective cooperativity upon interaction with potentially conserved allosteric sites. A second finding is the difference in the allosteric effect exerted by LY2033298 on various agonists and antagonists of the M_2 mAChR. This probe dependence has major implications for drug discovery, because the identification and subsequent classification of an allosteric ligand will depend on the orthosteric ligand that is used. Moreover, the potential clinical utility of combining an allosteric ligand with an existing orthosteric medicine for a given GPCR target must also take into account the possibility of probe dependent off-target effects at other GPCRs. Finally, a third finding was the differential effects of receptor mutations on the transmission of cooperativity with various M_2 mAChR agonists, suggesting that some of these residues play important roles in the ability of the M_2 mAChR to transition between multiple active states.

Previous studies have revealed that the mAChRs possess at least one allosteric site located extracellularly to the orthosteric site (Wess, 2005; Gregory *et al.*, 2007). This pocket is referred to as the "common" allosteric site, because prototypical modulators, such as gallamine, alcuronium and C_7/3-phth, interact with all five mAChR subtypes, albeit with different degrees of affinity / selectivity (Christopoulos *et al.*, 1999; Gregory *et al.*, 2007). In addition to demonstrating conclusively that LY2033298 interacts with the M_2 mAChR, our study has revealed that the binding of the modulator is very sensitive to mutation of Y177 within the E2L, a residue known to be important for the binding of prototypical modulators

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(Avlani *et al.*, 2007; Valant *et al.*, 2008). Given that mutation of F186 in the equivalent position of the M_4 mAChR E2L abolishes the binding of LY2033298 to that receptor (Nawaratne *et al.*, 2010), as well the fact that LY2033298 displays competitive behavior against the prototypical modulator, C_7/3-phth, at the M_4 mAChR (Leach *et al.*, 2010), it is reasonable to speculate that the interaction of LY2033298 with the M_2 mAChR also involves, at least in part, binding to the site utilized by other well-characterized mAChR modulators. We recently identified another conserved residue, $W^{3.28}$, as contributing to the binding of LY033298 at the M_4 mAChR, and proposed that this residue sits at the interface between orthosteric and allosteric sites (Leach *et al.*, 2011). In our current study, mutation of $W^{3.28}$A also reduced the affinity of LY2033298 for the M_2 mAChR, but to a lesser extent than at the M_4 mAChR. On the one hand, this supports the hypothesis that LY2033298 interacts with a common allosteric pocket at these two mAChRs, but on the other hand it suggests that the binding pose is sufficiently different at the two receptors such that not all mutations will affect the modulator to the same extent across subtypes. It is possible that this is a more general phenomenon warranting further study across other GPCRs.

The expectation that LY2033298 adopts a different orientation in the M_2 mAChR relative to the M_4 mAChR is convincingly demonstrated by the differences in the probe dependence between the modulator and orthosteric ligands. Whereas the positive cooperativity between ACh and LY2033298 is most marked at the M_4 mAChR, it is lower at the M_2 mAChR, and essentially neutral at the other mAChR subtypes (Chan *et al.*, 2008). Based on these observations, we conclude that the selectivity ascribed to the modulator arises predominantly as a consequence of differential cooperativity with ACh across mAChRs. This is also in accord with our recent study of species differences in the effects of LY2033298 at the M_4 mAChR; the lower potency of the modulator at the rodent M_4 mAChR relative to the human

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was again due to differences in cooperativity, not affinity, between species (Suratman *et al.*, 2011). Interestingly, the interaction between LY2033298 and antagonists at the M_2 mAChR was also different to that noted at the M_4 mAChR. Whereas the interaction with [^{3}H]NMS is positive at the M_2 mAChR, it is weakly negative at the M_4 mAChR (Leach *et al.*, 2010). For [^{3}H]QNB, the interaction is neutral at the M_4 (Leach *et al.*, 2010; 2011) but negative at the M_2 mAChR.

The study of probe-dependence is important for drug discovery, being particularly pertinent to situations where the endogenous agonist cannot be used for assessing the activity of putative allosteric ligands, perhaps due to metabolic instability or, for orphan GPCRs, where the endogenous agonist is not known; in both instances, surrogate orthosteric agonists (or antagonists) are required. It is also relevant for GPCRs that possess more than one endogenous agonist. The implications of our study are that an allosteric effect may be missed, if the probe dependence leads to neutral cooperativity, or misinterpreted, even across multiple endogenous agonists for a single GPCR (Koole *et al.*, 2010). For the mAChRs, it is common to see brain penetrant analogs of ACh, such as oxotremorine, used as surrogate probes to provide exogenous tone for *in vivo* studies (e.g., Chan *et al.* (2008); Leach *et al.*, (2010)). However, the fact that LY2033298 displays substantially higher positive cooperativity with oxotremorine than ACh means that *in vivo* studies ostensibly focused on M_4 mAChRs may inadvertently recruit M_2 mAChRs upon co-adminstration of compounds such as LY2033298 and oxotremorine, thus potentially confounding the interpretation of the experimental results. Indeed, this is likely why the effects of co-adminstration of these two compounds on the conditioned avoidance response in M_4 mAChR knockout mice, although markedly attenuated, are not completely abolished (Leach *et al.*, 2010). Finally, the effect of LY2033298 on compounds such as pilocarpine and xanomeline is particularly noteworthy,

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because in this situation the cooperativity is so highly negative that both agonists are essentially converted into antagonists in the presence of modulator. The finding that their binding is actually improved while their signaling efficacy is concomitantly abolished is reminiscent of the effects of the allosteric modulator, Org27569, at the CB_1 cannabinoid receptor (Price *et al.*, 2005), suggesting that this phenomenon may be more common that appreciated. Xanomeline has already shown clinical efficacy in improving cognitive deficits in schizophrenia (Shekhar *et al.*, 2008), but combination with LY2033298, which also demonstrates preclinical efficacy in a variety of antipsychotic models, may not prove useful due to the potential for introducing off-target antagonistic effects at the M_2 mAChR.

