

MOLPHARM-AR-2022-000591

Insights into the differential desensitization of $\alpha 4\beta 2$ nicotinic acetylcholine receptor isoforms obtained with positive allosteric modulation of mutant receptors

Roger L. Papke and Clare Stokes

Department of Pharmacology and Therapeutics, University of Florida, PO Box 100267
Gainesville, FL 32610 USA (RLP, CS)

MOLPHARM-AR-2022-000591

Running title: Desensitization of $\alpha 4\beta 2$ nAChR

To whom correspondence should be addressed:

Name: Roger L. Papke

Phone: 352-392-4712

Fax: 352-392-3558

E-mail: rlpapke@ufl.edu

Address: Department of Pharmacology and Therapeutics

University of Florida

P.O. Box 100267

Gainesville, FL 32610-0267

Number of text pages:41

Number of tables:5

Number of figures:9

Number of references:98

Number of words in Abstract:246

Number of words in Introduction:721

Number of words in Discussion:1354

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)

MOLPHARM-AR-2022-000591

Abstract

The development of highly efficacious positive allosteric modulators (PAMs) of $\alpha 7$ nicotinic acetylcholine receptors (nAChR) has proven useful in defining the ligand dependence of the conformational dynamics of $\alpha 7$ receptors. No such effective modulators are known to exist for the $\alpha 4\beta 2$ 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 $\beta 2$ subunits that allowed the use of the $\alpha 7$ PAM TQS to probe the desensitizing effects of nicotinic ligands on the two forms of $\alpha 4\beta 2$ receptors; high sensitivity (HS) (two $\alpha 4$ and three $\beta 2$ subunits) and low sensitivity (LS) (three $\alpha 4$ and two $\beta 2$ 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 $\alpha 4\beta 2$ receptors. Results confirm that HS $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ 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 $\alpha 7$ -selective agonists revealed varying patterns of $\alpha 4\beta 2$ cross-desensitization that were predictive of the effects of these drugs on the activation of wild-type $\alpha 4\beta 2$ receptors by ACh, supporting the utility of TQS-sensitive receptors for the development of focused therapeutics.

Keywords:

allosteric modulation, desensitization, addiction, therapeutics, nicotinic receptors

MOLPHARM-AR-2022-000591

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.

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 $\alpha 4$ and $\beta 2$ 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 $\alpha 4$ – $\beta 2$ interfaces, the fifth position can be occupied by either an $\alpha 4$ or a $\beta 2$ subunit (Nelson et al., 2003). While some expression systems may bias receptor expression toward the $\alpha 4(3)\beta 2(2)$ configuration, both types are present in the brain (Fasoli et al., 2016), and chronic nicotine favors the expression of the $\alpha 4(2)\beta 2(3)$ 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 $\alpha 4\beta 2$ nAChR differ greatly in their functional properties, with one notable difference being that receptors with the $\alpha 4(2)\beta 2(3)$ configuration respond to low concentrations of ACh or nicotine but saturate their responses when agonist concentrations are

raised to higher levels. They have therefore come to be referred to as a high sensitivity (HS) subtype. In contrast, receptors of $\alpha 4(3)\beta 2(2)$ 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 $\alpha 7$ nAChR (Uteshev et al., 2002), the second most abundant nAChR in brain (Millar and Gotti, 2009). Our understanding of $\alpha 7$ receptor desensitization has been greatly enhanced by the discovery of the type II class of $\alpha 7$ -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 $\alpha 7$ -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 $\alpha 7$ among nAChR, in the 15' position of the pore-forming second transmembrane domain (Young et al., 2008). The transfer of that residue into $\beta 2$ or $\beta 4$ 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 $\alpha 4$ and $\beta 2L15'M$ mutants, co-expressed in *Xenopus* oocytes with monomers of wild-type $\alpha 4$ or $\beta 2$ subunits to selectively form TQS-sensitive LS or HS $\alpha 4\beta 2$ receptors. Using TQS to reveal the extent of $\alpha 4\beta 2$ receptor desensitization, we demonstrate the great degree to which desensitization limits HS receptor

MOLPHARM-AR-2022-000591

responses to determine the degree to which their activities on the two $\alpha 4\beta 2$ isoforms are limited by TQS-sensitive desensitization. Finally, we tested the relevance of these observation to the functional activation of wild-type $\alpha 4\beta 2$ receptors by ACh in the presence of putative $\alpha 7$ -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), cytosine, 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 synthesized by 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-

MOLPHARM-AR-2022-000591

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 -20°C and diluted in Ringer's solution each day. Other compounds' stock solutions were prepared in Ringer's solution and held at 4°C 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 $\alpha 4\beta 2$ receptors independently of each other in *Xenopus* oocytes. One approach has been to inject the $\alpha 4$ and $\beta 2$ RNA at ratios that would favor the assembly of LS or HS receptors (usually 10:1, $\alpha 4$ to $\beta 2$ for LS receptors or 1:10 $\alpha 4$ to $\beta 2$ 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 $\alpha 4$ – $\beta 2$ subunits (Zhou et al., 2003) which permits the co-expression of the concatamer with monomeric $\alpha 4$ or $\beta 2$ 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 $\beta 2$ –6– $\alpha 4$ concatamer were obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia PA). The $\beta 2$ L15'M mutant in the concatamer was made as

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 $\alpha 4$ or $\beta 2$ 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 μ M 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 μ M racemic TQS and a final control application of ACh. The control ACh concentrations were 10 μ M for HS receptors and 100 μ M for LS receptors. The concentrations of the test compounds are provided

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 \pm 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 \pm 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.

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.

Results

The generation of TQS-sensitive HS and LS $\alpha 4\beta 2$ nAChR

The L15'M mutation (Stokes et al., 2019) was made in the $\beta 2$ subunit of the $\beta 2-6-\alpha 4$ concatamer (Zhou et al., 2003), so that by co-expressing this concatamer with monomers of either $\beta 2$ or $\alpha 4$, 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 μ M (Log error = -.39) and 0.11 μ M (Log error = -.1) for the wild-type and mutant receptors, respectively. For the LS receptors, the ACh Log EC_{50} values were 2.13 μ M (Log error = 1.39) and 2.27 μ M (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 μ M 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).

MOLPHARM-AR-2022-000591

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 $\alpha 7$ ago-PAM GAT107 (Papke et al., 2014b), and was observed with the TQS-sensitive $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ 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 μ M nicotine to HS and LS $\alpha 4\beta 2$ L15'M receptors and then co-applied 30 μ M nicotine and 30 μ M

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 μ M ACh controls was 2.47 μ A (SD = 1.03), while the average peak amplitude of the LS 100 μ M ACh controls was 15.9 μ A (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 $\alpha 4\beta 2$ receptors when applied alone. These were the $\alpha 7$ silent agonists, NS6740, 1-methylpyridinium (Papke et al., 2022b), and triethylmethylammonium (Papke et al., 2014a) (Supplemental Figure 2); the $\alpha 9$ -selective agonists, *p*CN diEPP, *p*CONH2 diEPP, and diMPiP (Papke et al., 2022a) (Supplemental Figure 3); and the LS $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ 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.

Effects of nAChR antagonists

The $\alpha 7$ -selective antagonist methyllycaconitine (MLA) (Turek et al., 1995) and the $\alpha 4\beta 2$ -selective antagonist dihydro- β -erythroidine (DH β E) (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. $> 10 \mu\text{M}$) 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 $125 \pm 22 \text{ nM}$ for ACh plus TQS compared to $1.39 \pm 0.07 \mu\text{M}$ 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.

LS receptor potentiation at higher drug concentrations

Given that the receptors with the $\alpha 4(3)\beta 2(2)$ 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 $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ 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).

MOLPHARM-AR-2022-000591

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 $\alpha 4\beta 2$ partial agonists

Four $\alpha 4\beta 2$ 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 $\alpha 4\beta 2^*$ 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 $\alpha 4\beta 2$ -selective agonists

Sazetidine-A and TC-2559, two agents that are potent activators of HS $\alpha 4\beta 2$ receptors with little or no efficacy for activating LS receptors, were tested (Figure 7B). Sazetidine-A was actually first published as a selective $\alpha 4\beta 2$ 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

receptor formation was enhanced by injection of oocytes with a ten-fold excess of $\beta 2$ relative to $\alpha 4$ 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).

$\alpha 7$ -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 $\alpha 7$ nAChR without affecting other subtypes like $\alpha 4\beta 2$ receptors (Papke and Horenstein, 2021), leading to the identification of several $\alpha 7$ -selective agonists. Among the first $\alpha 7$ -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 $\alpha 4\beta 2$ 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 $\alpha 7$ -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 $\alpha 4\beta 2$ 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 $\alpha 4$ and $\beta 2$ 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 $\alpha 7$ agonists. When co-applied with TQS to HS receptors, GTS-21 and choline gave responses that

MOLPHARM-AR-2022-000591

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 $\alpha 4\beta 2$ ACh responses by $\alpha 7$ -selective agonists

The data in Figure 9 suggest that some of the compounds proposed to activate $\alpha 7$ receptors would be effective desensitizing antagonists of $\alpha 4\beta 2$ receptors. To test this, cells expressing wild-type forms of HS and LS $\alpha 4\beta 2$ receptors were pre-exposed to the commercially developed $\alpha 7$ -selective agonists for 30 s, and then ACh was co-applied at the control concentration along with the $\alpha 7$ agonists at the test concentration. The ACh responses were compared with the control ACh responses obtained prior to the application of the $\alpha 7$ agonists (Figure 9). GTS-21 pre-application evoked a small response from the LS $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ 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 $\alpha 4\beta 2$ receptor subtype (Figure 9). These results with wild-type receptors are consistent with the TQS effects obtained on the receptors with L15'M mutant $\beta 2$ subunits (Figure 8).

Discussion

The results support the hypothesis that the responses of HS $\alpha 4\beta 2$ 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 $\alpha 7$ silent agonist and the $\alpha 9$ agonists, that appear to be free of $\alpha 4\beta 2$ activating or desensitizing effects. They also indicate that desensitization is a factor limiting the

MOLPHARM-AR-2022-000591

efficacy of $\alpha 4\beta 2$ 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 $\alpha 7$ 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 $\alpha 7$ 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 $\alpha 7$ receptors are similar to those of PNU-120596, (Palczynska et al., 2012; Quadri et al., 2019), while the effects of TQS on $\alpha 7$ ACh responses are somewhat less (Palczynska et al., 2012). However, the basic mechanisms of $\alpha 7$ desensitization are fundamentally different from those of $\alpha 4\beta 2$ receptors, so likewise the TQS effects on the TQS-sensitive $\alpha 4\beta 2$ receptors might be very different on the molecular level from the effects on $\alpha 7$ receptors. While $\alpha 7$ receptors show no activation at all with high agonist concentrations (Williams et al., 2011), $\alpha 4\beta 2$ receptors can smolder (Campling et al., 2013), occasionally opening under predominantly desensitizing conditions.

While TQS is considered strictly a PAM for $\alpha 7$, since we observed it to activate the TQS-sensitive $\alpha 4\beta 2$ 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 $\alpha 7$ 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 $\alpha 4\beta 2$ receptors by TQS was blocked by 10 μ M of the $\alpha 4\beta 2$ -selective antagonist DH β E as well as by 100 μ M MLA, a concentration at which the drug is no longer selective for $\alpha 7$

(Buisson et al., 1996). While the allosteric activation of $\alpha 7$ by GAT107 can be blocked by 10 μ M of the $\alpha 7$ selective antagonist MLA (Papke et al., 2014b), it is insensitive to 100 μ M DH β E (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 $\alpha 7$ -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 $\alpha 4\beta 2$ receptors in the brain. This is a particular concern in the case of Alzheimer's disease, since evidence suggests that $\alpha 4\beta 2$ receptor function is specifically impaired in this patient population (Court et al., 2001; Gotti et al., 2006). The results with the $\alpha 7$ -selective agonists tested indicate that, depending on the specific agent, a number of differing profiles of $\alpha 7$ agonism and $\alpha 4\beta 2$ antagonism may be available, with PNU-282987 being the least likely to affect $\alpha 4\beta 2$ function. As noted above, GTS-21 was the first synthetic $\alpha 7$ -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 $\alpha 7$ 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 $\alpha 7$ receptors. PNU-282987 is relatively potent and nearly a full agonist (Hajos et al., 2005), while

GTS-21 has lower potency and efficacy and additionally produces residual $\alpha 7$ receptor desensitization (Papke et al., 2009). Interestingly, the $\alpha 7$ 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 $\alpha 4\beta 2^*$ and $\alpha 7$ receptors, with GTS-21 capable of decreasing $\alpha 4\beta 2$ function as well as working on $\alpha 7$, and PNU-282987 affecting $\alpha 7$ 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 $\alpha 7$ nAChR, especially in the context of the CAP mediated by $\alpha 7$, and possibly $\alpha 9\alpha 10$, 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 $\alpha 7$ 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

MOLPHARM-AR-2022-000591

(Stokes et al., 2015), and interestingly the intracellular domain of $\alpha 4$ is the largest of any nAChR subunit, by a factor of three or more. Although not so well studied as $\alpha 7$ in this regard, there have been reports that $\alpha 4$ -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 $\alpha 4\beta 2$ receptors and may prove useful for the development of more focused therapeutics.

MOLPHARM-AR-2022-000591

Acknowledgments

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.

References

- Acharya S, Kundu D, Choi HJ and Kim KM (2020) Metabotropic signaling cascade involved in alpha4beta2 nicotinic acetylcholine receptor-mediated PKCbetaII activation. *Biochim Biophys Acta Mol Cell Res* **1867**(8): 118721.
- Aickin M and Gensler H (1996) Adjusting for multiple testing when reporting research results: the Bonferroni vs Holm methods. *American journal of public health* **86**(5): 726-728.
- Alijevic O, McHugh D, Rufener L, Mazurov A, Hoeng J and Peitsch M (2020) An electrophysiological characterization of naturally occurring tobacco alkaloids and their action on human alpha4beta2 and alpha7 nicotinic acetylcholine receptors. *Phytochemistry* **170**: 112187.
- Andersen ND, Nielsen BE, Corradi J, Tolosa MF, Feuerbach D, Arias HR and Bouzat C (2016) Exploring the positive allosteric modulation of human alpha7 nicotinic receptors from a single-channel perspective. *Neuropharmacology* **107**: 189-200.
- Badio B and Daly JW (1994) Epibatidine, a potent analgetic and nicotinic agonist. *Mol Pharmacol* **45**: 563-569.
- Bagdas D, Gurun MS, Flood P, Papke RL and Damaj MI (2018) New Insights on Neuronal Nicotinic Acetylcholine Receptors as Targets for Pain and Inflammation: A Focus on alpha7 nAChRs. *Curr Neuropharmacol* **16**(4): 415-425.
- Bodnar AL, Cortes-Burgos LA, Cook KK, Dinh DM, Groppi VE, Hajos M, Higdon NR, Hoffmann WE, Hurst RS, Myers JK, Rogers BN, Wall TM, Wolfe ML and Wong E (2005) Discovery and structure-activity relationship of quinuclidine benzamides as agonists of alpha7 nicotinic acetylcholine receptors. *J Med Chem* **48**(4): 905-908.
- Boyd ND (1987) Two distinct kinetic phases of desensitization of acetylcholine receptors of clonal rat PC12 cells. *J Physiol* **389**: 45-67.
- Briggs CA and McKenna DG (1998) Activation and inhibition of the human alpha7 nicotinic acetylcholine receptor by agonists. *Neuropharmacology* **37**(9): 1095-1102.
- Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP and Bertrand D (1996) Human alpha4beta2 neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study. *J Neurosci* **16**(24): 7880-7891.
- Campling BG, Kuryatov A and Lindstrom J (2013) Acute activation, desensitization and smoldering activation of human acetylcholine receptors. *PLoS One* **8**(11): e79653.
- Cannon CE, Puri V, Vivian JA, Egbertson MS, Eddins D and Uslaner JM (2013) The nicotinic alpha7 receptor agonist GTS-21 improves cognitive performance in ketamine impaired rhesus monkeys. *Neuropharmacology* **64**: 191-196.
- Chen L, Yamada K, Nabeshima T and Sokabe M (2006) alpha7 Nicotinic acetylcholine receptor as a target to rescue deficit in hippocampal LTP induction in beta-amyloid infused rats. *Neuropharmacology* **50**(2): 254-268.
- Coe JW, Brooks PR, Vetelino MG, Wirtz MC, Arnold EP, Huang J, Sands SB, Davis TI, Lebel LA, Fox CB, Shrikhande A, Heym JH, Schaeffer E, Rollema H, Lu Y, Mansbach RS, Chambers LK, Rovetti CC, Schulz DW, Tingley FD, 3rd and O'Neill BT (2005) Varenicline: an alpha4beta2 nicotinic receptor partial agonist for smoking cessation. *J Med Chem* **48**(10): 3474-3477.
- Corrie LW, Stokes C, Wilkerson JL, Carroll FI, McMahon LR and Papke RL (2020) Nicotinic Acetylcholine Receptor Accessory Subunits Determine the Activity Profile of Epibatidine Derivatives. *Mol Pharmacol* **98**(4): 328-342.

