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Insights into the differential desensitization of α4β2 nicotinic acetylcholine receptor isoforms obtained with positive allosteric modulation of mutant receptors

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Running title: Desensitization of α4β2 nAChR

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Abbreviations

acetylcholine (ACh), nicotinic acetylcholine receptors (nAChR), positive allosteric modulators (PAMs), high sensitivity (HS), low sensitivity (LS), 3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3H-cyclopentan[c]quinoline-8-sulfonamide (TQS)

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Abstract

The development of highly efficacious positive allosteric modulators (PAMs) of α7 nicotinic acetylcholine receptors (nAChR) has proven useful in defining the ligand dependence of the conformational dynamics of α7 receptors. No such effective modulators are known to exist for the α4β2 nAChR of the brain, limiting our ability to understand the importance of desensitization for the activity profile of specific ligands. In this study, we used mutant β2 subunits that allowed the use of the α7 PAM TQS to probe the desensitizing effects of nicotinic ligands on the two forms of α4β2 receptors; high sensitivity (HS) (two α4 and three β2 subunits) and low sensitivity (LS) (three α4 and two β2 subunits). A total of 28 different ligands of 8 different categories, based on activity and selectivity, were tested for their ability to induce TQS-sensitive desensitization of HS and LS α4β2 receptors. Results confirm that HS α4β2 receptor responses are strongly limited by desensitization, by at least an order of magnitude more so than the responses of LS receptors. The activation of α4β2 receptors by the smoking cessation drugs cytisine and varenicline is strongly limited by desensitization, as is the activation of LS receptors by the HS-selective agonists sazetidine-A and TC-2559. The evaluation of drugs previously identified as α7-selective agonists revealed varying patterns of α4β2 cross-desensitization that were predictive of the effects of these drugs on the activation of wild-type α4β2 receptors by ACh, supporting the utility of TQS-sensitive receptors for the development of focused therapeutics.

Keywords:

allosteric modulation, desensitization, addiction, therapeutics, nicotinic receptors

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Significance statement

To varying degrees, ligands regulate the balance of active and desensitized states of the two forms of the primary nAChR subtypes in brain. Using mutant beta subunits, an allosteric modulator can reverse ligand-induced desensitization, revealing the differential desensitization of the receptors by specific ligands. We show that drugs believed to be selective for therapeutic targets may cross-desensitize other targets and that, within a class of drugs, improved specificity can be achieved by using agents that reduce such cross-desensitization.

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Introduction

Any meaningful interpretation of the physiology and pharmacology of nicotinic acetylcholine receptors (nAChR) must include a consideration of the balance between activation and desensitization (Katz and Thesleff, 1957). It might be argued that the lifetime of the endogenous acetylcholine (ACh) signal at a mature neuromuscular junction is too brief for desensitization to play a large role (Land et al., 1981). However, in virtually any other context, desensitization should be considered as a factor shaping macroscopic responses (Papke, 2010). Typical *in vitro* approaches used to study heterologously expressed receptors rely on solution application/exchange methods that are slower than receptor desensitization rates, so drug application rates and receptor desensitization are both factors limiting the responses. Likewise, a balance between activation and desensitization must be important for the function of nAChR in the brain, where ACh is delivered by diffuse volume transmission (Descarries et al., 1997) and nicotine is delivered through relatively slow self-administration by smokers (Picciotto et al., 2008).

Most of the nAChR in vertebrate brain that bind ACh and nicotine with high affinity are pentameric complexes containing α4 and β2 subunits (Millar and Gotti, 2009). Pentamers composed of just two different subunits necessarily can vary in subunit stoichiometry, such that while two agonist binding sites are configured at α4–β2 interfaces, the fifth position can be occupied by either an α4 or a β2 subunit (Nelson et al., 2003). While some expression systems may bias receptor expression toward the α4(3)β2(2) configuration, both types are present in the brain (Fasoli et al., 2016), and chronic nicotine favors the expression of the α4(2)β2(3) configuration due to nicotine's ability to selectively chaperone receptors of that configuration to the membrane (Kuryatov et al., 2005; Nelson et al., 2003; Srinivasan et al., 2011).

The two configurations of α4β2 nAChR differ greatly in their functional properties, with one notable difference being that receptors with the α4(2)β2(3) configuration respond to low concentrations of ACh or nicotine but saturate their responses when agonist concentrations are

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raised to higher levels. They have therefore come to be referred to as a high sensitivity (HS) subtype. In contrast, receptors of α4(3)β2(2) configuration, in general, generate larger currents across a wider range of concentration; these are known as the low sensitivity (LS) subtype (Eaton et al., 2014; Lopez-Hernandez et al., 2004; Nelson et al., 2003). It is the core hypothesis of this study that desensitization is the primary factor limiting the responses of HS receptors to high concentrations of agonist (Corrie et al., 2020). We will utilize mutant forms of the receptors that are sensitive to positive allosteric modulators (PAMs) that activate desensitized receptors to test that hypothesis. Although it should be noted that our experiments do not necessarily fulfill the criteria for the statistical testing of a null hypothesis, we show that the use of a PAM which reverses desensitization selectively increases the response of HS receptors compared to LS receptors.

It is well established that desensitization profoundly limits the ion channel function of homomeric α7 nAChR (Uteshev et al., 2002), the second most abundant nAChR in brain (Millar and Gotti, 2009). Our understanding of α7 receptor desensitization has been greatly enhanced by the discovery of the type II class of α7-selective PAMs (Gronlien et al., 2007), which destabilize one of the nonconducting states, allowing desensitized receptors to reactivate (Papke et al., 2009; Williams et al., 2011).

The α7-selectivity of PAMs like 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea (PNU-120596) and 3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3H-cyclopentan[c]quinoline-8-sulfonamide (TQS) is due to the presence of a methionine, unique to α7 among nAChR, in the 15' position of the pore-forming second transmembrane domain (Young et al., 2008). The transfer of that residue into β2 or β4 allows for the formation of heteromeric nAChR that are sensitive to potentiation by TQS (Stokes et al., 2019). In the present study, we used a concatamer (Zhou et al., 2003) of α4 and β2L15'M mutants, co-expressed in *Xenopus* oocytes with monomers of wild-type α4 or β2 subunits to selectively form TQS-sensitive LS or HS α4β2 receptors. Using TQS to reveal the extent of α4β2 receptor desensitization, we demonstrate the great degree to which desensitization limits HS receptor

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responses to determine the degree to which their activities on the two α4β2 isoforms are limited by TQS-sensitive desensitization. Finally, we tested the relevance of these observation to the functional activation of wild-type α4β2 receptors by ACh in the presence of putative α7-selective agonists.

Materials and methods

Acetylcholine chloride, atropine, choline, methyllycaconitine citrate (MLA), dihydro-b-erythrodine hydrobromide (DHbE), N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide (PNU-282987), cytisine, arecoline, nicotine, cotinine, and other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). 3a,4,5,9b-Tetrahydro-4-(1-naphthalenyl)-3H-cyclopentan[c]quinoline-8-sulfonamide (TQS), 2-(3-Pyridinyl)-1-azabicyclo[3.2.2]nonane dihydrochloride (TC-1698), (3*S*)-Spiro[1-azabicyclo[2.2.2]octane-3,5'-oxazolidine]-2'-one hydrochloride (AR-R17779), 3-[3-(3-Pyridinyl)-1,2,4-oxadiazol-5-yl]benzonitrile (NS9283), varenicline, 6-[5-[(2*S*)-2-Azetidinylmethoxy]-3-pyridinyl]-5-hexyn-1-ol dihydrochloride (sazetidine-A), and epibatidine were purchased from Tocris, Minneapolis, MN. 4-(4-cyanophenyl)-1,1-diethylpiperazin-1-ium (*p*CN-diEPP) and 4-(4-carbamoylphenyl)-1,1-diethylpiperazin-1-ium (*p*CONH2-diEPP) were synthesized as previously reported (Quadri et al., 2016). 1,1-dimethylpiperidinium (diMPip) was synthesizedby Kinga Chojnacka (Papke et al., 2014a). 1,4-Diazabicyclo[3.2.2]non-4-yl[5-[3-(trifluoromethyl)phenyl]-2-furanyl]methanone hydrochloride (NS6740) and desformylflustrabromine (dFBr) were provided by Ganesh Thakur (Northeastern University). (±)-nornicotine (free base) was synthesized as previously described (Swango et al. 1999), a gift from Peter Crooks. Anabaseine was synthesized by Jingyi Wang in the Nicole Horenstein laboratory (University of Florida). Other compounds were sourced as follows: methyl pyridinium chloride (n-MP) from AK Scientific, Union City CA; triethylmethylammonium chloride (triEMA) from Tokyo Chemical Industry, Portland OR; 3-(2,4-Dimethoxybenzylidene)-anabaseine dihydrochloride (GTS-21) from Taiho Pharmaceuticals, Tokyo Japan;, (*E*)-*N*-Methyl-4-(3-pyridinyl)-3-buten-1-amine oxalate (TC-2403) and 4-(5-

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ethoxy-3-pyridinyl)-*N*-methyl-(3*E*)-3-buten-1-amine difumarate (TC-2559) from Targacept, Winston-Salem NC; and 1,2,3,6-tetrahydro-2,3'-bipyridine (anatabine) from Cayman Chemical, Ann Arbor MI.

Fresh ACh stock solutions were made in Ringer's solution each day of experimentation. Stock solutions of TQS, PNU-282987, NS6740, *p*CN-diEPP, *p*CONH2-diEPP, and dFBr were made in DMSO and kept at -20ºC and diluted in Ringer's solution each day. Other compounds' stock solutions were prepared in Ringer's solution and held at 4ºC and diluted in Ringer's solution each day.

Heterologous expression of nAChRs in Xenopus laevis oocytes

Two approaches have been developed to study HS and LS α4β2 receptors independently of each other in Xenopus oocytes. One approach has been to inject the α4 and β2 RNA at ratios that would favor the assembly of LS or HS receptors (usually 10:1, α4 to β2 for LS receptors or 1:10 α4 to β2 for HS receptors) (Zwart et al., 2008). However, this approach generates a heterogeneous population of receptors and if applied to the present study would give an unequal number of mutant β subunits in the LS and HS biased population. The alternative approach is to use linked α4–β2 subunits (Zhou et al., 2003) which permits the co-expression of the concatamer with monomeric α4 or β2 subunit to yield pure populations of defined subunit composition and furthermore by placement of L15'M mutation in the concatamer allows for LS and HS receptors to be formed with the same number of mutant subunits in both receptor types. The original publication of the concatamers (Zhou et al., 2003) provided a thorough validation of the constructs with Western blots and other analyses. The fidelity with which the concentration-response data of the receptors formed with the concatamer containing the L15'M mutation match the data obtained with the original concatamer, obtained and characterized by the Lindstrom lab, indicate that the mutation did not disrupt the function of the concatamers. The human nAChR clones and the original β2–6–α4 concatamer were obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia PA). The β2 L15'M mutant in the concatamer was made as

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previously described (Stokes et al., 2019). Subsequent to linearization and purification of the plasmid cDNAs, cRNAs were prepared using the mMessage mMachine in vitro RNA transfection kit (Ambion, Austin TX).

Oocytes were surgically removed from mature *Xenopus laevis* frogs (Nasco, Ft. Atkinson WI) and injected with appropriate nAChR subunit cRNAs as described previously (Papke and Stokes, 2010). Frogs were maintained in the Animal Care Service facility of the University of Florida, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee (approval #202002669). In brief, the frog was first anesthetized for 15-20 min in 1.5 L frog tank water containing 1 g of 3-aminobenzoate methanesulfonate buffered with sodium bicarbonate. The harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemicals, Freehold NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO3, 0.82 mM MgSO4, 15 mM HEPES, and 12 mg/l tetracycline, pH 7.6) to remove the follicular layer. Stage V oocytes were subsequently isolated and injected with 50 nl water containing 5 ng concatamer plus 5 ng α4 or β2 nAChR subunit cRNA. Recordings were carried out 2-7 days after injection.

Two-electrode voltage clamp electrophysiology

Experiments were conducted at room temperature (24°C) using OpusXpress 6000A (Molecular Devices, Union City, CA) (Papke and Stokes, 2010). Both the voltage and current electrodes were filled with 3 M KCl. Oocytes were voltage-clamped at -60 mV. The oocytes were bath-perfused with Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 10 mM HEPES, and 1 μM atropine, pH 7.2) at 4 ml/min. Drug applications were 6 s in duration followed by 241 s washout periods. A typical recording for each oocyte constituted two initial control applications of ACh, an application of the experimental compound applied alone, a follow-up control application of ACh, a co-application of the test compound with 30 μM racemic TQS and a final control application of ACh. The control ACh concentrations were 10 μM for HS receptors and 100 μM for LS receptors. The concentrations of the test compounds are provided

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in Table 1. All experiments began with 8 oocytes voltage clamped and treated in parallel; however, some cells lost voltage clamp or otherwise failed to remain viable through the series of drug applications and were thus excluded from the analyses. Final n values are also provided in Table 1.

The responses are reported as peak current amplitudes. The average responses of the two initial ACh controls from each cell were used for normalization. Data are presented as the averages ± standard deviations. Statistical analyses were conducted based on T-test comparisons of the normalized peak current data or one-way ANOVA. Bonferroni corrections were applied for multiple comparisons (Aickin and Gensler, 1996). When drug responses without and with TQS were obtained from the same cells, pairwise comparisons were made. However, it was noted that with the LS receptors, ACh control responses were inhibited by the applications of nicotine, TC-1698, varenicline, and epibatidine applied alone. Therefore, for these drugs on the LS receptors, the responses to the drugs alone and the drugs co-applied with TQS were obtained on separate sets of cells.

