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Structural Requirements of Transmembrane Domain 3 for Activation by the M_1 Muscarinic Receptor Agonists AC-42, AC-260584, Clozapine and N-desmethylclozapine: Evidence for three distinct modes of receptor activation

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Running Title

M_1 receptors are activated in at least three distinct ways

12 pages of text, 3 tables and 4 figures.

26 references.

Abstract: 250 words

Introduction: 749 words

Discussion: 1440 words

Abbreviations

AC-42: 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine hydrogen chloride

NMS: N-methyl scopolamine

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Abstract

Transmembrane domain three (TM3) plays a crucial role mediating muscarinic acetylcholine receptor activation by acetylcholine, carbachol, and other muscarinic agonists. We compared the effects of point mutations throughout TM3 on the interactions of carbachol, AC-42, a potent structural analogue of AC-42 called AC-260584, N-desmethylclozapine, and clozapine with the M_1 muscarinic receptor. The binding and activation profiles of these ligands fell into three distinct patterns; one exemplified by orthosteric compounds like carbachol, another by structural analogs of AC-42, and a third by structural analogs of N-desmethylclozapine. All mutations tested severely reduced carbachol binding and activation of M_1. In contrast, the agonist actions of AC-42 and AC-260584 were greatly potentiated by the W101A mutation, slightly reduced by Y106A and slightly increased by S109A. Clozapine and N-desmethylclozapine displayed substantially increased maximum responses at the Y106A and W101A mutants, slightly lower activity at S109A, but no substantial changes in potency. At L102A and N110A, agonist responses to AC-42, AC-260584, clozapine and N-desmethylclozapine were all substantially reduced, but usually less than carbachol. D105A showed no functional responses to all ligands. Displacement and dissociation rate experiments demonstrated clear allosteric properties of AC-42 and AC-260584, but not for N-desmethylclozapine and clozapine indicating they may contact different residues than carbachol to activate M_1, but occupy substantially overlapping spaces, in contrast to AC-42 and AC-260584, which occupy separable spaces. These results show M_1 receptors can be activated in at least three distinct ways and that there is no requirement for potent muscarinic agonists to mimic acetylcholine interactions with TM3.

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Introduction

Agonists that activate the M_1 muscarinic acetylcholine receptor have been shown to improve cognitive function in humans and other animals (Bodick et al, 1997, Weiss et al, 2000, Bartolomeo et al, 2000), making the M_1 receptor an attractive therapeutic target for treating cognitive dysfunction in Alzheimer's disease and psychosis (Bymaster et al, 2002). Unfortunately, the M_1 agonists that have been developed are not M_1-selective and retain significant dose-limiting side-effects such as sweating, vomiting and nausea (Bodick et al, 1997, Bartolemeo et al, 2000, Thal et al, 2000). Work with knockout animals suggests that these side-effects are likely to be caused by activation of the M_2 and M_3 muscarinic receptor subtypes (Bymaster et al, 2002, Wess et al, 2003).

Muscarinic M_1 receptors bind their endogenous agonist, acetylcholine, through a binding site embedded in the transmembrane domains of the receptor and involving TM3, TM4, TM5, TM6 and TM7 (Hulme et al, 2003). This is termed the *orthosteric* site, because it binds the endogenous ligand for the receptor. TM3 is a crucial part of this orthosteric site, and is thought to fulfill a central role in activation mechanism of muscarinic receptors, as well as many other G-protein coupled receptors (GPCRs) (Gether, 2000). A series of residues in TM3 have been shown to participate in binding and activation by muscarinic agonists (Lu and Hulme, 1999). The primary feature of acetylcholine binding is a salt bridge believed to exist between the choline headgroup of acetylcholine and aspartate 105 (D105) in TM3. This residue reacted with the affinity label acetylcholine mustard, where the onium headgroup of acetylcholine is replaced by a highly reactive aziridinium group (Spalding et al, 1994), demonstrating that this moiety was physically close to D105 when it bound the receptor. When D105 was replaced by the neutral amino acid alanine, thereby preventing the salt bridge from forming, the affinity of acetylcholine was reduced by 60-fold, and the compound no longer showed agonist activity (Lu and Hulme, 1999). Recently Hulme et al (2003) suggested that other residues in TM3 such as tryptophan 101 (W101), leucine 102 (L102) and tyrosine 106 (Y106), along with residues in TM6 and TM7 form a hydrophobic cage around D105 that closes around the acetylcholine molecule, thus triggering the isomerization of the receptor into an active conformation.

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We recently identified a novel agonist, AC-42, that potently activates the M_1 subtype but has no agonist activity on M_2 - M_5 subtypes. Using a series of chimeric receptors, we demonstrated that residues in the N-terminus/TM1 and the third outer loop (o3)/TM7 domains are required for AC-42 to elicit agonist activity at the M_1 receptor (Spalding et al, 2002). The residues in these regions are not conserved among the muscarinic subtypes, which probably accounts for the selectivity of AC-42. Recently, AC-42 was shown to act allosterically at M_1 receptors based on the observations that it did not completely displace NMS from M_1, that it retarded the dissociation of NMS from M_1, and that atropine antagonism of AC-42 induced functional responses yielded Schild slopes less than unity (Langmead et al, 2006).

Moreover, it has been shown that the active metabolite of the atypical antipsychotic clozapine, N-desmethylclozapine, is a potent M_1 receptor partial agonist (Sur et al, 2003; Weiner et al, 2004; Davies et al, 2005). We, and others (Li et al, 2005) have proposed that the M_1 agonist activity of N-desmethylclozapine may contribute to the pro-cognitive benefits of clozapine therapy. N-desmethylclozapine has also been suggested to bind to M_1 receptors at a site distinct from the acetylcholine-binding site (Sur et al, 2003).

We present data from a series of experiments examining whether the activation sites of AC-42, AC-260584 (a structurally related compound with substantially greater potency and efficacy than AC-42), N-desmethylclozapine and clozapine overlap with the orthosteric binding site on TM3. We investigated the interactions of these ligands with a series of receptors mutated at residues in TM3 crucial for interaction with orthosteric agonists. We show that unlike carbachol, the binding affinity and agonist activity of each of these other ligands is generally maintained, and in some cases greatly increased, with two activation patterns apparent, one for AC-42 and AC-260584, and the other for N-desmethylclozapine and clozapine. Displacement and dissociation rate experiments demonstrated clear allosteric properties of AC-42 and AC-260584, but not of N-desmethylclozapine and clozapine. Together these data suggest M_1 muscarinic receptors can be activated in at least three distinct ways; one exemplified by orthosteric compounds like carbachol, another by structural analogs of AC-42, and a third by structural analogs of N-desmethylclozapine.

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Materials and methods

Ligands. Carbachol (carbamylcholine), clozapine and atropine were obtained from Sigma-Aldrich (St. Louis, MO). *l*-[N-methyl-^{3}H]Scopolamine methyl chloride ([^{3}H]NMS) was obtained from Amersham Biosciences (Piscataway, NJ). AC-42 (4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine hydrogen chloride) was synthesized by Organic Consultants Inc. (Eugene, OR). AC-260584 (4-[3-(4-butylpiperidin-1-yl)-propyl]-7-fluoro-4H-benzo[1,4]oxazin-3-one) and N-desmethylclozapine (8-chloro-11-(1-piperazinyl)-5H-dibenzo [b,e] [1,4] diazepine) were synthesized at ACADIA. Compound structure was verified by NMR. Purity was greater than 99% measured by HPLC and gas chromatography.

DNA Constructs: The rat M_1 receptor and the W101A, L102A, D105A, Y106A, S109A and N110A mutants were the kind gift of Dr. E.C. Hulme, MRC National Institute for Medical Research, London, UK. All constructs were sequence verified.