- Court J, Martin-Ruiz C, Piggott M, Spurden D, Griffiths M and Perry E (2001) Nicotinic receptor abnormalities in Alzheimer's disease. *Biol Psychiatry* **49**(3): 175-184.
- Crooks PA, Li M and Dwoskin LP (1995) Determination of nicotine metabolites in rat brain after peripheral radiolabeled nicotine administration: detection of nornicotine. *Drug Metab Dispos* **23**(10): 1175-1177.
- D'Andrea MR and Nagele RG (2006) Targeting the alpha 7 nicotinic acetylcholine receptor to reduce amyloid accumulation in Alzheimer's disease pyramidal neurons. *Curr Pharm Des* **12**(6): 677-684.
- Damaj MI, Welch SP and Martin BR (1995) In vivo pharmacological effects of dihydro-beta-erythroidine, a nicotinic antagonist, in mice. *Psychopharmacology (Berl)* **117**(1): 67-73.
- de Fiebre CM, Meyer EM, Zoltewicz J, Henry JC, Muraskin S, Kem WR and Papke RL (1995) Characterization of a family of anabaseine-derived compounds reveals that the 3-(4)-dimethylaminocinnamylidene derivative (DMAC) is a selective agonist at neuronal nicotinic $\alpha 7/[^{125}\text{I}]\alpha$ -bungarotoxin receptor subtypes. *Mol Pharm* **47**: 164-171.
- de Jonge WJ and Ulloa L (2007) The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation. *Br J Pharmacol* **151**(7): 915-929.
- Descarries L, Gisiger V and Steriade M (1997) Diffuse transmission by acetylcholine in the CNS. *Prog Neurobiol* **53**(5): 603-625.
- Dilger JP and Liu Y (1992) Desensitization of acetylcholine receptors in BC3H-1 cells. *Pflugers Arch* **420**(5-6): 479-485.
- Eaton JB, Lucero LM, Stratton H, Chang Y, Cooper JF, Lindstrom JM, Lukas RJ and Whiteaker P (2014) The unique alpha4+/-alpha4 agonist binding site in (alpha4)3(beta2)2 subtype nicotinic acetylcholine receptors permits differential agonist desensitization pharmacology versus the (alpha4)2(beta2)3 subtype. *J Pharmacol Exp Ther* **348**(1): 46-58.
- Etter JF, Lukas RJ, Benowitz NL, West R and Dresler CM (2008) Cytisine for smoking cessation: a research agenda. *Drug Alcohol Depend* **92**(1-3): 3-8.
- Fasoli F, Moretti M, Zoli M, Pistillo F, Crespi A, Clementi F, Mc Clure-Begley T, Marks MJ and Gotti C (2016) In vivo chronic nicotine exposure differentially and reversibly affects upregulation and stoichiometry of alpha4beta2 nicotinic receptors in cortex and thalamus. *Neuropharmacology* **108**: 324-331.
- Feltz A and Trautmann A (1982) Desensitization at the frog neuromuscular junction: a biphasic process. *J Physiol* **322**: 257-272.
- Forman SA and Miller KW (1988) High acetylcholine concentrations cause rapid Inactivation before fast desensitization in nicotinic acetylcholine receptors. *Biophys J* **54**: 149-158.
- Gerzanich V, Peng X, Wang F, Wells G, Anand R, Fletcher S and Lindstrom J (1995) Comparative pharmacology of epibatidine: a potent agonist for neuronal nicotinic acetylcholine receptors. *Mol Pharm* **48**: 774-782.
- Godin JR, Roy P, Quadri M, Bagdas D, Toma W, Narendrula-Kotha R, Kishta OA, Damaj MI, Horenstein NA, Papke RL and Simard AR (2020) A silent agonist of alpha7 nicotinic acetylcholine receptors modulates inflammation ex vivo and attenuates EAE. *Brain Behav Immun* **87**: 286-300.
- Gotti C, Moretti M, Bohr I, Ziabreva I, Vailati S, Longhi R, Riganti L, Gaimarri A, McKeith IG, Perry RH, Aarsland D, Larsen JP, Sher E, Beattie R, Clementi F and Court JA (2006) Selective nicotinic acetylcholine receptor subunit deficits identified in Alzheimer's

- disease, Parkinson's disease and dementia with Lewy bodies by immunoprecipitation. *Neurobiol Dis* **23**(2): 481-489.
- Gronlien JH, Haakerud M, Ween H, Thorin-Hagene K, Briggs CA, Gopalakrishnan M and Malysz J (2007) Distinct profiles of alpha7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. *Mol Pharmacol* **72**(3): 715-724.
- Gupta PC and Warnakulasuriya S (2002) Global epidemiology of areca nut usage. *Addict Biol* **7**(1): 77-83.
- Hajos M, Hurst RS, Hoffmann WE, Krause M, Wall TM, Higdon NR and Groppi VE (2005) The selective alpha7 nicotinic acetylcholine receptor agonist PNU-282987 enhances GABAergic synaptic activity in brain slices and restores auditory gating deficits in anesthetized Rats. *J Pharmacol Exp Ther* **312**(3): 1213-1222.
- Hajos M and Rogers BN (2010) Targeting alpha7 nicotinic acetylcholine receptors in the treatment of schizophrenia. *Curr Pharm Des* **16**(5): 538-554.
- Haydar SN and Dunlop J (2010) Neuronal nicotinic acetylcholine receptors - targets for the development of drugs to treat cognitive impairment associated with schizophrenia and Alzheimer's disease. *Curr Top Med Chem* **10**(2): 144-152.
- Horenstein NA and Papke RL (2017) Anti-inflammatory Silent Agonists. *ACS Med Chem Lett* **8**(10): 989-991.
- Kabbani N and Nichols RA (2018) Beyond the Channel: Metabotropic Signaling by Nicotinic Receptors. *Trends Pharmacol Sci* **39**(4): 354-366.
- Katz B and Thesleff S (1957) A study of the "desensitization" produced by acetylcholine at the motor end-plate. *Journal of Physiology (London)* **138**: 63-80.
- Kem WR, Mahnir VM, Papke RL and Lingle CJ (1997) Anabaseine is a potent agonist upon muscle and neuronal alpha-bungarotoxin sensitive nicotinic receptors. *J Pharm Exp Ther* **283**: 979-992.
- King JR, Gillevet TC and Kabbani N (2017) A G protein-coupled alpha7 nicotinic receptor regulates signaling and TNF-alpha release in microglia. *FEBS Open Bio* **7**(9): 1350-1361.
- King JR, Ullah A, Bak E, Jafri MS and Kabbani N (2018) Ionotropic and Metabotropic Mechanisms of Allosteric Modulation of alpha7 Nicotinic Receptor Intracellular Calcium. *Mol Pharmacol* **93**(6): 601-611.
- Kong W, Kang K, Gao Y, Liu H, Meng X, Cao Y, Yang S, Liu W, Zhang J, Yu K and Zhao M (2018) GTS-21 Protected Against LPS-Induced Sepsis Myocardial Injury in Mice Through alpha7nAChR. *Inflammation* **41**(3): 1073-1083.
- Kuryatov A, Luo J, Cooper J and Lindstrom J (2005) Nicotine acts as a pharmacological chaperone to up-regulate human alpha4beta2 acetylcholine receptors. *Mol Pharmacol* **68**(6): 1839-1851.
- Land BR, Salpeter EE and Salpeter MM (1981) Kinetic parameters for acetylcholine interaction in intact neuromuscular junction. *Proc Natl Acad Sci USA* **78**(11): 7200-7204.
- Leiser SC, Bowlby MR, Comery TA and Dunlop J (2009) A cog in cognition: how the alpha7 nicotinic acetylcholine receptor is geared towards improving cognitive deficits. *Pharmacol Ther* **122**(3): 302-311.
- Lester RA (2004) Activation and desensitization of heteromeric neuronal nicotinic receptors: implications for non-synaptic transmission. *Bioorg Med Chem Lett* **14**(8): 1897-1900.
- Levin ED, Bettegowda C, Blosser J and Gordon J (1999) AR-R17779, an alpha7 nicotinic agonist, improves learning and memory in rats. *Behavioural pharmacology* **10**(6-7): 675-680.

MOLPHARM-AR-2022-000591

- Lopez-Hernandez GY, Sanchez-Padilla J, Ortiz-Acevedo A, Lizardi-Ortiz J, Salas-Vincenty J, Rojas LV and Lasalde-Dominicci JA (2004) Nicotine-induced up-regulation and desensitization of alpha4beta2 neuronal nicotinic receptors depend on subunit ratio. *J Biol Chem* **279**(36): 38007-38015.
- Marrero MB, Papke RL, Bhatti BS, Shaw S and Bencherif M (2004) The neuroprotective effect of 2-(3-pyridyl)-1-azabicyclo[3.2.2]nonane (TC-1698), a novel alpha7 ligand, is prevented through angiotensin II activation of a tyrosine phosphatase. *J Pharmacol Exp Ther* **309**(1): 16-27.
- Millar NS and Gotti C (2009) Diversity of vertebrate nicotinic acetylcholine receptors. *Neuropharmacology* **56**(1): 237-246.
- Nelson ME, Kuryatov A, Choi CH, Zhou Y and Lindstrom J (2003) Alternate stoichiometries of alpha4beta2 nicotinic acetylcholine receptors. *Mol Pharmacol* **63**(2): 332-341.
- Nordman JC, Muldoon P, Clark S, Damaj MI and Kabbani N (2014) The alpha4 nicotinic receptor promotes CD4+ T-cell proliferation and a helper T-cell immune response. *Mol Pharmacol* **85**(1): 50-61.
- Palczynska MM, Jindrichova M, Gibb AJ and Millar NS (2012) Activation of alpha7 nicotinic receptors by orthosteric and allosteric agonists: influence on single-channel kinetics and conductance. *Mol Pharmacol* **82**(5): 910-917.
- Papke RL (2002) Enhanced inhibition of a mutant neuronal nicotinic acetylcholine receptor by agonists: protection of function by (E)-N-methyl-4-(3-pyridinyl)-3-butene-1-amine (TC-2403). *J Pharmacol Exp Ther* **301**(2): 765-773.
- Papke RL (2010) Tricks of Perspective: Insights and limitations to the study of macroscopic currents for the analysis of nAChR activation and desensitization. *Journal of Molecular Neuroscience* **40**(1-2): 77-86.
- Papke RL, Andleeb H, Stokes C, Quadri M and Horenstein NA (2022a) Selective Agonists and Antagonists of alpha9 Versus alpha7 Nicotinic Acetylcholine Receptors. *ACS chemical neuroscience* **13**(5): 624-637.
- Papke RL, Bencherif M and Lippiello P (1996) An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the a7 subtype. *Neurosci Lett* **213**: 201-204.
- Papke RL, Chojnacka K and Horenstein NA (2014a) The minimal pharmacophore for silent agonism of alpha7 nAChR. *Journal of Pharmacology and Experimental Therapeutics* **350**(3): 665-680.
- Papke RL, Dwoskin LP and Crooks PA (2007) The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery. *J Neurochem* **101**(1): 160-167.
- Papke RL and Horenstein NA (2021) Therapeutic targeting of alpha7 nicotinic acetylcholine receptors. *Pharm Reviews* **73**(3): 1118-1149.
- Papke RL, Horenstein NA, Kulkarni AR, Stokes C, Corrie LW, Maeng CY and Thakur GA (2014b) The activity of GAT107, an allosteric activator and positive modulator of alpha7 nicotinic acetylcholine receptors (nAChR), is regulated by aromatic amino acids that span the subunit interface. *J Biol Chem* **289**(7): 4515-4531.
- Papke RL, Horenstein NA and Stokes C (2015) Nicotinic Activity of Arecoline, the Psychoactive Element of "Betel Nuts", Suggests a Basis for Habitual Use and Anti-Inflammatory Activity. *PLoS One* **10**(10): e0140907.

MOLPHARM-AR-2022-000591

- Papke RL, Karaffa M, Horenstein NA and Stokes C (2022b) Coffee and cigarettes: Modulation of high and low sensitivity alpha4beta2 nicotinic acetylcholine receptors by n-MP, a biomarker of coffee consumption. *Neuropharmacology* **216**: 109173.
- Papke RL, Kem WR, Soti F, López-Hernández GY and Horenstein NA (2009) Activation and desensitization of nicotinic alpha7-type acetylcholine receptors by benzyldiene anabaseines and nicotine. *J Pharmacol Exp Ther* **329**(2): 791-807.
- Papke RL and Stokes C (2010) Working with OpusXpress: methods for high volume oocyte experiments. *Methods* **51**(1): 121-133.
- Parker MJ, Beck A and Luetje CW (1998) Neuronal nicotinic receptor beta2 and beta4 subunits confer large differences in agonist binding affinity. *Mol Pharmacol* **54**(6): 1132-1139.
- Picciotto MR, Addy NA, Mineur YS and Brunzell DH (2008) It is not "either/or": activation and desensitization of nicotinic acetylcholine receptors both contribute to behaviors related to nicotine addiction and mood. *Prog Neurobiol* **84**(4): 329-342.
- Quadri M, Garai S, Thakur GA, Stokes C, Gulsevin A, Horenstein NA and Papke RL (2019) Macroscopic and microscopic activation of alpha7 nicotinic acetylcholine receptors by the structurally unrelated ago-PAMs B-973B and GAT107. *Mol Pharmacol* **95**(1): 43-61.
- Quadri M, Papke RL and Horenstein NA (2016) Dissection of N,N-diethyl-N'-phenylpiperazines as alpha7 nicotinic receptor silent agonists. *Bioorganic & medicinal chemistry* **24**(2): 286-293.
- Quick MW and Lester RA (2002) Desensitization of neuronal nicotinic receptors. *J Neurobiol* **53**(4): 457-478.
- Richter K, Mathes V, Fronius M, Althaus M, Hecker A, Krasteva-Christ G, Padberg W, Hone AJ, McIntosh JM, Zakrzewicz A and Grau V (2016) Phosphocholine - an agonist of metabotropic but not of ionotropic functions of alpha9-containing nicotinic acetylcholine receptors. *Sci Rep* **6**: 28660.
- Rosas-Ballina M and Tracey KJ (2009) Cholinergic control of inflammation. *J Intern Med* **265**(6): 663-679.
- Simasko SM, Soares JR and Weiland GA (1986) Two components of carbamylcholine-induced loss of nicotinic acetylcholine receptor function in the neuronal cell line PC12. *Mol Pharmacol* **30**(1): 6-12.
- Sine SM and Steinbach JH (1987) Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by high concentrations of agonist. *J Physiol* **385**: 325-359.
- Srinivasan R, Pantoja R, Moss FJ, Mackey ED, Son CD, Miwa J and Lester HA (2011) Nicotine up-regulates alpha4beta2 nicotinic receptors and ER exit sites via stoichiometry-dependent chaperoning. *J Gen Physiol* **137**(1): 59-79.
- Stokes C, Garai S, Kulkarni AR, Cantwell LN, Noviello CM, Hibbs RE, Horenstein NA, Abboud KA, Thakur GA and Papke RL (2019) Heteromeric Neuronal Nicotinic Acetylcholine Receptors with Mutant beta Subunits Acquire Sensitivity to alpha7-Selective Positive Allosteric Modulators. *J Pharmacol Exp Ther* **370**(2): 252-268.
- Stokes C, Treinin M and Papke RL (2015) Looking below the surface of nicotinic acetylcholine receptors. *Trends Pharmacol Sci* **36**(8): 514-523.
- Tan X, Vrana K and Ding ZM (2021) Cotinine: Pharmacologically Active Metabolite of Nicotine and Neural Mechanisms for Its Actions. *Front Behav Neurosci* **15**: 758252.
- Thomsen MS and Mikkelsen JD (2012) The alpha7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-alpha release from microglia. *J Neuroimmunol* **251**(1-2): 65-72.