Within a simple two-state receptor model, it is expected that the degree of allosteric modulation would correlate with the intrinsic efficacy of the ligands and the degree of stimulus-response amplification (Hall, 2000; Keov *et al.*, 2011). If this were the case, then we would have expected a greater degree of allosteric potentiation of ERK1/2 activity over [^{35}S]GTPγS binding at the WT M_2 mAChR, which was not observed. The fact that the probe dependence was, instead, characterized by both positive and negative modulation in an agonist- and pathway-dependent manner is consistent with the notion that GPCRs can adopt multiple biologically active states that can be differentially stabilized by orthosteric and allosteric ligands (Leach *et al.*, 2007; Keov *et al.*, 2011). Because for most diseases there is currently no definitive link between a given cellular pathway and a desired therapeutic outcome, allosteric modulator screening should be as broad as possible, and ideally benchmarked iteratively against appropriate *in vivo* disease models during the drug candidate selection process. This will increase the likelihood of correlating *in vitro* "fingerprints" (e.g., positive modulation of G protein activation, negative modulation of ERK1/2 phosphorylation etc.) with *in vivo* efficacy.

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Finally, it was interesting to note the effects of mutations on the bias imposed by LY2033298. Both Y104A$^{3.33}$ and (especially) Y403A$^{6.51}$ were sensitive to functional rescue by the allosteric modulator, but in a manner that was consistent with a simple two-state mechanism, i.e., in all instances where functional modulation was observed, the ERK1/2 pathway was potentiated to a greater extent than the more proximal [^{35}S]GTPγS response. It is possible that the two amino acids participate in a key network of interactions that facilitate the ability of the receptor to transition between multiple active states, perhaps in a sequential fashion as proposed for other biogenic amine GPCRs (Liapakis *et al.*, 2004). Disruption of the network as a consequence of mutation may thus limit the transition to fewer, but more abundant, substates.

In conclusion, we have identified LY2033298 as a robust allosteric modulator of the M_2 mAChR, in addition to the M_4 mAChR. Key novel findings arising from this work include the fact that allosteric selectivity between GPCR subtypes can arise due to differences in cooperativity with the endogenous agonist, rather than differences in allosteric binding pockets; that opposite effects can be exerted by an allosteric modulator on agonist affinity versus agonist efficacy; that this profile of effects need not be retained when switching agonists or signaling pathways. These examples of probe dependence also have broader implications for the detection, classification and validation of allosteric modulators of other GPCRs, and highlight how investigations of the phenomenon should be incorporated into allosteric modulator discovery efforts.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Valant, Felder, Sexton and Christopoulos

Conducted experiments: Valant

Contributed new reagents or analytic tools: Felder

Performed data analysis: Valant and Christopoulos

Wrote or contributed to the writing of the manuscript: Valant, Felder, Sexton and Christopoulos

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FOOTNOTES

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Numbered footnotes:

[1] Ballesteros and Weinstein numbers are provided (in superscript) to indicate relative position of residues within the transmembrane domain.

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FIGURE LEGENDS

Figure 1. Ligands and mutants investigated in this study. (A) mAChR agonists used in this study. (B) Snake diagram of the M_2 mAChR highlighting residues mutated in alanine, and agonist structures of ligands used in this study.

Figure 2. LY2033298 interacts allosterically at the M_2 mAChR. (A) Effect of LY2033298 on the equilibrium binding of [^{3}H]QNB at the WT and four mutant receptors. (B) Effect of LY2033298 on the equilibrium binding of [^{3}H]NMS at the wild type (WT) the $Y^{177}A$ M_2 mAChR. (C) Concentration-dependent slowing by LY2033298 of the dissociation at the WT and Y177A M_2 mAChR, and apparent association rate of [^{3}H]NMS at the WT and M_2 mAChR. Kinetic rate constants were determined in the presence of increasing concentrations of LY2033298 at three times points and normalized as a percentage of the rate constants determined in the absence of modulator. In all instances, data points represent the mean ± S.E.M of three experiments performed in duplicate.

Figure 3. LY2033298 differentially modulates agonist binding at the M_2 mAChR. The competition between 0.5 nM [^{3}H]NMS and an IC_{20} concentration of agonist was determined in presence of increasing concentrations of LY2033298. The curves drawn through the points represent the best global fit of an allosteric ternary complex model (Equation 1) to each pair of datasets (ACh competition of [^{3}H]QNB binding and the IC_{20} concentration of ACh in the presence of LY2033298). Data points represent the mean ± S.E.M of three experiments performed in duplicate.