R-SAT™. R-SAT™ functional assays were carried out essentially as described in Spalding et al, 2002. NIH-3T3 cells were grown in 96-well tissue culture plates to 70 to 80% confluence in Dulbecco's modified essential media (DMEM) supplemented with 100 units/ml penicillin, 100 micrograms/ml streptomycin, 0.3 mg/ml L-glutamine (1% PSG, GIBCO) and 10% calf serum (Sigma-Aldrich). Cells were transfected for 18 h with DMEM containing 0.08 micrograms/ml receptor DNA and 0.3 micrograms/ml pSI-beta-galactosidase (Promega, Madison WI), 0.5% v/v Polyfect (Qiagen, Valencia CA). Medium was replaced with DMEM containing 1% PSG, 0.5% calf serum, 25% Ultraculture synthetic supplement (Cambrex, Walkersville, MD) instead of calf serum, and varying concentrations of ligand. Carbachol was tested at concentrations up to 100 micromolar, AC-42 was tested up to 5 micromolar, and AC-260584, N-desmethylclozapine and clozapine were tested up to 10 micromolar. Higher concentrations of AC-42 have been shown to nonspecifically inhibit cell growth (data not shown). Cells were grown in a humidified atmosphere with 5% ambient CO2 for 5 days. Medium was removed from the plates, and beta-galactosidase activity was measured by the addition of o-

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nitrophenyl -d-galactopyranoside in phosphate-buffered saline with 5% Nonidet P-40. The resulting colorimetric reaction was measured in a spectrophotometric plate reader (Titertek, Huntsville, AL) at 420 nm. The data was fitted to the following equation using GraphPad Prism software (San Diego, CA):

Response = Basal Response + (Maximum Response-Basal Response) x [Ligand] / (EC_{50} + [Ligand])

Phosphotidyl Inositol Hydrolysis Assays. Phosphotidyl Inositol (PI) Hydrolysis Assays were performed essentially as follows: TsA cells (a HEK 293 cell derivative) were seeded at 10,000 cells/well in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum, penicillin (100units/ml) and streptomycin (100mg/ml) in a 37^{O}C humidified atmosphere containing 5% CO_2. Eighteen hours later, the cells were transfected as described above with the indicated plasmid DNAs (30ng/well of a 96-well plate). Approximately 20-24 hours post-transfection, the cells were washed, and labeled overnight with DMEM culture medium containing 0.2uCi 2-[3H]-myo-inositol (NET114, 37MBg/ml, PerkinElmer) per well (0.1ml). The cells were washed and incubated with Hank's Balanced Salt Solutions (Invitrogen) supplemented with 1mM CaCl2, 1mM MgCl2, 10mM LiCl and 0.2% BSA for 45min. The buffer was removed, and the cells were incubated for another 45min at 37^{O}C in the same buffer with the indicated concentrations of freshly made ligands. The reaction was stopped by exchange with ice-cold 20mM formic acid, and the total [3H]inositol phosphate (IP_1, IP_2 and IP_3) formation was determined by ion-exchange chromatography on 1 ml-minicolumns loaded with 200ul of a 50% suspension of AG 1-X8 resin (200-400 mesh, Formate form, Bio-Rad, Hercules, CA). The columns were washed with 1ml 40mM ammonium hydroxide (pH9) after loading the cell extracts and then eluted with 0.4ml 2M ammonium formate in 0.1M formic acid. The eluates (0.1 ml) were loaded on LumaPlate-96 plates (Yttrium silicate scintillator coated, PerkinElmer), air-dried overnight, and counted on a Microplate Scintillation & Luminescence Counter (TopCount NXT, PerkinElmer).

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Radioligand Binding Assays. Radioligand binding assays were carried out as described by Wess et al, 1991. To determine ligand potency, washed membranes were prepared from HEK293 cells transfected with 10 micrograms plasmid DNA per 15 cm plate and stored at -80°C. Radioligand binding assays were carried out in 25 mM sodium phosphate, 5 mM magnesium chloride, 0.01% BSA (binding buffer). Incubations were for two hours at room temperature, and reactions were stopped by rapid filtration onto GF/B filters. To determine the K_d of ^{3}H-NMS, membranes were incubated in 0.2 ml (Y106A), 1 ml (M_1 wild-type and W101A) or 1.5 ml (S109A) buffer with eight ^{3}H-NMS dilutions between 8 and 1,000 pM (M_1 wild-type), 18 and 2,600 pM (W101A), 160 and 20,000 pM (Y106A) or 4 and 500 pM (S109A) in the presence or absence of 1 micromolar (M_1 wild-type, W101A, S109A) or 30 micromolar (Y106A) atropine. The pKd values of ^{3}H-NMS were (Mean +/- S.D.., N=2): M_1 wild-type: 10.0 +/- 0.3; W101A: 9.4 +/- 0.2; Y106A: 8.2 +/- 0.2; S109A: 9.7 +/- 0.1. To determine the IC_{50} of AC-42, AC-260584, clozapine, N-desmethylclozapine, carbachol and atropine, membranes were incubated with ligand in 0.2 ml buffer in the presence of ^{3}H-NMS at up to 3 times its K_d on that receptor. ^{3}H-NMS concentrations were: M_1 wild-type: 160 pM; W101A: 640 pM; Y106A 1,300 pM; S109A 160 pM. Expression levels for all receptors used were published by Lu and Hulme (1999). To examine whether or not compounds could simultaneously occupy M_1 receptors, these equilibrium binding assays were repeated as described above, using CHO-M_1 cell membranes and ^{3}H-NMS concentrations of 0.2 nM and 2 nM.

^{3}H-NMS Dissociation Rate Assays. For dissociation studies 1 ug/well of CHO-M_1 cell membranes were preincubated with 200 pM ^{3}H-NMS and binding buffer or atropine (1 uM) for at least 60 min. Binding buffer containing 1 uM atropine and the indicated ligands was then added. Total and non-specific binding was determined as described above at the indicated time points. Data were fitted to the following equation using GraphPad Prism software (San Diego, CA): $^3\text{H-NMS bound} = (^3\text{H-NMS bound}_{t=0})\, e^{(-kt)}$ where k is the dissociation rate constant.

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Results

The muscarinic agonists examined in this study comprise three structural classes, orthosteric ligands such as carbachol; AC-42 and its analog AC-260584, and the antipsychotic clozapine and its active metabolite, N-desmethylclozapine (**Figure 1).** The functional activity of each of these compounds was measured on wild-type rat M_1 and six receptors with mutations in TM3 using the R-SAT functional assay (Brauner-Osborne and Brann, 1996) (**Table 1**, **Figure 2**). The wild-type rat M_1 receptor gave a robust signal in this assay when exposed to carbachol with a maximum response typically 7-fold over basal (assigned a value of 100%, see **Table 1**). Compared with carbachol, AC-42 and N-desmethylclozapine were each partial agonists, and clozapine displayed weak but reproducible agonist activity at M_1 as reported previously (Spalding et al, 2002, Sur et al, 2003, Weiner et al, 2004, Davies et al, 2005). AC-260584 displayed nearly full efficacy compared with carbachol, and substantially increased potency and efficacy compared to AC-42 (**Figure 2A**, **2B**, and **Table 1**). Like AC-42, AC-260584 retains high selectivity for M_1 over the other muscarinic receptor subtypes, with greater than 50-fold selectivity versus M_2, and no significant agonist activity at M_3, (manuscript in preparation). As expected, all the TM3 mutants tested were severely compromised in their responses to carbachol (**Table 1** and **Figure 2**). The potency of carbachol was reduced 33-fold for W101A, 51-fold for S109A, and over 100-fold for L102A and N110A. Mutation of Y106 to alanine completely eliminated functional responses to carbachol at concentrations up to 100 uM. None of these mutated receptors showed significantly increased basal activity. The basal activities of the WT receptor and the W101A receptor were decreased slightly by atropine to ~7% and ~6% of their maximal responses to carbachol, respectively, and no significant responses were seen of any other mutant to atropine (data not shown). In contrast to the results observed with carbachol, responses to AC-42, AC-260584, clozapine and N-desmethylclozapine were maintained at many of the mutant receptors, and greatly increased at some.

The most striking differences observed were at the W101A mutant (**Figure 2C**) where 50-fold and 33-fold <u>increases</u> were seen in the potencies of AC-42 and AC-260584, respectively, whereas this same mutation caused a greater than 20-fold decrease in the potency of carbachol (see **Figure 2C**, **Table**

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1). The maximum response to AC-42 was also greatly increased at W101A, to over twice that observed at the wild-type receptor. Similarly, the maximum response to clozapine was increased almost 5-fold over that observed at the wild-type receptor, to a level comparable to carbachol (**Figure 2D**). In contrast to AC-42 and AC-260584, the potencies of clozapine and N-desmethylclozapine were not changed significantly.