- Tracey KJ (2007) Physiology and immunology of the cholinergic antiinflammatory pathway. *The Journal of clinical investigation* **117**(2): 289-296.
- Turek JW, Kang CH, Campbell JE, Arneric SP and Sullivan JP (1995) A sensitive technique for the detection of the alpha 7 neuronal nicotinic acetylcholine receptor antagonist, methyllycaconitine, in rat plasma and brain. *J Neurosci Methods* **61**(1-2): 113-118.
- Uteshev VV, Meyer EM and Papke RL (2002) Activation and inhibition of native neuronal alpha-bungarotoxin-sensitive nicotinic ACh receptors. *Brain Res* **948**(1-2): 33-46.
- Walling D, Marder SR, Kane J, Fleischhacker WW, Keefe RS, Hosford DA, Dvergsten C, Segreti AC, Beaver JS, Toler SM, Jett JE and Dunbar GC (2016) Phase 2 Trial of an Alpha-7 Nicotinic Receptor Agonist (TC-5619) in Negative and Cognitive Symptoms of Schizophrenia. *Schizophr Bull* **42**(2): 335-343.
- Wang H, Cai D, Chen Z and Wang Y (2020) GTS-21 Promotes alpha7 nAChR to Alleviate Intestinal Ischemia-Reperfusion-Induced Apoptosis and Inflammation of Enterocytes. *Med Sci Monit* **26**: e921618.
- Wang J, Kuryatov A, Sriram A, Jin Z, Kamenecka TM, Kenny PJ and Lindstrom J (2015) An Accessory Agonist Binding Site Promotes Activation of alpha4beta2* Nicotinic Acetylcholine Receptors. *J Biol Chem* **290**(22): 13907-13918.
- Weltzin MM and Schulte MK (2010) Pharmacological characterization of the allosteric modulator desformylflustrabromine and its interaction with alpha4beta2 neuronal nicotinic acetylcholine receptor orthosteric ligands. *J Pharmacol Exp Ther* **334**(3): 917-926.
- Wheeler JW, Olubajo O, Storm CB and Duffield RM (1981) Anabaseine: venom alkaloid of aphaenogaster ants. *Science* **211**(4486): 1051-1052.
- Williams DK, Wang J and Papke RL (2011) Investigation of the Molecular Mechanism of the Alpha7 nAChR Positive Allosteric Modulator PNU-120596 Provides Evidence for Two Distinct Desensitized States. *Mol Pharmacol* **80**(6): 1013-1032.
- Wu W, Ashley DL and Watson CH (2002) Determination of nicotine and other minor alkaloids in international cigarettes by solid-phase microextraction and gas chromatography/mass spectrometry. *Anal Chem* **74**(19): 4878-4884.
- Xiao Y, Fan H, Musachio JL, Wei ZL, Chellappan SK, Kozikowski AP and Kellar KJ (2006) Sazetidine-A, a novel ligand that desensitizes alpha4beta2 nicotinic acetylcholine receptors without activating them. *Mol Pharmacol* **70**(4): 1454-1460.
- Young GT, Zwart R, Walker AS, Sher E and Millar NS (2008) Potentiation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proc Natl Acad Sci U S A* **105**(38): 14686-14691.
- Yuan F, Jiang L, Li Q, Sokulsky L, Wanyan Y, Wang L, Liu X, Zhou L, Tay HL, Zhang G, Yang M and Li F (2020) A Selective alpha7 Nicotinic Acetylcholine Receptor Agonist, PNU-282987, Attenuates ILC2s Activation and Alternaria-Induced Airway Inflammation. *Front Immunol* **11**: 598165.
- Zhang J, Xiao YD, Jordan KG, Hammond PS, Van Dyke KM, Mazurov AA, Speake JD, Lippiello PM, James JW, Letchworth SR, Bencherif M and Hauser TA (2012) Analgesic effects mediated by neuronal nicotinic acetylcholine receptor agonists: correlation with desensitization of alpha4beta2* receptors. *Eur J Pharm Sci* **47**(5): 813-823.
- Zhou L, Zheng LF, Zhang XL, Wang ZY, Yao YS, Xiu XL, Liu CZ, Zhang Y, Feng XY and Zhu JX (2021) Activation of alpha7nAChR Protects Against Gastric Inflammation and Dysmotility in Parkinson's Disease Rats. *Front Pharmacol* **12**: 793374.

MOLPHARM-AR-2022-000591

- Zhou Y, Nelson ME, Kuryatov A, Choi C, Cooper J and Lindstrom J (2003) Human alpha4beta2 acetylcholine receptors formed from linked subunits. *J Neurosci* **23**(27): 9004-9015.
- Zwart R, Broad LM, Xi Q, Lee M, Moroni M, Bermudez I and Sher E (2006) 5-I A-85380 and TC-2559 differentially activate heterologously expressed alpha4beta2 nicotinic receptors. *Eur J Pharmacol* **539**(1-2): 10-17.
- Zwart R, Carbone AL, Moroni M, Bermudez I, Mogg AJ, Folly EA, Broad LM, Williams AC, Zhang D, Ding C, Heinz BA and Sher E (2008) Sazetidine-A is a potent and selective agonist at native and recombinant alpha 4 beta 2 nicotinic acetylcholine receptors. *Mol Pharmacol* **73**(6): 1838-1843.

MOLPHARM-AR-2022-000591

Footnotes

This work was support by NIH grant GM57481

Neither author has an actual or perceived conflict of interest with the contents of this article

Figure Legends

Figure 1. TQS-sensitive HS and LS $\alpha 4\beta 2$ receptors. **A)** Subunit configuration of the receptors composed of $\alpha 4$ – $\beta 2$ concatamers with the $\beta 2$ L15'M mutation (represented by the star) in the β subunits. When co-expressed with wild-type $\beta 2$ subunits, they yield HS $\alpha 4(2)\beta 2$ L15'M(2) $\beta 2$ receptors (left). When co-expressed with wild-type $\alpha 4$ subunits, they yield LS $\alpha 4(2)\beta 2$ L15'M(2) $\alpha 4$ 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 (\pm SD), normalized to preceding control ACh responses obtained from the same cells. ACh controls were 10 μ M for the HS receptors and 100 μ M 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 $\alpha 4(2)\beta 2$ L15'M(2) $\beta 2$ receptors (left) and LS $\alpha 4(2)\beta 2$ L15'M(2) $\alpha 4$ receptors (right). **A)** Cells were treated with control applications of ACh (red bars) and then after washout ACh co-applied with 30 μ M TQS (blue bars) and then another application of ACh. ACh controls were 10 μ M for the HS receptors and 100 μ M 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 μ M 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

MOLPHARM-AR-2022-000591

X-axis. All points are averages (\pm 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) $\alpha 4\beta 2$ L15'M receptors to a 30-second pre-application of 30 μ M nicotine followed by a co-application of 30 μ M nicotine and 30 μ M 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 μ Amps (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 μ M TQS (red symbols, right y-axis) of 5-8 cells (\pm 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 μ M 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 I_{\max} 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),

compared to the responses to drugs co-applied with 30 μ M 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 $\alpha 4\beta 2$ partial agonists when applied alone (circles), compared to the responses to drugs co-applied with 30 μ M 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 $\alpha 4\beta 2$ selective agonists when applied alone (circles), compared to the responses to drugs co-applied with 30 μ M TQS, indicated by the drug name with a plus sign, and plotted as half-color diamonds.

Figure 8. Effects of $\alpha 7$ -selective agonists (Table 4). **A)** Dot plot of the peak current responses of TQS-sensitive HS receptors to the $\alpha 7$ -selective agonists when applied alone, compared to ACh control responses and the responses to 30 μ M TQS applied alone. Although GTS-21 appeared to give detectable responses, these were not statistically significant compared to the other $\alpha 7$ -selective agonists (see Supplemental Data for ANOVA results). **B)** Responses of TQS-sensitive HS $\alpha 4\beta 2$ receptors to $\alpha 7$ -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 $\alpha 4\beta 2$ receptors to the $\alpha 7$ -selective agonists when applied alone, compared to ACh control responses and the responses to 30 μ M TQS applied alone. **D)** Responses of TQS-sensitive LS $\alpha 4\beta 2$ receptors to $\alpha 7$ -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).

MOLPHARM-AR-2022-000591

Figure 9. Effects of $\alpha 7$ -selective agonists on the ACh responses of wild-type HS and LS $\alpha 4\beta 2$ receptors. Averaged data were prepared as described (Methods). Following the ACh control responses (red bars), the $\alpha 7$ agonists were pre-applied for 30 s (colored bars). Then without washout the $\alpha 7$ agonists were co-applied with ACh at the control concentration (10 μ M for HS and 100 μ M 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.

MOLPHARM-AR-2022-000591

Table 1. Test compounds concentrations and n values

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

MOLPHARM-AR-2022-000591

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.

MOLPHARM-AR-2022-000591

Table 3. Antagonists

3a. Responses	HS activation	HS TQS	LS activation	LS TQS
	Average \pm SD	Average \pm SD	Average \pm SD	Average \pm SD
MLA	0.007 \pm 0.003	0.016 \pm 0.009 [#]	0.003 \pm 0.003	0.003 \pm 0.003
DH β E	0.046 \pm 0.032 [#]	0.085 \pm 0.046 [#]	0.004 \pm 0.002	0.004 \pm 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
DH β E	0.0201	2.0367	0.0209 [#]	0.8528

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

MOLPHARM-AR-2022-000591

Table 4. α 7-selective agonists

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

See Supplemental Data for ANOVA results

MOLPHARM-AR-2022-000591

Table 5. Pre-applications of $\alpha 7$ agonists to wild-type receptors

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

MOLPHARM-AR-2022-000591

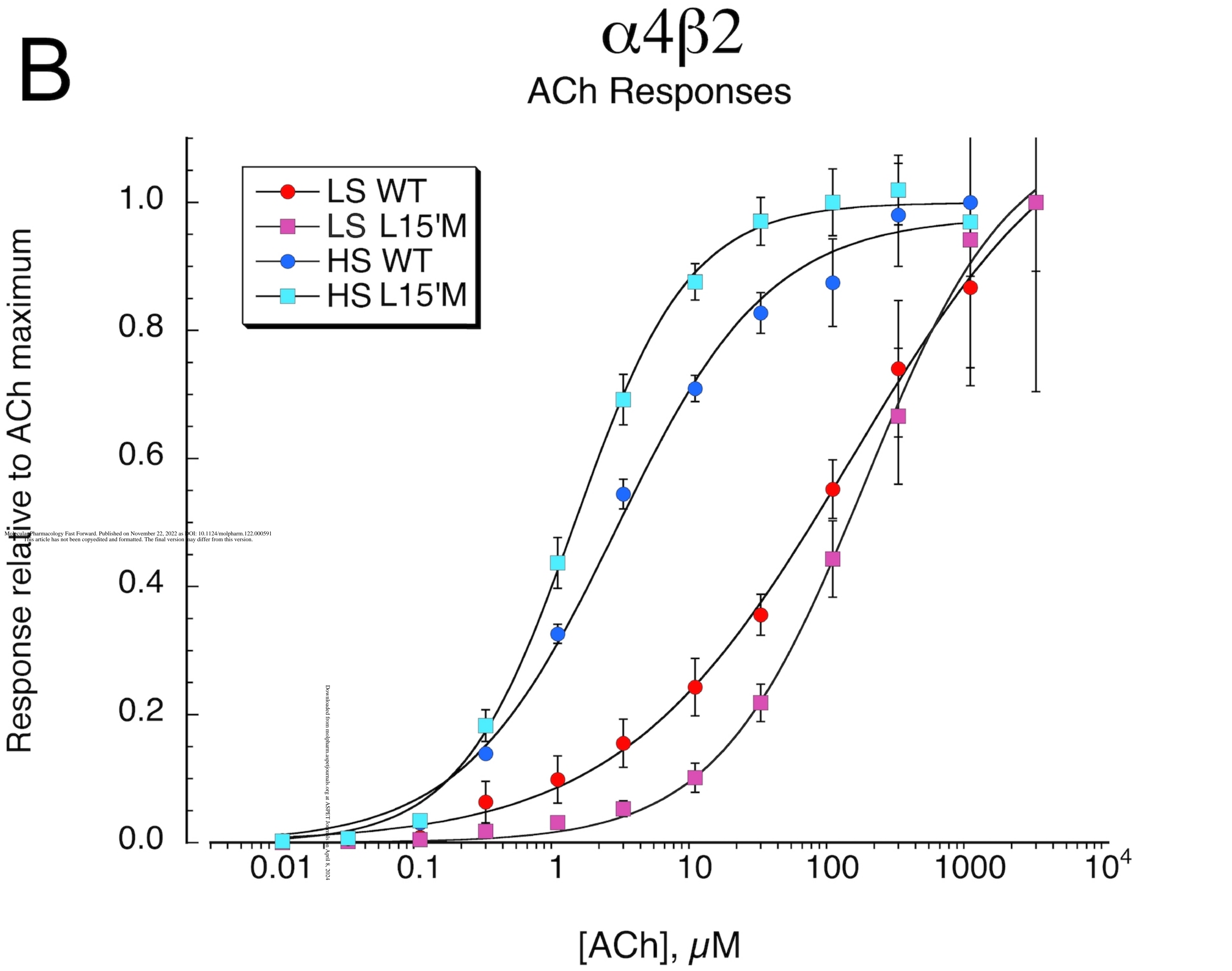
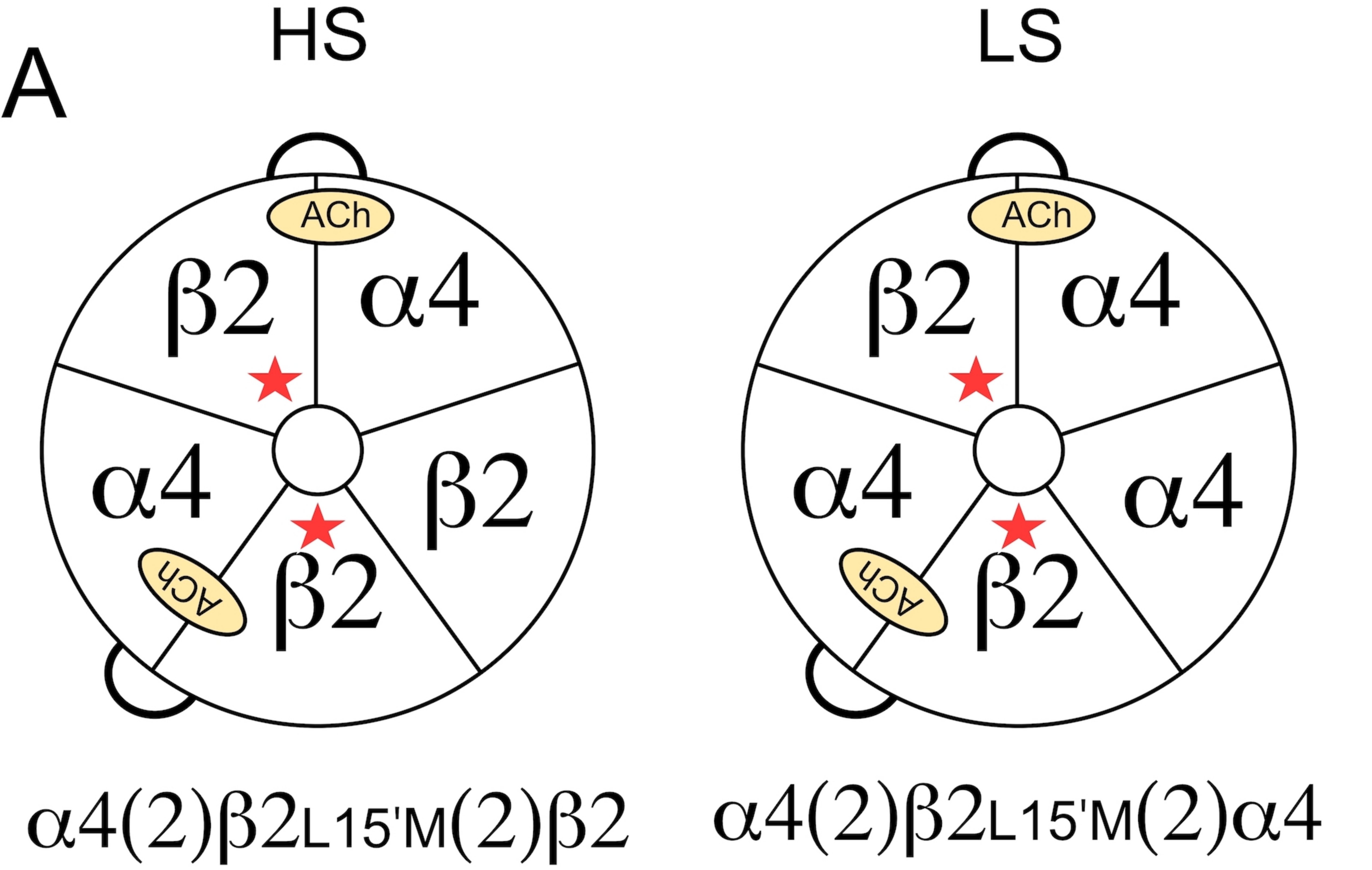
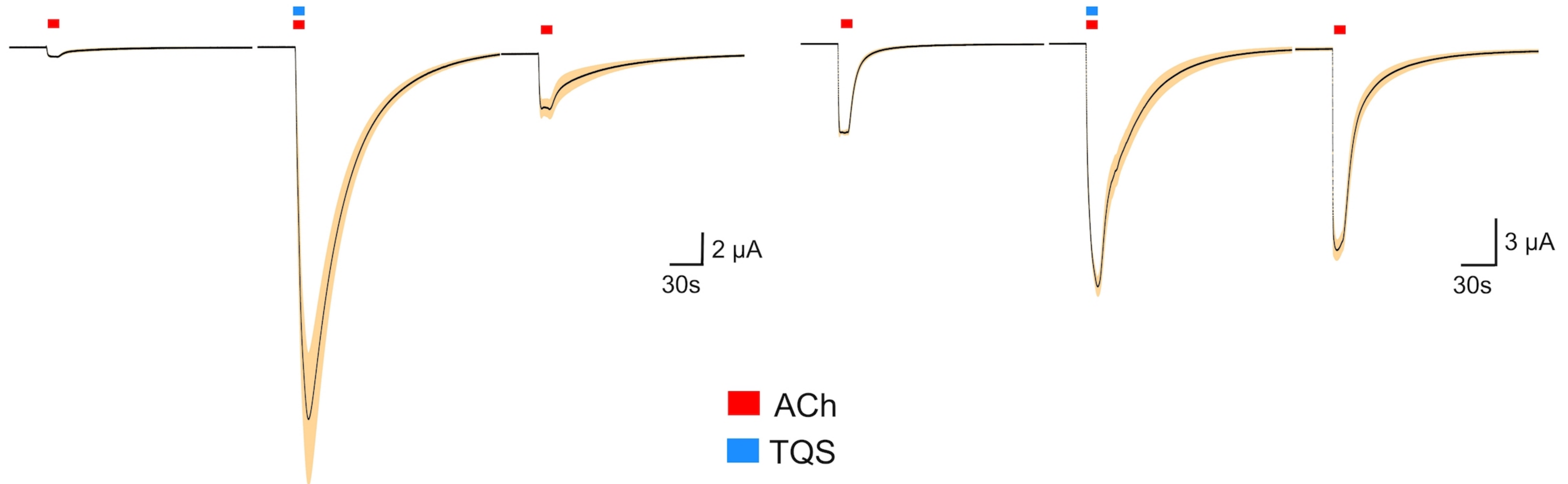