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Figure 4. LY2033298 mediates both positive and negative allosteric effects on M_2 mAChR-mediated ERK1/2 phosphorylation in an agonist-dependent manner. Concentration-response curves to the indicated agonist were generated in the absence or presence of increasing concentrations of LY2033298. Data points represent the mean ± S.E.M of three experiments performed in duplicate. The curves drawn through the points represent the best global fit of an operational model of allosterism (Equation 2 for pilocarpine, xanomeline and McN-A-343 or Equation 3 for ACh, oxotremorine, oxotremorine-M and TMA), as described in the Methods and Results.

Figure 5. LY2033298 mediates both positive and negative allosteric effects on M_2 mAChR-mediated [^{35}S]GTPγS binding in an agonist-dependent manner. The curves drawn through the points represent the best global fit of an operational model of allosterism (Equation 2 for TMA, pilocarpine, xanomeline and McN-A-343 or Equation 3 for ACh, oxotremorine and oxotremorine-M). All other details as for Figure 4.

Figure 6. LY2033298 displays divergent cooperativity estimates with different agonists at the wild type M_2 mAChR. (A) Functional cooperativity estimates (composite αβ values) derived from analysis of the data in Figures 4 and 5. Values are also shown in Table 3. (B) Individual estimates of α and β values calculated by subtraction of the cooperativity factors determined in the radioligand binding studies from the composite estimates determined from the functional assays.

Figure 7. Functional cooperativity estimates of LY2033298 with different agonists at mutant M_2 mAChRs. Functional cooperativity estimates (composite αβ values) derived from analysis of the data in Supplementary Figures 2-7. Values are also shown in Table 3.

Table 1 Allosteric model parameter estimates for the interaction between LY2033298 and [^{3}H]QNB at M_2 mAChRs. Values represent the mean ± S.E.M. from at least three experiments performed in duplicate.

	[^{3}H]QNB				
	WT	**W99A$^{3.28}$**	**Y104A$^{3.33}$**	**Y177A**	**Y403A$^{6.51}$**
pK_B [a]	4.74 ± 0.06	4.20 ± 0.07*	3.79 ± 0.17*	N.D.	4.44 ± 0.10
logα' [b]	-3[d]	-3 [d]	1.18 ± 0.12	N.D.	0.64 ± 0.04
α' [c]	0.001	0.001	15	N.D.	4.4

[a] Negative logarithm of the allosteric modulator equilibrium dissociation constant.

[b] Logarithm of the cooperativity factor for the interaction between LY2033298 and the orthosteric radioligand.

[c] Antilogarithm of the cooperativity factor.

[d] Values not significantly different from -3 and were therefore constrained to such value. Indicative of a negative allosteric interaction that is indistinguishable from competition ($\alpha' \rightarrow 0$).

N.D. Not determined.

* Significantly different from the corresponding value at the wild type ($P < 0.05$).

Table 2 Allosteric model parameter estimates for the interaction between LY2033298 and various agonists at the wild type M_2 mAChR. Values represent the mean ± S.E.M. from at least three experiments performed in duplicate.

	ACh	Oxotremorine	Oxotremorine M	TMA	Pilocarpine	Xanomeline	McN-A-343
pK_I [a]	5.54 ± 0.06	6.18 ± 0.06	5.48 ± 0.06	3.01 ± 0.03	5.33 ± 0.07	6.84 ± 0.24	4.60 ± 0.07
$\log\alpha$ [b]	1.21 ± 0.05	2.59 ± 0.08	2.28 ± 0.07	1.57 ± 0.04	0.45 ± 0.10	1.56 ± 0.13	0.95 ± 0.08
α [c]	16	390	190	37	2.8	36	8.9

[a] Negative logarithm of the unlabeled orthosteric ligand equilibrium dissociation constant.

[b] Logarithm of the cooperativity factor for the interaction between LY2033298 and each orthosteric ligand.

[c] Antilogarithm of the cooperativity factor.

Table 3 Functional cooperativity estimates for the interaction between LY2033298 and various agonists at M_2 mAChRs. Values represent the mean ± S.E.M. from at least three experiments performed in duplicate.