Data were collected at 50 Hz, filtered at 5 Hz, and analyzed by Clampfit 9.2 or 10.3 (Molecular Devices) and Excel (Microsoft, Redmond WA). All experiments began with eight voltage-clamped oocytes set up for parallel analysis in the Opus-Xpress system. However, due to the fact that PAM-potentiated currents were sometimes very large, some cells could not be held in voltage clamp and were therefore excluded from the subsequent analyses. If more than three cells were excluded due to inadequate voltage clamp, the entire experiment was repeated. Results are expressed as means ± standard deviation (SD) from at least five oocytes for each experiment or as dot plots generated by Kaleidagraph 4.5.2 (Synergy Software, Reading PA). ANOVA and other statistical comparisons were calculated in Kaleidagraph 4.5.2. The values for the curve fits were generated using the Levenberg-Marquardt algorithm to obtain the best Chi-Square fit to the Hill equation using the Kaleidagraph 4.5.2 plotting program. The errors reported for the fit parameters are based on the goodness of fit.

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We display multi-cell averages of the raw data for visual comparisons of complex responses. The averages of normalized data were calculated using an Excel (Microsoft) template for each of the 10,322 points in each of the 206.44 s traces (acquired at 50 Hz). Following subtraction of the basal holding current, data from each cell, including the ACh controls, were normalized by dividing each point by the peak of the ACh control from the same cell. The normalized data were then averaged and standard errors of the mean (SEM) for the multi-cell averages calculated on a point-by-point basis. The dark lines represent the average normalized currents and the shaded areas the range of the SEM. Scale bars in the figures of averaged traces reflect the scaling factor relative to the average peak current amplitude of the ACh controls used for the normalization procedures. These plots effectively illustrate the differences in peak currents, net charge, the kinetics of the responses, and the variability throughout the entire time course of the responses.

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Results

The generation of TQS-sensitive HS and LS α4β2 nAChR

The L15'M mutation (Stokes et al., 2019) was made in the β2 subunit of the β2–6–α4 concatamer (Zhou et al., 2003), so that by co-expressing this concatamer with monomers of either β2 or α4, we could obtain receptors with the subunit configuration shown in Figure 1A. These receptors show the expected differences in ACh sensitivity previously reported for wild-type HS and LS receptors (Figure 1B). For the HS receptors, the Log ACh EC_{50} values were 0.431 μM (Log error = -.39) and 0.11 μM (Log error = -.1) for the wild-type and mutant receptors, respectively. For the LS receptors, the ACh Log EC_{50} values were 2.13 μM (Log error = 1.39) and 2.27 μM (Log error = 1.27) for the wild-type and mutant receptors, respectively.

As expected, the ACh responses of oocytes expressing these constructs were strongly potentiated by co-application of ACh with 30 μM TQS (Figure 2A). The control ACh responses of HS receptors increased by a factor of 43 (with a standard deviation of 19.76), while the LS ACh responses were increased by a factor of only 2.546 (with a standard deviation of 0.237).

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We compared responses obtained with our co-application protocol to responses obtained when TQS was pre-applied for 30 seconds prior to the co-application of ACh and TQS. Responses were essentially identical with or without pre-applications (Supplemental Figure 1).

As noted in the earlier work with L15'M mutants (Stokes et al., 2019), the effects of TQS persist after the washout of the drug from the bath, so that responses to ACh alone after the TQS application were also increased relative to the initial ACh control responses. This sort of priming is similar to what has been described for the TQS-related α7 ago-PAM GAT107 (Papke et al., 2014b), and was observed with the TQS-sensitive α4β2 receptors, regardless of the test compound initially co-applied with TQS (not shown). For this reason, oocytes were not used for repeated measurements following an application of TQS.

With our standard protocol involving two initial responses to ACh alone, the application of TQS alone also evoked small currents (Figure 2B). Similar results were obtained when TQS was given prior to the ACh controls (data not shown). The responses to TQS alone were larger with the HS receptors than the LS receptors, although in both cases they were smaller than the ACh controls (Figure 2C). This observation raised the concern that while evaluating the effects of TQS on responses to ligands expected to produce little α4β2 activation, we could only consider there to be TQS potentiation if the co-application responses were larger than the sum of the responses to the ligand and to TQS alone.

We evaluated a total of 28 drugs for their activity alone and when co-applied with TQS, and it was consistently observed that for active compounds, regardless of the class of compound, the potentiation of HS responses was greater than that of LS receptors ($p < 0.001$), typically by at least an order of magnitude (Figure 3), consistent with TQS-sensitive desensitization being a factor limiting HS receptor responses.

Dynamic conversion of steady-state desensitization to PAM-potentiated currents.

In order to promote a progression toward steady-state desensitization, we pre-applied 30 μM nicotine to HS and LS α4β2L15'M receptors and then co-applied 30 μM nicotine and 30 μM

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TQS (Figure 4A). The upper traces show the averaged responses (see Methods) of seven cells of each type, normalized to their initial ACh controls (not shown). The average peak amplitude of the HS 10 μM ACh controls was 2.47 μA (SD = 1.03), while the average peak amplitude of the LS 100 μM ACh controls was 15.9 μA (SD = 11.8). The nicotine phases of the responses are shown below the main traces, scaled as indicated. The peak of the HS nicotine response was only 330 nA (SD = 67 nA), while the peak of the LS nicotine response was 982 nA (SD = 283 nA). A comparison of the normalized responses to the TQS co-applications (Figure 4B) would indicate a larger TQS response for the HS receptors ($p < 0.01$). However, comparison of the responses without normalization (Figure 4C) is consistent with TQS selectively increasing the HS response up to the level of the LS TQS responses (see Supplemental Data for statistics). Note that both experiments were conducted on the same day with cells from the same injection set.

Inactive compounds

Since one of our goals was to probe compounds that would be equivalent to silent agonists, i.e. give currents only when co-applied with the PAM, we tested three classes of compounds that were not expected to activate α4β2 receptors when applied alone. These were the α7 silent agonists, NS6740, 1-methylpyridinium (Papke et al., 2022b), and triethylmethylammonium (Papke et al., 2014a) (Supplemental Figure 2); the α9-selective agonists, *p*CN diEPP, *p*CONH2 diEPP, and diMPiP (Papke et al., 2022a) (Supplemental Figure 3); and the LS α4β2 modulators, NS9283 (Wang et al., 2015) and dFBr (Weltzin and Schulte, 2010) (Supplemental Figure 4). As expected, none of these compounds produced activation of either α4β2 receptor when applied alone, and when co-applied with TQS using our standard protocol, none of these compounds produced responses greater than those seen to TQS applied alone.

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Effects of nAChR antagonists

The α7-selective antagonist methyllycaconitine (MLA) (Turek et al., 1995) and the α4β2-selective antagonist dihydro-β-erythroidine (DHβE) (Damaj et al., 1995) were tested applied alone and in co-application with TQS on the L15'M receptors. As expected, neither compound produced any activation when applied alone (Table 3). Interestingly, both compounds suppressed any response when co-applied with TQS using our standard protocol. Due to the larger responses of the HS receptors to TQS alone, this effect was most obvious for that isoform ($p < 0.05$, Supplemental Figure 5). It may be the case that the nicotinic antagonists have selectivity for the inactive state of the receptor and inhibit the effect of TQS allosterically. Although the co-application of TQS with the antagonists generated no responses, subsequent responses to ACh alone were primed by the co-applications (not shown).

TQS effects on HS ACh responses across a range of ACh concentrations

As noted earlier, it has been proposed that HS responses to high concentrations of agonist are specifically limited by desensitization. If this is the case, then it might be possible for HS receptors to continue to show progressive increases in TQS-potentiated responses in a range of ACh concentrations (i.e. > 10 μM) where applications of ACh alone show little further increase, effectively causing a rightward shift in the ACh response curve, making them more LS-like in that regard. Therefore, to determine whether the inability of HS receptors to show increased responses to higher concentrations of ACh was due to progressively larger amounts of TQS-sensitive desensitization, co-applications of TQS with ACh were conducted across a wide range of ACh concentrations (Figure 5A). The TQS-potentiated ACh responses showed a concentration sensitivity that was similar to, or even greater than, the responses to ACh alone, with an EC_{50} of 125 ± 22 nM for ACh plus TQS compared to 1.39 ± 0.07 μM for ACh alone. These data suggest that TQS-sensitive desensitization is a limiting factor even at the lowest ACh concentrations, and not a factor especially limiting HS responses to higher concentrations of agonist.

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LS receptor potentiation at higher drug concentrations

Given that the receptors with the α4(3)β2(2) configuration are characterized as low sensitivity, the L15'M receptors with this configuration were also tested with eleven of the active compounds at 10-fold higher concentrations to determine if the effects of TQS were systematically underestimated by testing the compounds on both the HS and LS receptors at the same concentration. For seven of the eleven compounds tested at higher concentrations (see ANOVA results Table 2), there were no statistically significant differences in the TQS-potentiated responses at the two concentrations (Figure 5B). However, responses to sazetidine-A, nicotine, and epibatidine co-applied at the higher concentration with TQS were roughly 50% smaller ($p < 0.0001$) than the responses to the lower concentrations co-applied with TQS. Only responses to 1 mM arecoline co-applied with TQS were larger ($p < 0.001$, Table 2), by roughly a factor of two than when TQS was co-applied with the ten-fold lower concentration of arecoline.

Potentiation of non-selective nAChR agonists

We tested a selection of drugs considered relatively non-selective cholinergic agonists including nicotine, its primary metabolite cotinine (Briggs and McKenna, 1998), and its primary metabolite in brain, nor-nicotine (Crooks et al., 1995). While nor-nicotine has been shown to be a relatively potent α4β2 agonist (Papke et al., 2007), cotinine is generally thought of as primarily being a biomarker for nicotine use with very low potency as an agonist (Tan et al., 2021). We also tested the minor tobacco alkaloid, anatabine (Wu et al., 2002), previously reported to be an α4β2 agonist (Alijevic et al., 2020). We also tested carbachol (Parker et al., 1998), an agonist for both nicotinic and muscarinic AChR, along with epibatidine (Badio and Daly, 1994) a toxin isolated from frogs that is amongst the most potent of all nicotinic agonists (Gerzanich et al., 1995), and anabaseine, an alkaloid toxin produced by Nemertine worms and Aphaenogaster ants (Kem et al., 1997; Wheeler et al., 1981).

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When applied alone at the test concentration (Table 1), these compounds had varying levels of activity (Figure 6), and when normalized to the respective ACh controls, the responses of the HS and LS receptors were not different, except in the case of epibatidine, which was more active on the LS receptors than the HS receptors ($p < 0.001$, see Supplemental Data for statistical analysis). The TQS effects also differed somewhat for the HS and LS receptors. TQS did not potentiate the low responses to cotinine for either subtype.

Activity and potentiation of α4β2 partial agonists

Four α4β2 partial agonists, including the smoking cessation drugs cytisine (Etter et al., 2008) and varenicline (Coe et al., 2005), as well as arecoline (Papke et al., 2015), an active agent in areca associated with betel quid addiction (Gupta and Warnakulasuriya, 2002), and TC-2403 (Papke, 2002) were tested. As expected, responses to these agents were low when applied alone, especially for the HS receptors. Normalized to their respective ACh controls, the responses of LS receptors to varenicline and TC-2403, were greater compared to those of HS receptors ($p < 0.001$, see Supplemental Data for ANOVA and t-tests). TQS produced potentiation (Figure 7A) at varying levels of statistical significance for all these agents on both receptor subtypes (Supplemental Data), supporting the hypotheses that receptor desensitization is at least in part a factor that limits the efficacy of these agents for α4β2* receptors. Comparison of the data in Figures 6 and 7A suggests that the TQS-potentiated responses of the partial agonists are roughly equivalent to those of the non-selective agonists.

Potentiation of HS α4β2-selective agonists

Sazetidine-A and TC-2559, two agents that are potent activators of HS α4β2 receptors with little or no efficacy for activating LS receptors, were tested (Figure 7B). Sazetidine-A was actually first published as a selective α4β2 desensitizer, since it was shown to primarily desensitize receptors in an expression system that was biased toward the formation of LS-type receptors (Xiao et al., 2006), and only later shown to be an HS-selective agonist when HS

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receptor formation was enhanced by injection of oocytes with a ten-fold excess of β2 relative to α4 RNA (Zwart et al., 2008). The same approach was also used to demonstrate the increased efficacy of TC-2559 for HS receptors (Zwart et al., 2006). As expected, when applied alone, these agents stimulated large responses for HS receptors with very little response in the LS receptors ($p < 0.0001$, Supplemental Data). However, when co-applied with TQS, both compounds were strong activators of both receptor types, confirming that they are indeed subtype-selective silent agonists (Figure 7B).

α7-selective agonists

One of the most frequently sought-after goals in the pre-clinical developments on nicotinic drugs has been to identify drugs that will target α7 nAChR without affecting other subtypes like α4β2 receptors (Papke and Horenstein, 2021), leading to the identification of several α7-selective agonists. Among the first α7-selective agonists to be published, and one of the most widely used, is GTS-21 (DMXB) (de Fiebre et al., 1995), although even in the first publication it was noted to also antagonize α4β2 responses. Subsequently, several large pharmaceutical companies developed agents that were proposed to be more selective than GTS-21, including AR-R17779 (Levin et al., 1999), TC-1698 (Marrero et al., 2004), and PNU-282987 (Bodnar et al., 2005). The ACh precursor choline was also identified as an α7-selective agonist (Papke et al., 1996), although its low potency and ubiquitous presence in the brain and blood generally precludes its consideration as a therapeutic agent.

As expected, none of these drugs evoked much activation of the α4β2 receptors, although there were small responses of HS receptors to GTS-21 (Table 4 and Figure 8). This activity may have been missed in earlier studies that were based on the expression of α4 and β2 injected at equal ratios in *Xenopus* oocytes and might have biased expression toward the LS form. In any case, these responses were smaller than responses to ACh or TQS alone ($p < 0.0001$, see Supplemental Data for ANOVA results) and were not larger than the responses to the other α7 agonists. When co-applied with TQS to HS receptors, GTS-21 and choline gave responses that

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were larger than those of TQS alone. AR-R17779 and TC-1698 gave measurable responses, but the ANOVA results did not indicate that they were statistically larger than responses to TQS alone. For the LS receptors, GTS-21 ($p < 0.0001$), TC-1698 ($p < 0.01$), and choline ($p < 0.0001$) co-applied with TQS gave responses larger than to TQS alone (see Supplemental Data).