Striking differences in the effects of mutations on carbachol and the other tested ligands were also seen on the Y106A and S109A mutants (**Figure 2G-2J**, and **Table 1**). On Y106A, the maximum responses of N-desmethylclozapine and clozapine were increased 1.4 fold and over 7-fold respectively, compared to their responses at the wild-type receptor, whereas no response to carbachol could be detected. Small, but clear functional responses to AC-42 were observed, and robust functional responses to AC-260584 were observed at Y106A receptors. On S109A, the potencies of AC-42, AC-260584 and N-desmethylclozapine were hardly affected, while the potency of carbachol was reduced over 50-fold. The maximal response to clozapine on S109A was not increased as it was at several of the other mutant receptors.

The L102 and N110 mutations caused significant impairment to responses induced by each of the tested ligands, but even here there were some apparent differences between carbachol and the other ligands (see **Figure 2E**, **2F**, **2K**, **2L** and **Table 1**). For example, the maximum response to clozapine was increased over 2-fold on both L102A and N110A compared with wild-type receptor, whereas the maximal response to carbachol was reduced at these mutants compared with wild-type. In general, the potencies for all ligands were significantly reduced on L102A and N110A, though more for carbachol (>100 fold in each case) and less for the other ligands (typically ~30-fold in most cases).

To confirm the differential effects of these mutations upon ligand activity, we tested several of the most interesting ligand-receptor combinations in conventional phosphatidyl inositol (PI) hydrolysis assays. The potencies of carbachol, AC-260584, and N-desmethylclozapine at wild-type M_1 were very similar to that observed in RSAT (**Table 2** and **Figure 3**). AC-260584 displayed full activity relative to carbachol, N-desmethylclozapine was a partial agonist, and clozapine displayed minimal responses.

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On W101A, we again observed that the potency of AC-260584, and the maximal response to clozapine were each strongly increased, while the potency of carbachol was dramatically decreased (**Table 2** and **Figure 3B**). On Y106A, the maximal response to clozapine was equal to N-demethylclozapine and greater than AC-260584, and carbachol was totally inactive (**Figure 3C**). On S109A, the potency of carbachol was reduced over 50-fold, while the potencies of AC-260584 and N-desmethylclozapine were unaffected (**Figure 3D**). AC-42 displayed qualitatively similar activities as AC-260584 on wild-type M_1 and these mutant receptors (data not shown). These results are highly consistent with the RSAT results.

To assess the effect of these mutations on receptor affinity, radioligand binding studies were carried out using the antagonist radioligand ^{3}H-N-methyl scopolamine (^{3}H-NMS) (see **Table 3**). The binding affinity of carbachol was significantly reduced on Y106A and S109A, though it was unchanged on W101A. Similarly, the binding affinities of the orthosteric antagonists NMS and atropine were strongly reduced at Y106A. In contrast, the affinities of AC-42, AC-260584, clozapine and N-desmethylclozapine were only slightly affected on Y106A and S109A, and the affinities of AC-42 and AC-260584 were greatly increased at W101A.

To directly examine if N-desmethylclozapine, clozapine, AC-42 and AC-260584 act allosterically at M_1 receptors, ^{3}H-NMS-inhibition binding studies were performed using increased amounts of ^{3}H-NMS (**Figure 4**). N-desmethylclozapine, clozapine, and AC-260584 were each able to completely displace ^{3}H-NMS, with AC-260584 requiring significantly higher concentrations than either N-desmethylclozapine or clozapine to achieve this. In contrast, AC-42 was unable to completely displace ^{3}H-NMS up to concentrations of 300 uM. We cannot rule out the possibility that AC-42 would completely displace ^{3}H-NMS at higher concentrations than 300 uM, however the compound was not soluble above 300 uM. As expected, gallamine only partially displaced ^{3}H-NMS, and this effect became much more pronounced as the concentration of ^{3}H-NMS was increased.

To further explore the possible allosteric interactions of N-desmethylclozapine, clozapine, AC-42 and AC-260584 at M_1 receptors, dissociation-rate experiments from CHO-M_1 cell membranes were

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performed using ^{3}H-NMS. As shown in **Figure 5A**, both AC-42 and AC-260584 significantly retarded the dissociation rate of ^{3}H-NMS, as did the classic muscarinic allosteric ligand gallamine. In contrast, under similar conditions, neither clozapine nor N-desmethylclozapine retarded dissociation of ^{3}H-NMS, and may have very slightly accelerated it (**Figure 5B**). The observed k_{off} values in the presence of AC-42, AC-260584, clozapine, N-desmethylclozapine and gallamine were 0.105+0.003 min^{-1}, 0.090+0.009 min^{-1}, 0.205+0.009 min^{-1}, 0.203+0.016 min^{-1}, and 0.004+0.003 min^{-1}, respectively, compared with atropine alone, which was 0.181+ 0.002 min^{-1} (mean+S.E.M.).

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Discussion

We have compared the binding and activation patterns of three structural classes of muscarinic ligands at M_1 receptors mutated throughout TM3. The first class was exemplified by orthosteric ligands like carbachol, atropine and NMS; the second class by the M_1 selective agonist AC-42, and a more potent, more efficacious structural analog of AC-42 called AC-260584; and the third class by the antipsychotic clozapine, and its active metabolite, N-desmethylclozapine. The TM3 mutations tested in this paper were chosen because they were previously found to participate in the orthosteric-binding site of the M_1 receptor (Lu and Hulme, 1999, Hulme et al, 2003). Mutation of all of these residues profoundly reduces the ability of orthosteric ligands such as carbachol to bind and activate M_1, confirming earlier findings (Fraser et al 1989, Page et al 1995, Lu and Hulme 1999).

AC-42 and AC-260584 activity was affected by the mutations very differently. Most strikingly, mutation of W101 to alanine substantially increased the potency of AC-42 and AC-260584 (50 and 30-fold, respectively) while causing a greater than 20-fold decrease in carbachol potency. Similarly, the binding affinities of AC-42 and AC-260584 increased 50-fold, and over 100-fold, respectively. Mutations to Y106 and S109 also had strikingly different effects on these ligands. M_1-Y106A was not activated by carbachol, and its affinity for carbachol was reduced over 40-fold. In contrast, M_1-Y106A retained the ability to be activated by AC-42 and AC-260584. Similarly, on M_1-S109A, the potencies of AC-42 and AC-260584 were unchanged, and their maximal responses were increased, while the potency of carbachol for M_1-S109A was decreased 50-fold in functional assays and 9-fold in radioligand binding, and its maximal responses decreased. Mutation of L102 and N110 impaired responses to AC-42, AC-260584, and carbachol, though the reduction in potency of AC-260584 (30-fold) was less than that of carbachol (>100-fold). Possibly AC-42 can activate M_1-L102A and M_1-N110A at doses that could not be tested due to dose-limiting cytotoxicity (unpublished observations).

A third pattern of activation was observed for clozapine and N-desmethylclozapine. In contrast to AC-42 and AC-260584, the potencies of neither clozapine, nor N-desmethylclozapine were increased at M_1-W101A, though their maximum responses were, especially for clozapine. At M_1-Y106A, the

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maximum responses to clozapine and N-desmethylclozapine were dramatically increased, whereas both the maximum response and potency of AC-42 and AC-260584 were reduced. Conversely, the maximum responses to N-desmethylclozapine and especially clozapine were reduced at M_1-Y109A, while the maximum responses to AC-42 and AC-260584 were increased. Responses to N-desmethlyclozapine and clozapine were impaired to similar degrees as AC-42 and AC-260584 at M_1-L102A and M_1-N110A, though responses to N-desmethylclozapine were slightly less affected than responses to AC-260584 at M_1-N110A, and the maximum response to clozapine actually increased at M_1-L102A and M_1-N110A, in contrast to all the other ligands tested.

Mutations such as W101A that induce large potency increases are rare; frequently they are caused by increases in constitutive activity (Spalding et al, 1995, 1998, Burstein et al, 1998, Lu and Hulme, 1999) as would be predicted by increasing *J*, the isomerization constant defining interconversion of receptors between active and inactive conformations (Samama et al 1993). This is unlikely in this case, because the constitutive activity of the mutant and wild-type receptors were similar (see Results above), thus their *J* values are likely to be similar, and the receptors were expressed at similar levels (Lu and Hulme, 1999). We therefore suggest that the W101A mutation may directly strengthen interactions between AC-42 and AC-260584, and the M_1 receptor. Possibly steric constraints are removed upon replacement of a large tryptophan residue with a smaller alanine residue at this position, allowing AC-42 and AC-260584 to bind more tightly to the receptor. This strengthened interaction could involve other residues in TM3 or residues elsewhere in the receptor that are revealed by a change in receptor conformation.

