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Development of a radioligand, [^{3}H]-LY2119620, to probe the human M_2 and M_4 muscarinic receptor allosteric binding sites

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Running Title: [^{3}H]-LY2119620, a novel M_2 and M_4 mAChR allosteric probe

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Abbreviations: [^{3}H]-NMS, [^{3}H]-N-methylscopolamine; LY2119620, 3-amino-5-chloro-N-cyclopropyl-4-methyl-6-[2-(4-methylpiperazin-1-yl)-2-oxoethoxy] thieno[2,3-b]pyridine-2-carboxamide; LY2033298, 3-amino-5-chloro-6-methoxy-4-methyl-thieno(2,3-b)pyridine-2-carboxylic acid cyclopropylamide; WIN62,577, 17-β-Hydroxy-17-α-ethynyl-δ-4-androstano[3,2-b]pyrimido[1,2-a]benzimidazole; VU152100, 3-Amino-N-(4-methoxybenzyl)-4,6-dim ethylthieno[2,3-b]pyridine carboxamide; VU10010, 3-Amino-N-[(4-chlorophenyl)methyl]-4,6-dimethylthieno[2,3-b]pyridine-2-carboxamide; CHO, Chinese Hamster Ovary

Abstract

In this study, we described the characterization of a muscarinic acetylcholine receptor (mAChR) potentiator, LY2119620 (3-amino-5-chloro-N-cyclopropyl-4-methyl-6-[2-(4-methylpiperazin-1-yl)-2-oxoethoxy]thieno[2,3-b]pyridine-2-carboxamide) as a novel probe of the human M_2 and M_4 allosteric binding sites. Since the discovery of allosteric binding sites on G-protein coupled receptors (GPCRs), compounds targeting these novel sites are starting to emerge. For example, LY2033298 (Chan et al., 2008) and a derivative of this chemical scaffold, VU152100 (Brady et al., 2008), bind to the human M_4 mAChR allosteric pocket. In the current study we characterized LY2119620, a compound similar in structure to LY2033298 that binds to the same allosteric site on the human M_4 mAChRs. However, LY2119620 also binds to an allosteric site on the human M_2 subtype. [^{3}H]-NMS binding experiments confirm that LY2119620 does not compete for the orthosteric binding pocket at any of the 5 muscarinic receptor subtypes. Dissociation kinetic studies using [^{3}H]-NMS further support that LY2119620 binds allosterically to the M_2 and M_4 mAChRs and was positively cooperative with muscarinic orthosteric agonists. In order to directly probe the allosteric sites on M_2 and M_4, we radiolabelled LY2119620. Cooperativity binding of [^{3}H]-LY2119620 with mAChR orthosteric agonists detect significant changes in B_{max} values with little change in K_d suggesting a G-protein dependent process. Furthermore, [^{3}H]-LY2119620 was displaced by compounds of similar chemical structure but not by previously described mAChR allosteric compounds such as gallamine or WIN 62,577. Our results therefore demonstrate the development of a radioligand, [^{3}H]-LY2119620, to probe specifically the human M_2 and M_4 muscarinic receptor allosteric binding sites.

Introduction

Acetylcholine activates two families of receptors; the nicotinic ligand-gated ion channel receptors (nAChRs) and the G protein-coupled muscarinic receptors (mAChRs) classified initially based on their differential activation by nicotine (Lindstrom, 1997) and muscarine (Wess, 1996) respectively. The wide distribution of mAChRs in the CNS and periphery support their involvement in physiological processes such as arousal, cognition, pain, exocrine gland secretion, smooth muscle and vascular contraction (Wess et al., 2003). Muscarinic acetylcholine receptors have long been viewed as viable targets for developing therapeutic agents to treat Alzheimer's disease (AD) and other CNS disorders. The muscarinic agonist, xanomeline, for example was developed to treat AD (Bodick et al., 1994), but was also found to induce improvements in positive, negative, and cognitive symptoms associated with schizophrenia (Shekhar et al., 2008). However, the lack of selectivity of xanomeline led to peripheral side effects that prohibited it from advancing in the clinic. Due to the highly conserved sequence within the acetylcholine binding domain (Heinrich et al., 2009), targeting the orthosteric site for small molecule development resulted in a number of muscarinic agonist compounds with poor selectivity.

However, the discovery of allosteric sites on GPCRs is allowing more selective small molecule modulators to emerge, offering a unique approach to treating CNS diseases (Christopoulos, 2002; May et al., 2007). Allosteric modulators bind to novel sites distinct from the natural transmitter orthosteric binding site. Positive allosteric modulators (PAMs) enhance the affinity and/or efficacy of the endogenous ligand and have a number of therapeutic advantages compared with direct acting agonists such as xanomeline. Besides the observation of improved receptor selectivity, PAMs offer physiologically relevant spatial and temporal

signaling that may limit undesirable side-effects compared to direct acting agonists that can lead to desensitization and long-term changes in receptor densities (Christopoulos, 2002).

Several *in vitro* pharmacological methods can be used to assess allosteric binding and functional signaling modulation. Typically muscarinic allosteric agents have been identified by the effect they induced on radioligand competition experiments at the orthosteric site (Birdsall et al., 1997). For example, allosteric modulators can be revealed by their inability to fully block radiolabeled orthosteric probes such as [^{3}H]-N-methylscopolamine (NMS). Positive allosteric modulation is typically assessed by functional signal transduction studies using various assay formats including measurement of cAMP generation and GTPg^{35}S binding. Because previous methods for studying allosteric binding mechanisms has been limited to indirect measurements using orthosteric binding, we describe here the development of a radiotracer from LY2119620 which allows direct labeling of the muscarinic allosteric site. In addition, we discuss the possibility of being able to discern muscarinic allosteric binding sites in native tissue. Our data support the hypothesis that allosteric selectivity between M_2 and M_4 mAChR subtypes with LY2119620 is a result of differences in cooperativity and not affinity of the orthosteric agonist similar to what has been previously described for thiochrome (Lazareno et al., 2004) and LY2033298 (Leach et al., 2010) at the M_4 mAChR.