Inhibition of wild-type α4β2 ACh responses by α7-selective agonists

The data in Figure 9 suggest that some of the compounds proposed to activate α7 receptors would be effective desensitizing antagonists of α4β2 receptors. To test this, cells expressing wild-type forms of HS and LS α4β2 receptors were pre-exposed to the commercially developed α7-selective agonists for 30 s, and then ACh was co-applied at the control concentration along with the α7 agonists at the test concentration. The ACh responses were compared with the control ACh responses obtained prior to the application of the α7 agonists (Figure 9). GTS-21 pre-application evoked a small response from the LS α4β2 receptors and suppressed the ACh responses of both subtypes, with a greater effect on HS than on LS (Table 5). TC-1698 produce a nearly complete block of the ACh responses of both α4β2 subtypes, while AR-R17779 produced a 50% block of the HS responses with no effect on the LS ACh response. PNU-282987 pre-application and co-application caused no block of either α4β2 receptor subtype (Figure 9). These results with wild-type receptors are consistent with the TQS effects obtained on the receptors with L15'M mutant β2 subunits (Figure 8).

Discussion

The results support the hypothesis that the responses of HS α4β2 receptors are strongly limited by desensitization, even at low agonist concentrations. They also show that the desensitization is not specifically a factor limiting the response of the HS receptors to high agonist concentrations. The survey of the several classes of ligands identified some types of compounds, like the α7 silent agonist and the α9 agonists, that appear to be free of α4β2 activating or desensitizing effects. They also indicate that desensitization is a factor limiting the

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efficacy of α4β2 partial agonists like cytisine and varenicline and that selective desensitization of LS receptors may tune the efficacy of agents like sazetidine-A and TC-2559 so that they are functionally HS receptor selective agonists and functionally LS receptor desensitizers.

The desensitization of nAChR is a complex and multiphasic process (Boyd, 1987; Dilger and Liu, 1992; Feltz and Trautmann, 1982; Forman and Miller, 1988; Lester, 2004; Papke et al., 2009; Quick and Lester, 2002; Simasko et al., 1986; Sine and Steinbach, 1987), and the effect of type II PAMs on α7 receptors is selective for only some form(s) of desensitization (Williams et al., 2011). Although the effects of some type II PAMs can be quite large, they do not reverse all the receptor desensitization or affect all receptors equally. Indeed, the effects of the α7 PAM PNU-120596 are to enormously increase the activation of a small fraction of receptors while the majority of receptors remain in desensitized states (Andersen et al., 2016; Williams et al., 2011). The single-channel effects of the ago-PAM 4BP-TQS (GAT107) on α7 receptors are similar to those of PNU-120596, (Palczynska et al., 2012; Quadri et al., 2019), while the effects of TQS on α7 ACh responses are somewhat less (Palczynska et al., 2012). However, the basic mechanisms of α7 desensitization are fundamentally different from those of α4β2 receptors, so likewise the TQS effects on the TQS-sensitive α4β2 receptors might be very different on the molecular level from the effects on α7 receptors. While α7 receptors show no activation at all with high agonist concentrations (Williams et al., 2011), α4β2 receptors can smolder (Campling et al., 2013), occasionally opening under predominantly desensitizing conditions.

While TQS is considered strictly a PAM for α7, since we observed it to activate the TQS-sensitive α4β2 receptors (particularly the HS receptors) when applied alone, it might be classified as a weak ago-PAM or allosteric agonist for these receptors, behaving like the TQS analog (+)4BP-TQS (GAT107) on α7 receptors. By definition, "ago-PAMs" potentiate the responses evoked by agonists but also produce activation on their own and may also prime the potentiation of subsequent agonist application. The direct allosteric activation of HS α4β2L15'M receptors by TQS was blocked by 10 μM of the α4β2-selective antagonist DHβE as well as by 100 μM MLA, a concentration at which the drug is no longer selective for α7

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(Buisson et al., 1996). While the allosteric activation of α7 by GAT107 can be blocked by 10 μM of the α7 selective antagonist MLA (Papke et al., 2014b), it is insensitive to 100 μM DHβE (Papke, unpublished data not shown). It seems unlikely that TQS itself is a suitable ligand for the ACh binding sites, since it lacks a positively charged nitrogen common to most nicotinic agonists, so it is possible that, especially for the HS receptors, there was an incomplete washout of ACh from the previous control application of ACh and that residual ACh facilitated the effects of TQS when nominally applied alone. Alternatively, TQS may actually function as a weak ago-PAM for these receptors, and occupancy of the ACh sites by the competitive antagonists might be sufficient to inhibit the allosteric activation by TQS.

The therapeutic development of α7-selective agonists for indications such as schizophrenia (Cannon et al., 2013; Hajos and Rogers, 2010; Haydar and Dunlop, 2010; Walling et al., 2016) or Alzheimer's disease (Chen et al., 2006; D'Andrea and Nagele, 2006; Leiser et al., 2009) is largely predicated on the assumption that these drugs will not impair the normal functions of α4β2 receptors in the brain. This is a particular concern in the case of Alzheimer's disease, since evidence suggests that α4β2 receptor function is specifically impaired in this patient population (Court et al., 2001; Gotti et al., 2006). The results with the α7-selective agonists tested indicate that, depending on the specific agent, a number of differing profiles of α7 agonism and α4β2 antagonism may be available, with PNU-282987 being the least likely to affect α4β2 function. As noted above, GTS-21 was the first synthetic α7-selective agonist identified, and since 1994 it has been cited in 248 PubMed publications, including 41 since 2019. PNU-282987, has been cited in 202 PubMed publications since it was first reported in 2005, and it is also in current use, with 39 citations since 2019. The α7 agonists TC-1698 and AR-R17779 are far less commonly used, with only 3 and 50 total PubMed citations, respectively. Two studies related to CAP activity reported comparable effects with GTS-21 and PNU-282987 (Yuan et al., 2020; Zhou et al., 2021). However, it is important to note that from an electrophysiological perspective, these compounds have very different activity profiles for α7 receptors. PNU-282987 is relatively potent and nearly a full agonist (Hajos et al., 2005), while

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GTS-21 has lower potency and efficacy and additionally produces residual α7 receptor desensitization (Papke et al., 2009). Interestingly, the α7 desensitizing activity of GTS-21 may be important for its CAP activity (Horenstein and Papke, 2017; Thomsen and Mikkelsen, 2012). While these two agents may have similar CAP activity, it is likely that they would have distinctly different profiles in the brain, where cholinergic activity is associated with dynamically balanced function of α4β2* and α7 receptors, with GTS-21 capable of decreasing α4β2 function as well as working on α7, and PNU-282987 affecting α7 exclusively.

The perspective of nAChR as mediators of fast synaptic transmission, which is their function at neuromuscular junctions and autonomic ganglia, relegates desensitization to the background as perhaps nothing more than a safety valve to prevent overstimulation. However, as modulators of neurotransmission in the brain, often as presynaptic receptors on neurons that release other neurotransmitters, desensitization must be accounted for in the cholinergic control of brain function. Consideration of desensitization is even more important when considering the effects of self-administered nicotine, especially after a smoker's very first cigarette puff of the day (Picciotto et al., 2008). However, does desensitization just move receptors to the sidelines, or is it possible that desensitized receptors serve other functions, independent of ion channel activity? Recent studies of α7 nAChR, especially in the context of the CAP mediated by α7, and possibly α9α10, receptors in immune cells (Rosas-Ballina and Tracey, 2009; Tracey, 2007) have suggested that the nonconducting (i.e. desensitized) conformations of those receptors function as metabotropic receptors regulating intracellular signal transduction and the release of pro- and anti-inflammatory cytokines (de Jonge and Ulloa, 2007; Kabbani and Nichols, 2018; King et al., 2017; King et al., 2018). This has led to the proposed development of weak α7 agonists like GTS-21 (Kong et al., 2018; Wang et al., 2020) and even silent agonists (Bagdas et al., 2018; Godin et al., 2020; Horenstein and Papke, 2017; Papke and Horenstein, 2021; Richter et al., 2016) for the treatment of inflammatory and neuropathic pain.

Additionally, an exclusive focus on the ion channel activity of nAChR largely ignores potential functions for the variable, and often large intracellular domains of these receptors

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(Stokes et al., 2015), and interestingly the intracellular domain of α4 is the largest of any nAChR subunit, by a factor of three or more. Although not so well studied as α7 in this regard, there have been reports that α4-containing nAChR also play roles in the regulation of inflammatory and neuropathic pain (Acharya et al., 2020; Nordman et al., 2014) and that these effects are correlated with receptor desensitization (Zhang et al., 2012).

In conclusion, the use of TQS-sensitive receptors provides a way to probe the ligand dependence of the conformational equilibrium of the two primary forms of the brain's α4β2 receptors and may prove useful for the development of more focused therapeutics.

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Oocyte recordings were conducted by Lu Wenchi Corrie.

Authorship Contributions

Participated in research design: Papke, R.

Performed data analysis: Papke, R. and Stokes, C.

Wrote the manuscript: Papke, R. and Stokes, C.

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Footnotes

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Figure Legends

Figure 1. TQS-sensitive HS and LS α4β2 receptors. **A)** Subunit configuration of the receptors composed of α4–β2 concatamers with the β2L15'M mutation (represented by the star) in the β subunits. When co-expressed with wild-type β2 subunits, they yield HS α4(2)β2L15'M(2)β2 receptors (left). When co-expressed with wild-type α4 subunits, they yield LS α4(2)β2L15'M(2)α4 receptors (right). **B)** ACh concentration-response data for wild-type (circles) and mutant (squares) receptors. Points represent the average of 4-8 oocyte responses at each concentration (± SD), normalized to preceding control ACh responses obtained from the same cells. ACh controls were 10 μM for the HS receptors and 100 μM for the LS receptors. The Levenberg-Marquardt algorithm was used in Kaleidagraph to generate curves based on the Hill equation that best fit the data.

Figure 2. Averaged data from HS α4(2)β2L15'M(2)β2 receptors (left) and LS α4(2)β2L15'M(2)α4 receptors (right). **A)** Cells were treated with control applications of ACh (red bars) and then after washout ACh co-applied with 30 μM TQS (blue bars) and then another application of ACh. ACh controls were 10 μM for the HS receptors and 100 μM for the LS receptors. The data are the averages of 7 cells for each receptor subtype. Scale bars are based on the average initial ACh controls that were used for normalization (see Methods). **B)** Averaged responses of HS and LS receptors to ACh and then 30 μM TQS applied alone, followed with another ACh application. The data are the averages of 8 cells for the HS configuration and 7 cells for the LS configuration. **C)** Superimposition of the ACh pre-application controls and the responses to TQS alone taken from the traces in B.

Figure 3. The TQS potentiated responses to all of the test compounds. The HS receptor responses, normalized to their ACh controls, are plotted relative to the scale on the Y-axis. The LS receptor responses, normalized to their ACh controls, are plotted relative to the scale on the

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X-axis. All points are averages (± SD). The n values are provided in Table 1. The various classes of drugs are color-coded as indicated.

Figure 4. **A)** Averaged raw data traces (n = 7 in each case) of HS (on left) and LS (on right) α4β2L15'M receptors to a 30-second pre-application of 30 μM nicotine followed by a co-application of 30 μM nicotine and 30 μM TQS. The nicotine-only phases of the responses are shown as inserts below the main traces, scaled as indicated. **B)** Peaks of TQS responses normalized to the initial ACh controls (see Supplemental Data for statistics). **C)** Peaks of TQS responses measured in μAmps (see Supplemental Data for statistics).

Figure 5. Agonist concentration dependence of TQS-potentiated responses. **A)** TQS potentiation of ACh HS receptor responses across a range of ACh concentrations. Plotted are the average peak current responses of HS receptors to co-applications of ACh and 30 μM TQS (red symbols, right y-axis) of 5-8 cells (± SD) at each concentration, compared to the responses to ACh alone (black symbols, left y-axis, data from Figure 1B). In both cases the responses were normalized and expressed relative to the initial peak currents of the 10 μM ACh controls from the same cells. The estimated I_{max} for ACh alone was only 1.14 ± 0.012 the ACh controls (r = 0.999), while for the TQS-potentiated current the Imax was 82.7 ± 2.6 (r = 0.988). **B)** TQS potentiation of LS receptors at two different concentrations. Circles represent the average normalized peak current responses obtained with TQS co-applied with the test compounds at the concentrations indicated in Table 1. Diamonds represent the average normalized peak current responses obtained with TQS co-applied with the test compounds at 10-fold higher concentrations than those indicated in Table 1. See Table 3 for ANOVA results. ** indicates $p < 0.001$ for comparisons between the low and high concentration responses.

Figure 6. Effects of non-selective agonists. **A)** Dot plot of the peak current responses of HS (left) and LS receptors (right) to the non-selective agonists when applied alone (circles),

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compared to the responses to drugs co-applied with 30 μM TQS, indicated by the drug name with a plus sign, and plotted as half-color diamonds.

Figure 7. Effects of selective agonists. **A)** Dot plot of the peak current responses of HS (left) and LS receptors (right) to the α4β2 partial agonists when applied alone (circles), compared to the responses to drugs co-applied with 30 μM TQS, indicated by the drug name with a plus sign, and plotted as half-color diamonds. **B)** Dot plot of the peak current responses of HS (left) and LS receptors (right) to the HS α4β2 selective agonists when applied alone (circles), compared to the responses to drugs co-applied with 30 μM TQS, indicated by the drug name with a plus sign, and plotted as half-color diamonds.