Ligand	[^{35}S]GTPγS logαβ (αβ)				
	Wild Type	**W99A$^{3.28}$**	**Y104A$^{6.51}$**	**Y177A**	**Y403A$^{6.51}$**
ACh	1.15 ± 0.09 (14)	0.90 ± 0.09 (7.9)	1.65 ± 0.12 (45)*	N.D.	1.30 ± 0.07 (20)
Oxo	1.88 ± 0.06 (76)	2.10 ± 0.05 (126)	2.06 ± 0.06 (115)	N.D.	1.83 ± 0.10 (68)
OxoM	1.94 ± 0.05 (87)	1.82 ± 0.06 (66)	1.97 ± 0.07 (93)	N.D.	1.71 ± 0.07 (51)
TMA	1.61 ± 0.07 (41)	1.20 ± 0.06 (16)	N.A.	N.D.	1.73 ± 0.09 (54)
Pilocarpine	-3 (0.001) [b]	-3 (0.001)[b]	N.A.	N.D.	1.20 ± 0.14 (16)*
Xanomeline	-3 (0.001) [b]	-3 (0.001)[b]	N.A.	N.D.	0.63 ± 0.09 (4.3)*
McN-A-343	0.88 ± 0.16 (4.7)	0.13 ± 0.16 (1.3)*	N.A.	N.D.	1.12 ± 0.04 (13)
Ligand	**pERK1/2 logαβ (αβ)[a]**				
	Wild Type	**W99A$^{3.28}$**	**Y104A$^{3.33}$**	**Y177A**	**Y403A$^{6.51}$**
ACh	0.55 ± 0.14 (3.5)	0.86 ± 0.14 (7.2)	2.52 ± 0.07 (331)*	N.D.	2.23 ± 0.09 (170)*
Oxo	2.94 ± 0.09 (870)	2.89 ± 0.07 (776)	2.66 ± 0.11 (457)	N.D.	2.56 ± 0.09 (363)
OxoM	2.03 ± 0.08 (107)	2.25 ± 0.09 (178)	2.40 ± 0.07 (251)	N.D.	3.24 ± 0.08 (1737)*
TMA	2.43 ± 0.10 (270)	1.99 ± 0.08 (98)	N.A.	N.D.	2.91 ± 0.17 (813)
Pilocarpine	-3 (0.001)[b]	-3 (0.001) [b]	N.A.	N.D.	2.15 ± 0.32 (141)*
Xanomeline	-3 (0.001)[b]	-3 (0.001) [b]	N.A.	N.D.	2.72 ± 0.12 (525)*
McN-A-343	1.96 ± 0.18 (90)	0.44 ± 0.17 (2.7)*	N.A.	N.D.	3.38 ± 0.33 (2399)*

[a] Logarithm of the αβ factor for the interaction between LY2033298 and each agonist; antilogarithm is shown in parentheses.

[b] Values not significantly different from -3 and were therefore constrained to such value. Indicative of a negative allosteric interaction that is indistinguishable from complete abolition of signaling efficacy ($\alpha\beta \rightarrow 0$).

N.A., not applicable.

N.D., not determined.

* Significantly different from the corresponding value at the wild type ($P < 0.05$).

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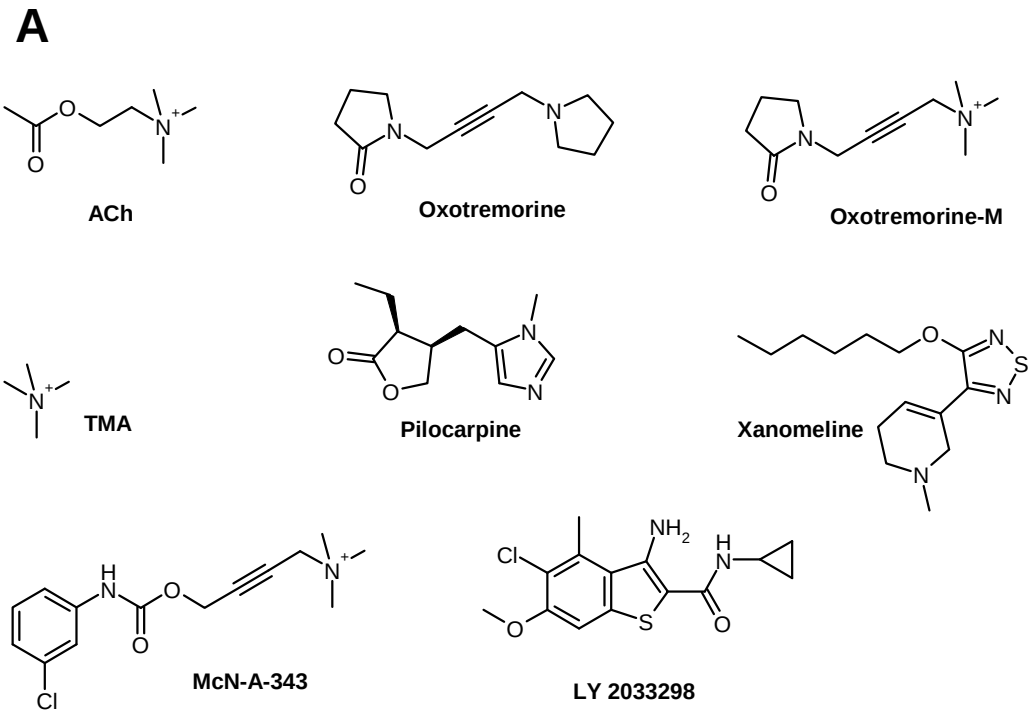
Figure 1.