Materials and Methods

CHO cell line stably expressing human M_1 (B_{max} NMS=4.4 pmol/mg membrane), M_2 (B_{max} NMS =11.0 pmol/mg membrane), M_3 (B_{max} NMS =7.64 pmol/mg membrane), M_4 (B_{max} NMS =3.3 pmol/mg membrane) or M_5 (B_{max} NMS =4.2 pmol/mg membrane) were purchased from Perkin Elmer. Chemicals and ligands were purchased from the following sources: Oxotremorine-M, VU152100, and VU10010 from Tocris; Acetylcholine, WIN 62,577, and Gallamine triethiodide (Sigma); [^{3}H]-NMS (GE Healthcare); [^{3}H]-LY2119620, [^{35}S] GTP-γ-S (Perkin Elmer), LY2119620 and LY2033298 (Lilly)

[^{3}H]-NMS binding Assays

[^{3}H]-NMS binding assays were performed in HEPES buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4) as described previously (Chan et al., 2008) with the following modifications. Briefly, frozen membrane preparations were thawed and resuspended in assay buffer and approximately 25 µg protein was added to each well and incubated with [^{3}H]-NMS for 2 h at room temperature in a total volume of 200 µL in polypropylene 96-deep well plates. Non-specific binding was determined using 1 µM atropine. Membranes were collected by rapid filtration, using a Tomtec cell harvester (Tomtec, Inc., Hamden, CT, USA) through GF/A filters that had been presoaked in 0.3% polyethyleneimine. The filters were washed with 5 mL ice-cold 50 mM Tris buffer (pH 7.4) and air-dried overnight. The dried filters were treated with MeltiLex A melt-on scintillator sheets, and the radioactivity retained on the filters was counted using a Wallac 1205 Betaplate scintillation counter (Perkin-Elmer, Wallac, Gaithersburg, MD, USA). Displacement experiments for [^{3}H]-NMS were carried out at in the presence of various concentrations of compounds for all 5 human muscarinic receptor subtypes. More specifically, in the potentiation experiments with [^{3}H]-NMS, various concentrations of orthosteric agonists

were used to displace the radioligand, but in the presence of either 10, 1, 0.1, or 0 μM LY2119620. The dissociation kinetic binding assays were performed using a reverse time protocol. For these experiments, P1 membrane preparations of a CHO cell line stably expressing either the human M_2 or M_4 muscarinic mAChR were utilized. Membranes were added to ~1.0 nM [^{3}H]-NMS in the presence or absence of LY2119620 and allowed to equilibrate for 2 h at room temperature. Once equilibrated, 1.0 μM oxotremorine-M was added in a time-staggered approach to allow 1-60 minute time-point collection. Statistical Analyses: K_i values were determined from the Cheng-Prusoff relationship $K_i = IC_{50}/ 1 + [ligand]/K_d$, where IC_{50} is determined from a four parameter fit of displacement curves, [ligand] = 1 nM [^{3}H]-NMS and K_d is the equilibrium dissociation constant of [^{3}H]-NMS for each mAChR subtype determined by saturation binding experiments carried out by the membrane supplier.

[^{3}H]-LY2119620 binding Assays

[^{3}H]-LY2119620 saturation binding assays were performed in HEPES buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$, pH 7.4). Saturation binding was initiated by incubating 15 μg of muscarinic-containing membranes (hM_1-M_5, Perkin Elmer), orthosteric ligand (100 μM unless otherwise noted; oxotremorine-M or acetylcholine) and various concentrations of hot-ligand [^{3}H]-LY2119620 (0.2-60 nM) for 1 h at room temperature, although equilibrium was achieved within 15 min (data not shown). [^{3}H]-LY2119620 displacement assays were performed in HEPES buffer as described previously. Muscarinic-containing membranes M_2 or M_4 receptors were incubated with 100 μM oxotremorine-M and [^{3}H]-LY2119620 at approximately the K_d concentration of the receptor, and varying concentrations (0.1 nM-10 μM) of allosteric ligands gallamine, VU152100, VU10010, WIN 62,577, LY2033298 and LY2119620. Incubations were carried out for 1 h at room temperature. All reactions were stopped by rapid

filtration on a TOMTEC 96-well cell harvester. Non-specific binding was determined using 10 μM LY2033298. Radioactivity retained on the filtermats was counted on a Wallac 1205 Betaplate. Statistical Analyses: The specific binding vs. time data was fit to a one-site specific binding model using GraphPad Prism 6.7 and the B_{max} and K_d for the allosteric molecule was calculated for each orthosteric ligand. K_i values were determined using the Cheng-Prusoff relationship for [^{3}H]-LY2119620 displacement studies.

Autoradiographic studies using [^{3}H]-LY2119620

Male cynomolgus monkey brains were supplied from Covance (Greenfield, IN). Brains were rapidly removed, placed in ice-cold PBS for 5 minutes and then stored at -80°C. The brains were mounted onto chucks and sectioned at 12 μm using a cryostat (Zeiss, Thornwood, NY). Sagittal sections were thaw mounted onto gelatin coated slides, and stored at -80°C until assayed. Sections were initially pre-incubated for 10 min in PBS at room temperature. The sections were then placed into polypropylene containers containing ~5.0 nM [^{3}H]-LY2119620 either 100 μM acetylcholine or oxotremorine-M. Some near adjacent sections were also incubated with 10 μM LY2033298 to define non-specific binding. Following a 1 h incubation, the sections were rinsed with fresh ice-cold PBS on ice for 10 min each and dried rapidly. The labeled sections were exposed to Fujifilm Imaging Plate for 15 days. The plate was read in Fuji BAS-5000 and analyzed using MCID Software (Cambridge, England).

Results

Previous studies have revealed that the mAChRs possess at least one allosteric site located extracellularly to the orthosteric site (Wess, 2005). 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). In this study we describe the identification of a novel positive allosteric modulator, LY2119620 for common allosteric site on the human M_2 and M_4 mAChR. For comparisons sake, we also evaluated other muscarinic allosteric compounds including LY2033298, VU10010, VU152100, gallamine and WIN 62,577 (Figure.1).

Binding analysis of LY2119620 using [^{3}H]-NMS

Shown in Figure 2, LY2119620 displays little to no binding affinity for all 5 human mAChRs to the orthosteric pocket when interacting with the non-selective antagonist radioligand, [^{3}H]-NMS. In contrast, the orthosteric non-selective antagonist, atropine, caused a concentration-dependent inhibition in the binding of [^{3}H]-NMS for all 5 mAChRs (Figure 2 A-E). The competition for atropine and LY2119620 for M_1-M_5 mAChRs is summarized in Table 1. To address whether LY2119620 interacts with the human mAChRs in an allosteric manner, radioligand dissociation experiments were performed to see if co-incubating LY2119620 with the non-selective muscarinic agonist, oxotremorine-M, changed the off-rate ($t_{1/2}$) of [^{3}H]-NMS, since altered dissociation rates can be indicative of an allosteric interaction. Figure 3 illustrates the dissociation of [^{3}H]-NMS by oxotremorine-M in the presence of various concentrations of LY2119620 for both the human M_2 and M_4 mAChRs. The off-rate of [^{3}H]-NMS in the presence of oxotremorine-M was significantly reduced for both M_2 (Figure 3A) and M_4 (Figure 3B) by LY2119620, and could be fitted to a one phase exponential decay model. The $t_{1/2}$ for M_2