Figure 8. Effects of α7-selective agonists (Table 4). **A)** Dot plot of the peak current responses of TQS-sensitive HS receptors to the α7-selective agonists when applied alone, compared to ACh control responses and the responses to 30 μM TQS applied alone. Although GTS-21 appeared to give detectable responses, these were not statistically significant compared to the other α7-selective agonists (see Supplemental Data for ANOVA results). **B)** Responses of TQS-sensitive HS α4β2 receptors to α7-selective agonists co-applied with TQS. GTS-21 and choline gave responses that were larger than those of TQS alone. AR-R17779 and TC-1698 gave measurable responses, but the ANOVA results did not indicate that they were statistically greater than TQS alone (see Supplemental Data for ANOVA results). **C)** The lack of responses of TQS-sensitive LS α4β2 receptors to the α7-selective agonists when applied alone, compared to ACh control responses and the responses to 30 μM TQS applied alone. **D)** Responses of TQS-sensitive LS α4β2 receptors to α7-selective agonists co-applied with TQS. GTS-21 ($p < 0.0001$), TC-1698 ($p < 0.01$) and choline ($p < 0.0001$) co-applied with TQS gave responses greater than to TQS alone (see Supplemental Data for ANOVA results).

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Figure 9. Effects of α7-selective agonists on the ACh responses of wild-type HS and LS α4β2 receptors. Averaged data were prepared as described (Methods). Following the ACh control responses (red bars), the α7 agonists were pre-applied for 30 s (colored bars). Then without washout the α7 agonists were co-applied with ACh at the control concentration (10 μM for HS and 100 μM for LS). The n values for the GTS-21 experiments were 8 for the HS receptors and 7 for the LS receptors. The n values for the TC-1698 experiments were 7 for the HS receptors and 7 for the LS receptors. The n values for the AR-R17779 experiments were 7 for the HS receptors and 4 for the LS receptors. The n values for the PNU-282987 experiments were 8 for the HS receptors and 6 for the LS receptors.

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Table 1. Test compounds concentrations and n values

Drug	Concentration	n_{HS}	n_{LS}
Antagonists			
MLA	100 μM	8	4
DHβE	10 μM	8	7
α7 silent agonists			
NS6740	30 μM	8	8
n-MP	300 μM	7	8
triEMA	100 μM	7	8
α9 agonists			
*p*CN diEPP	100 μM	8	7
*p*CONH2 diEPP	100 μM	7	7
diMPiP	100 μM	8	7
α4β2 modulators			
NS9283	30 μM	8	8
dFBr	30 μM	8	7
Non-selective agonists			
carbachol	100 μM	6	8
epibatidine	3 μM	8	6
anabaseine	100 μM	7	8
nicotine	10 μM	7	8
cotinine	100 μM	6	8
nor-nicotine	10 μM	5	6
anatabine	100 μM	4	8
HS selective agonists			
sazetidine-A	30 μM	7	7
TC-2559	30 μM	6	8
α4β2 partial agonists			
TC-2403	10 μM	8	8
cytisine	100 μM	6	6
varenicline	10 μM	7	7
arecoline	100 μM	8	7
α7-selective agonists			
GTS-21	100 μM	7	7
PNU-282987	10 μM	8	8
TC-1698	10 μM	8	8
AR-R17779	10 μM	7	5
choline	1 mM	7	8

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Table 2. Analysis of Variance high vs low concentrations

Source	DF	SS	MS	F	p
Total	164	275.44943	1.6795697		
A	21	222.04497	10.57357	28.312629	< .0001
Error	143	53.404455	0.37345773		

Comparison	Mean Difference	|t|	p	95% CL
sazetidine-A low vs high	2.15358	6.8091	< .0001	0.95292 to 3.3542
nicotine low vs high	2.33651	7.6468	< .0001	1.1766 to 3.4965
TC-1698 low vs high	-0.100207	0.328	1	-1.2601 to 1.0597
varenicline low vs high	-0.253913	0.7773	1	-1.4939 to 0.98612
epibatidine low vs high	2.09233	6.1541	< .0001	0.80166 to 3.383
PNU-282987 low high	0.0842623	0.2553	1	-1.1686 to 1.3371
Choline low vs high	-0.408172	1.3358	1	-1.5681 to 0.75177
arecoline low vs high	-1.64859	5.2124	0.0001	-2.8492 to -0.44794
TC-2403 low vs high	-0.817367	2.5843	1	-2.018 to 0.38329
anatabine low vs high	-0.0802815	0.2538	1	-1.2809 to 1.1204
anabaseine low vs high	0.35324	1.1561	1	-0.8067 to 1.5132

Data were extracted from a larger ANOVA with Bonferroni correction for multiple comparisons and are not based on pairwise tests.

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Table 3. Antagonists

3a. Responses	HS activation	HS TQS	LS activation	LS TQS
	Average ± SD	Average ± SD	Average ± SD	Average ± SD
MLA	0.007 ± 0.003	0.016 ± 0.009#	0.003 ± 0.003	0.003 ± 0.003
DHβE	0.046 ± 0.032#	0.085 ± 0.046#	0.004 ± 0.002	0.004 ± 0.002

3b. Corrected p values

	Drug vs Drug plus TQS		Drug plus TQS vs TQS alone	
	p value HS	p value LS	p value HS	p value LS
MLA	0.0492	0.1879	0.0053#	1.4235
DHβE	0.0201	2.0367	0.0209#	0.8528

#p < 0.05, drugs co-applied with TQS reduced response compared to TQS alone

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Table 4. α7-selective agonists

4a. Responses	HS activation Average ± SD	HS TQS Average ± SD	LS activation Average ± SD	LS TQS Average ± SD
GTS-21	0.103 ± 0.023	34.514 ± 11.300	0.012 ± 0.004	0.753 ± 0.173
PNU-282987	0.014 ± 0.018	0.566 ± 0.298	0.009 ± 0.012	0.142 ± 0.147
TC-1698	0.013 ± 0.003	7.197 ± 1.694	0.009 ± 0.002	0.545 ± 0.199
AR-R17779	0.008 ± 0.006	3.017 ± 0.847	0.007 ± 0.007	0.109 ± 0.039
choline	0.022 ± 0.005	12.702 ± 6.048	0.024 ± 0.009	0.885 ± 0.237

See Supplemental Data for ANOVA results

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Table 5. Pre-applications of α7 agonists to wild-type receptors

Drug	HS	n	LS	n	HS vs LS	HS vs ACh	LS vs ACh
GTS-21	0.064 ± 0.038	8	0.263 ± 0.122	7	$p < 0.05$	$p < 0.01$	$p < 0.05$
TC-1698	0.012 ± 0.011	6	0.042 ± 0.028	7	N.S.	$p < 0.01$	$p < 0.05$
AR-R17779	0.518 ± 0.02	7	1.002 ± 0.039	4	$p < 0.0001$	$p < 0.01$	N.S.
PNU-282987	0.800 ± 0.074	8	0.973 ± 0.137	6	N.S.	$p < 0.05$	N.S.

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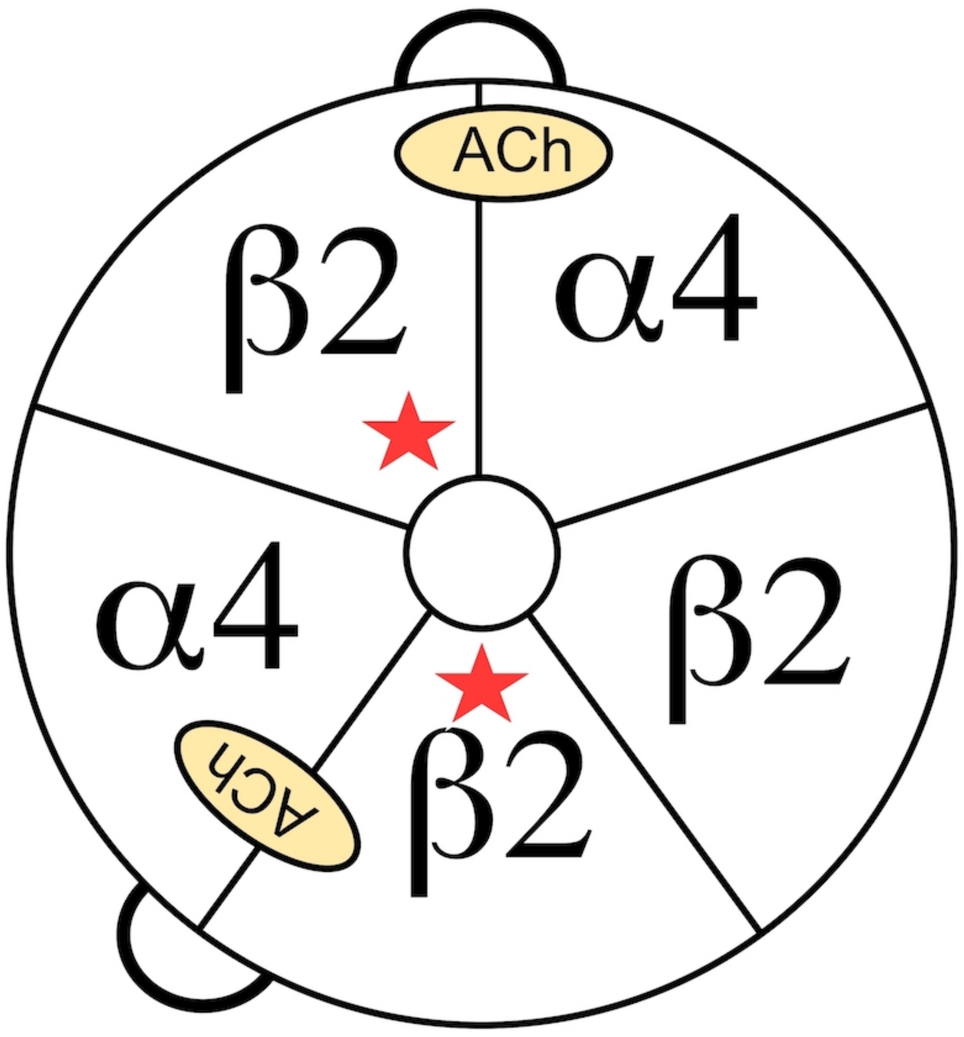
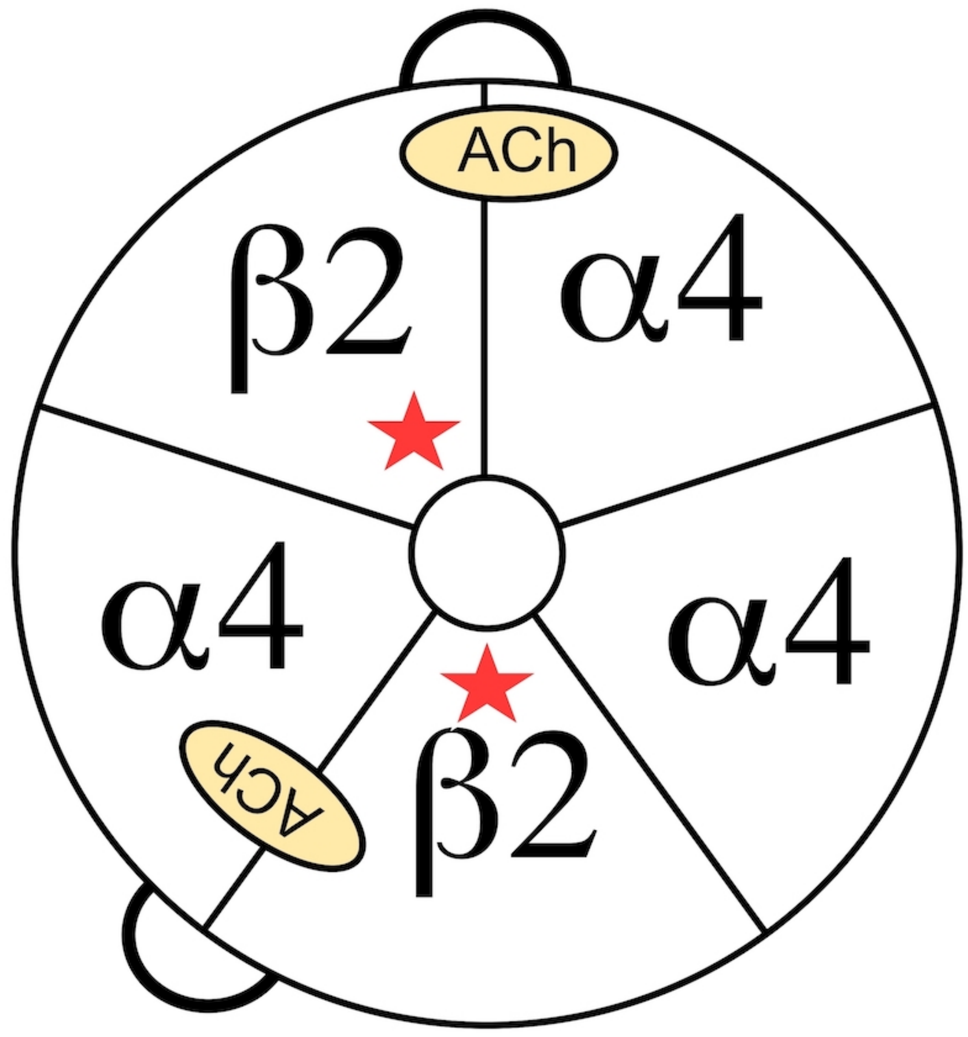
A