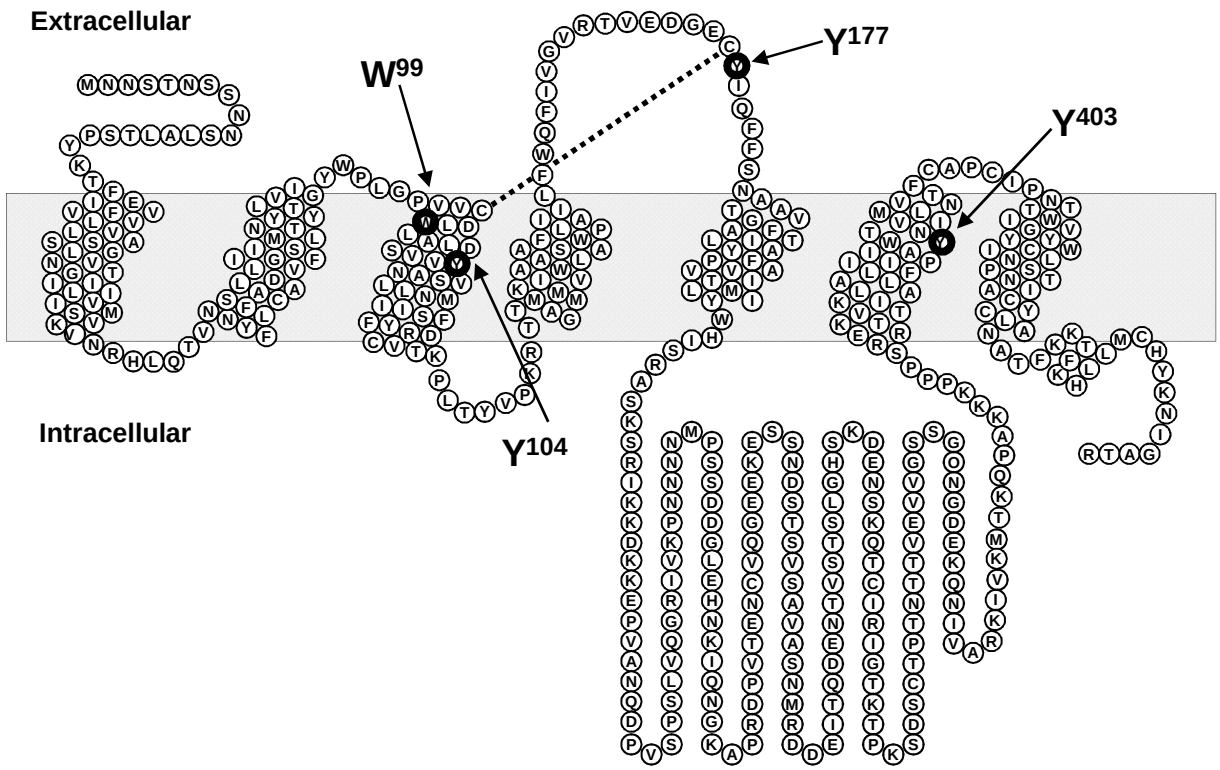


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Figure 2.

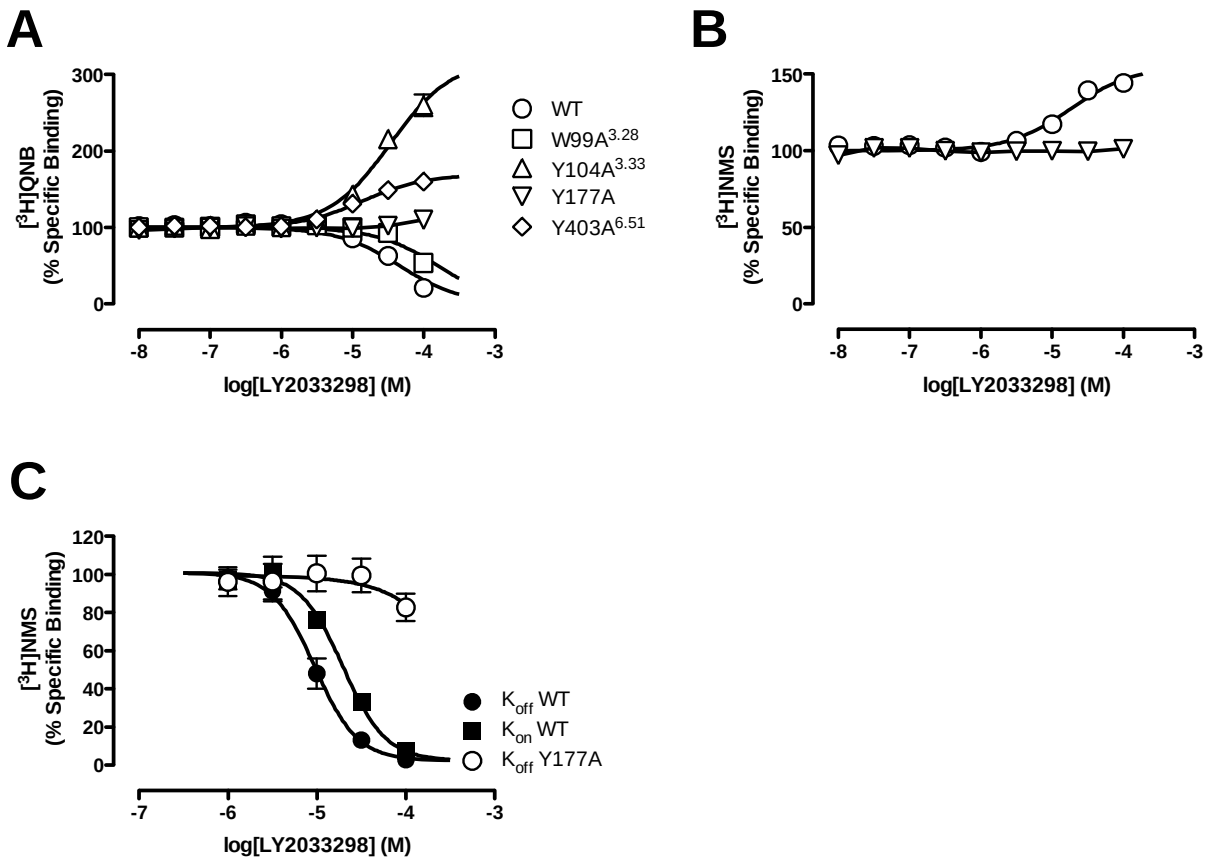

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Figure 3.