According to modern models of receptor activation (Samama et al, 1993; Spalding et al, 1997; Christopoulos and Kenakin, 2002), agonists preferentially bind to active receptor conformations; inverse agonists preferentially bind to inactive receptor conformations, and neutral antagonists have equal affinity for active and inactive receptor conformations. Therefore, clozapine is likely to bind the inactive and active conformations of M_1 with similar affinity since it is an extremely weak partial agonist at the wild-type M_1 receptor. The large increase in maximal responses to clozapine caused by most mutations in

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TM3 suggests that the primary effect of these mutations is to increase the relative affinity of clozapine for an active conformation of M_1. Another mechanism for increasing the maximal response of partial agonists is increased receptor reserve, however given that the maximal response of the partial agonist AC-42 decreased at M_1-Y106A, and that both M_1-W101A and M_1-Y106A are expressed at similar levels to wild-type M_1 (Lu and Hulme, 1999), this seems unlikely.

Current models of rhodopsin-like receptor activation propose movement of TM3 and TM6 as crucial to attaining an active conformation, and in addition to acetylcholine, the positively charged head groups of dopamine, serotonin, histamine, epinephrine and norepinephrine are all thought to interact directly with the aspartate analogous to D105 (AspIII.08$^{3.32}$) that is conserved in all biogenic amine receptors (Gether, 2000). We did not observe functional responses to any of the ligands tested here at the M_1-D105A (**Table 1**). Possibly interactions of AC-42, AC-260584, clozapine or N-desmethylclozapine with D105 essential for receptor activation are lost when this residue is mutated to alanine. Alternatively, D105 may be essential for signaling by M_1, and that mutation of this residue to alanine disrupts receptor activation regardless of where agonists bind the receptor, and/or that M_1-D105A achieves insufficient cell surface expression to mediate functional responses (Lu and Hulme, 1999).

TM3 is believed to form an alpha-helix based on mutagenesis and affinity labeling data (Lu and Hulme 1999, Javitch et al, 1995; Spalding et al, 1998) and by inference from the 3D structure of rhodopsin (Palzewski et al, 2000, Hulme et al, 2003). To approximate their positions in the 3D structure of the M_1 receptor, the residues tested in this study were mapped onto a helical net (**Figure 6**). W101 is predicted to lie one turn above D105, Y106 is adjacent to D105, and S109 is predicted to lie one turn below D105. As described above, the side-chains of Y106 and S109 are essential for carbachol activity, suggesting that carbachol makes interactions well into the transmembrane domain of the receptor. In contrast, AC-42, AC-260584, and N-desmethylclozapine were substantially less affected by these mutations, and the activity of clozapine was dramatically increased on M_1-Y106A, suggesting that these ligands bind closer to the extracellular space. This is consistent with data showing that carbachol, but not AC-42 or N-desmethylclozapine activity is strongly impaired, and clozapine activity is greatly increased

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by mutations of tyrosine 381 and asparagine 382 in TM6 (Spalding et al, 2002, Sur et al, 2003), which are also believed to lie well into the transmembrane domain. The strong potentiating effect of W101A on AC-42 and AC-260584 activity could be explained as an allosteric effect propagated to an AC-42 binding site located elsewhere, however it is more likely that AC-42 and AC-260584 interact with the extracellular regions of TM3. This is consistent with the observation that AC-42 agonist activity is dependant on M_1 sequence in the extracellular parts of the receptor such as the N-terminus and the third extracellular loop (Spalding et al, 2002).

The strikingly different effects of these mutations on orthosteric ligands like carbachol, structural analogs of AC-42, and structural analogs of N-desmethylclozapine suggest that M_1 receptors can be activated in at least three different ways. AC-42 and AC-260584 display clear allosteric properties (Langmead et al, 2006; this paper), whereas we were unable to demonstrate that N-desmethylclozapine and clozapine bind allosterically, i.e. through a non-overlapping (with respect to carbachol) site. AC-42 and AC-260584 exhibit high functional selectivity for M_1 over the other muscarinic subtypes (Spalding et al, 2002 and see above), possibly because these ligands act through non-conserved amino acid residues, consistent with an allosteric mechanism of action. In contrast, N-desmethylclozapine and clozapine are substantially less selective for M_1 over the other muscarinic subtypes (Weiner et al, 2004; Davies et al, 2005), possibly reflecting interactions more similar to those utilized by orthosteric (and non-selective) ligands like carbachol. We conclude that although N-desmethylclozapine and clozapine contact different residues than carbachol to activate M_1, they may occupy a substantially overlapping space with carbachol, whereas AC-42 and AC-260584 appear to occupy separable spaces.

These observations demonstrate that GPCRs do not have a single agonist-binding site, where a ligand must bind to activate the receptor. Instead, receptors appear to spontaneously adopt active conformations, and ligands that stabilize one of these active conformations will act as agonists, irrespective of the site where they bind the receptor.

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Figure Legends

Figure 1. Structures of carbachol, AC-42, AC-260584, clozapine and N-desmethylclozapine.

Figure 2. Functional Activity of AC-42 (inverted triangles), AC-260584 (triangles), N-desmethylclozapine (circles), Clozapine (diamonds) and Carbachol (open circles) on rat M_1 receptors containing mutations in TM3. Assays were carried out using R-SAT. Response values were normalized relative to the maximum response of the wild-type receptor to carbachol. Points represent the mean +/- S.D. of duplicate determinations. Lines represent the fit to a logistical function. Data shown are typical of at least two or more independent experiments.

Figure 3. Functional Activity of AC-260584 (circles), N-desmethylclozapine (triangles), Clozapine (inverted triangles) and Carbachol (open circles) on rat M_1 receptors containing mutations in TM3. Assays were carried out using phosphatidyl inositol hydrolysis. Response values were normalized relative to the maximum response of the wild-type receptor to carbachol. Points represent the mean +/- S.D. of duplicate determinations. Lines represent the fit to a logistical function. Data shown are typical of at least two or more independent experiments.

Figure 4. Inhibition of 0.2 and 2 nM ^{3}H-NMS binding to CHO-M_1 cell membranes by atropine (open circles), gallamine (diamonds), AC-42 (circles), AC-260584 (squares), clozapine (triangles) and N-desmethylclozapine (inverted triangles). Data points are means of three determinations, and are representative of at least three independent experiments.

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Figure 5. Effect of AC-42 (300 uM, circles), AC-260584 (300 uM, squares) and gallamine (1 mM, diamonds) (**A**), or N-desmethylclozapine (100 uM, inverted triangles), clozapine (10 uM, triangles), and gallamine (1 mM, diamonds) (**B**) on the atropine (1 uM) induced dissociation of ^{3}H-NMS from CHO-M_1 cell membranes. Control time courses were run with atropine (1 uM, open circles) alone. The k_{off} values in the presence of AC-42, AC-260584, clozapine, N-desmethylclozapine and gallamine were 0.105+0.003 min^{-1}, 0.090+0.009 min^{-1}, 0.205+0.009 min^{-1}, 0.203+0.016 min^{-1}, and 0.004+0.003 min^{-1}, respectively, compared with atropine alone, which was approximately 0.181+ 0.002 min^{-1} (mean+S.E.M.). Data points are means of three determinations, and are representative of three to eight independent experiments.

Figure 6. Helical net showing the positions of residues mutated in this study. Letters represent amino acid type using the single letter code. Large black circle: Carbachol activity was reduced, all other ligands gained activity, AC-42 and AC-260584 potency were each substantially increased. Gray circles: Carbachol activity was reduced or abolished, AC-42 and AC-260584 activities were each retained; clozapine and N-desmethylclozapine maximal responses were each substantially increased. White circles: Potency reduced for all ligands tested, maximal responses reduced for all ligands except clozapine. Small black circles: Residues that were not tested in this study.

Table 1 Functional actbivity in RSAT assays of muscarinic agonists on rat M_1 receptors containing mutations in TM3. Eff (%) represents the maximum response of the receptor to each ligand normalized relative to the maximum response of carbachol at the wild-type receptor. Values represent the mean of 3-5 separate determinations +/- S.E.M. in each case. Shift represents the EC_{50} for the mutant receptor divided by the EC_{50} for the wild-type receptor. Where the response was too low, pEC_{50} was not determined (denoted nd). Where a plateau in the concentration-response curve was not reached at the maximum ligand concentration that could be tested (100 uM for carbachol, 10 uM for clozapine and N-desmethylclozapine, and 5 uM for AC-42), estimates of the minimum efficacy and the maximum pEC_{50} as denoted by greater than or less than signs, respectively, are noted. No resp denotes where the measured response was not significantly different to baseline.