Figure 1

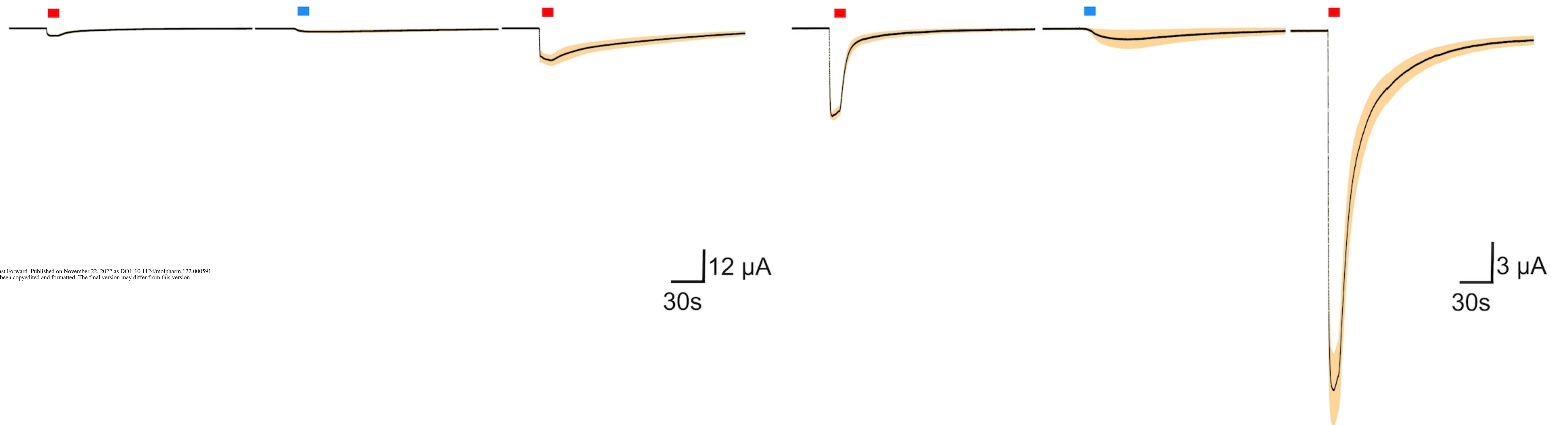
$\alpha 4(2) \beta 2 L 15' M(2) \beta 2$

$\alpha 4(2) \beta 2 L 15' M(2) \alpha 4$

A



B



C



Figure 2

TQS-potentiated responses of HS and LS $\alpha 4\beta 2$ L15'M receptors

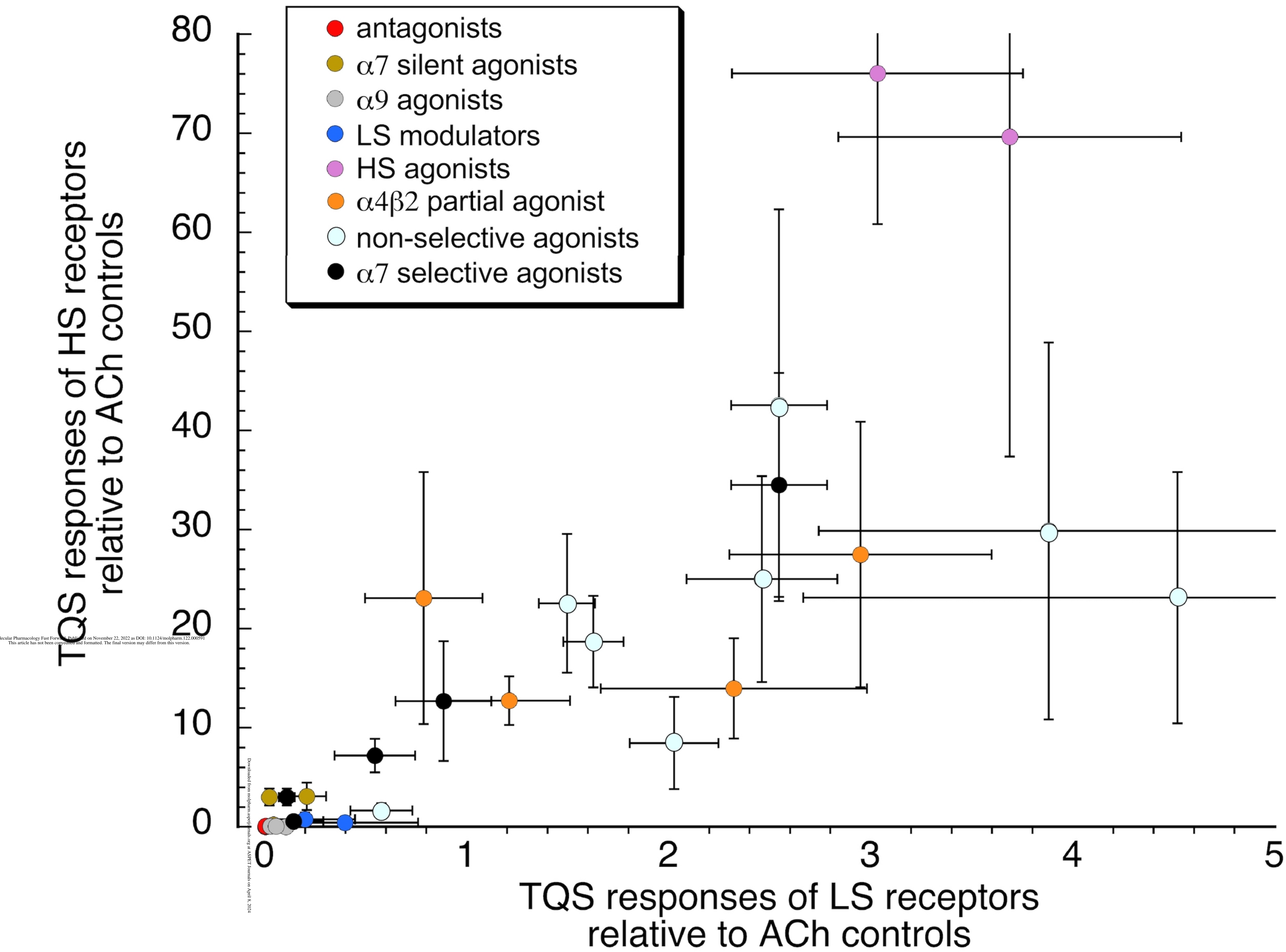
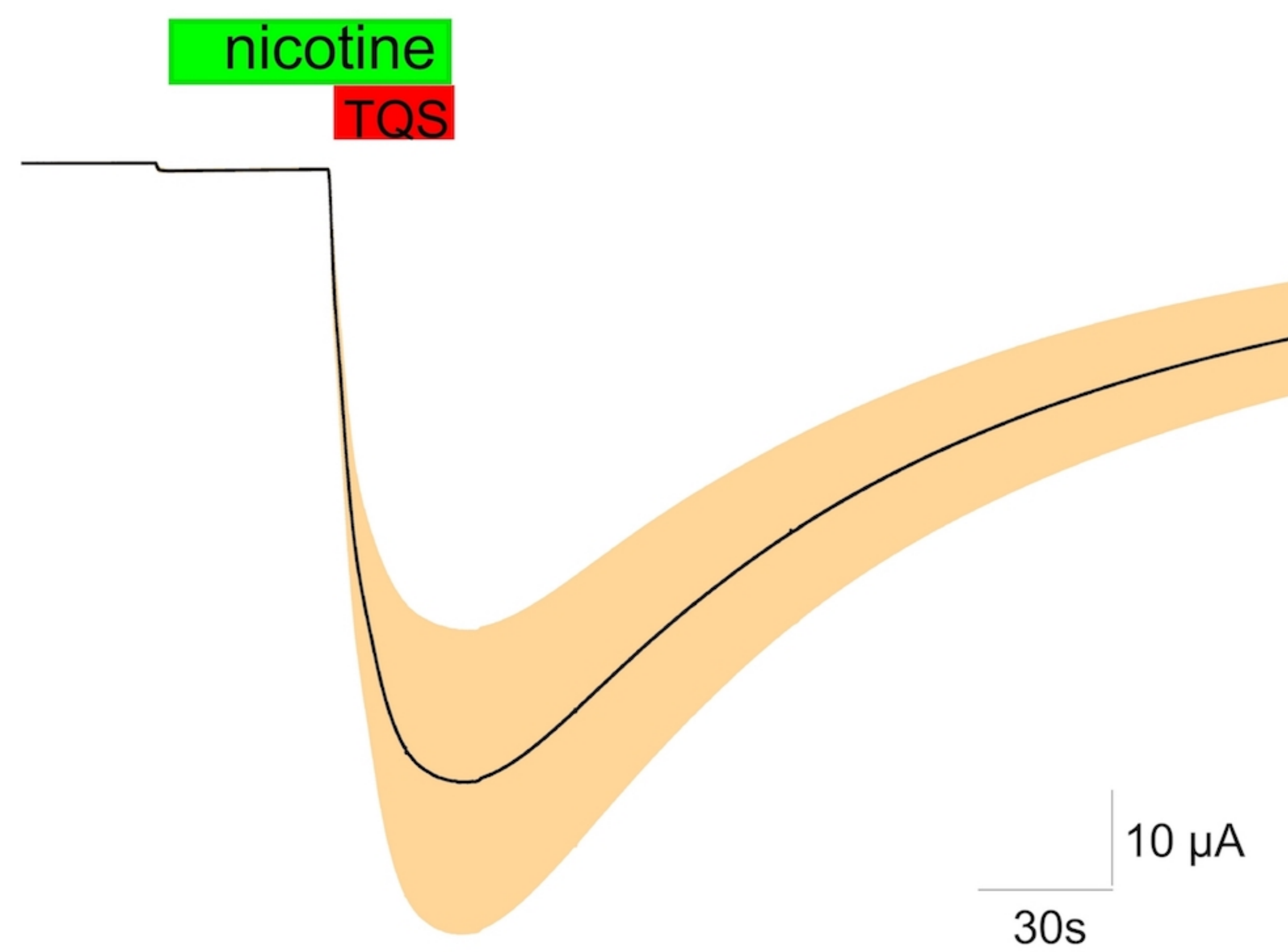