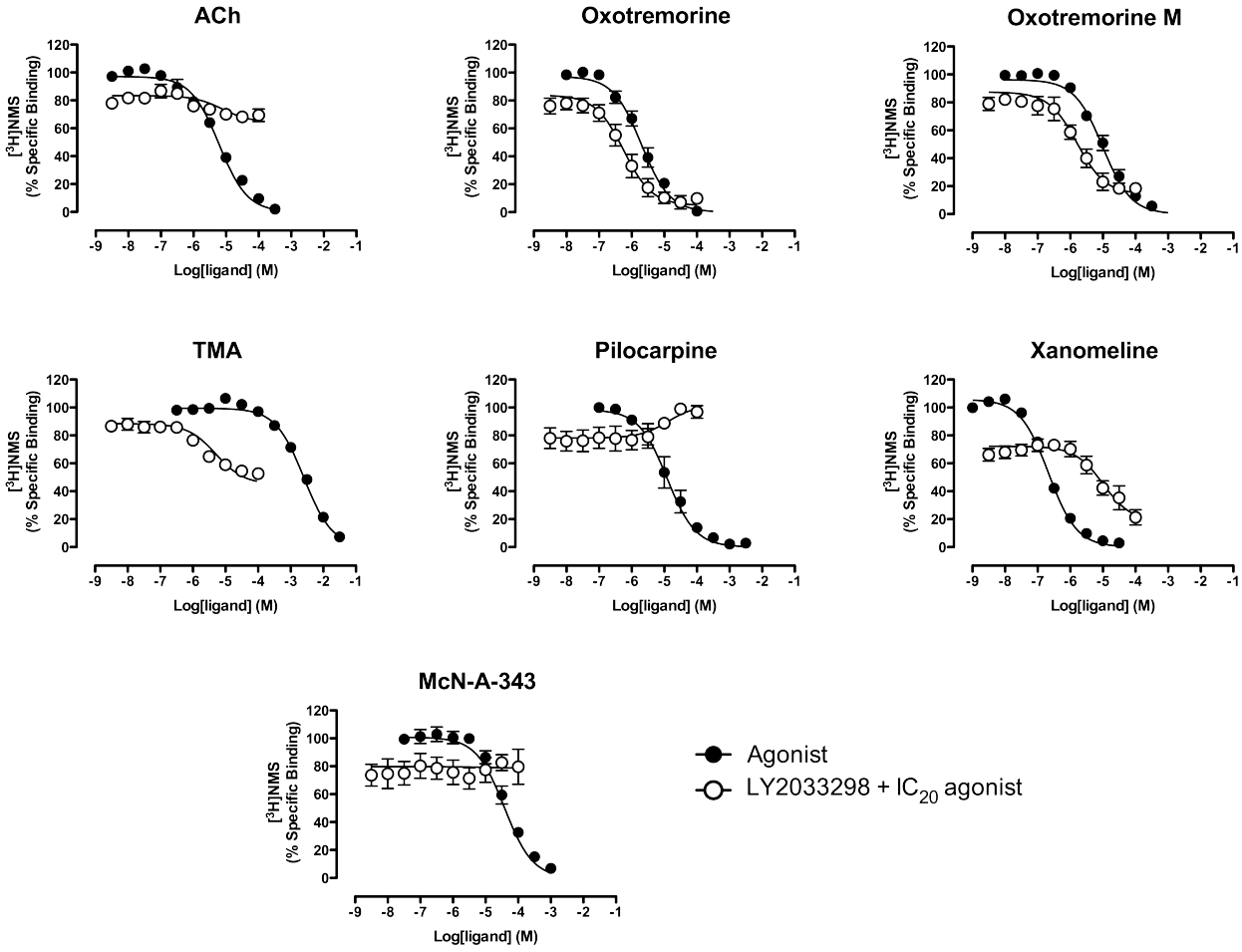

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Figure 4.