mAChR alone was 2.5 minutes and in the presence 10 μM LY2119620 doubled to 5.8 minutes. The $t_{1/2}$ of the radioligand at M_2 mAChR decreased in a concentration dependent manner as more LY2119620 was added. The $t_{1/2}$ at 20 μM was 15.2 minutes and at 40 μM increased to 51.8 minutes. The $t_{1/2}$ for the dissociation of [^{3}H]-NMS by oxotremorine-M for the M_4 mAChR was 11.5 minutes, however in the presence 10 μM LY2119620 nearly quadrupled to 44.6 minutes. Like the M_2 mAChR, the $t_{1/2}$ of [^{3}H]-NMS at the M_4 decreases in a concentration dependent manner. The $t_{1/2}$ at 5 μM was 18.7 minutes and at 20 μM increased to 116.7 minutes. Clearly, the dissociation kinetic studies indicated that LY2119620 binds allosterically to the human M_2 and M_4 mAChRs and was positively cooperative with orthosteric ligand binding. To test an alternative approach to determine if LY2119620 can be positively cooperative with orthosteric agonist binding, we measured the influence of LY2119620 on the ability of acetylcholine or oxotremorine-M to displace [^{3}H]-NMS (Figure 4 A-D). LY2119620 was positively cooperative in its enhancement of orthosteric agonist competition for [^{3}H]-NMS binding as shown by a leftward shift in the binding curve for both agonists. LY2119620 was significantly more cooperative at the M_4 mAChR compared to its cooperativity at M_2 for both acetylcholine and oxotremorine-M. Application of an allosteric ternary complex model (Christopoulos and Kenakin, 2002) using the equation built in to the GraphPad Prism program yielded the logarithm of cooperativity factor (logα) for LY2119620 in the presence of acetylcholine, which was 0.7 and 1.9 for M_2 and M_4, respectively. LY2119620 increased oxotremorine-M affinity at the M_2 receptor with a logα of 1.5 whereas M_4 increased by a logα of 2.3.

Binding analysis of [^{3}H]-LY2119620 as a radioligand for human muscarinic acetylcholine receptors

To address if [^{3}H]-LY2119620 bound specifically to any of the 5 mAChRs, we performed saturation binding studies with membranes stably expressing the human M_1-M_5 mAChRs. A summary of these results can be found in Table 2. Confirming unlabeled binding studies, [^{3}H]-LY2119620 did not bind to the M_1, M_3 or M_5 mAChRs. However, [^{3}H]-LY2119620 bound to the human M_2 and M_4 mAChRs with relativity high affinity. Depending on the orthosteric agonist used, both mAChRs bound with similar affinity (K_d), but very different B_{max} values (number of binding sites). In the absence of orthosteric agonists, no specific binding of [^{3}H]-LY2119620 was detected further, indicating a robust cooperativity between the orthosteric and allosteric sites (data not shown). The K_d values for [^{3}H]-LY2119620 at the human M_2 were not significantly different (p=0.89, n=3, Student's t-Test) 12.9±3.24 nM and 14.4±2.7 nM in the presence of 100 μM acetylcholine or oxotremorine-M, respectively. The B_{max} value for [^{3}H]-LY2119620 binding to the human M_2 was 160±34 fmol/mg protein in the presence of 100 μM acetylcholine. However, in the presence of 100 oxotremorine-M, the number of binding sites increased 17 fold (2700±383 fmol/mg protein). The M_4 mAChR was similar to M_2 in that the K_d values for 100 μM acetylcholine and oxotremorine-M were not significantly different (p=0.57, n= 3, Student's T-Test), 2.54±0.39 nM and 2.73±0.08 nM, respectively. The B_{max} value for the M_4 mAChR were significantly higher in the presence of 100 μM oxotremorine-M (1110±157 fmol/mg protein) compared to acetylcholine (468±54 fmol/mg protein) (p>0.02, n=3, Student's T-Test). The concentrations of acetylcholine and oxotremorine-M were tittered from 0-1000 μM and 100 μM was shown to yield a maximal response for both M_2 and M_4 mAChRs (data not shown). Displacement studies were conducted with [^{3}H]-LY2119620 at the human M_2 or M_4 mAChRs to elucidate whether this compound bound to a unique allosteric site on these receptors or to previously published sites using key allosteric tool

compounds. Figure 5 illustrates that [^{3}H]-LY2119620 binding in the presence of 100 μM oxotremorine-M was potently displaced by unlabeled LY2119620 at both the M_2 (K_i = 15.3 ± 1.36 nM) and M_4 (K_i = 1.03 ± 0.08 nM) mAChRs. A structurally similar compound, LY2033298, displaced [^{3}H]-LY2119620 binding to the human M_2 mAChR with a K_i of 87.1 ± 20.9 nM and M_4 with a K_i of 2.14 ± 0.28 nM (Figure 5 A-B). We also investigated whether LY2119620 bound to either the putative strychnine or staurosporine allosteric sites by displacing [^{3}H]-LY2119620 with gallamine and WIN 62,577, respectively. Neither gallamine nor WIN 62,577 displaced [^{3}H]-LY2119620 from the human M_2 or M_4 mAChRs under these assay conditions (Table 3). Other M_4 allosteric modulators similar to LY2033298, VU152100 and VU10010 were also able to displace [^{3}H]-LY2119620 from the human M_4 mAChR but not M_2. This result was expected based on previous work that has demonstrated that these compounds are selective for the M_4 receptor (Brady et al., 2008; Shirey et al., 2008)

Autoradiographic localization of the M_4 allosteric binding sites in cynomolgus monkey brain using [^{3}H]-LY2119620

A series of sagittal sections through cynomolgus monkey brains were incubated with ~5 nM [^{3}H]-LY2119620 and 100 μM acetylcholine to examine the distribution of labeling in different brain structures. Under certain assay conditions, one can favor M_4 binding over M_2. We took advantage of probe dependence to label mostly M_4 receptors, because [^{3}H]-LY2119620 does not label large numbers of M_2 receptors in the presence of 100 μM acetylcholine (supplemental Figure 1). Because LY2119620 has relatively low affinity for rodent mAChRs similar to LY2033298, we relied on the distribution of the M_4 allosteric binding sites in the cynomolgus monkey to give us insight into the distribution in higher species. Cynomolgus monkey has nearly identical M_2 and M_4 mAChRs sequences as humans. In general, [^{3}H]-

LY2119620 binding in monkey was broadly distributed in the cortex and devoid in the cerebellum (Figure 6). Some of the highest levels of binding with [^{3}H]-LY2119620 were observed in the caudate-putamen and the superficial (I-III) laminae of the cerebral cortex (Figure 6A). [^{3}H]-LY2119620 binding was almost completely eliminated by the presence of 10 μM LY2033298 (Figure 6B). Therefore, the amount of radioligand binding remaining in Figure 6B represents non-specific binding.