	Carbachol			AC-42			AC-260584		
Receptors	Eff (%)	pEC50	Shift	Eff (%)	pEC50	Shift	Eff (%)	pEC50	Shift
M1 WT	100 ± 9	6.3 ± 0.1	**1**	47 ± 5	6.8 ± 0.1	**1**	89 ± 6	7.8 ± 0.1	**1**
W101A	>80 ± -	<5 ± -	**>20**	95 ± 10	8.5 ± 0.1	**0.02**	100 ± 8	9.4 ± 0.1	**0.03**
L102A	>30 ± -	<4 ± -	**>100**	6 ± 0	nd ± -	**-**	37 ± 7	6.3 ± 0.1	**30**
D105A	no resp.	nd ± -	**-**	no resp.	nd ± -	**-**	no resp.	nd ± -	**-**
Y106A	no resp.	nd ± -	**-**	>30 ± -	<6 ± -	**>8**	69 ± 3	7.1 ± 0.0	**6**
S109A	>65 ± -	<4.5 ± -	**>50**	59 ± 5	6.8 ± 0.1	**1**	104 ± 12	7.8 ± 0.1	**1**
N110A	>35 ± -	<4 ± -	**>100**	7 ± 1	nd ± -	**-**	38 ± 6	6.2 ± 0.1	**43**

	Clozapine			N-desmethylclozapine		
	Eff (%)	pEC50	Shift	Eff (%)	pEC50	Shift
M1 WT	13 ± 2	8.1 ± 0.2	**1**	78 ± 4	7.3 ± 0.1	**1**
W101A	63 ± 7	7.6 ± 0.1	**3**	88 ± 12	6.7 ± 0.1	**5**
L102A	32 ± 3	6.6 ± 0.1	**32**	>35 ± -	<5 ± -	**>100**
D105A	no resp.	nd ± -	**-**	no resp.	nd ± -	**-**
Y106A	96 ± 11	8.3 ± 0.1	**0.7**	112 ± 15	7.0 ± 0.1	**2**
S109A	7 ± 3	nd ± -	**-**	68 ± 9	7.0 ± 0.1	**2**
N110A	>30 ± -	<5 ± -	**>100**	66 ± 16	6.1 ± 0.1	**21**

Table 2 Functional activity in phosphatidyl inositol hydrolysis assays of muscarinic agonists on rat M_1 receptors containing mutations in TM3. Max Resp represents the maximum response of the receptor to each ligand normalized relative to the maximum response of the wild-type receptor to that ligand. Values represent the mean of 3-6 separate determinations +/- S.E.M. in each case. Shift represents the EC_{50} for the mutant receptor divided by the EC_{50} for the wild-type receptor. Where the response was too low, pEC_{50} was not determined (denoted nd). No resp denotes where the measured response was not significantly different to baseline.

	Carbachol			AC-260584		
	Eff (%)	**pEC50**	**Shift**	**Eff (%)**	**pEC50**	**Shift**
M1 WT	100 ± 4	6.3 ± 0.2	**1**	100 ± 7	7.3 ± 0.1	**1**
W101A	>75 ± -	<4.5 ± -	**>30**	112 ± 6	8.8 ± 0.1	**0.03**
Y106A	no resp.	nd ± -	**-**	110 ± 22	6.6 ± 0.1	**4**
S109A	>100 ± -	<4.5 ± -	**>30**	85 ± 11	7.1 ± 0.3	**1**

	Clozapine			N-desmethylclozapine		
	Eff (%)	**pEC50**	**Shift**	**Eff (%)**	**pEC50**	**Shift**
M1 WT	no resp.	nd ± -	**-**	55 ± 8	7.0 ± 0.2	**1**
W101A	57 ± 11	8.1 ± 0.1	**-**	54 ± 11	6.5 ± 0.2	**6**
Y106A	95 ± 21	8.9 ± 0.2	**-**	40 ± 4	6.7 ± 0.1	**3**
S109A	no resp.	nd ± -	**-**	47 ± 17	6.7 ± 0.6	**4**

Table 3. Inhibition of ^{3}H-NMS binding by muscarinic agonists and muscarinic antagonists on rat M_1 receptors containing mutations in TM3. Values represent the mean of 3-9 determinations +/- S.E.M. in each case. Shift represents the IC_{50} for the mutant receptor divided by the IC_{50} for the wild-type receptor. ^{3}H-NMS concentrations were: M_1 wild-type: 160 pM; W101A: 640 pM; Y106A 1,300 pM; S109A 160 pM.

	Carbachol		AC-42		AC-260584	
Receptor	-log (IC50)	Shift	-log (IC50)	Shift	-log (IC50)	Shift
M1 WT	3.7 ± 0.2	**1**	5.3 ± 0.0	**1**	5.9 ± 0.1	**1**
W101A	3.5 ± 0.3	**1**	7.2 ± 0.1	**0.01**	8.4 ± 0.1	**0.003**
Y106A	2.7 ± 0.4	**9**	5.3 ± 0.1	**1**	5.5 ± 0.0	**3**
S109A	2.9 ± 0.1	**6**	5.6 ± 0.1	**0.5**	5.8 ± 0.3	**1**

	^{3}H-NMS		N-desmethylclozapine		Clozapine	
Receptor	-log (Kd)	Shift	-log (IC50)	Shift	-log (IC50)	Shift
M1 WT	10.0 ± 0.2	**1**	6.8 ± 0.2	**1**	7.7 ± 0.4	**1**
W101A	9.4 ± 0.2	**4**	6.9 ± 0.2	**1**	7.7 ± 0.1	**1**
Y106A	8.2 ± 0.2	**68**	7.3 ± 0.1	**0.3**	8.1 ± 0.2	**0.4**
S109A	9.7 ± 0.1	**2**	6.2 ± 0.0	**4**	7.1 ± 0.1	**4**

Figure 1.