α4(2)β2L15'M(2)β2



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B

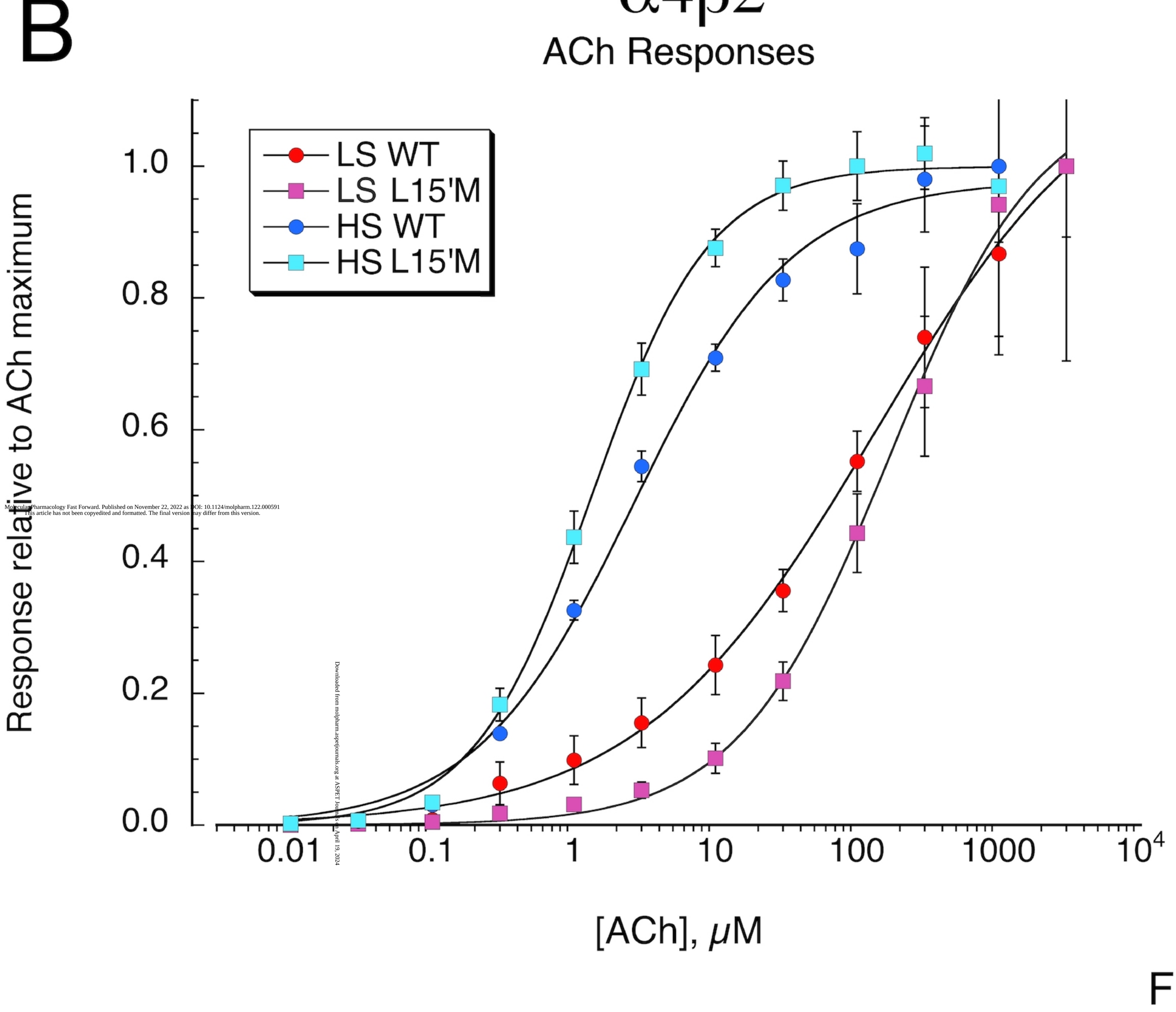

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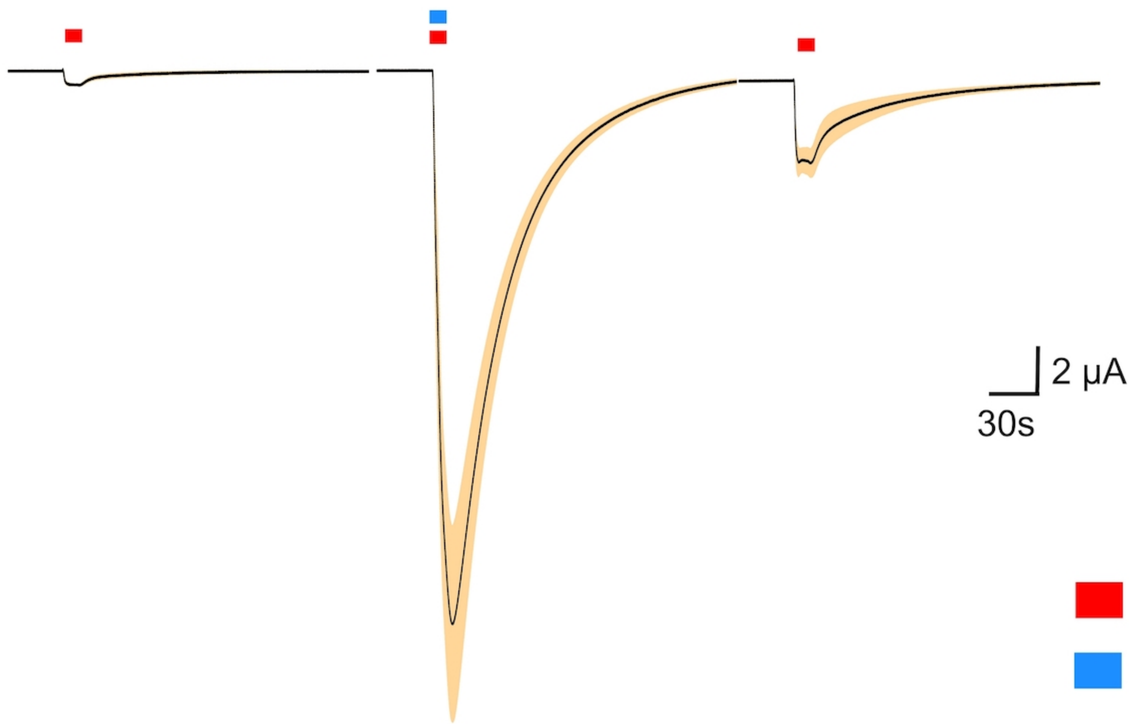
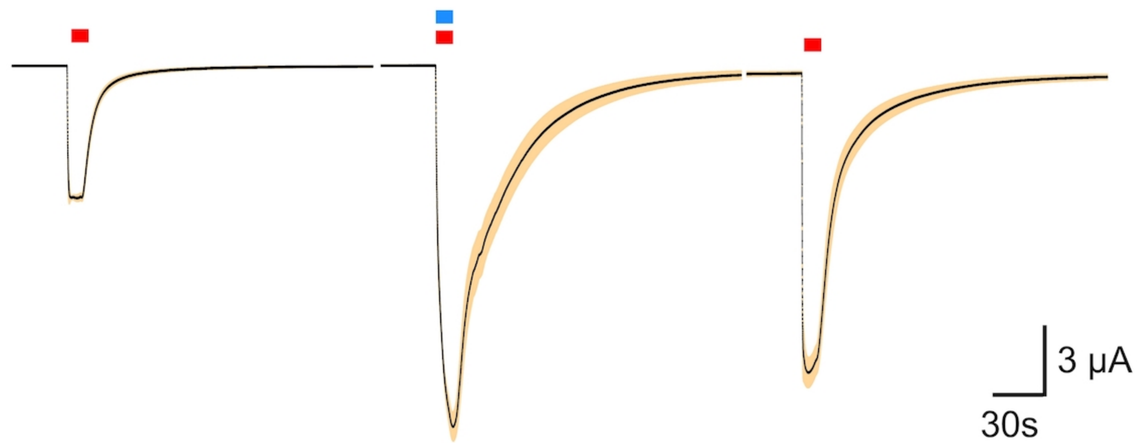
Figure 1

α4(2)β2L15'M(2)β2

α4(2)β2L15'M(2)α4

A

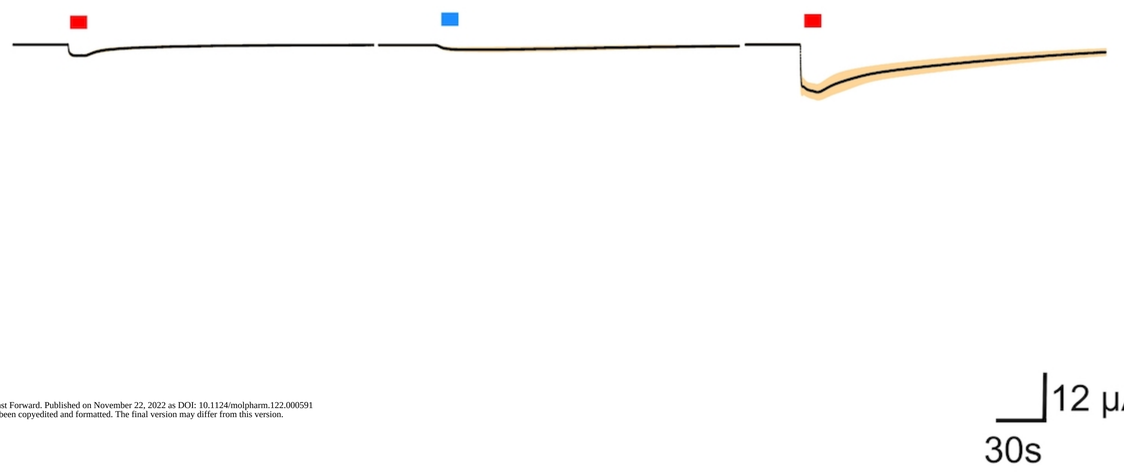


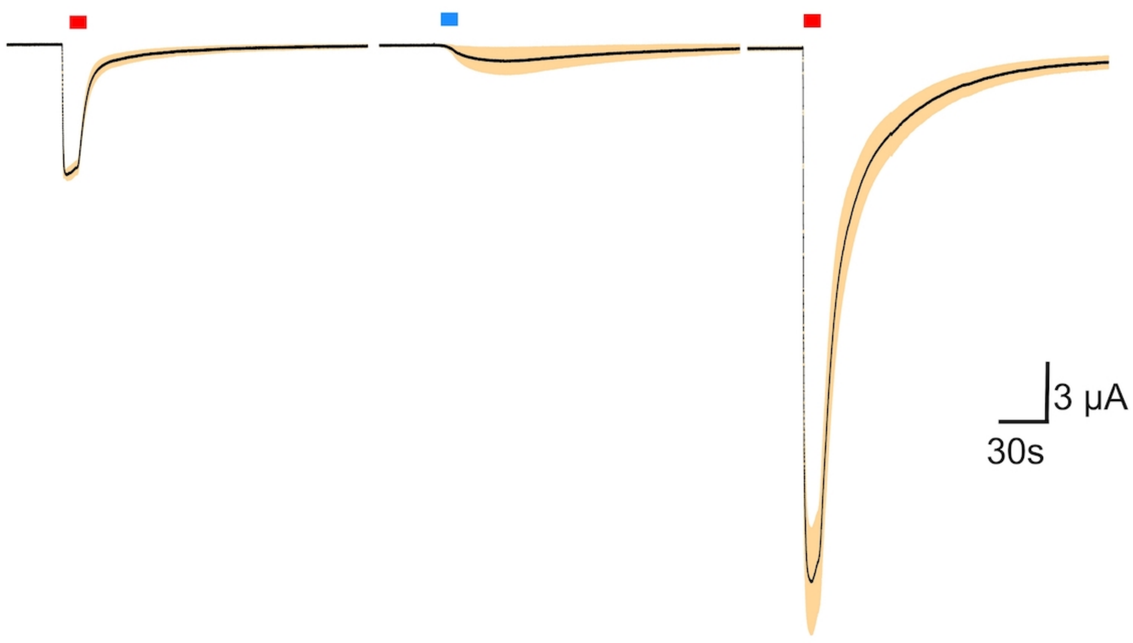


ACh

TQS

B





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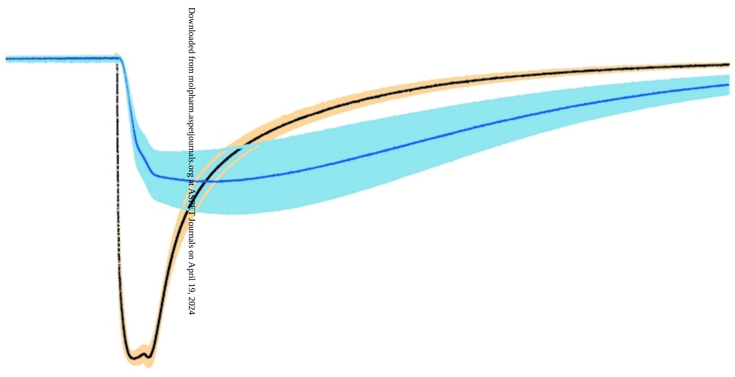