Discussion

The five subtypes of mAChRs are members of the superfamily of G-protein coupled receptors (Caulfield and Birdsall, 1998) and are now known to have allosteric binding sites that provide significant modulation of functional signaling (Christopoulos, 2002; Christopoulos and Kenakin, 2002; Christopoulos et al., 1998). In the present study we have shown that the allosteric modulator, LY2119620, exerts its modulator effects through a common site on the M_4 mAChR, similar to LY2033298 (Chan et al., 2008) which has been extensively studied using mutagenesis (Leach et al., 2010). In agreement with LY2033298, our findings clearly indicate that LY2119620 is a novel allosteric compound that does not interact with the orthosteric site , similar to the properties of known allosteric compounds such as staurosporine (Lazareno et al., 2000) or strychnine (Ellis et al., 1991). To investigate whether LY2119620 bound to mAChRs in a bitopic manner (a ligand engaging both the orthosteric and allosteric sites at the same time) we employed [^{3}H]-NMS displacement studies. Unlike the M_2 bitopic ligand, McN-A-343 (Valant et al., 2008), LY2119620 does not displace the classic orthosteric pocket labeled with [^{3}H]-NMS. This is in contrast to the orthosteric antagonist, atropine, which readily displaces [^{3}H]-NMS from the M_1-M_5 mAChRs (Figure 2). A radiolabeled allosteric modulator of mAChRs was first described for the M_2 mAChR using [^{3}H]dimethyl-W84 (Tränkle et al., 1998). Prototype muscarinic allosteric agents, alcuronium and gallamine displaced in a concentration dependent manner the high affinity site of [^{3}H]dimethyl-W84 binding, These data lead Tränkle *et al* to conclude that this radioligand bound to the "common" allosteric site on M_2. In contrast, [^{3}H]-LY2119620 was not displaced by previously described mAChR allosteric compounds such as gallamine or WIN 62,577. The greatest distinction between [^{3}H]dimethyl-W84 and [^{3}H]-LY2119620 appears to be in how these radioligands interact with the allosteric site.

[^{3}H]dimethyl-W84 was shown to negatively modulate the M_2 allosteric site while [^{3}H]-LY2119620 demonstrates positive cooperativity with this site.

The dissociation rate of [^{3}H]-NMS was significantly reduced for both the M_2 and M_4 mAChRs in the presence of LY2119620 (Figure 3). We have taken the ability of LY2119620 to slow the off-rate of [^{3}H]-NMS as a measure of its allosteric effect (the binding of the allosteric ligand to the allosteric site which alters the affinity of the muscarinic orthosteric agonist to the bind to the orthosteric binding pocket on the receptor). The cooperative effect was dependent on the muscarinic ligand it interacts with which can be either positive, negative or neutral. In the [^{3}H]-NMS competition-binding, the interaction of LY2119620 with either acetylcholine or oxotremorine-M was positively cooperative for both M_2 and M_4 mAChRs (Figure 4). Interestingly, the affinity (K_d) of [^{3}H]-LY2119620 for the mAChR was similar while the B_{max} varied considerably whether acetylcholine or oxotremorine-M was used. This finding of probe dependence (the interaction between allosteric and orthosteric sites changing depending on the orthosteric ligand used) was evident in these studies because we used saturating concentrations of acetylcholine or oxotremorine-M. Probe dependence was also shown for the structurally similar compound, LY2033298, at the mouse M_4 mAChR (Suratman et al., 2011). In that study, it was speculated that the probe dependence was due to different cooperativities between modulator and orthosteric ligands, because LY2033298 had similar affinities for both human and mouse M_4 allosteric sites. The positive cooperativity between acetylcholine and LY2033298 was most evident at the human M_4 and was lower at the human M_2 and essentially neutral at the other mAChR subtypes (Chan et al., 2008). We see similar cooperativity differences between M_2 and M_4 with acetylcholine or oxotremorine-M using [^{3}H]-NMS binding (Figure 4). Therefore, one could speculate that the difference in the B_{max} values between orthosteric agonists

with [^{3}H]-LY2119620 was due a similar mechanism. That is, increased modulator binding was directly proportion of the number of active state receptors since it is well known that GPCRs exist in two states, active (RG) and inactive (R). Although the exact mechanism remains unclear, a common interpretation is that this somehow reflects the coupling of the GPCR to the G-protein(s) to promote RG (Christopoulos and El-Fakahany, 1999). Not yet tested was whether the functional positive allosteric modulation by either LY2033298 or LY2119620 can be driven by increasing the cooperativity between orthosteric ligand and G-protein binding thus increasing the number of G-protein bound mAChRs and thereby increasing the functional output of the signaling being measured. This interaction could be inferred as functional GTPg^{35}S binding was positively modulated by LY2119620 (Croy et al., In preparation). Furthermore, emerging crystal structure studies with allosteric compounds might shed some light on probe dependence. Recently the M_2 mAChR was crystalized in the active-state with iperoxo docked in the orthosteric binding pocket in the presence of LY2119620 (Kruse et al., 2013). The M_2 crystal structure revealed LY2119620 induces additional albeit subtle structural changes as compared to those seen with just the orthosteric agonist.

Like its predecessor LY2033298, LY2119620 is also subject to species variability. In the initial characterization of LY2033298, it was noted that this compound had reduced *in vitro* potency as a modulator at the rat when compared to the human M_4 mAChR (Chan et al., 2008) and later for the mouse (Suratman et al., 2011). However, the affinity for LY2033298 across species has been shown to be very similar (Leach et al., 2010; Suratman et al., 2011; Valant et al., 2012) in several assay formats. In contrast, any attempt to get [^{3}H]-LY2119620 to bind to either recombinantly expressed rodent muscarinic or native tissue in the presence of any orthosteric agonist was not successful. However, it should be pointed out that the concentrations

of radioligand used were limited due to reagent costs as well as increasing non-specific binding at higher concentrations. These direct labeling experiments with a radiolabeled allosteric probe contradicts previous reports using various functional assays that these allosteric modulators have similar affinity across species (Leach et al., 2010; Suratman et al., 2011; Valant et al., 2012). In addition, we used [^{3}H]-LY2119620 to probe the distribution of these allosteric sites in the brain. We used non-human primate (NHP) as the gene sequences between NHP and human are nearly identical. We found the distribution of [^{3}H]-LY2119620 was similar to the distribution of M_2 and M_4 mAChRs using [^{3}H]AF-DX 384, a selective M_2 and M_4 antagonist of the muscarinic acetylcholine receptors (Quirion et al., 1993). [^{3}H]AF-DX 384 binds preferentially to the striatum, cortex, thalamus and cerebellum.

In summary, we have identified LY2119620 as an allosteric modulator of the human M_2 and M_4 mAChRs. Our data support the hypothesis that allosteric selectivity between M_2 and M_4 mAChR subtypes was the result of differences in cooperativity with the endogenous agonist, exemplifying probe dependence. Cooperativity governing selectivity, rather than affinity for a unique allosteric site, has been seen previously reported with thiochrome (Lazareno et al., 2004) and LY2033298 (Leach et al., 2010). This supports our hypothesis that the allosteric modulators can recruit of G-protein coupling, because the number of high affinity binding sites labeled with [^{3}H]-acetylcholine significantly increase in the presence of thiochrome and LY2119620 increased the number of high-affinity binding sites using [^{3}H]-Oxotremorine-M (Croy et al., In preparation). Likewise, we report in this study that the affinity of [^{3}H]-LY2119620 was similar between orthosteric agonists, but the total number of binding sites was significantly different between agonists. So in conclusion, not all orthosteric agonists recruit high-affinity binding sites

to the same extent and allosteric binding governs G-protein recruitment cooperatively with orthosteric agonist binding.

Authorship Contributions

Conducted experiments: Douglas A Schober, Carrie H Croy and Hongling Xiao.

Performed data analysis: Douglas A Schober, Carrie H Croy, Arthur Christopoulos and Christian C Felder.

Wrote or contributed to the writing of the manuscript: Douglas A Schober, Carrie H Croy, Christian C Felder and Arthur Christopoulos.