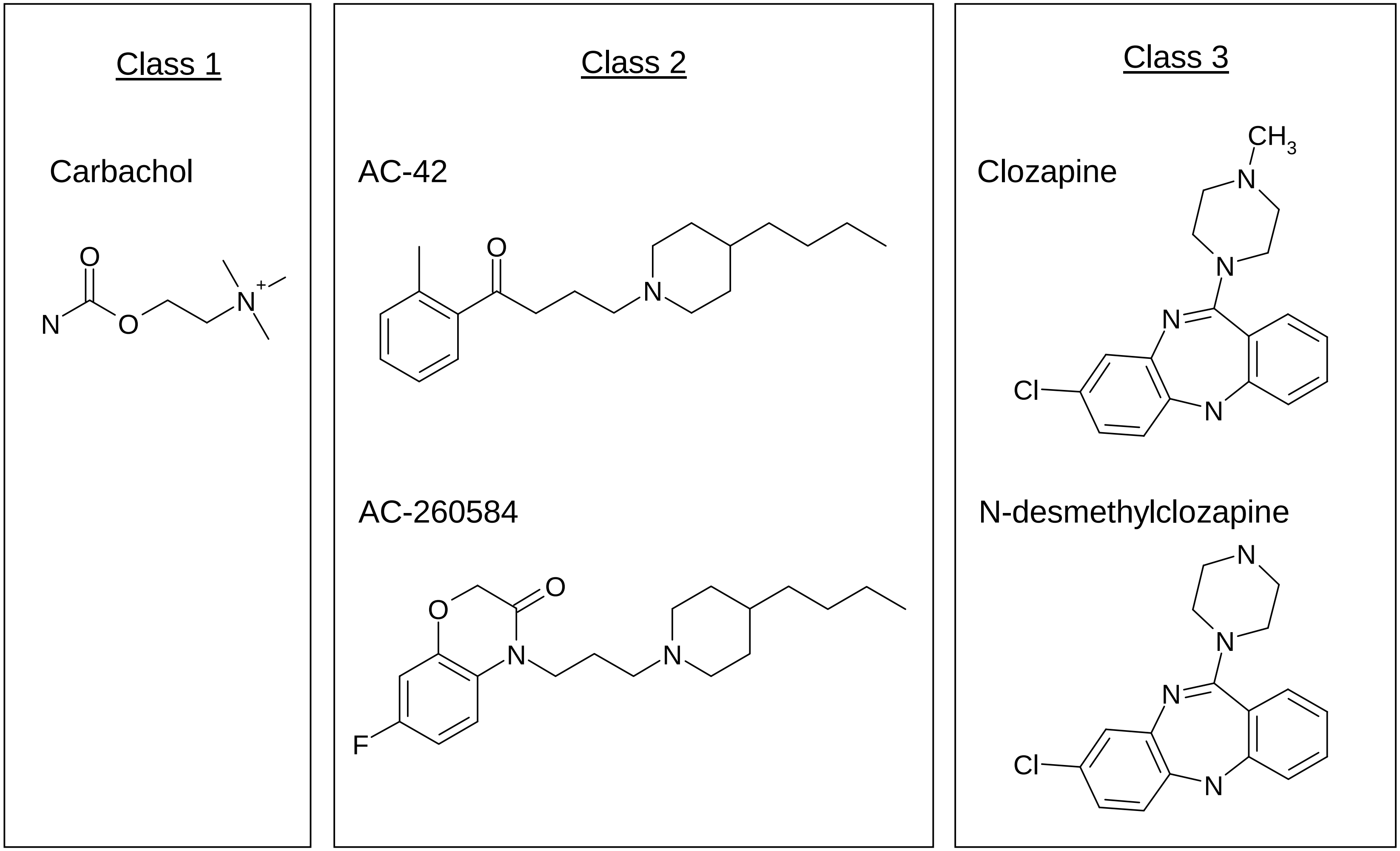

Class 1
Carbachol
O
N
O
N+
Class 2
AC-42
O
N
AC-260584
O
O
N
N
F
Class 3
Clozapine
CH3
N
N
N
Cl
N
N-desmethylclozapine
N
N
N
Cl
N

Figure 2A-2F.

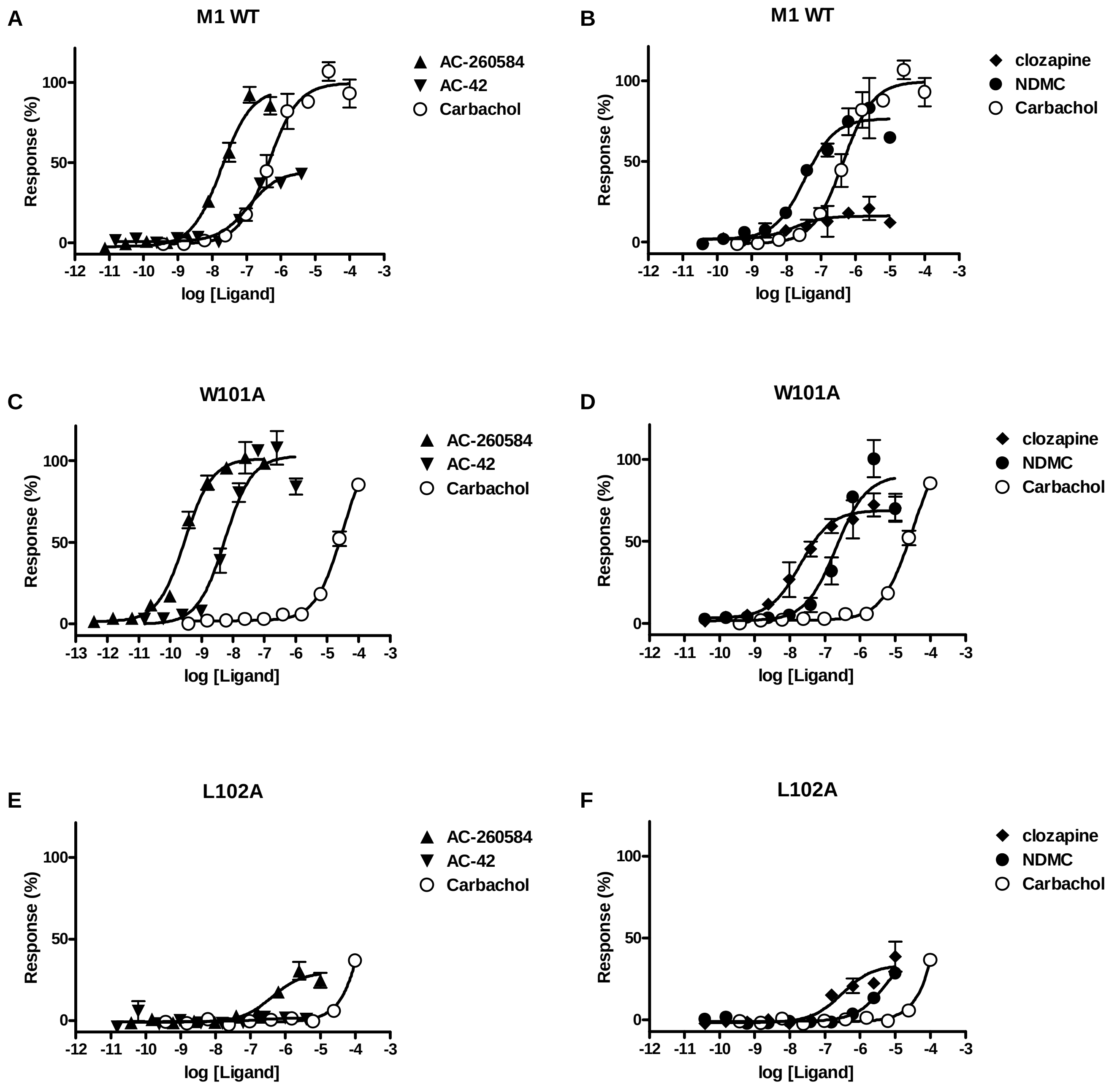
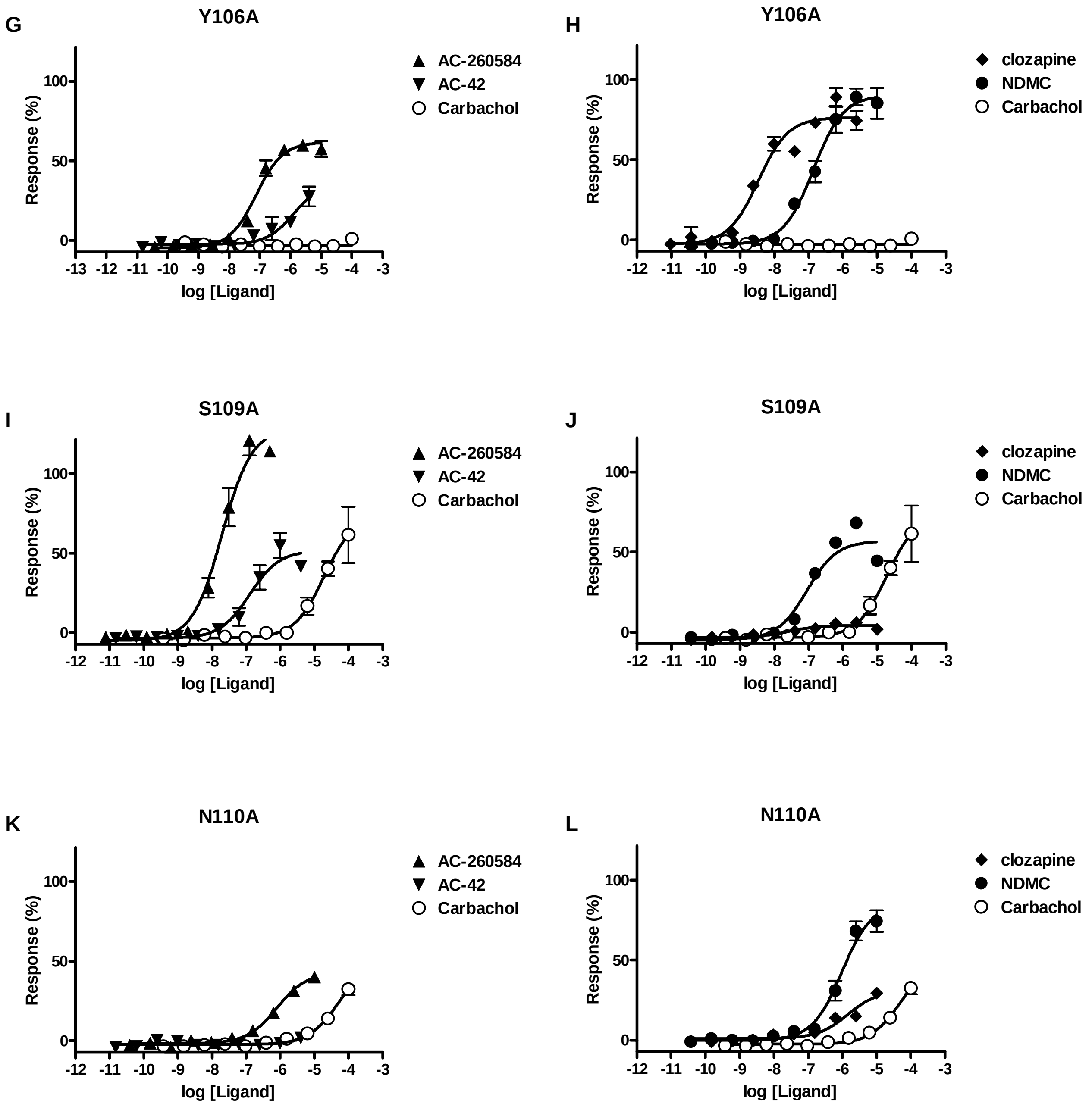

A
M1 WT
AC-260584
AC-42
Carbachol
Response (%)
log [Ligand]
B
M1 WT
clozapine
NDMC
Carbachol
C
W101A
D
W101A
E
L102A
F
L102A

Figure 2G-1L.