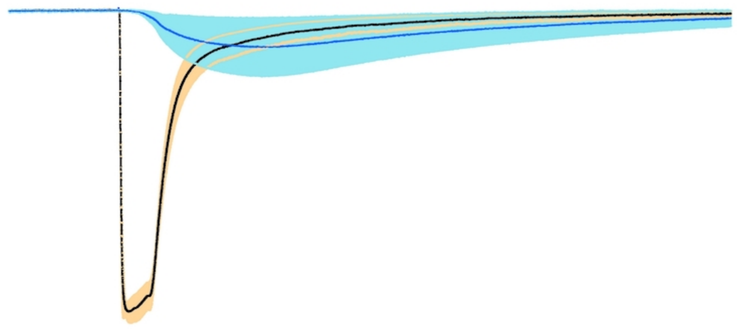

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Figure 2

TQS-potentiated responses of HS and LS α4β2 L15'M receptors

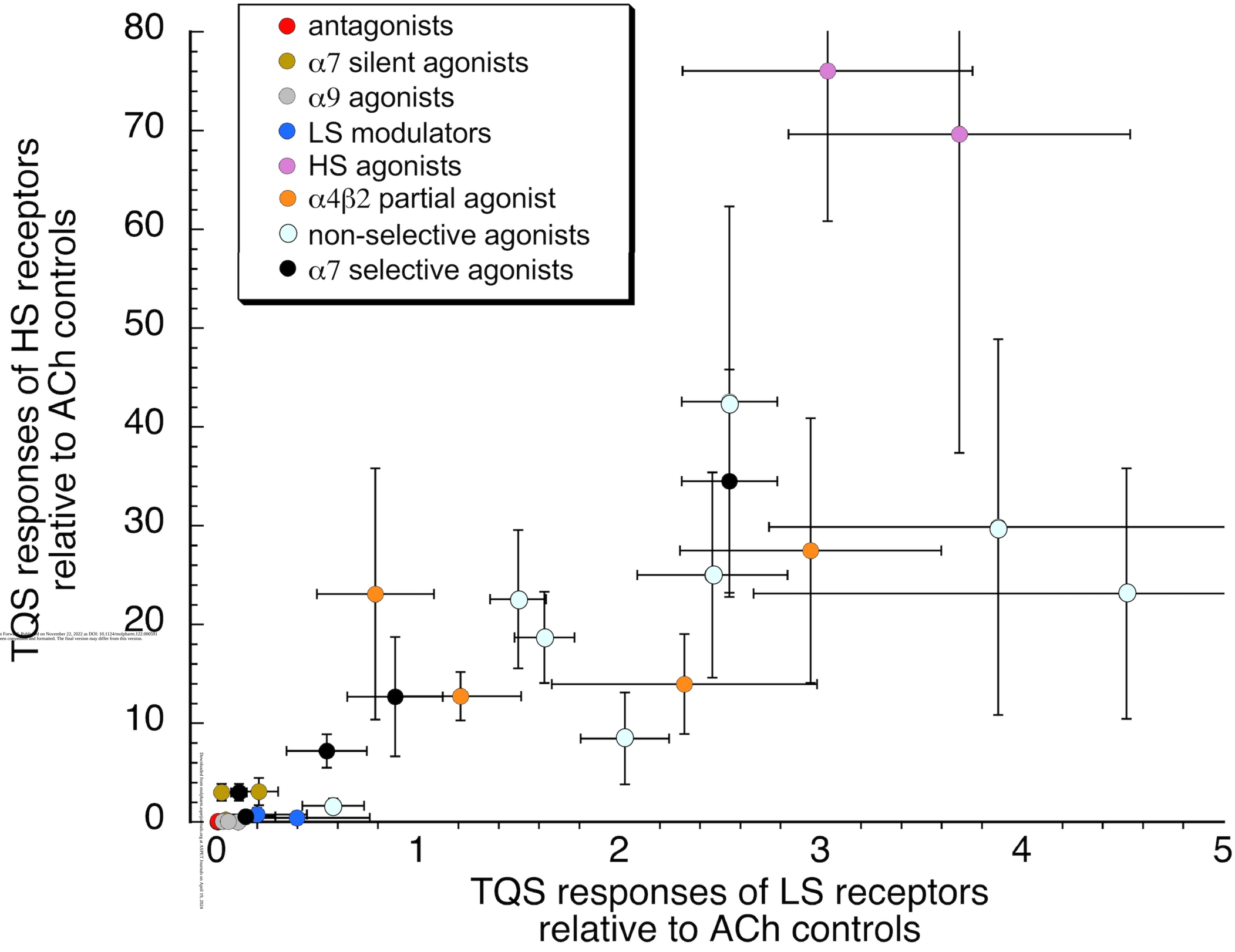

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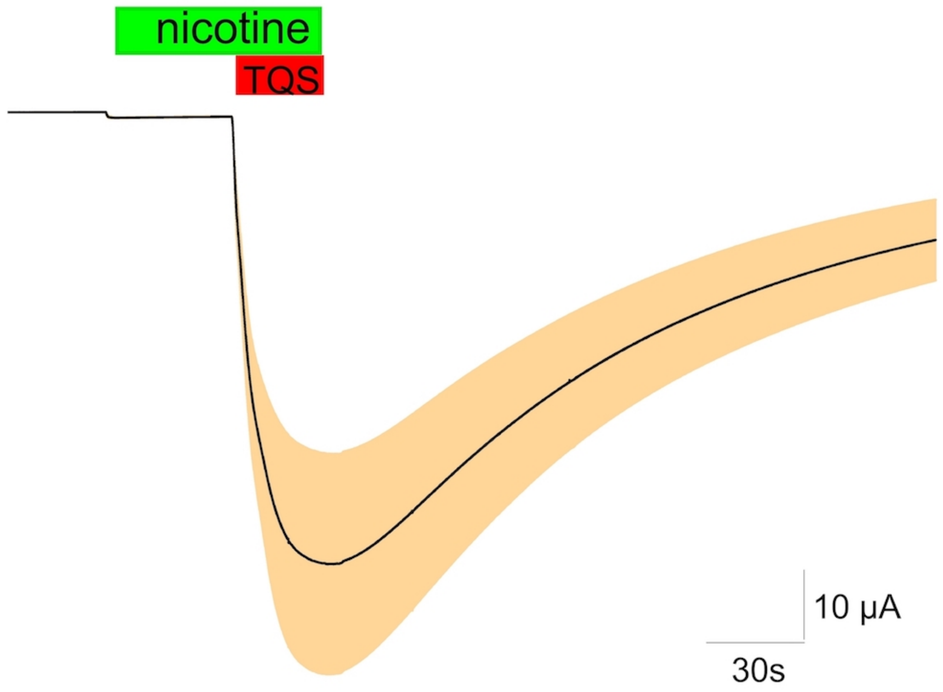
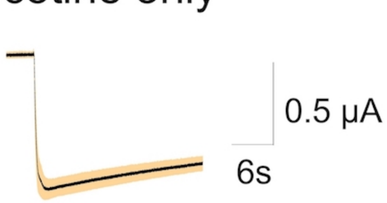
Figure 3

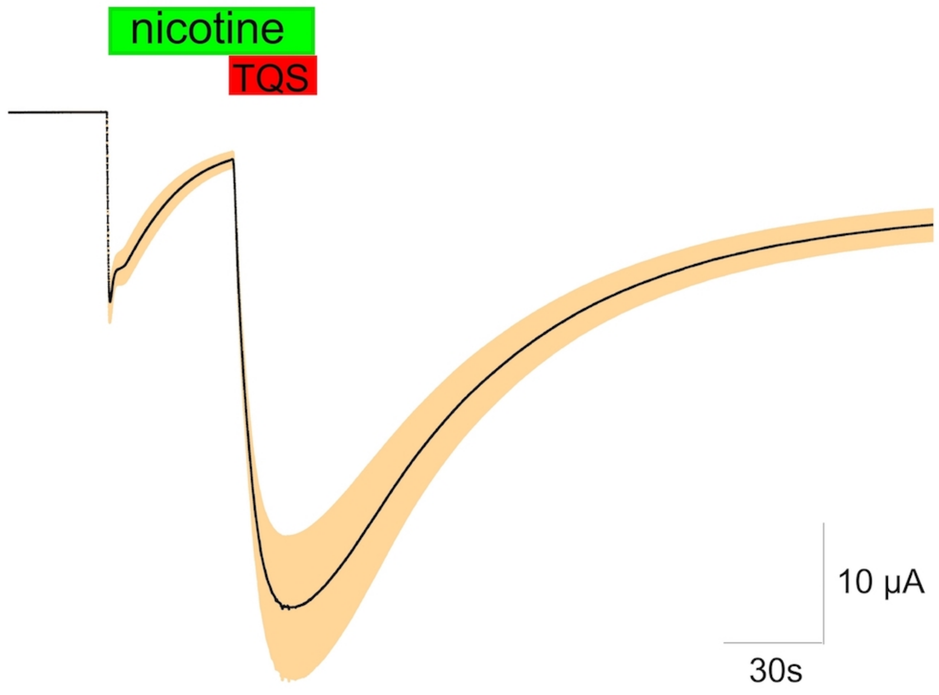
A

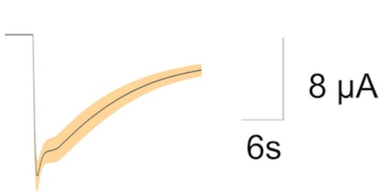
α4(2)β2L15'M(2)β2



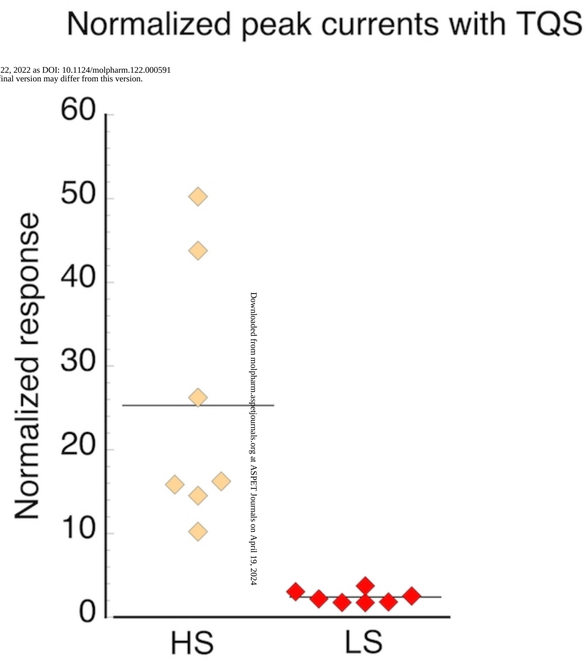


α4(2)β2L15'M(2)α4





B



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C

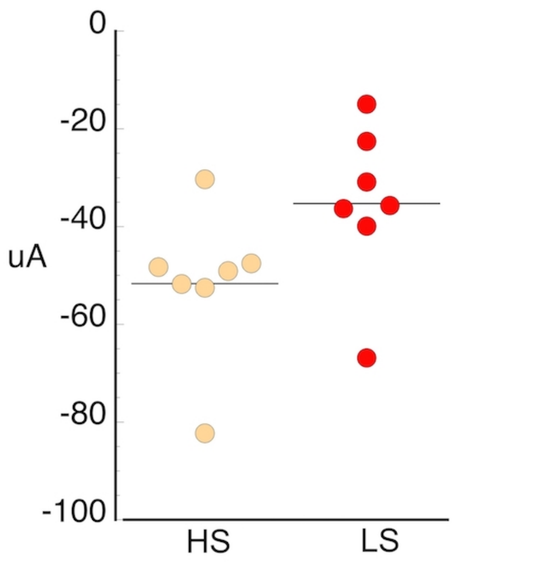
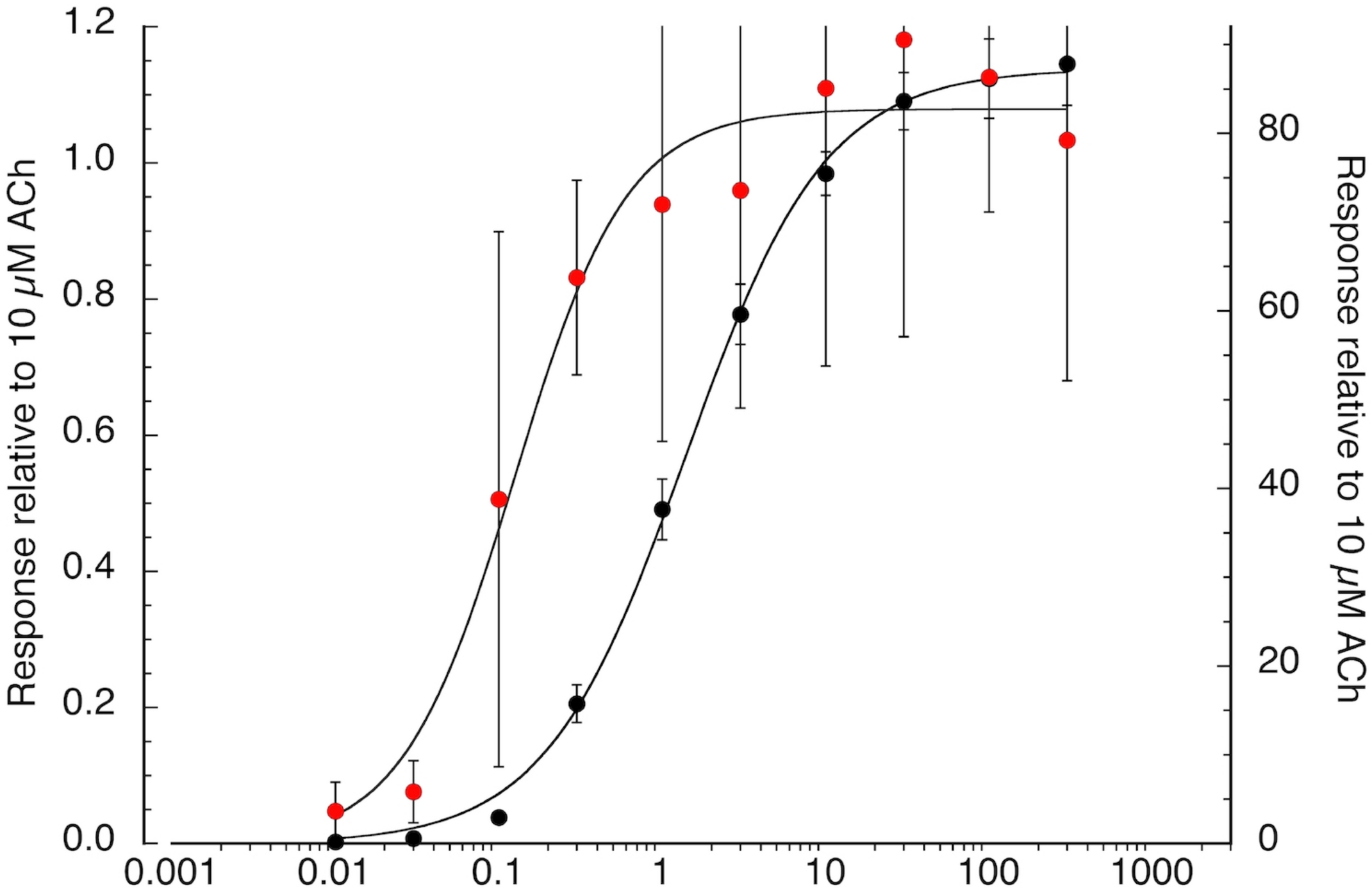