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Figure Legends

Fig. 1. Structures of key pharmacological tools. Structures of M_4 allosteric modulators LY2119620, LY2033298, VU152100 and VU10010 as well as other putative muscarinic allosteric compounds gallamine and WIN 62,577.

Fig. 2. Pharmacological characterization of LY2119620 (■) and atropine (●) to CHO membrane homogenates incubated with ~0.5 nM [^{3}H]-NMS for 2 h at 22°C. Membranes are from CHO cells stably expressing human mAChRs (**A)** M_1 (**B)** M_2 (**C)** M_3 (**D)** M_4 and (**E)** M_5. Data shown are the result of four independent experiments performed in duplicate. The symbols and error bars are expressed as the mean ± S.E.M., respectively.

Fig. 3. Dissociation rates for CHO homogenates stably expressing the human mAChRs M_2 **(A)** or M_4 **(B)**. Membranes were incubated with ~1.0 nM concentration of [^{3}H]-NMS and various concentrations of LY2119620 for 2 h prior to the addition of 1.0 μM oxotremorine-M. The membrane homogenates were incubated at room temperature for the duration of the experiment. A single representative graph is shown performed, from three separate experiments. Data shown are the result of three independent experiments performed in duplicate. The symbols and error bars are expressed as the mean ± S.E.M., respectively.

Fig. 4. Concentration-dependent effects of LY2119620 on [^{3}H]-NMS displacement binding to the M_2 and M_4 mAChRs in the presence of various concentrations of either oxotremorine-M **(A-B)** or acetylcholine **(C-D)**. M_2 and M_4 CHO membrane homogenates were incubated with ~1.0

nM [^{3}H]-NMS for 2 h at room temperature. Data shown are the result of three independent experiments performed in duplicate and expressed as percent of control. The symbols and error bars are expressed as the mean ± S.E.M., respectively.

Fig. 5. Displacement [3H]-LY2119620 by known muscarinic allosteric compounds at the human mAChRs M_2 **(A)** and M_4 **(B)**. Membranes were incubated with ~5.0 nM concentrations of [^{3}H]-LY2119620 for 1 h. The addition of 10 µM LY2033298 was used to define non-specific binding. Data shown are the result of three independent experiments performed in duplicate and expressed as percent of control. The symbols and error bars are expressed as the mean ± S.E.M., respectively.

Fig. 6. Autoradiography of the cynomolgus monkey brain. Male monkey coronal brain sections were radiolabelled with [^{3}H]-LY2119620 in the presence of 100 µM acetylcholine. Twelve µm sagittal section were incubated with ~5.0 nM of [^{3}H]-LY2119620 as described in the Methods section. In addition, some sections were incubated with the addition of 10 µM LY2033298 to define non-specific binding. Representative autoradiograms for total specific binding are presented in panel A represent the amount of binding in the presence of 100 µM acetylcholine. Non-specific binding are in panel B. A binding density scale illustrates highest levels in orange and lowest in blue. Abbreviations are: CTX, cortex; CPu, caudate-putamen.

Table 1 Displacement of [^{3}H]-NMS by Atropine and LY2119620 to human M_1-M_5 mAChRs. The following IC_{50} values are calculated for the displacement of [^{3}H]-NMS by Atropine or LY2119620. The IC_{50} values were determined using log(inhibitor) vs. response (three parameters) using GraphPad Prism. For those compounds that did not inhibit LY2119620 response at highest concentration tested (10 μM) are represented as >10000 nM. The values are expressed as the averages ± S.E.M three independent experiments performed in duplicate.

Receptor Subtype	Atropine IC_{50} (nM)	LY2119620 IC_{50} (nM)
M_1	1.78 ± 0.13	>10000
M_2	4.51 ± 0.22	>10000
M_3	1.78 ± 0.13	>10000
M_4	5.01 ± 0.79	>10000
M_5	5.02 ± 1.01	>10000

Table 2 Saturation binding of [^{3}H]-LY2119620 to human M_1-M_5 mAChRs in the presence of 100 μM of the orthosteric agonists acetylcholine or oxotremorine-M Membranes were incubated with various concentrations of [^{3}H]-LY2119620 for 1 h at room temperature. BDL (below detectable levels). Data shown are the result of three independent experiments performed in duplicate.

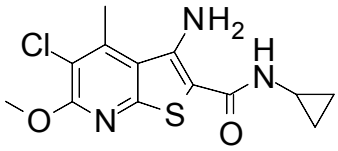
Receptor Subtype	Acetylcholine (100 μM)		Oxotremorine-M (100 μM)	
	B_{max} (fmol/mg)	K_d (nM)	B_{max} (fmol/mg)	K_d (nM)
M_1	BDL	BDL	BDL	BDL
M_2	160 ± 34.0	12.9 ± 3.24	2700 ± 383	14.4 ± 2.70
M_3	BDL	BDL	BDL	BDL
M_4	468 ± 53.8	2.54± 0.389	1110 ± 157	2.73 ± 0.0774
M_5	BDL	BDL	BDL	BDL

Table 3 Defining the allosteric binding site by displacing [^{3}H]-LY2119620 with other allosteric compounds in the presence of 100 μM oxotremorine-M. Displacement of [^{3}H]-LY2119620 by other allosteric compounds in the presence of 100 μM Oxotremorine-M. The K_i values shown are the result of three independent experiments performed in duplicate. The values are expressed as the averages ± S.E.M.

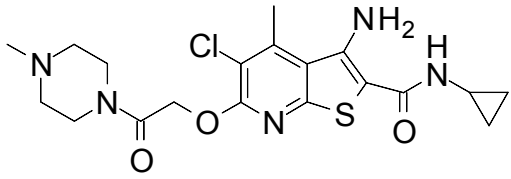
Receptor Subtype	LY2119620	LY2033298	VU10010	VU152100	Gallamine	WIN 62,577
M_2	15.3 ± 1.36	87.1 ± 20.9	>10 000	>10 000	>10 000	>10 000
M_4	1.03 ± 0.0776	2.14 ± 0.279	42.6 ± 16.4	48.3 ± 10.7	>10 000	>10 000

Figure 1

LY2033298



LY2119620



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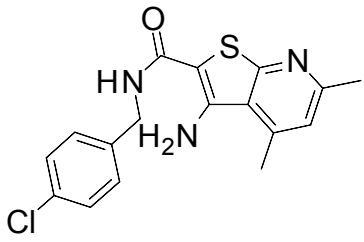

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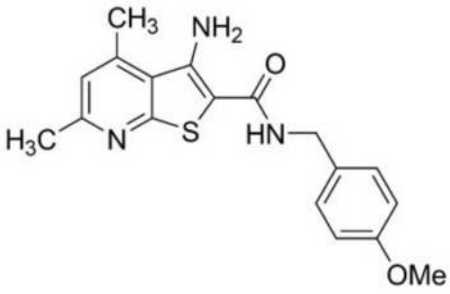
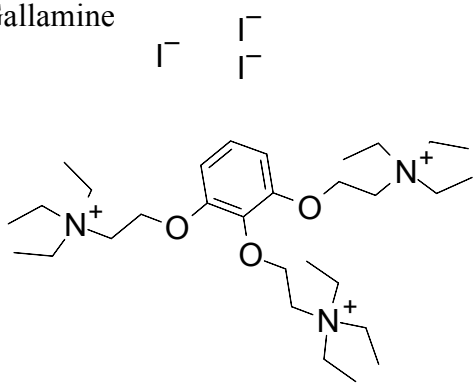