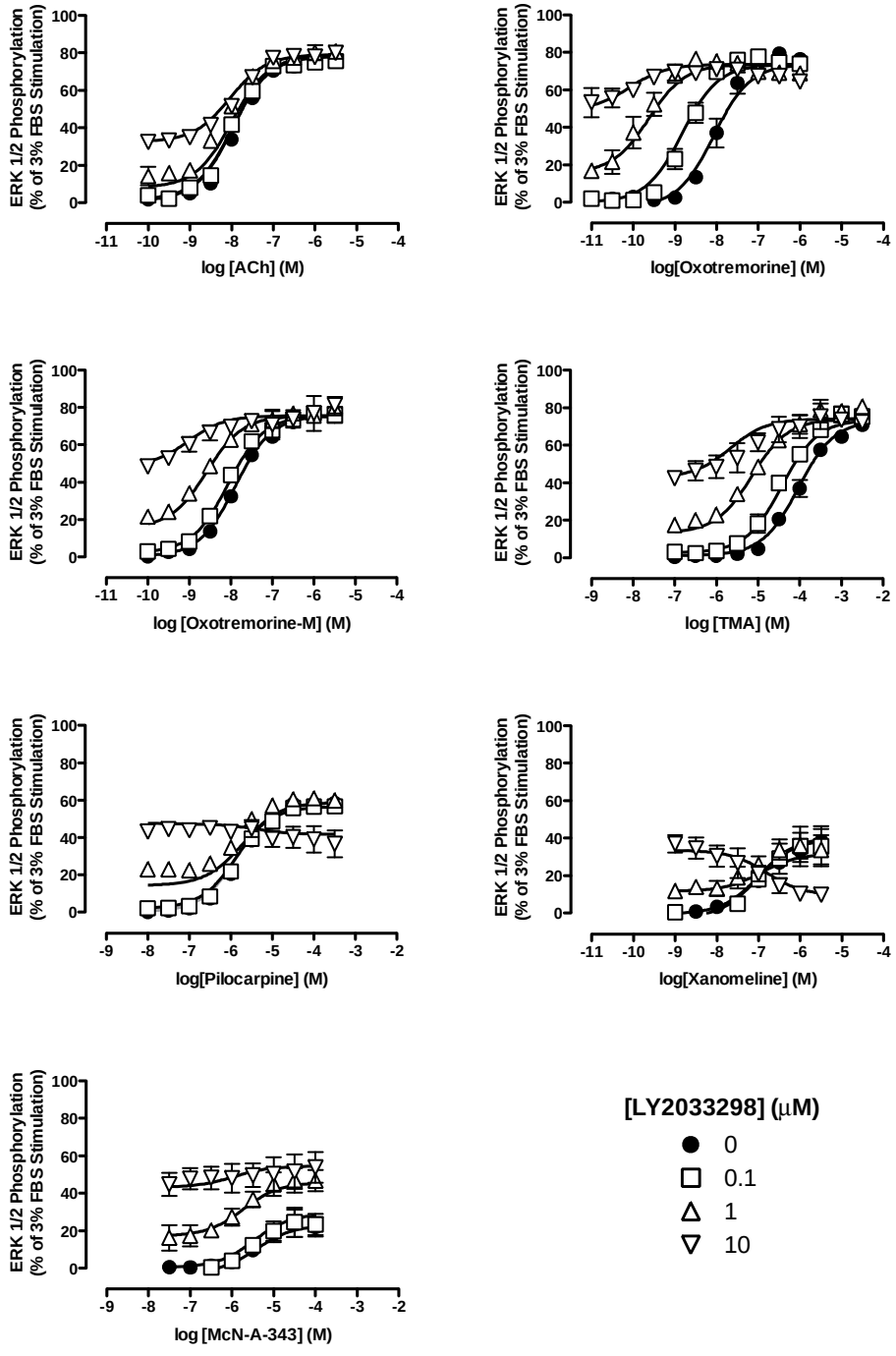

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Figure 5.