Figure 4

A

α4(2)β2L15'M(2)β2

ACh response of HS receptors ± TQS



HS alone

[ACh], μM

HS plus TQS

B

α4(2)β2L15'M(2)α4

Low and high concentrations on LS receptors

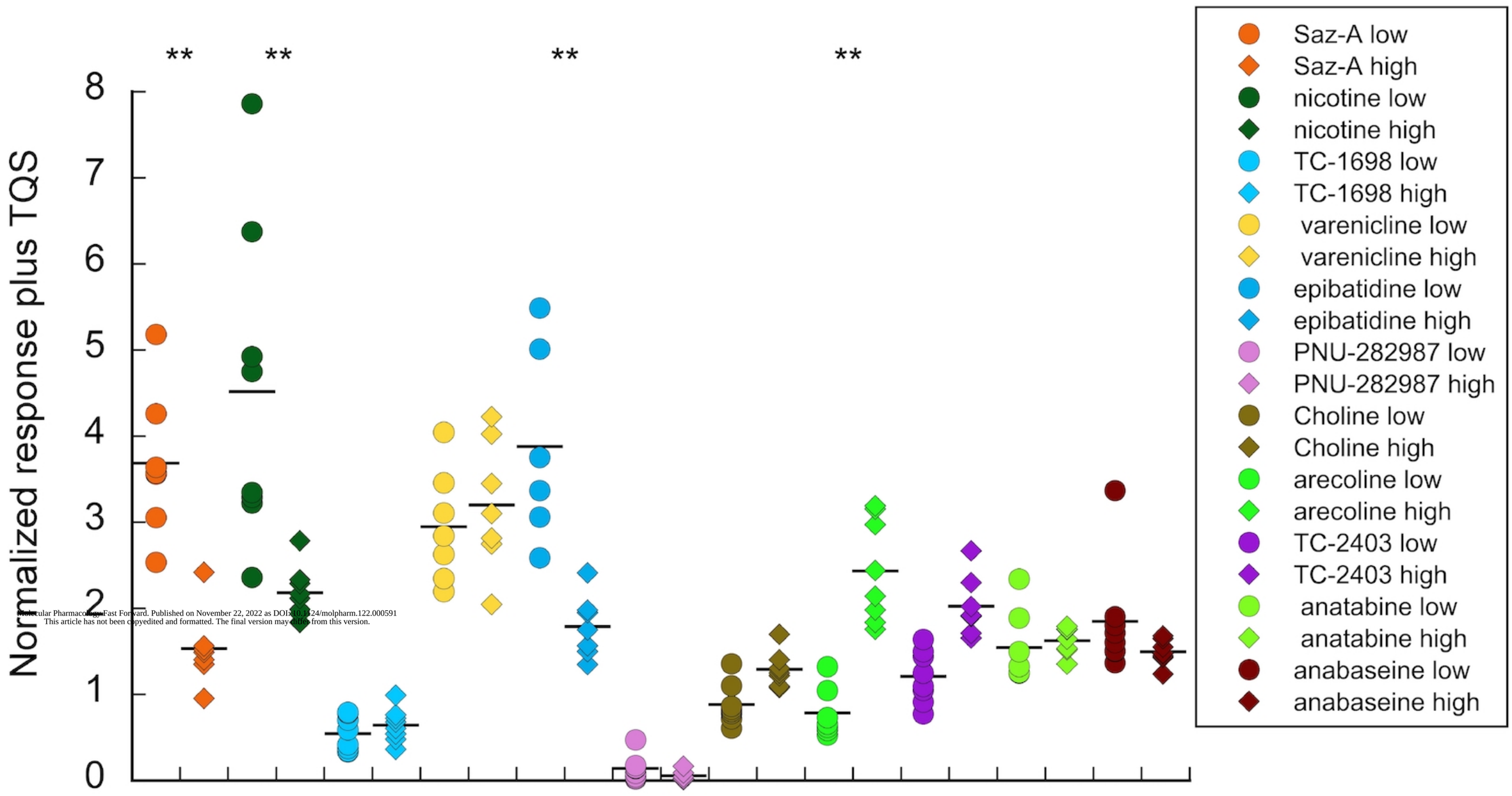


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Figure 5

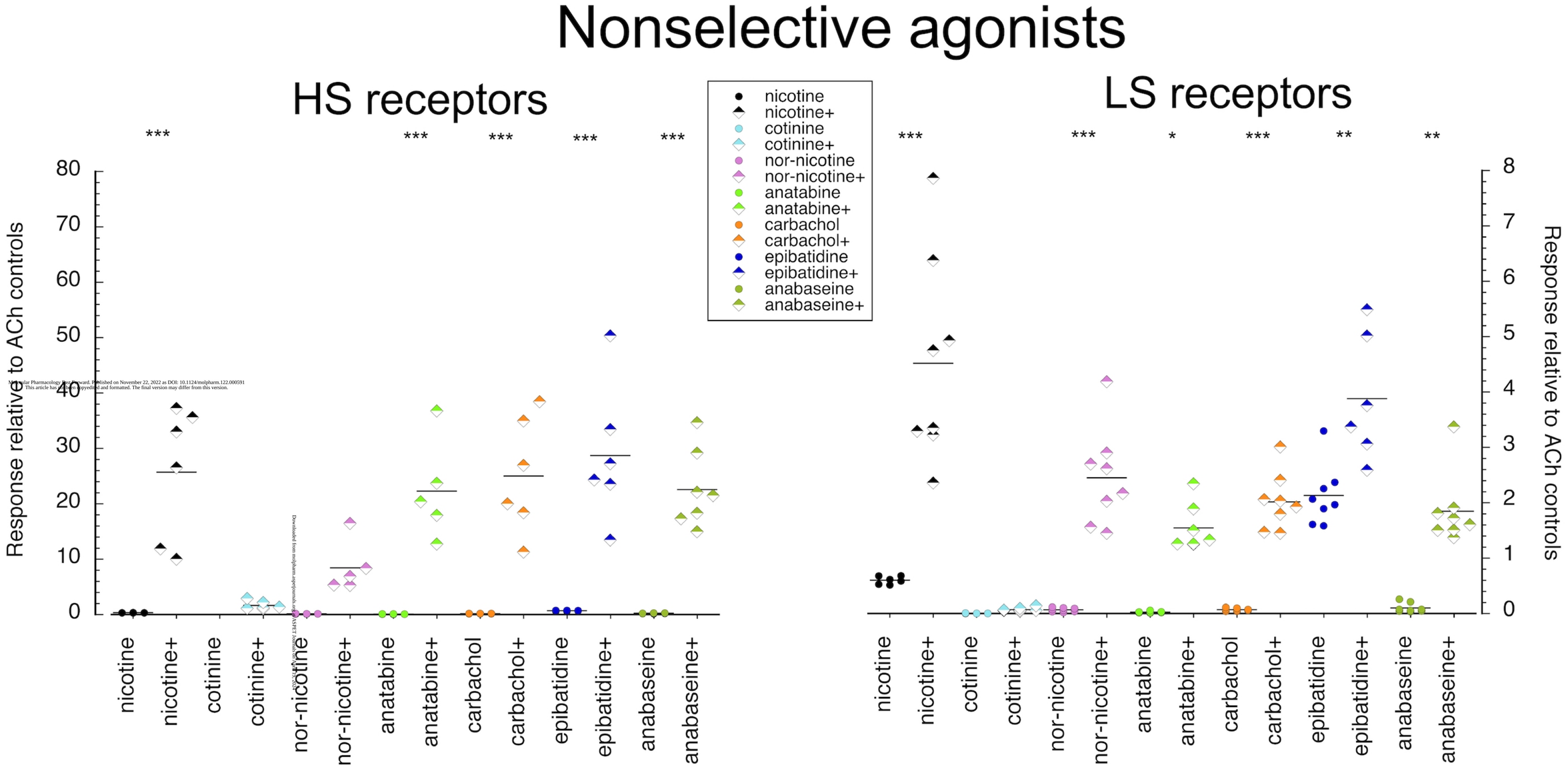

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Figure 6

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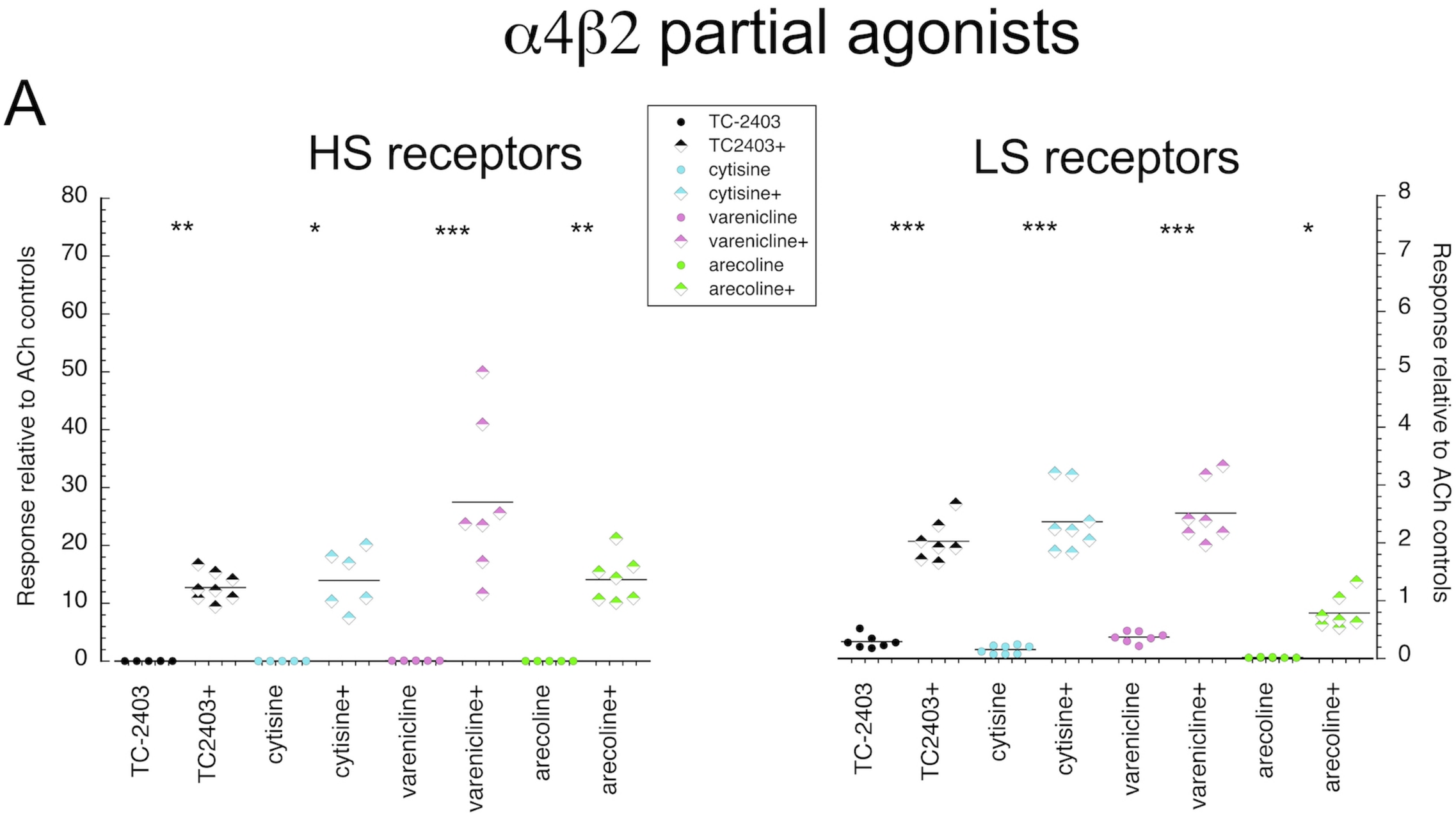