Gallamine



WIN 62,577

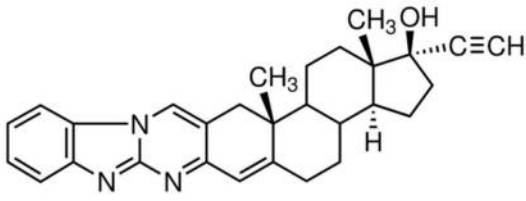


Figure 2

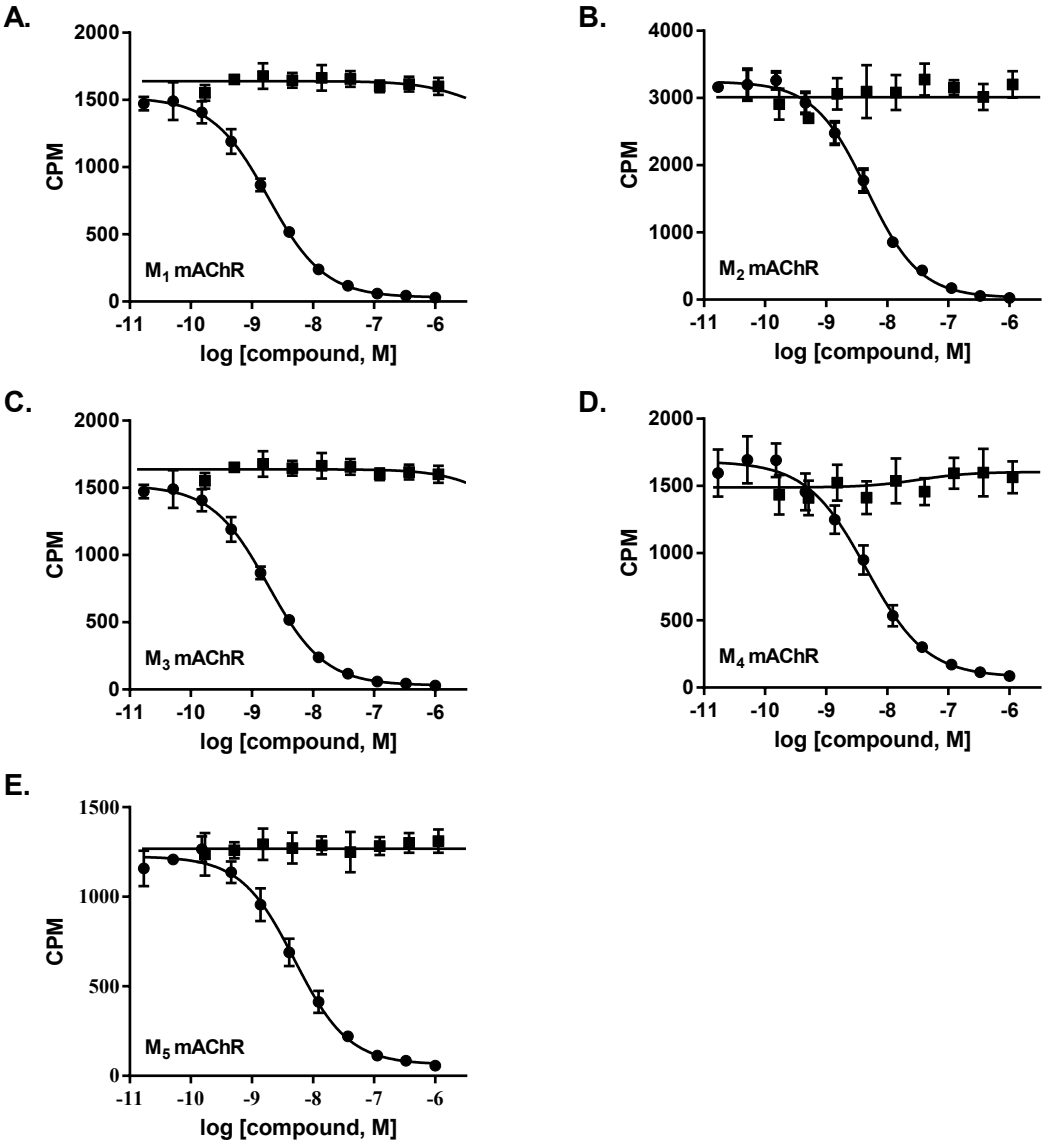

A.
2000
1500
1000
500
0
CPM
M1 mAChR
-11
-10
-9
-8
-7
-6
log [compound, M]
B.
4000
3000
2000
1000
0
CPM
M2 mAChR
-11
-10
-9
-8
-7
-6
log [compound, M]
C.
2000
1500
1000
500
0
CPM
M3 mAChR
-11
-10
-9
-8
-7
-6
log [compound, M]
D.
2000
1500
1000
500
0
CPM
M4 mAChR
-11
-10
-9
-8
-7
-6
log [compound, M]
E.
1500
1000
500
0
CPM
M5 mAChR
-11
-10
-9
-8
-7
-6
log [compound, M]

Figure 3

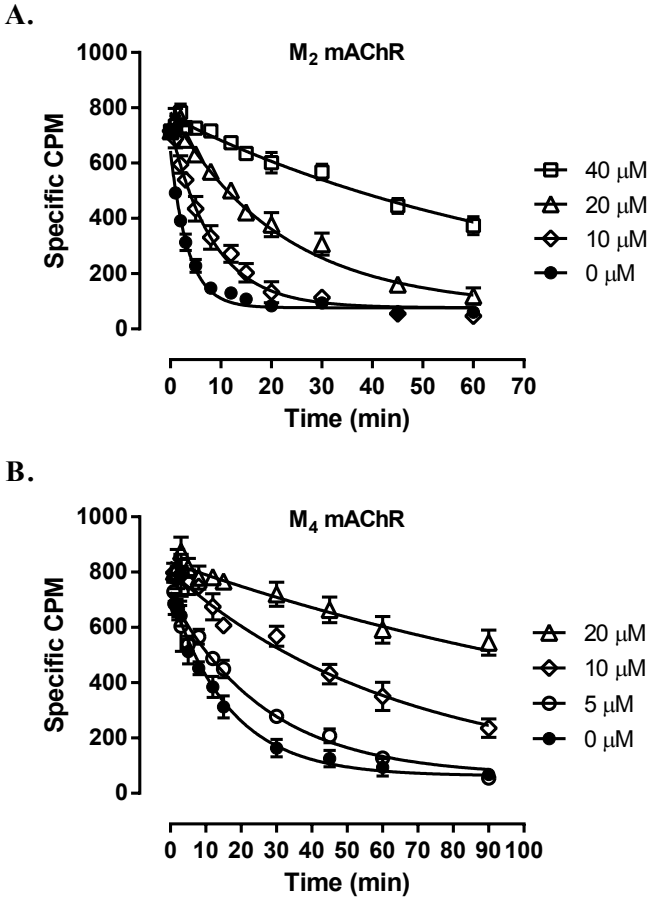

A.
M2 mAChR
Specific CPM
1000
800
600
400
200
0
0 10 20 30 40 50 60 70
Time (min)
40 μM
20 μM
10 μM
0 μM
B.
M4 mAChR
Specific CPM
1000
800
600
400
200
0
0 10 20 30 40 50 60 70 80 90 100
Time (min)
20 μM
10 μM
5 μM
0 μM