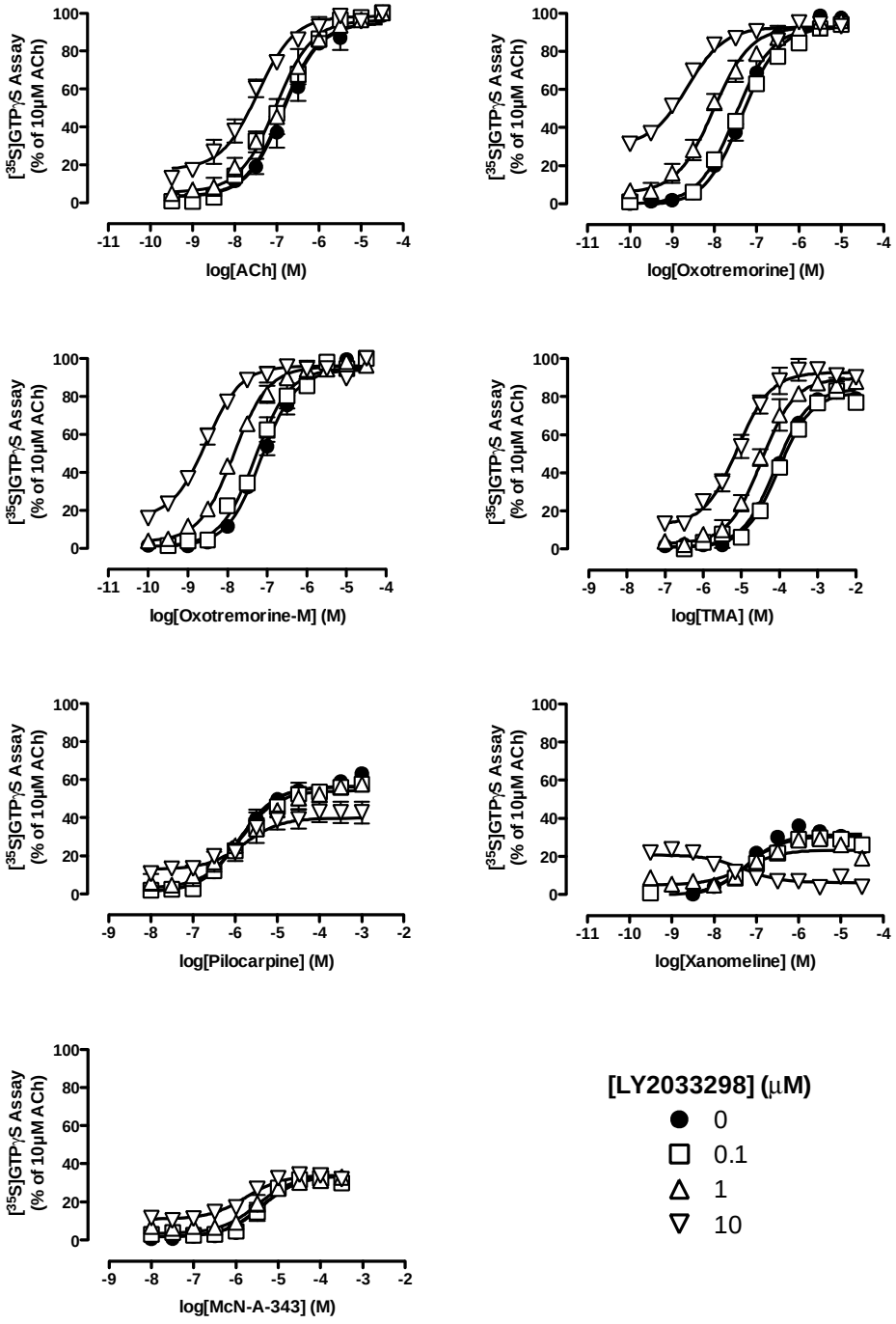

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Figure 6.

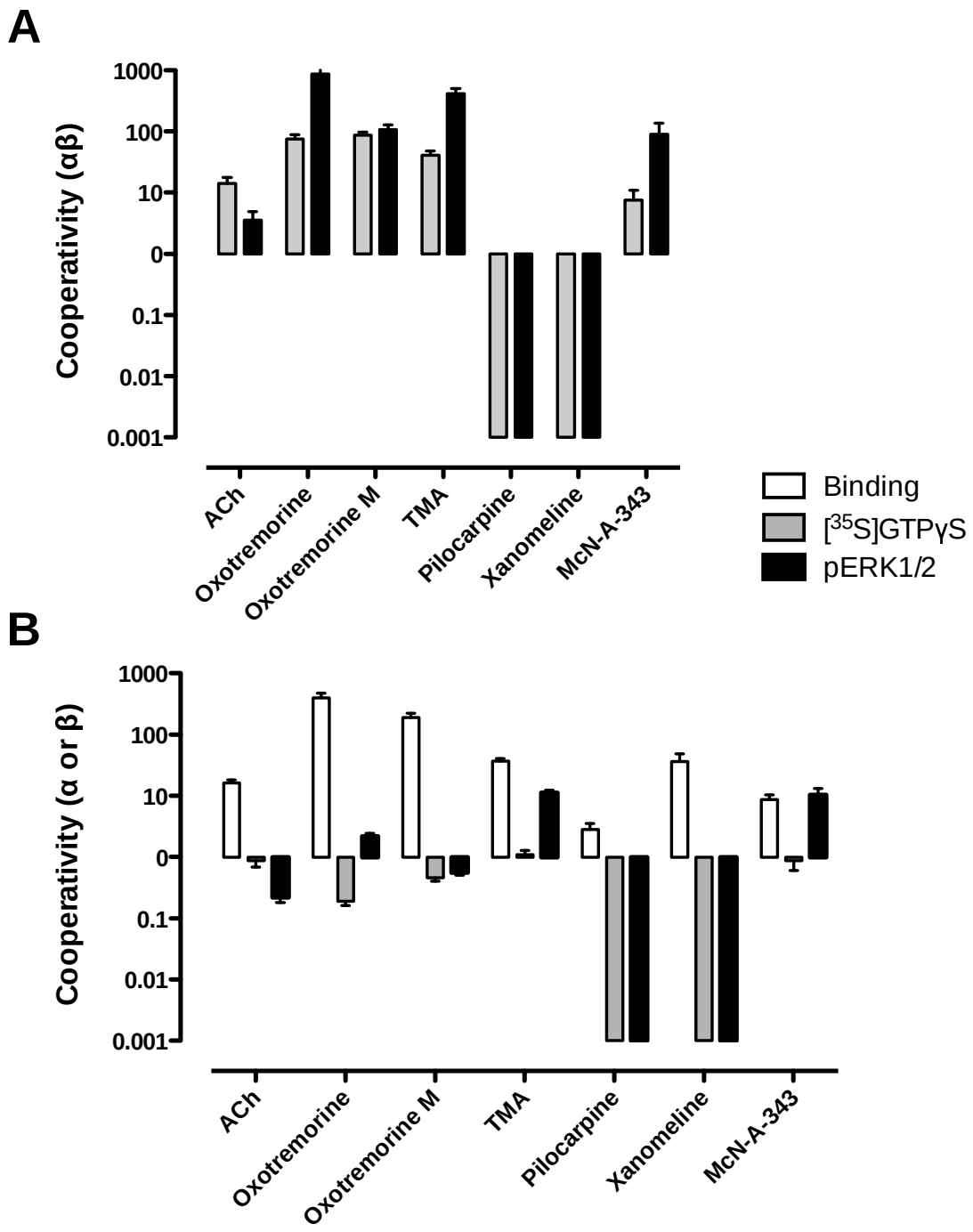

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Figure 7.