G
Y106A
AC-260584
AC-42
Carbachol
Response (%)
log [Ligand]
H
Y106A
clozapine
NDMC
Carbachol
I
S109A
J
S109A
K
N110A
L
N110A

Figure 3.

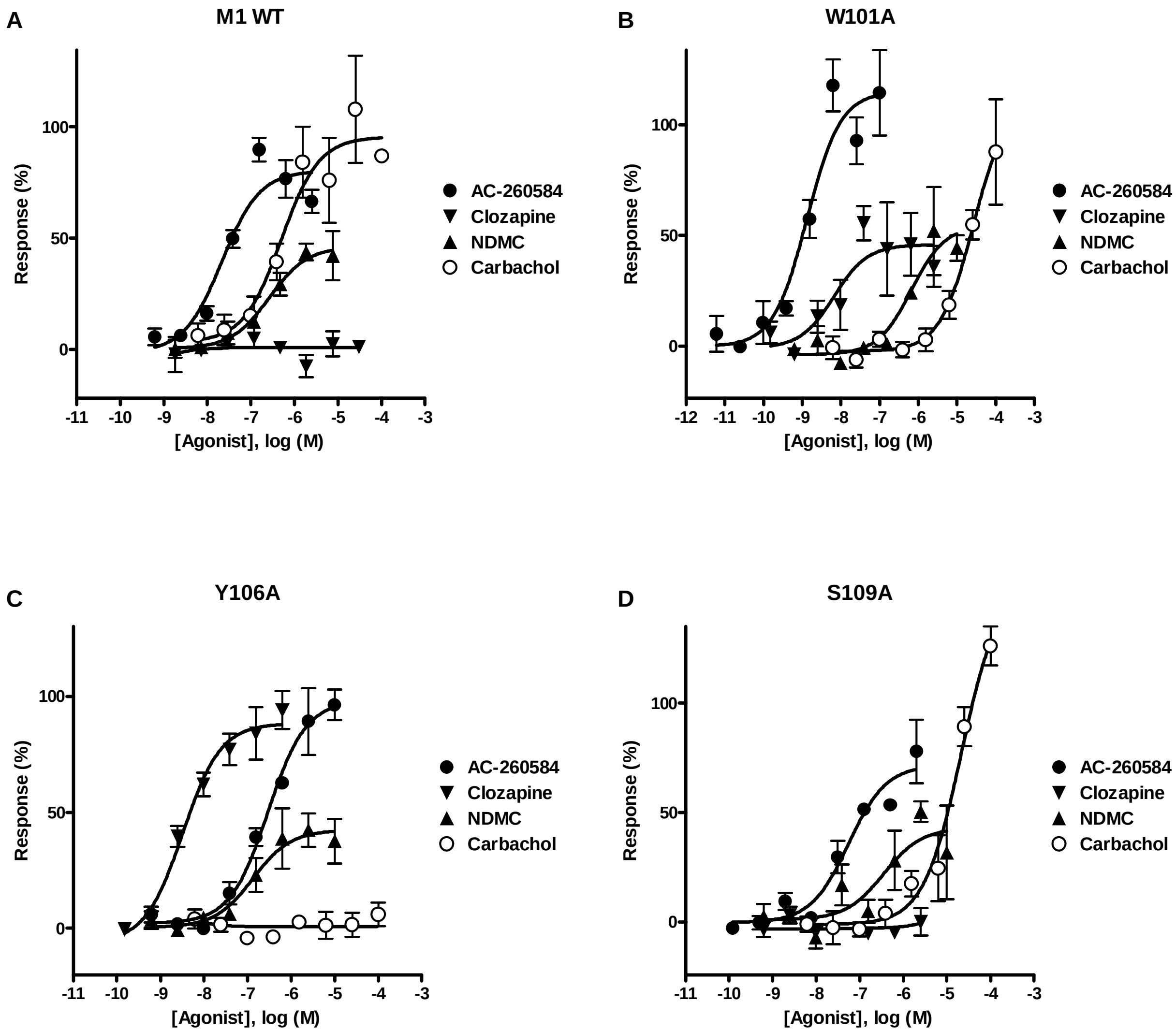


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Figure 4.

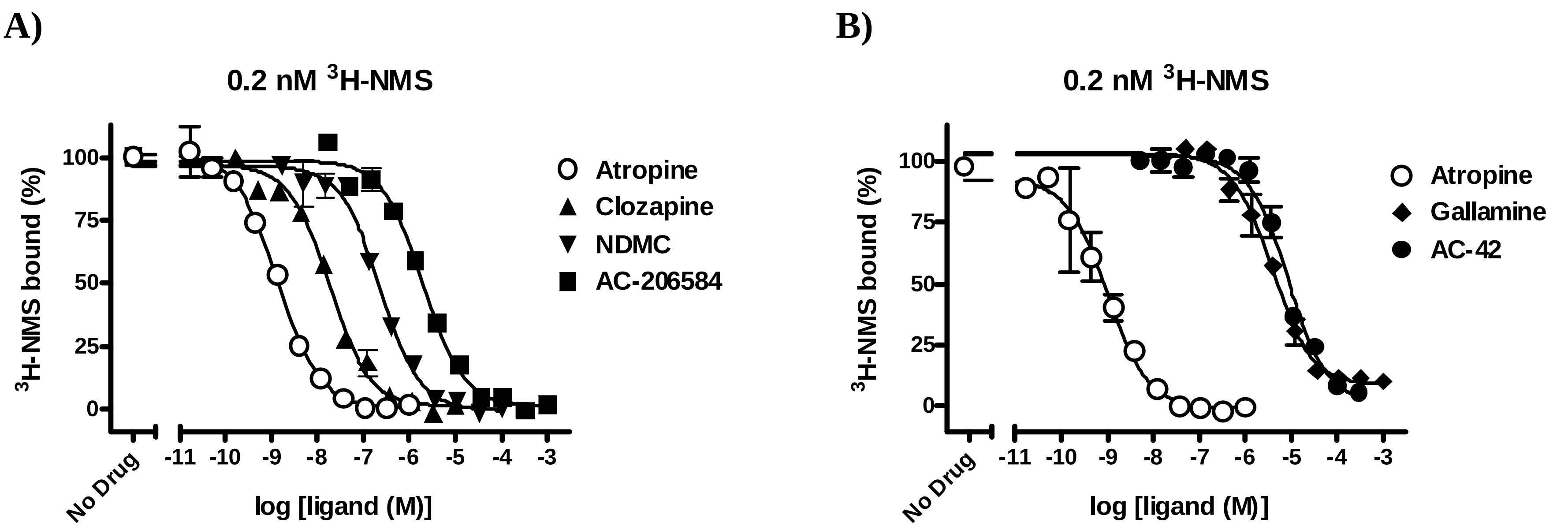

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Figure 4.