Figure 4

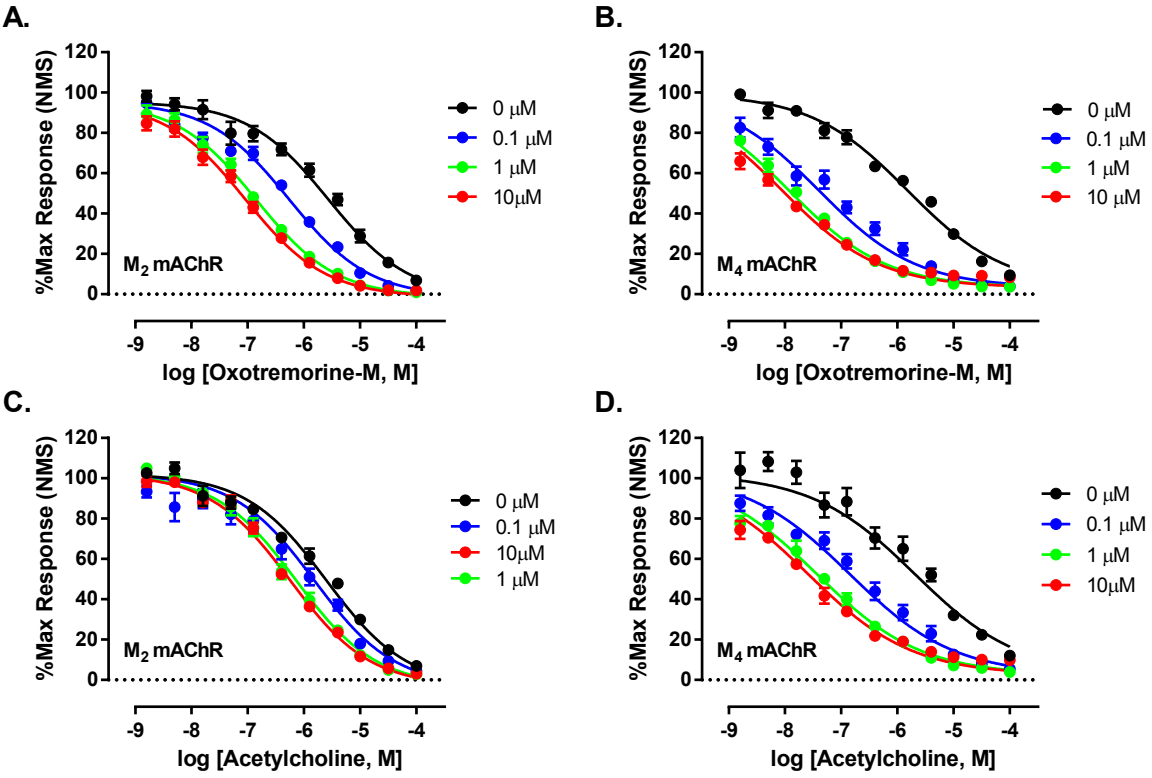
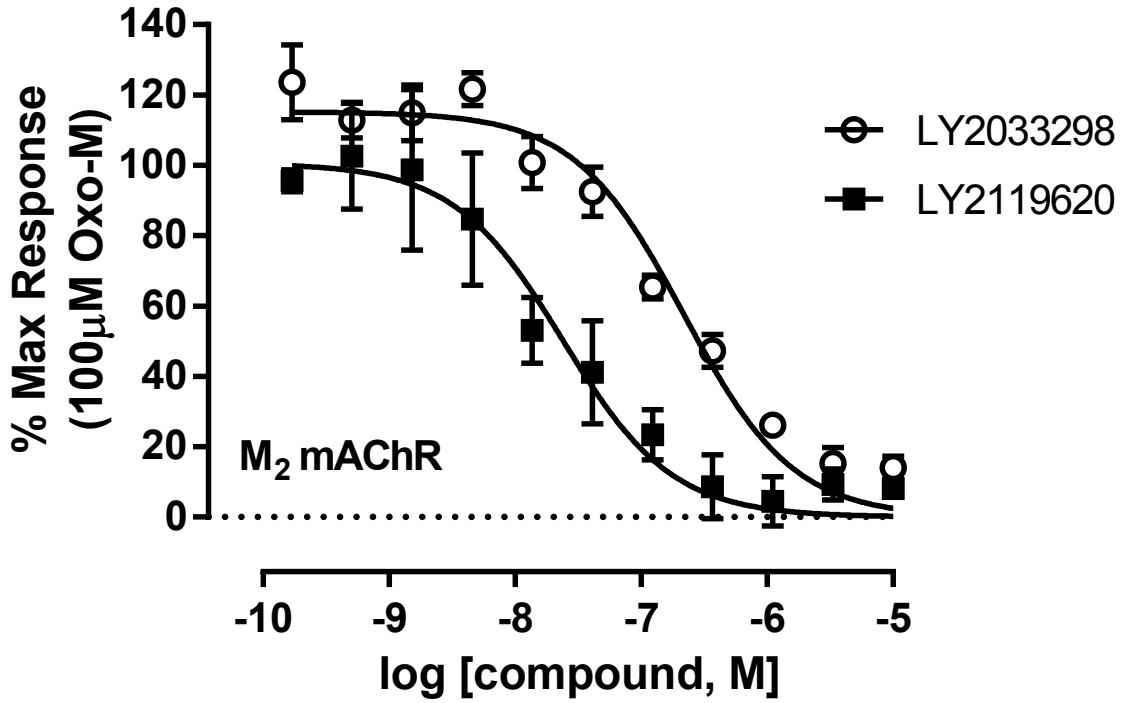

A.
%Max Response (NMS)
120
100
80
60
40
20
0
M2 mAChR
0 μM
0.1 μM
1 μM
10μM
-9
-8
-7
-6
-5
-4
log [Oxotremorine-M, M]
B.
%Max Response (NMS)
M4 mAChR
0 μM
0.1 μM
1 μM
10 μM
log [Oxotremorine-M, M]
C.
%Max Response (NMS)
M2 mAChR
0 μM
0.1 μM
10μM
1 μM
log [Acetylcholine, M]
D.
%Max Response (NMS)
M4 mAChR
0 μM
0.1 μM
1 μM
10μM
log [Acetylcholine, M]

Figure 5

A.