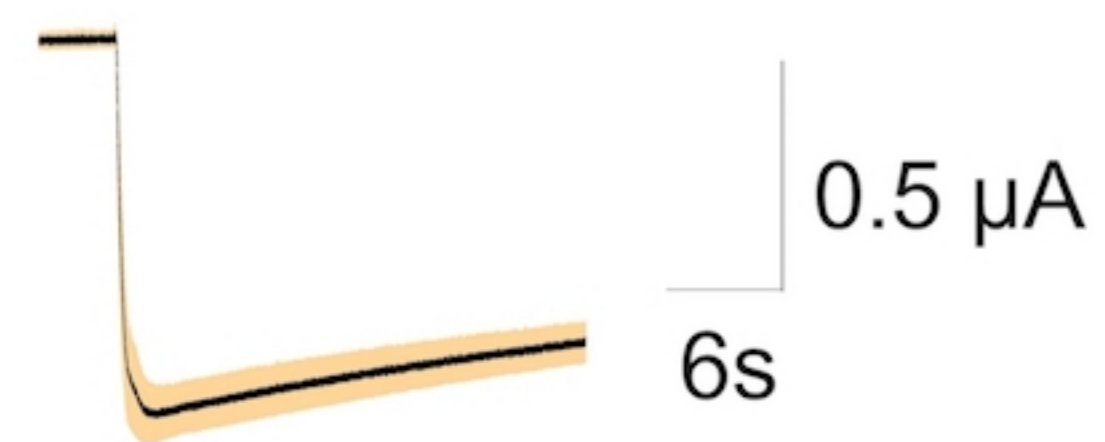
Figure 3

A

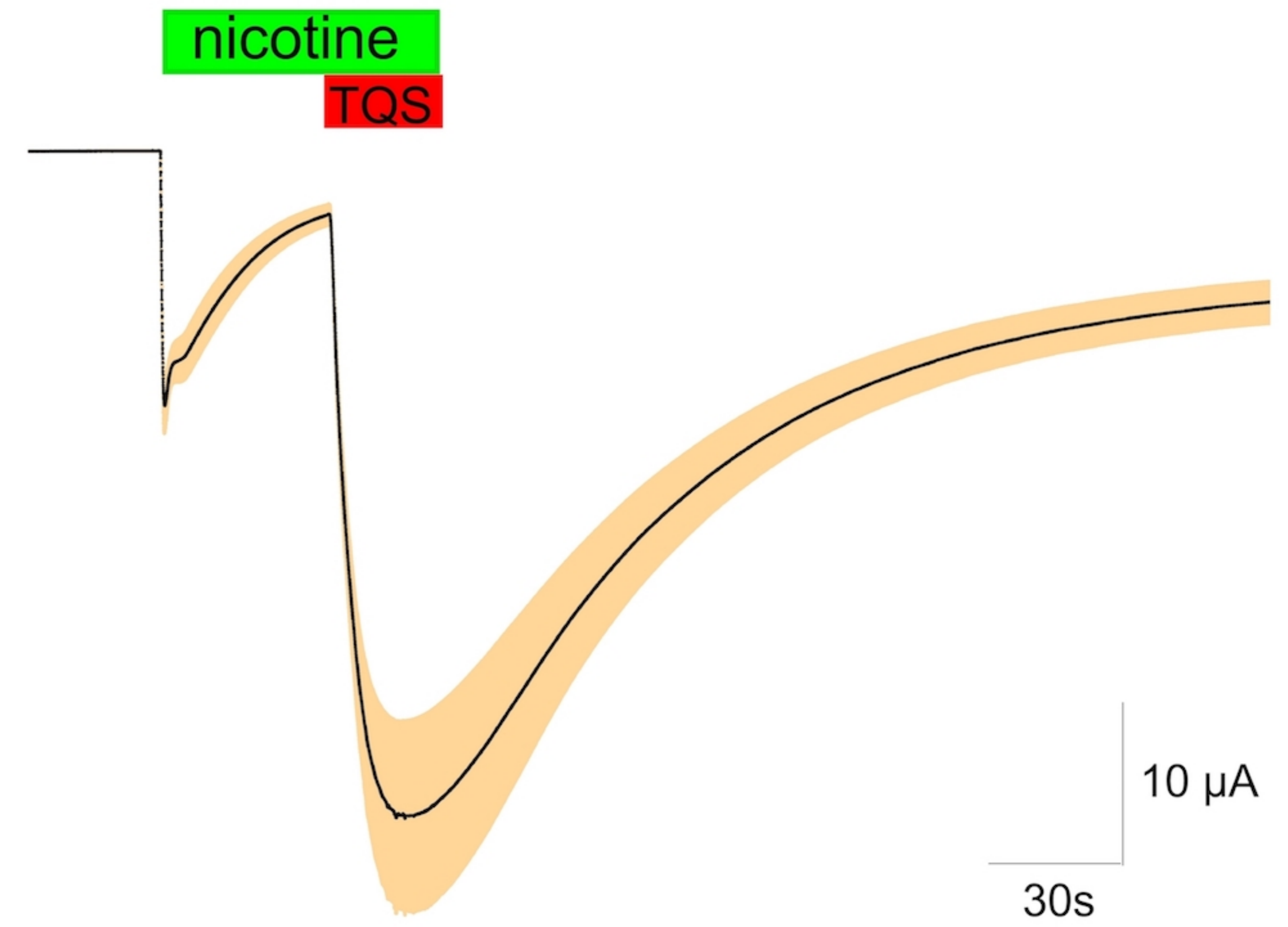
$\alpha 4(2) \beta 2 L 15' M(2) \beta 2$



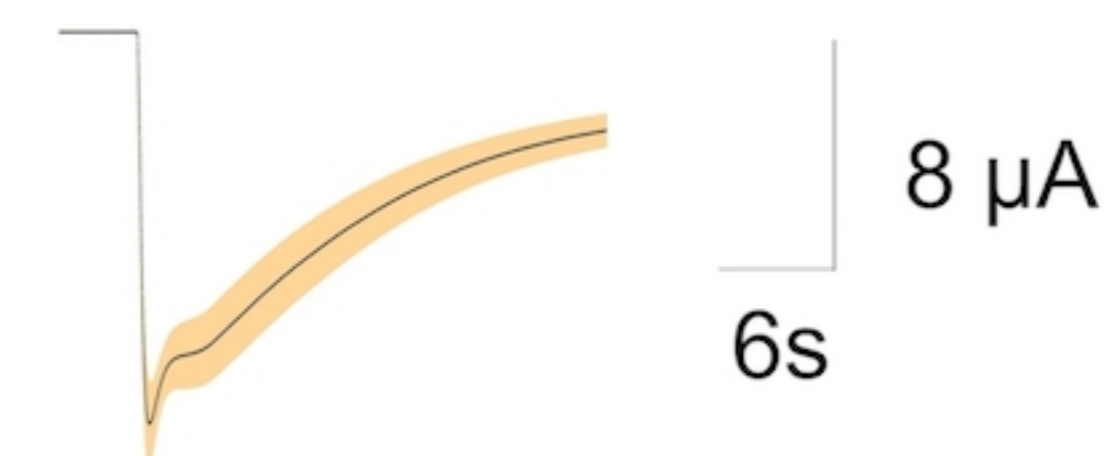
Nicotine only



$\alpha 4(2) \beta 2 L 15' M(2) \alpha 4$



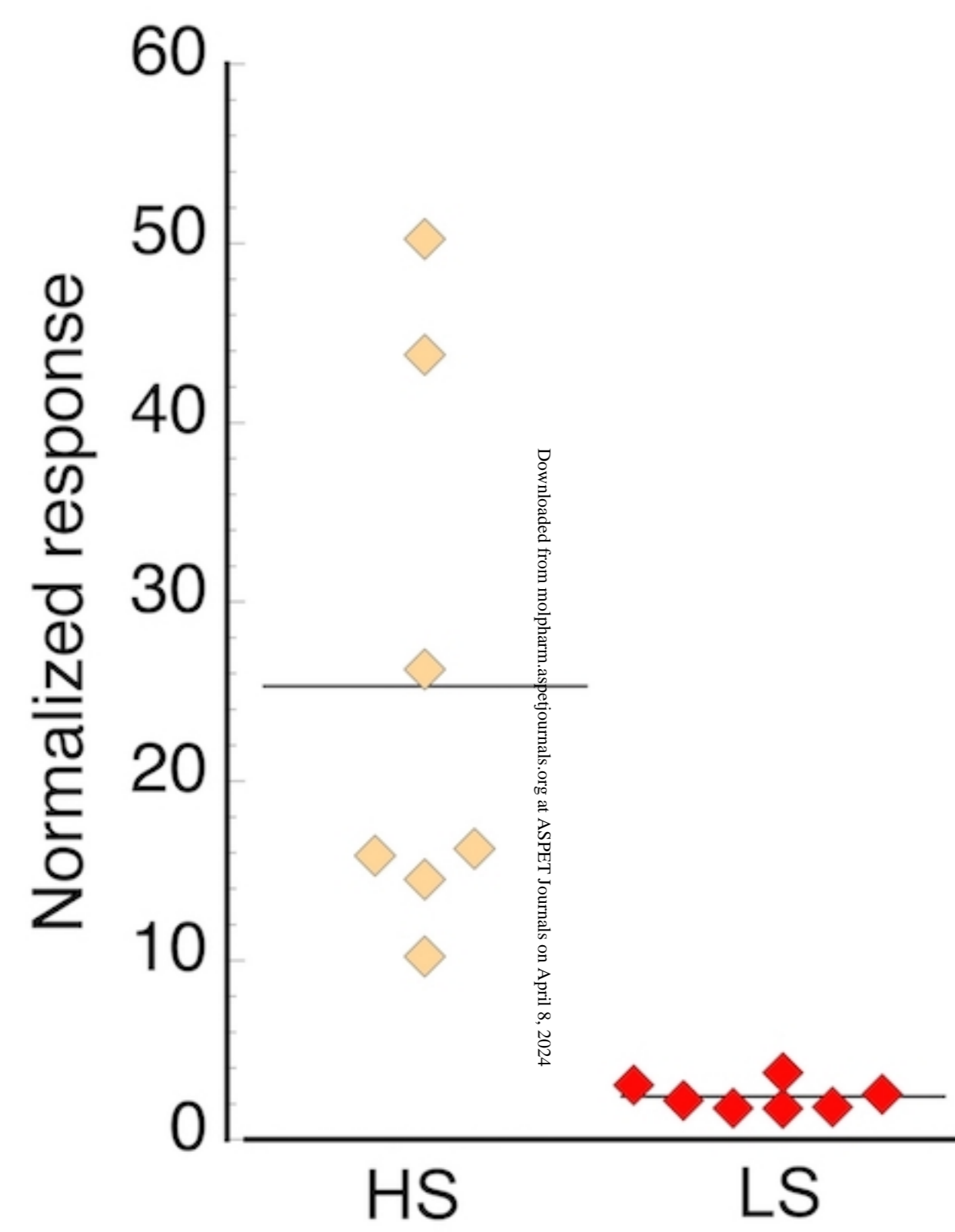
Nicotine only



B

Normalized peak currents with TQS

Molecular Pharmacology Fast Forward. Published on November 22, 2022 as DOI: 10.1124/molpharm.122.000591
This article has not been copyedited and formatted. The final version may differ from this version.