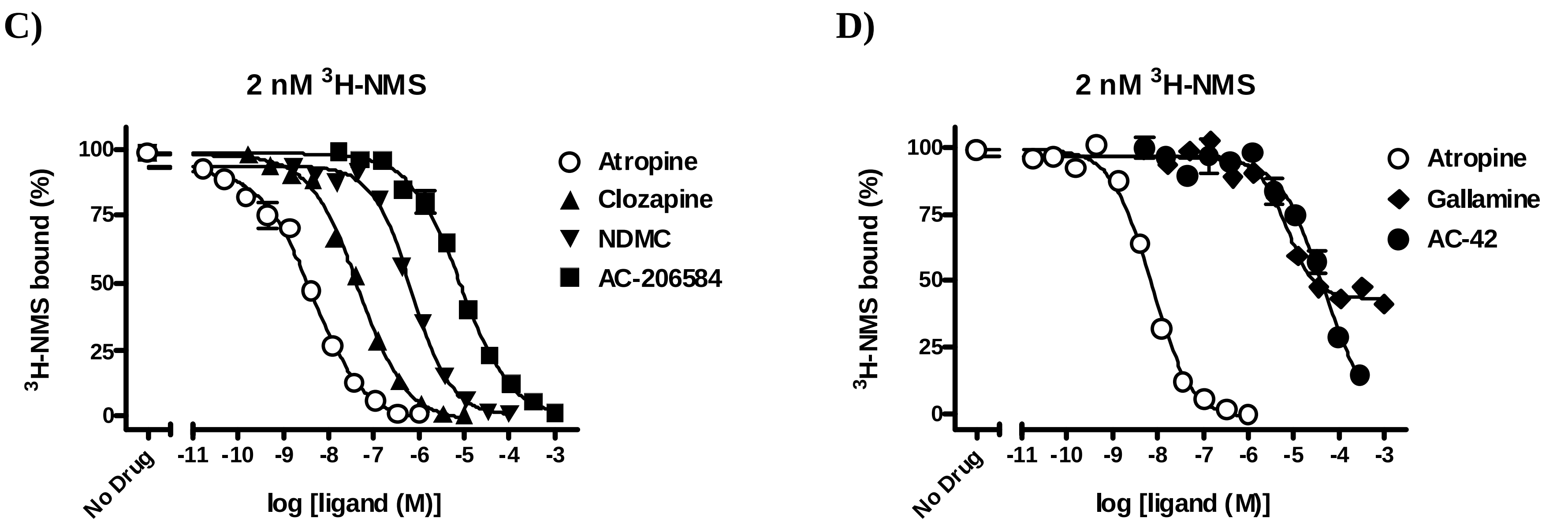
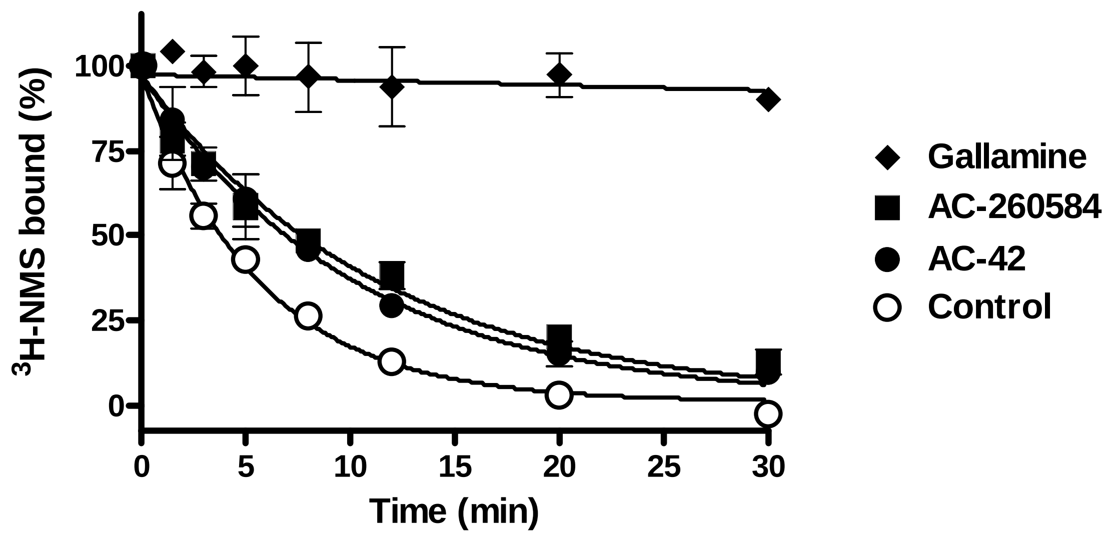
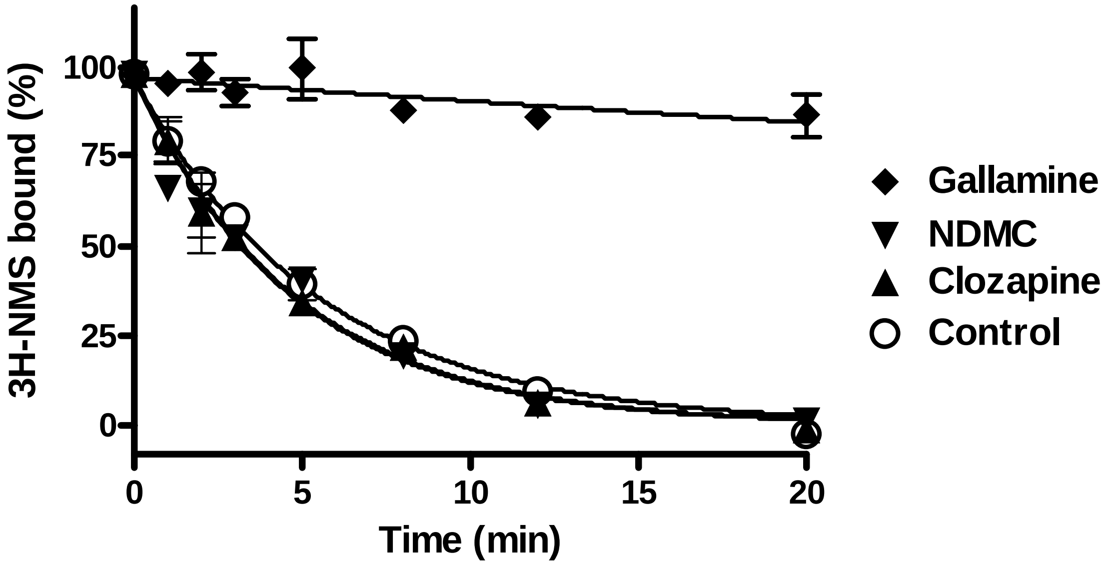

C)
2 nM 3H-NMS
3H-NMS bound (%)
100
75
50
25
0
No Drug
-11 -10 -9 -8 -7 -6 -5 -4 -3
log [ligand (M)]
Atropine
Clozapine
NDMC
AC-206584
D)
2 nM 3H-NMS
3H-NMS bound (%)
100
75
50
25
0
No Drug
-11 -10 -9 -8 -7 -6 -5 -4 -3
log [ligand (M)]
Atropine
Gallamine
AC-42

Figure 5A.



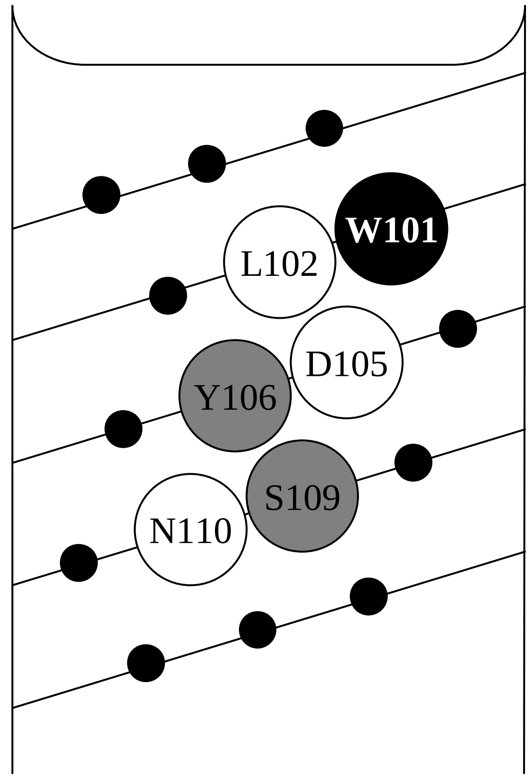
100
75
50
25
0
^{3}H-NMS bound (%)
0
5
10
15
20
25
30
Time (min)
Gallamine
AC-260584
AC-42
Control

Figure 5B.



3H-NMS bound (%)
100
75
50
25
0
0
5
10
15
20
Time (min)
Gallamine
NDMC
Clozapine
Control

Figure 6.



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