% Max Response
(100μM Oxo-M)
140
120
100
80
60
40
20
0
M2 mAChR
-10
-9
-8
-7
-6
-5
log [compound, M]
LY2033298
LY2119620

B.

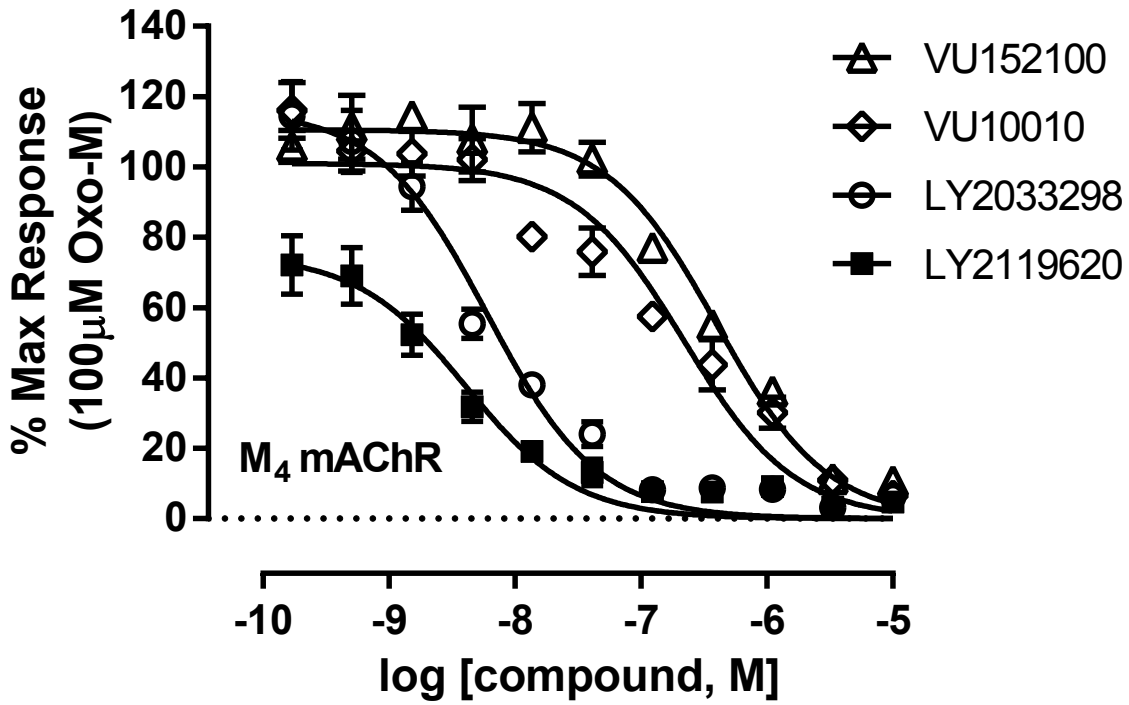
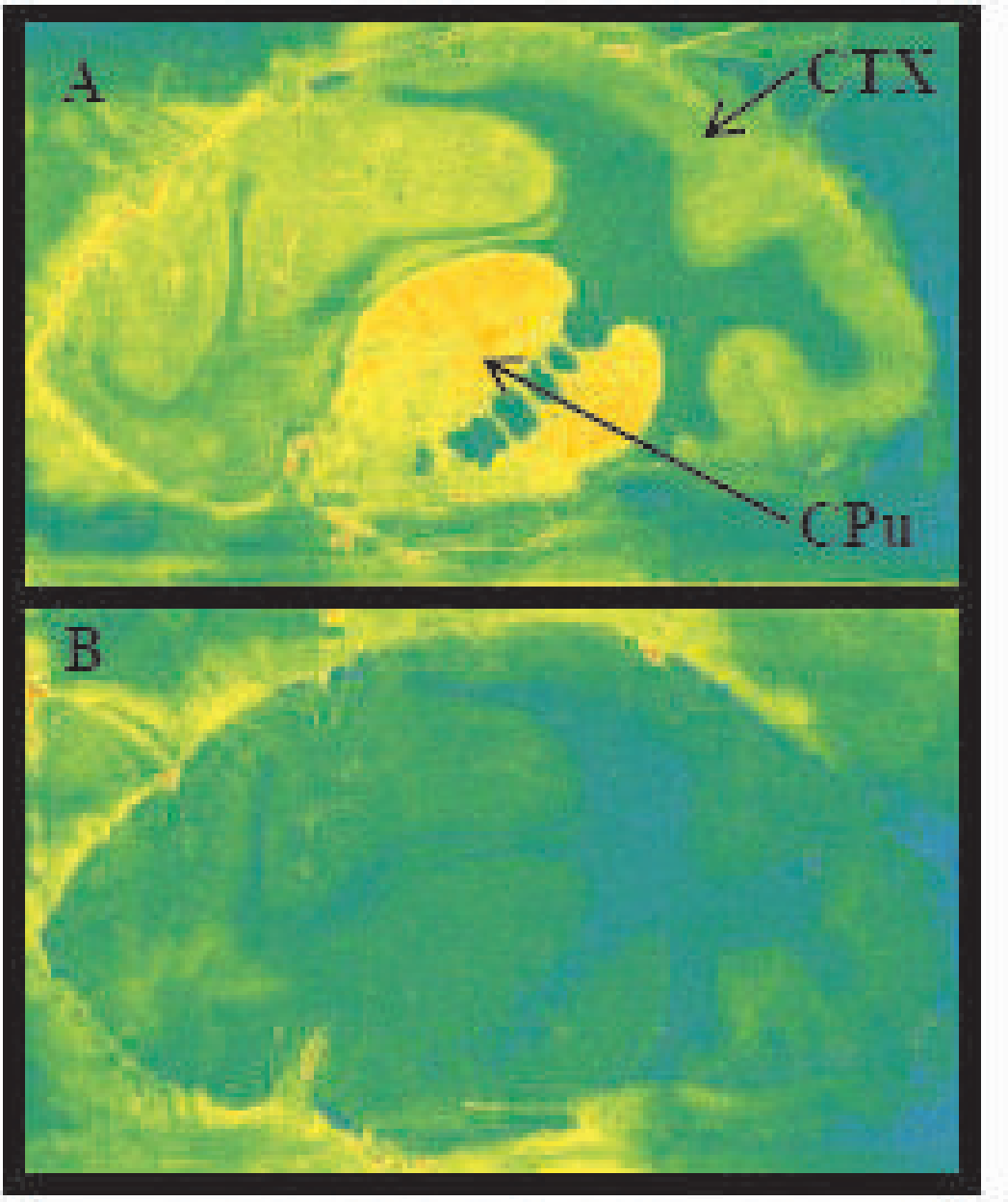
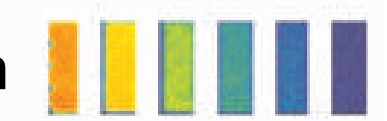

% Max Response
(100μM Oxo-M)
140
120
100
80
60
40
20
0
M4 mAChR
-10
-9
-8
-7
-6
-5
log [compound, M]
VU152100
VU10010
LY2033298
LY2119620

Figure 6





Receptor density scale