C

Peak currents (μA) with TQS

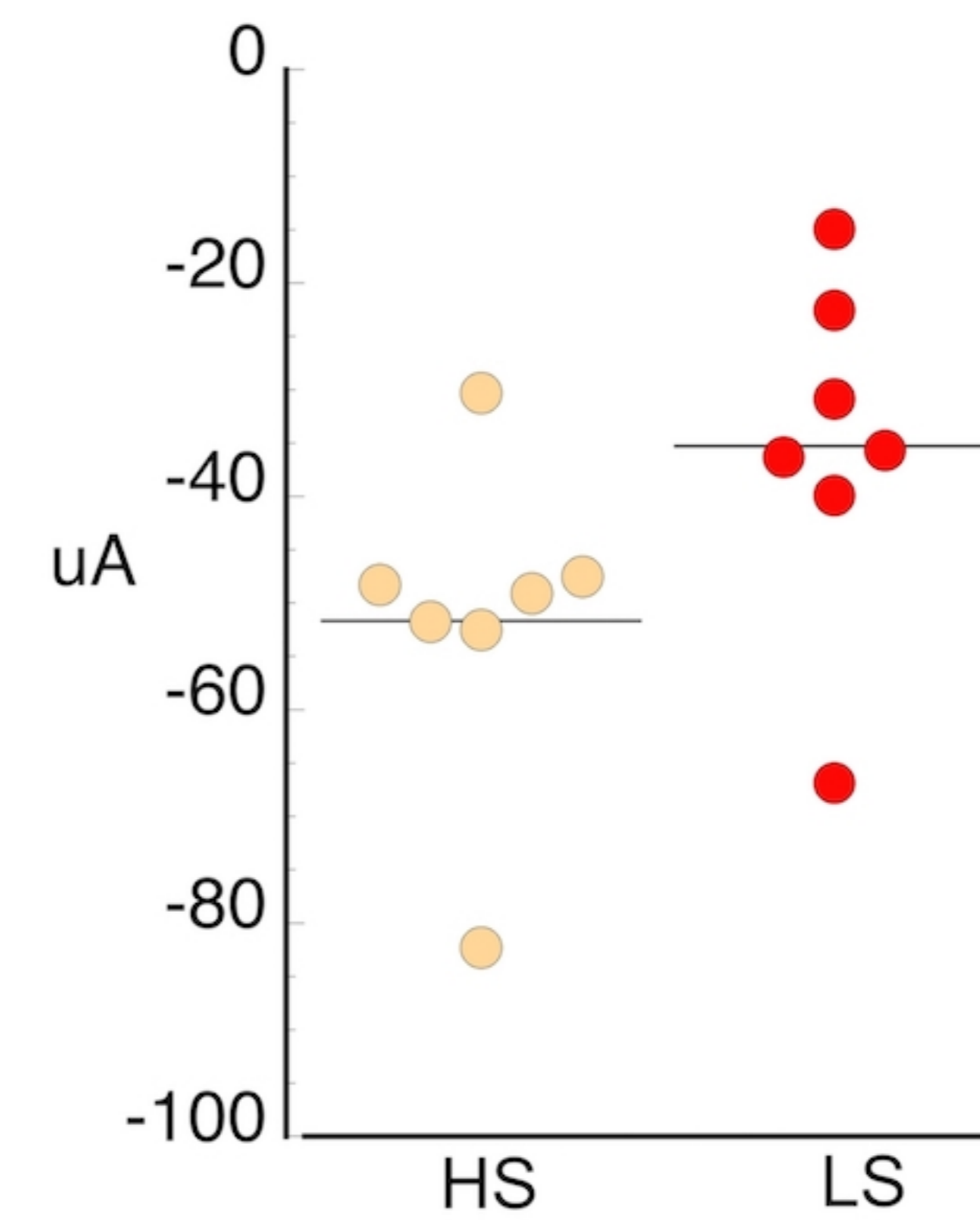


Figure 4

Nonselective agonists

HS receptors

LS receptors

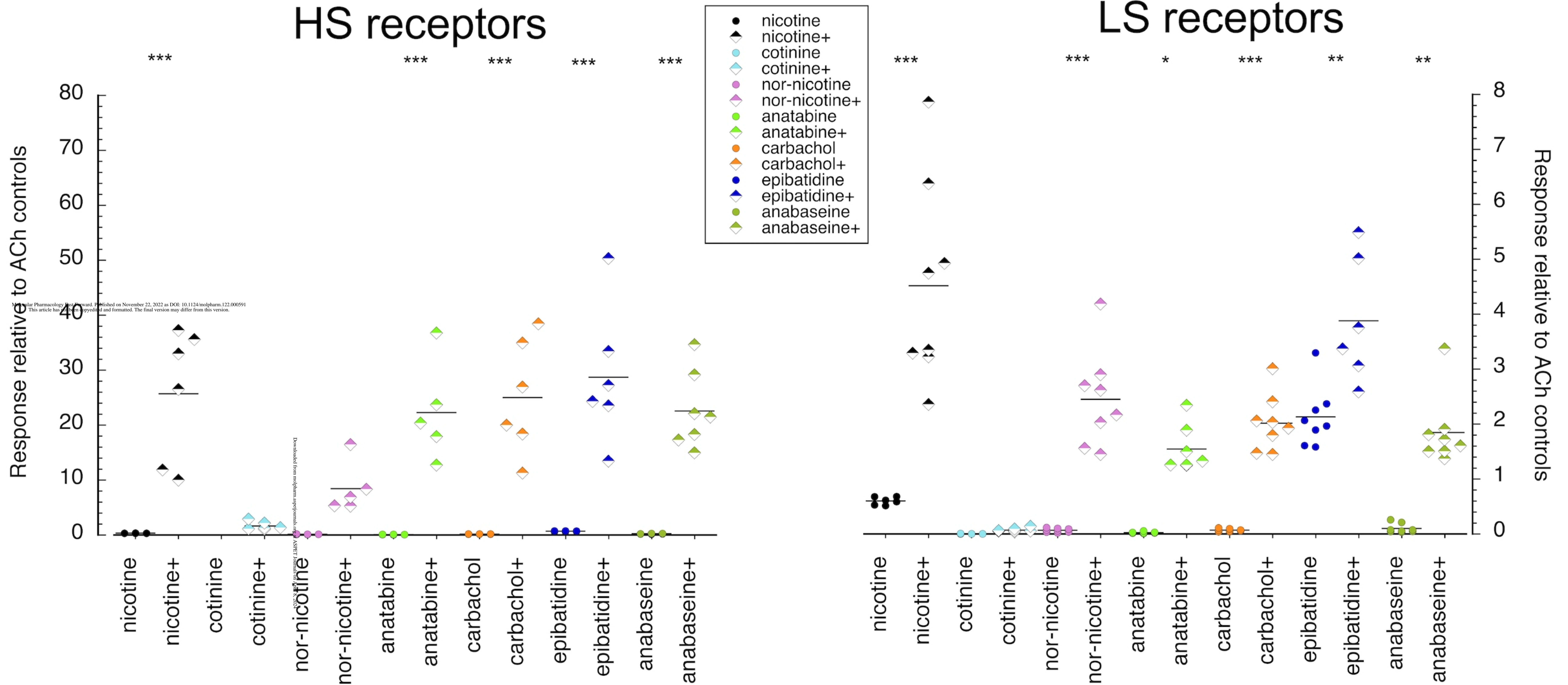
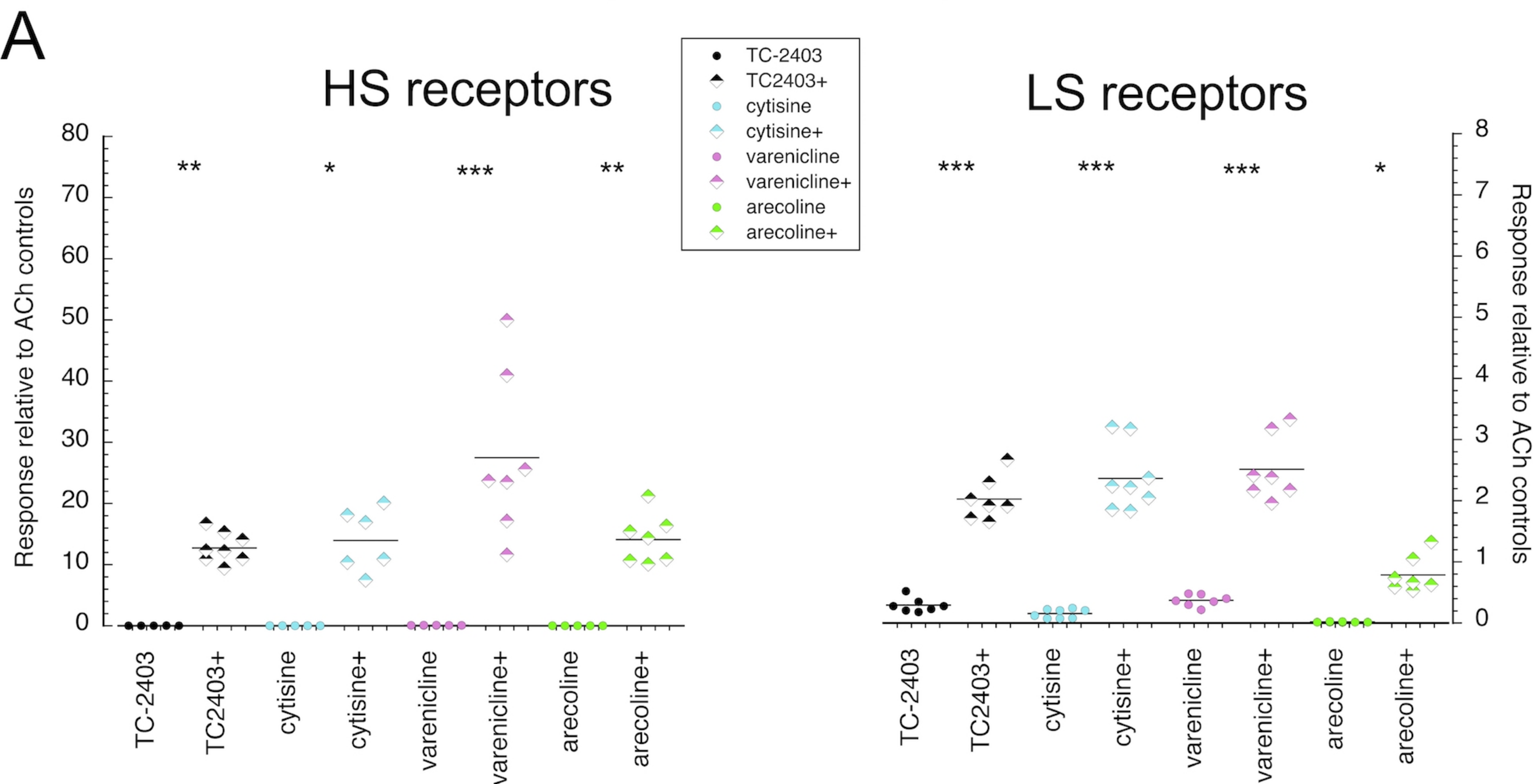


Figure 6

$\alpha 4 \beta 2$ partial agonists



B

HS- $\alpha 4 \beta 2$ selective agonists

HS receptors

LS receptors

Response relative to ACh controls

Response relative to ACh controls

Legend:

- sazetidine-A
- sazetidine-A +
- TC-2559
- TC-2559 +

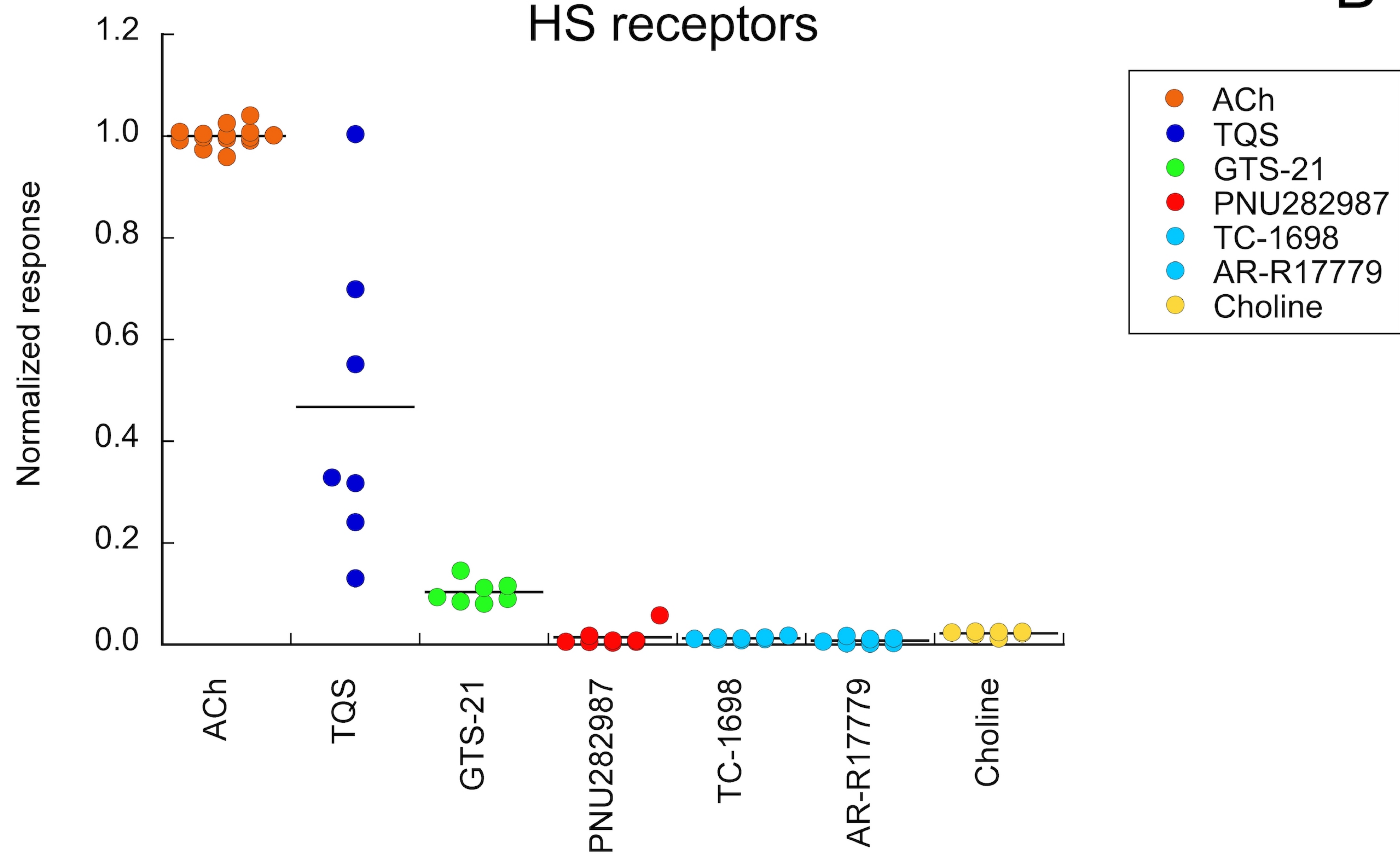
Agonist	HS receptors (Response relative to ACh controls)	LS receptors (Response relative to ACh controls)
sazetidine-A	~0	~0.5
sazetidine-A +	~68	~3.5
TC-2559	~0	~0.5
TC-2559 +	~75	~3.5

Figure 7

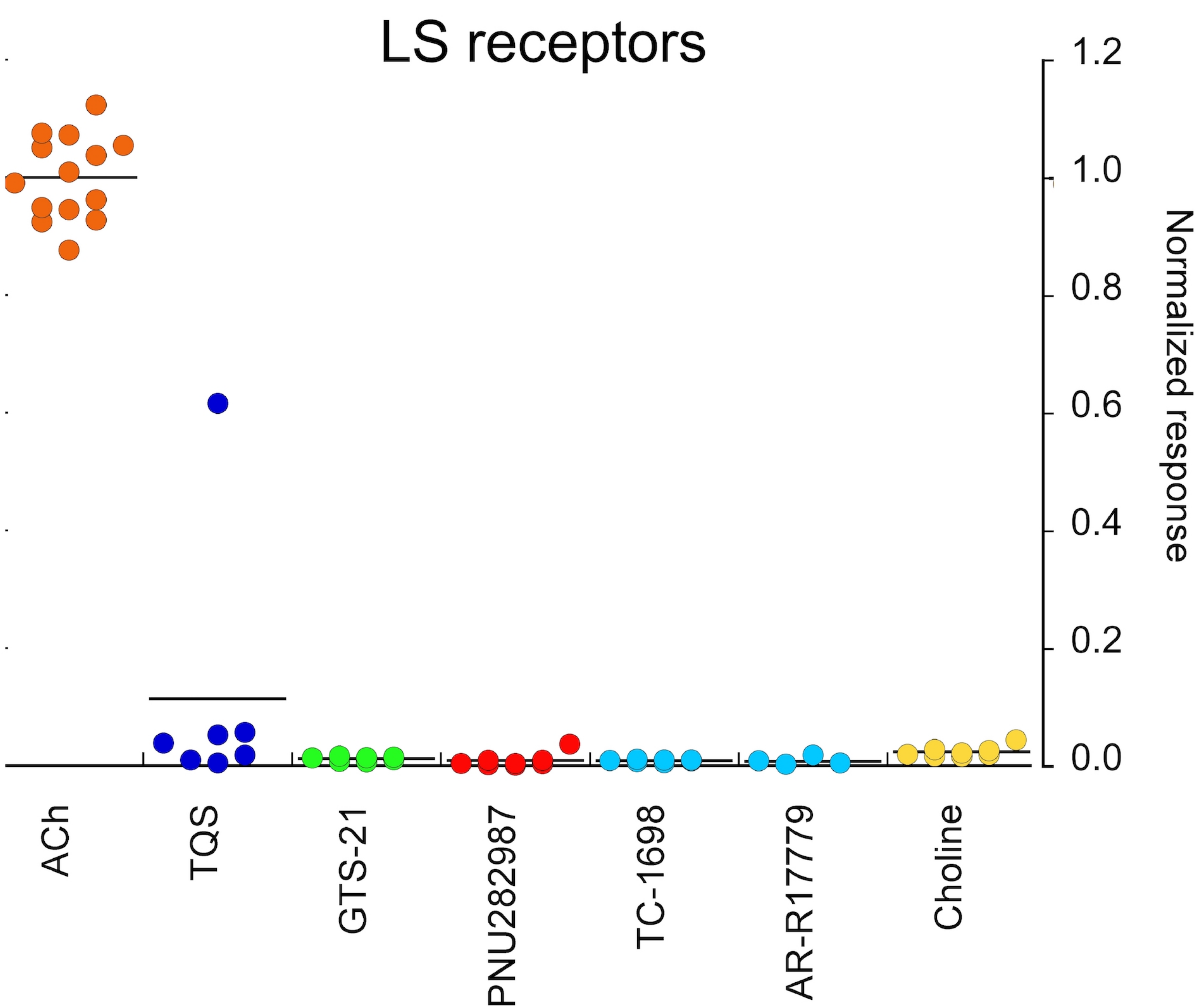
Effects of $\alpha 7$ -selective agonists on $\alpha 4\beta 2$ L15'M receptors