Figure 7

Effects of α7-selective agonists on α4β2L15'M receptors

Drugs applied alone

A

HS receptors

Normalized response

1.2
1.0
0.8
0.6
0.4
0.2
0.0

ACh
TQS
GTS-21
PNU282987
TC-1698
AR-R17779
Choline

B

LS receptors

ACh
TQS
GTS-21
PNU282987
TC-1698
AR-R17779
Choline

Normalized response

1.2
1.0
0.8
0.6
0.4
0.2
0.0

Drugs co-applied with TQS

C

HS receptors

Normalized response

60.0
50.0
40.0
30.0
20.0
10.0
0.0

ACh
TQS
GTS-21
PNU282987
TC-1698
AR-R17779
Choline

D

LS receptors

ACh
TQS
GTS-21
PNU282987
TC-1698
AR-R17779
Choline

Normalized response

3.0
2.5
2.0
1.5
1.0
0.5
0.0

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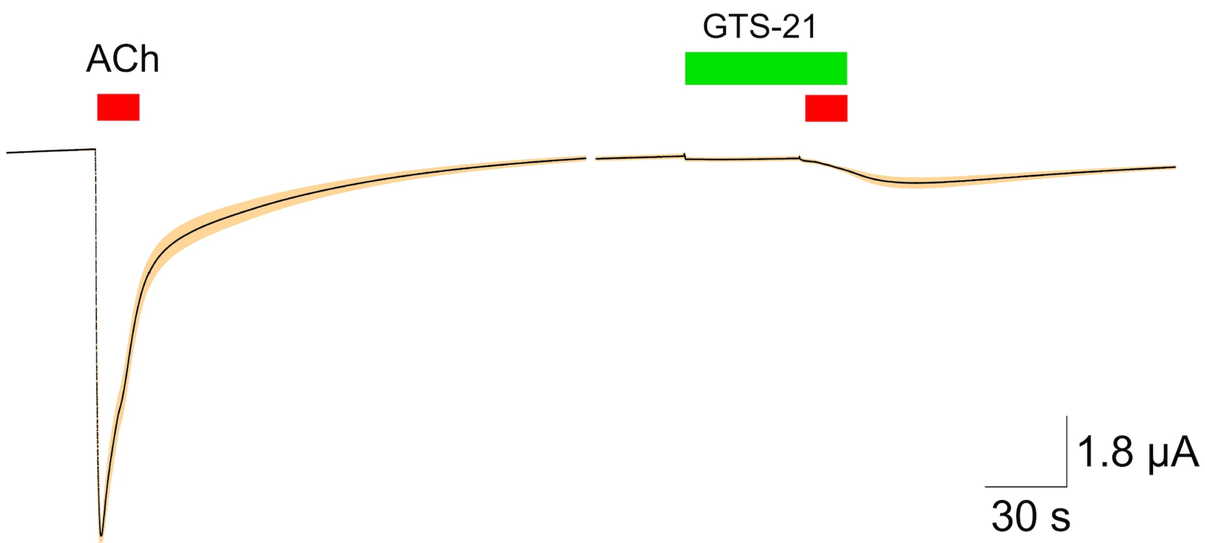
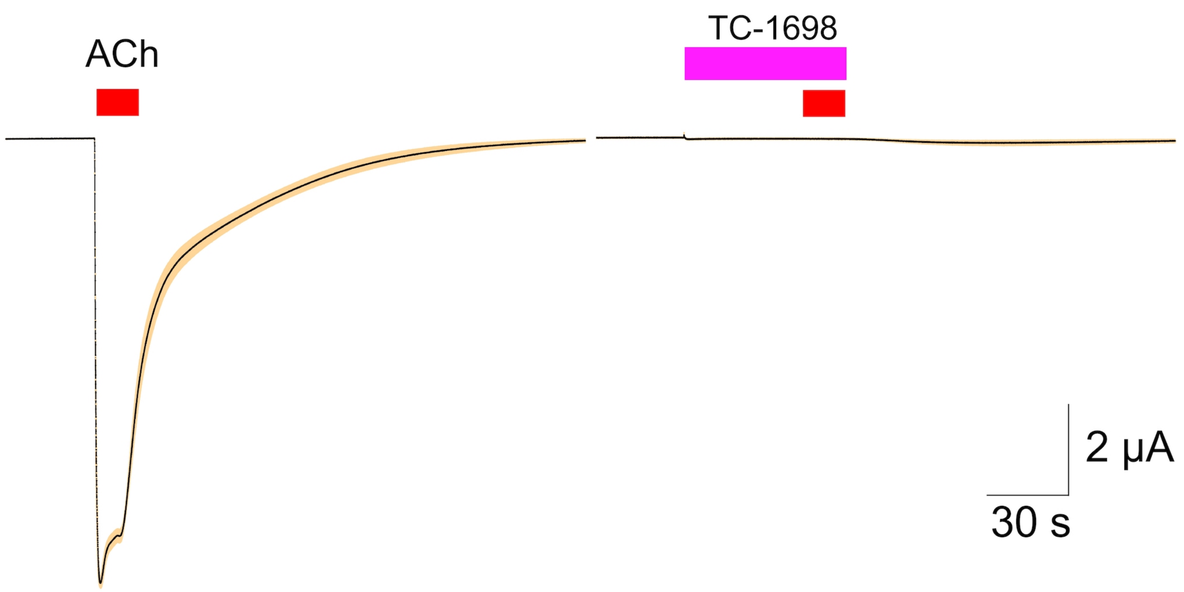
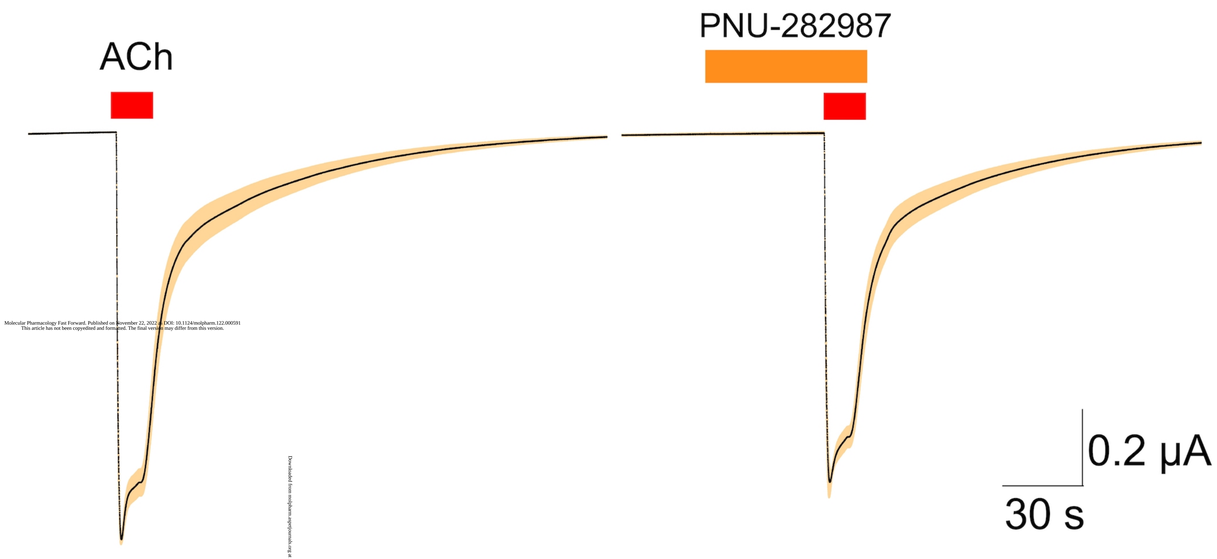
Figure 8

Wild-type receptors

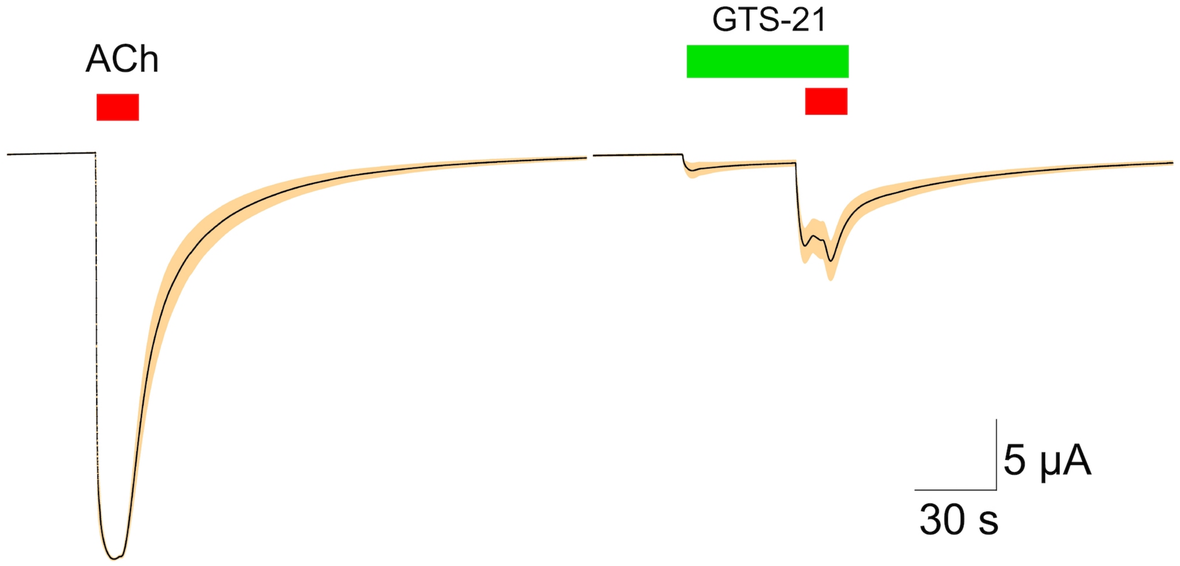
HS
α4(2)β2(3)

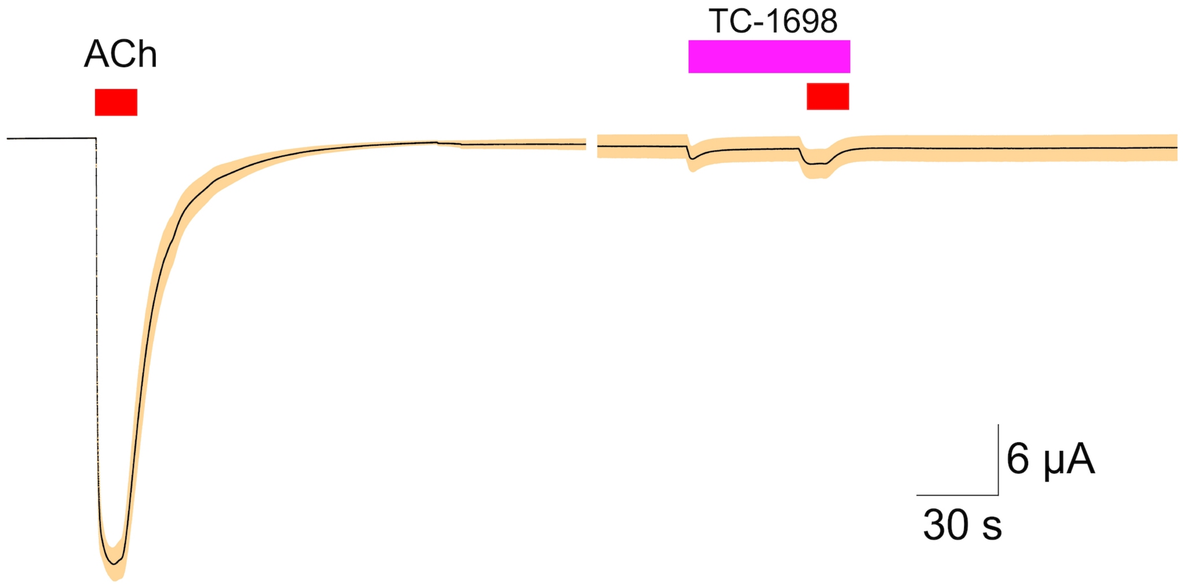


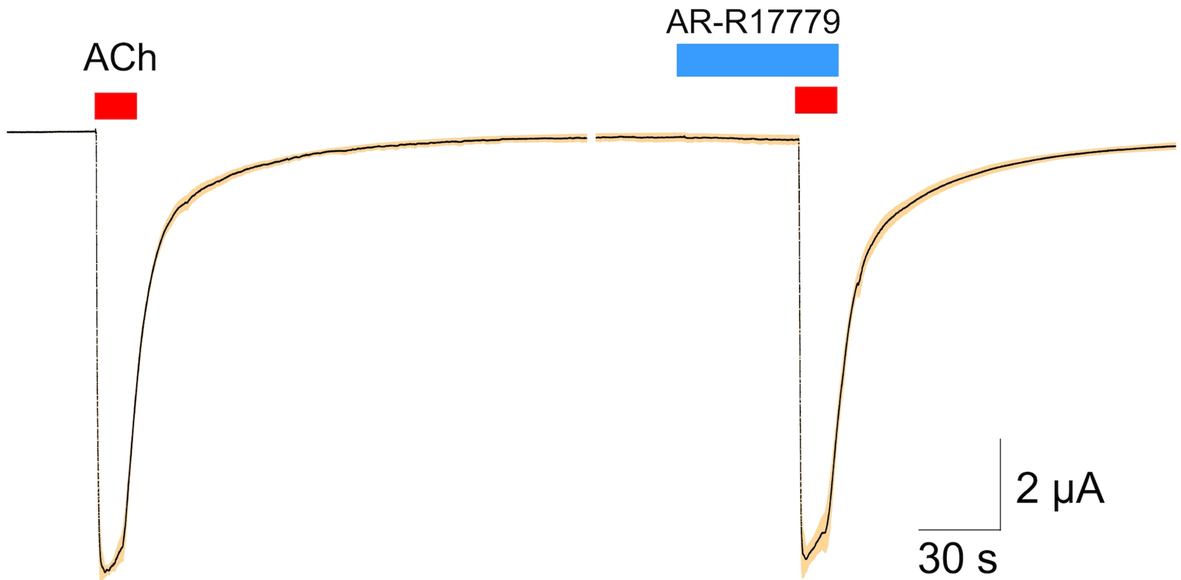




LS
α4(3)β2(2)







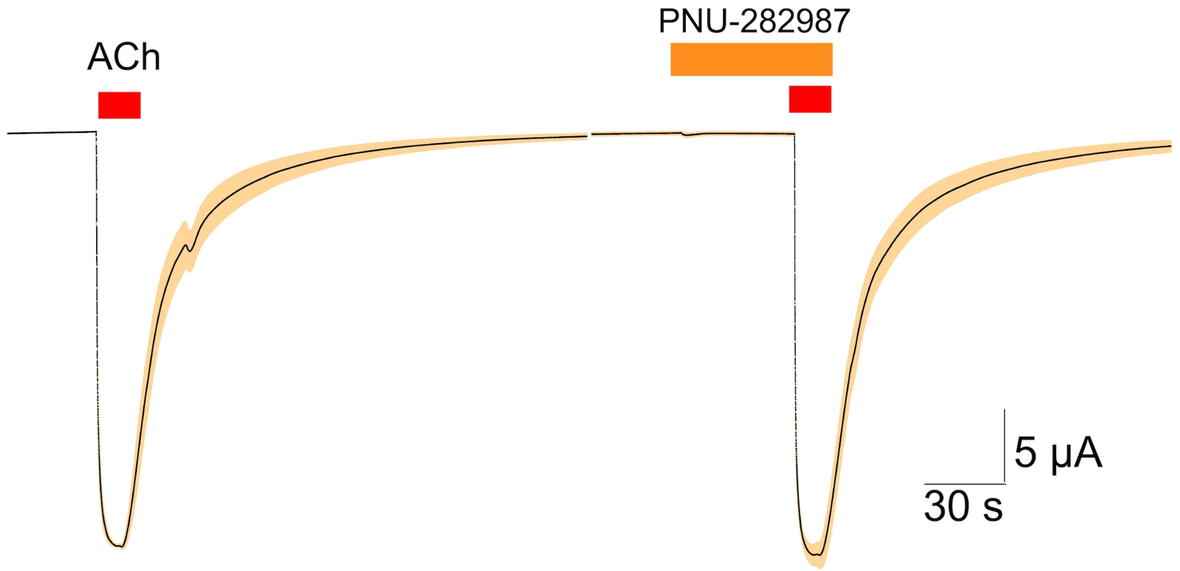


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Figure 9