Drugs applied alone

A

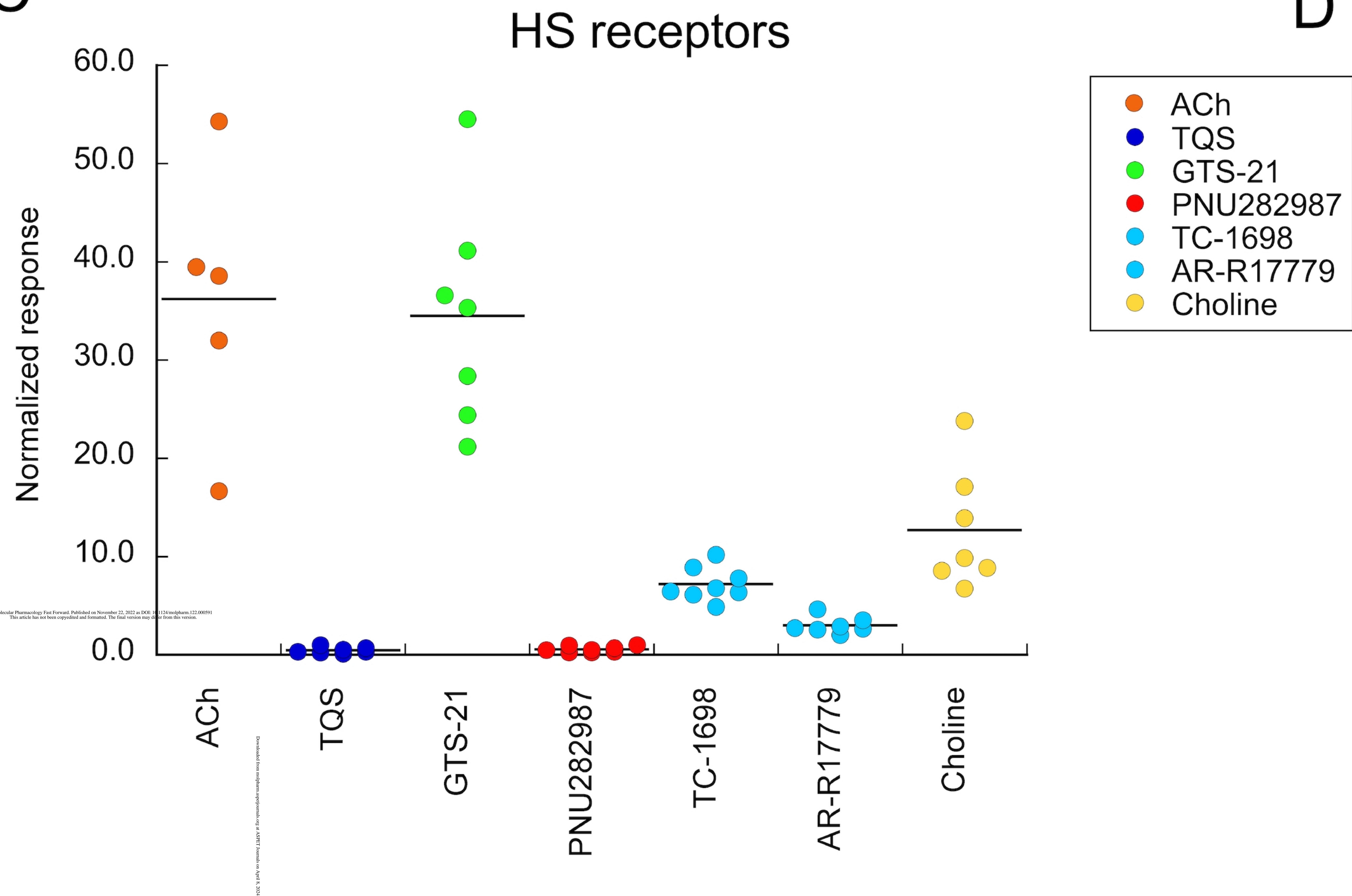


B



Drugs co-applied with TQS

C



D

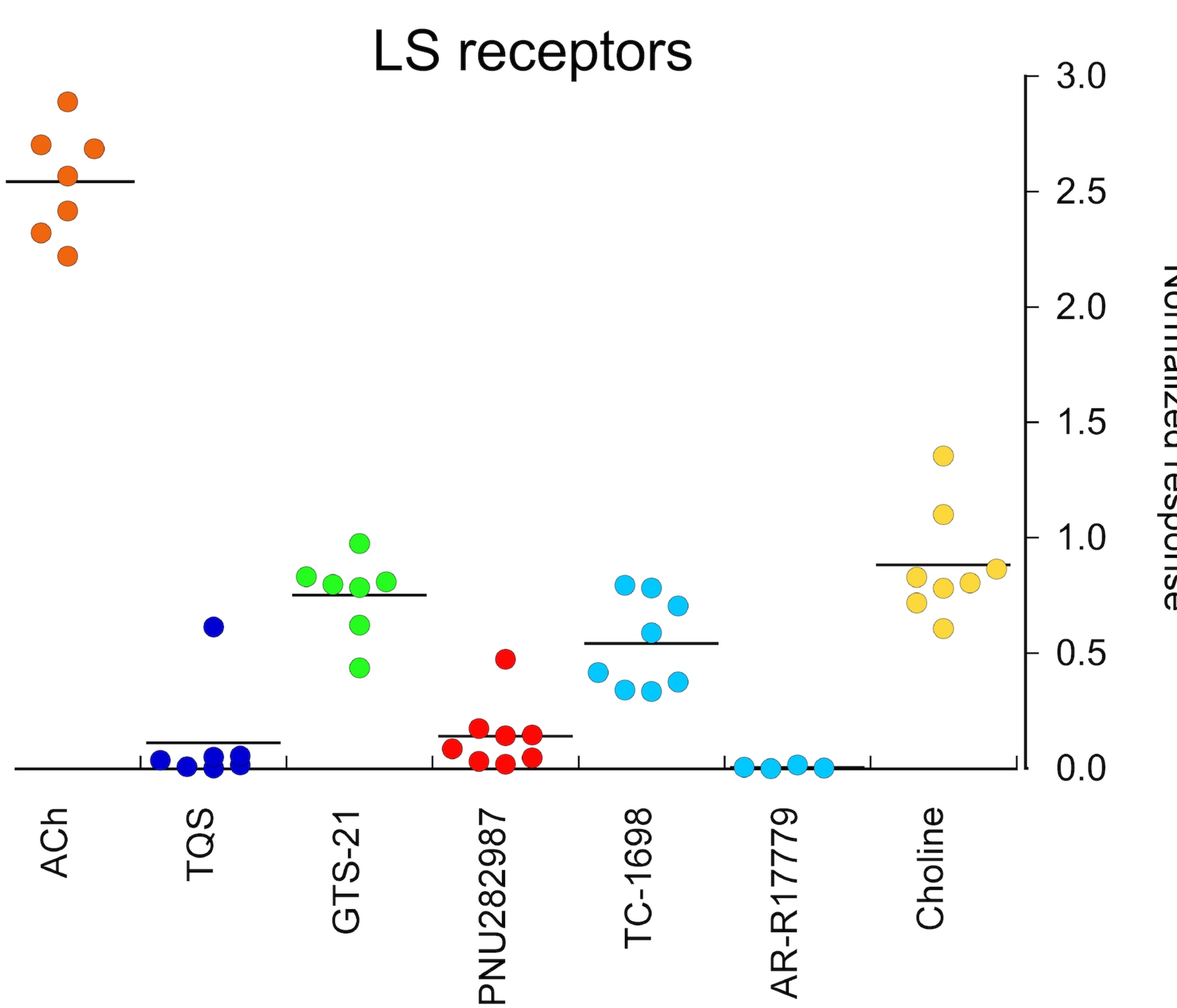


Figure 8

Wild-type receptors

HS
 $\alpha 4(2)\beta 2(3)$

LS
 $\alpha 4(3)\beta 2(2)$

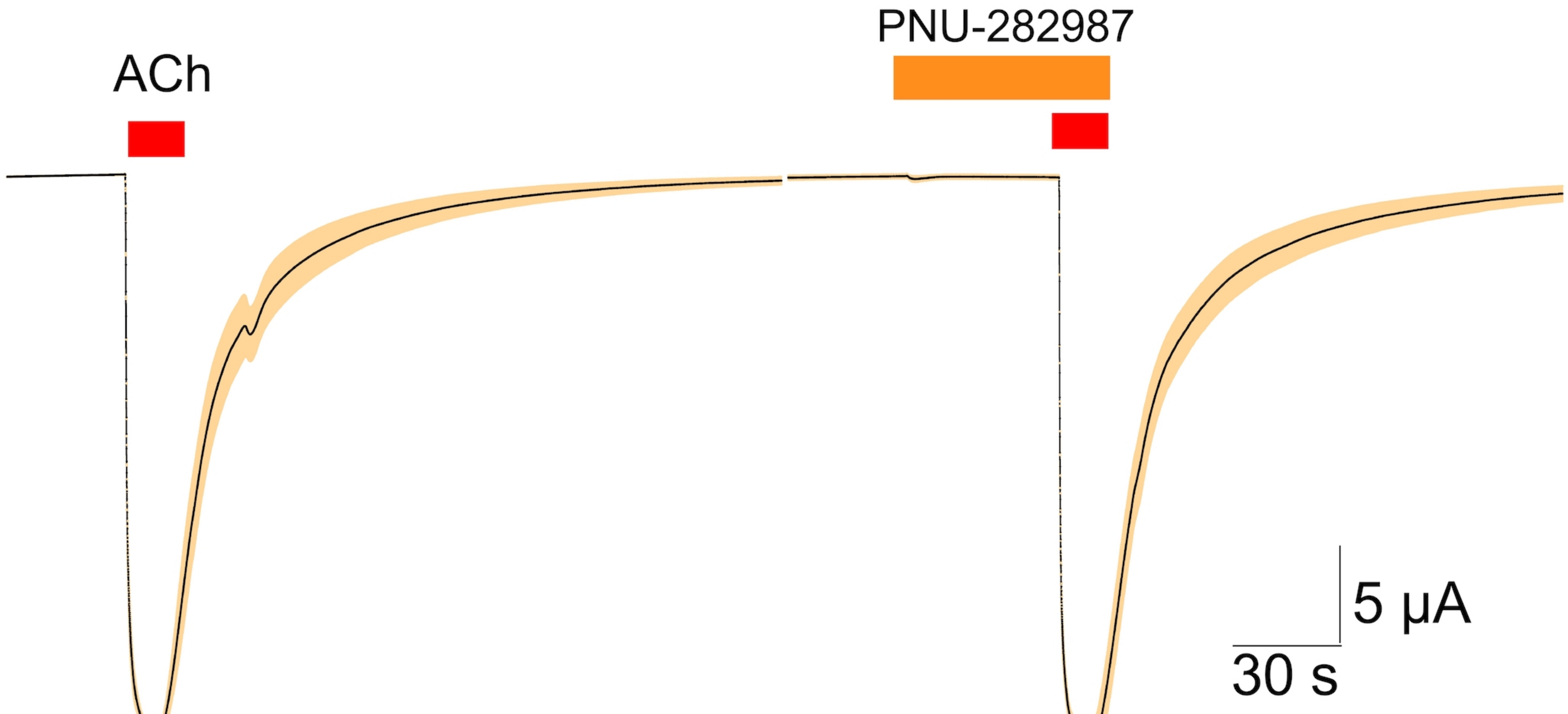
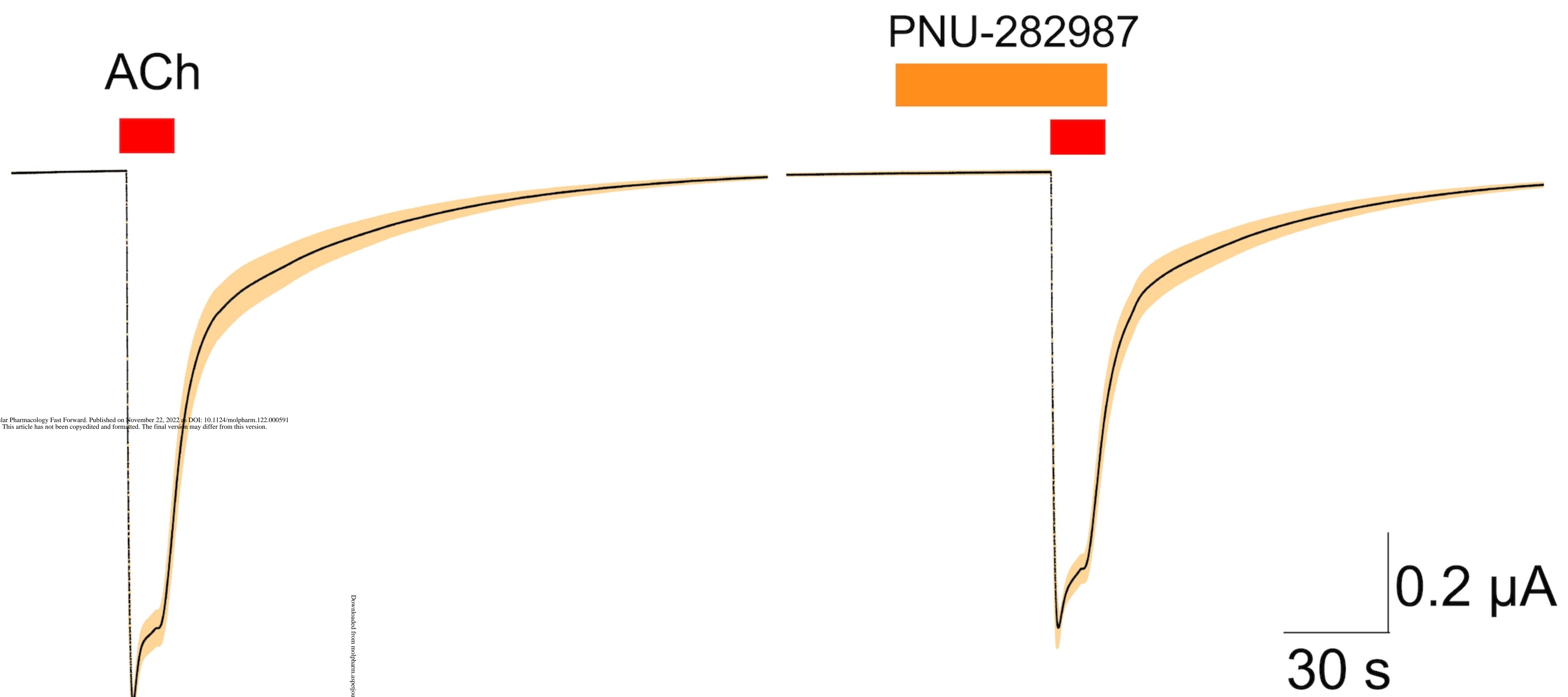
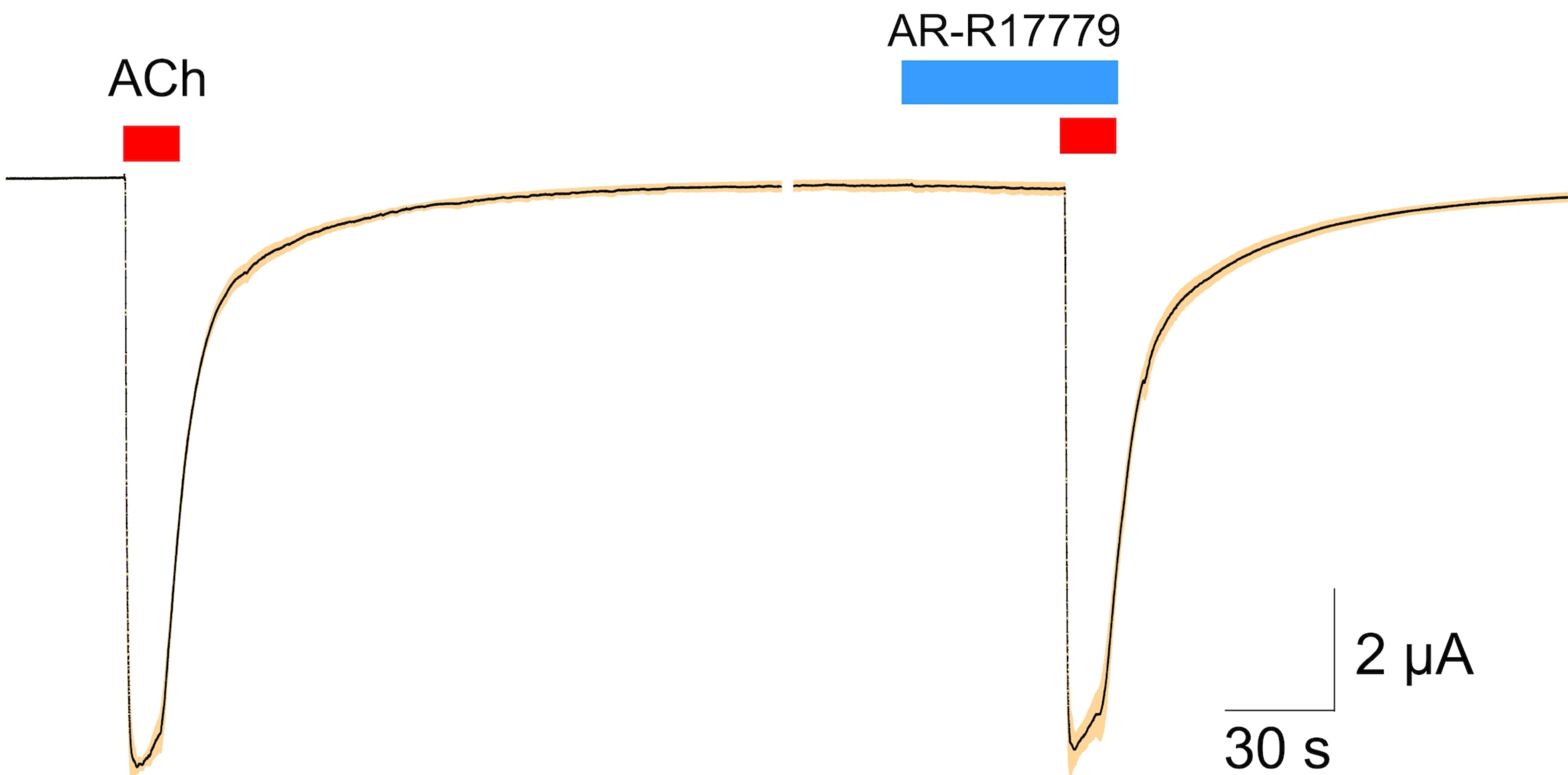
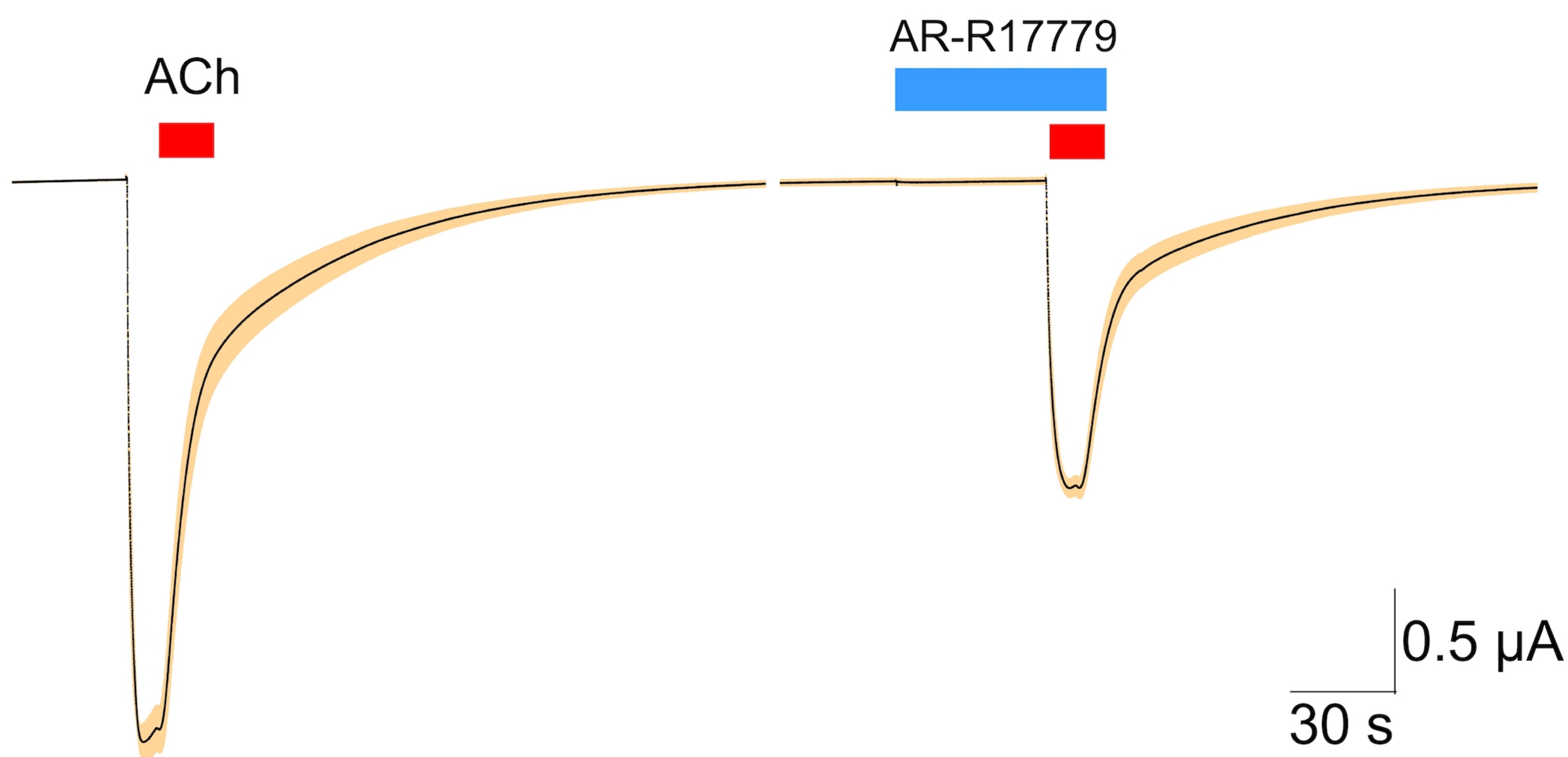
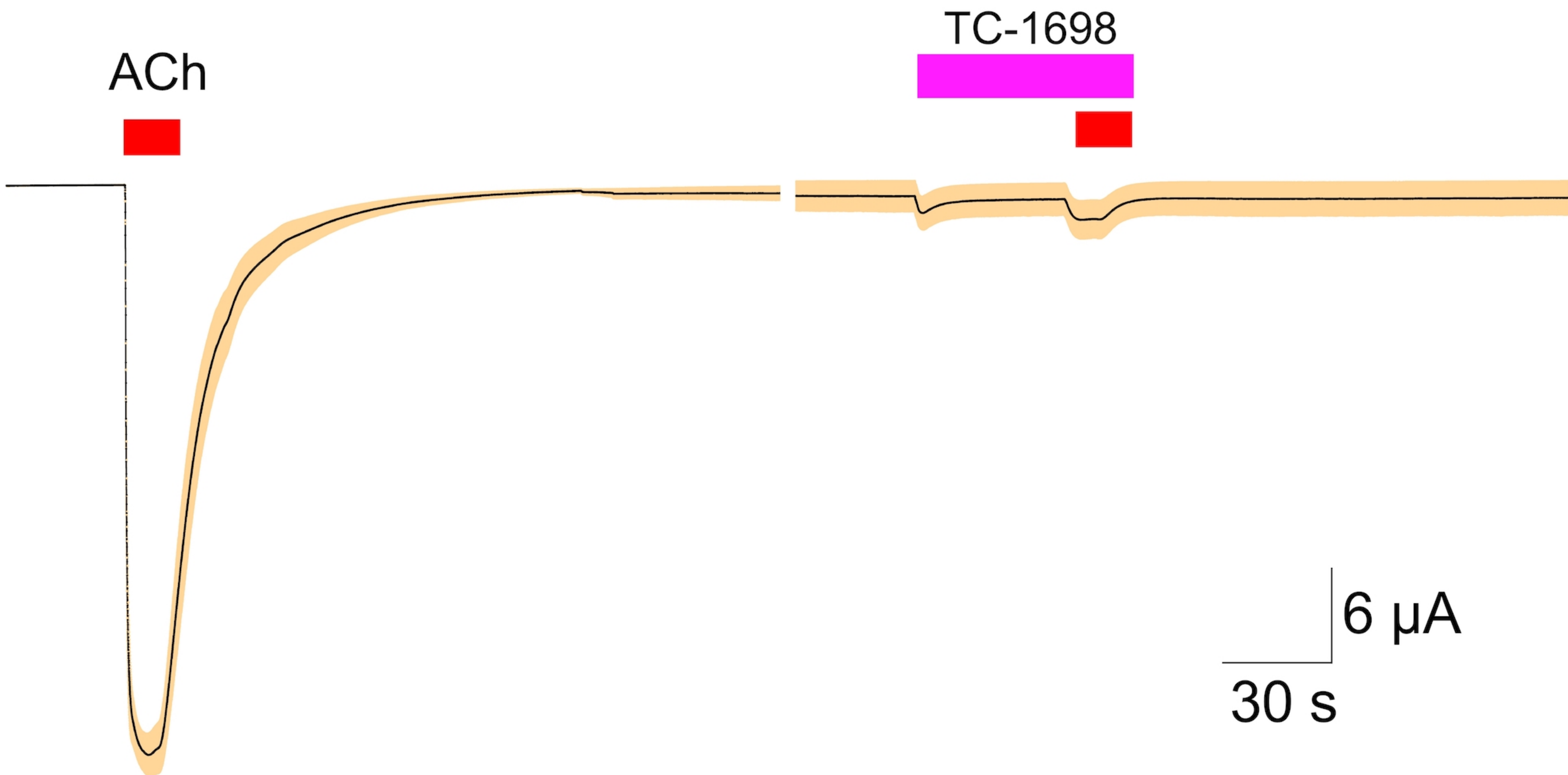
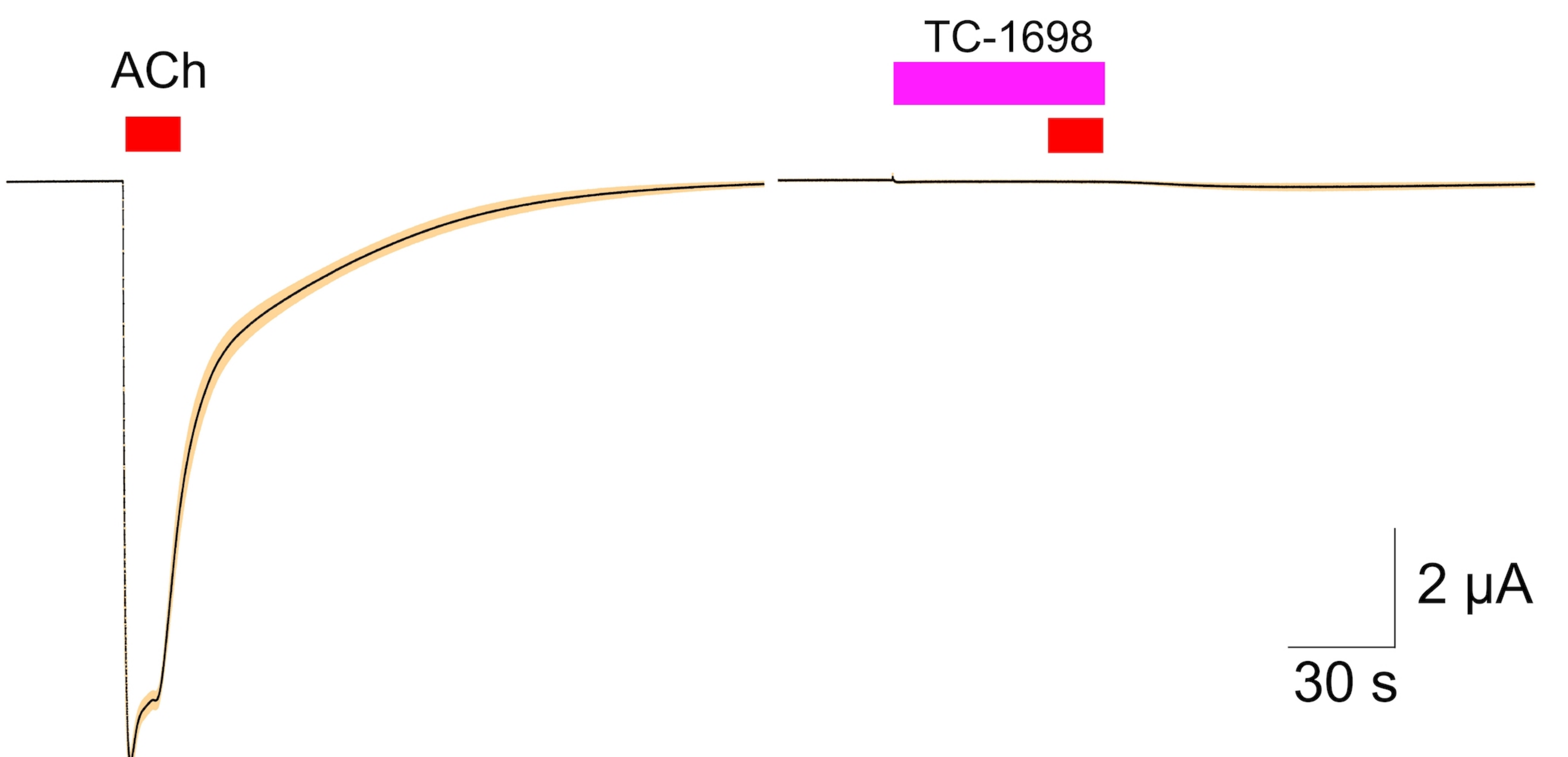
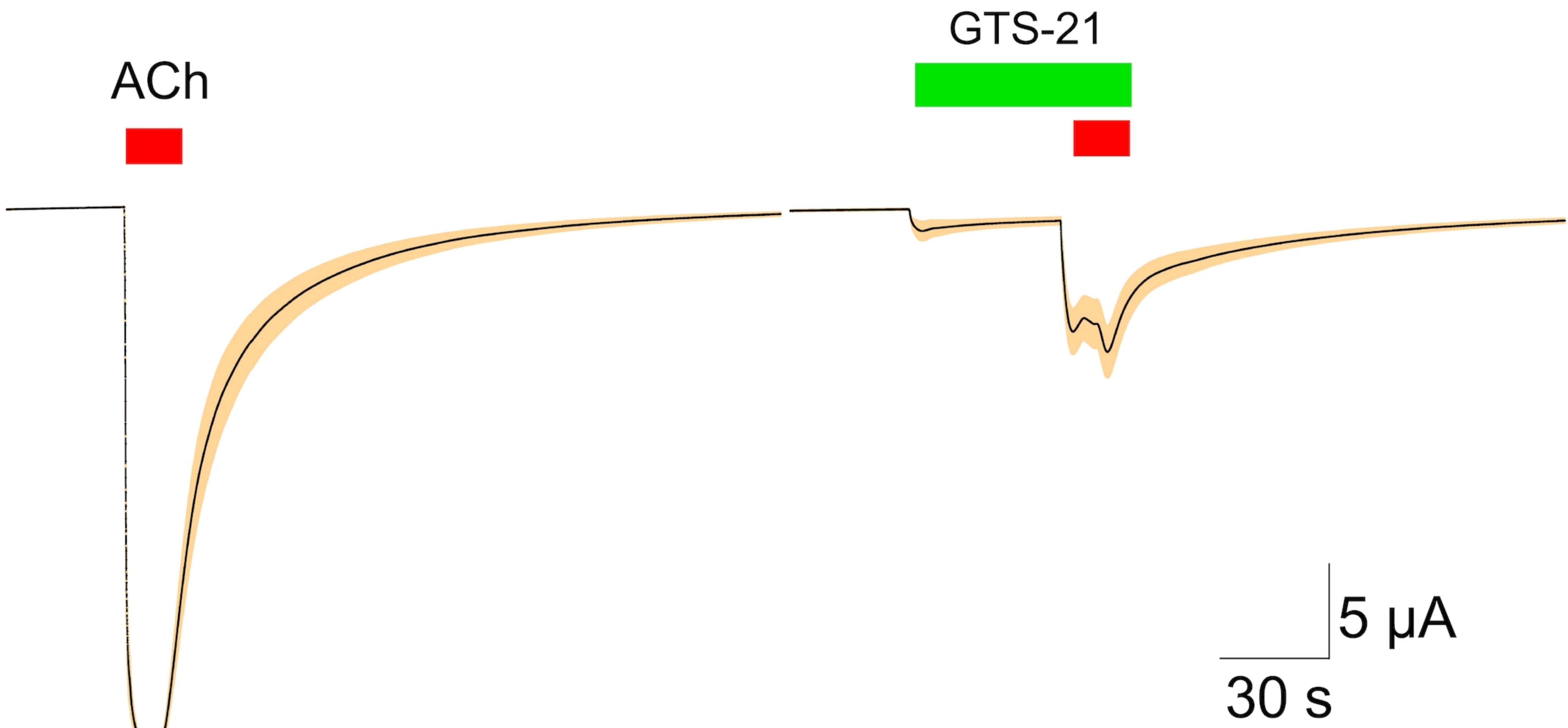


Figure 9

Supplemental Data

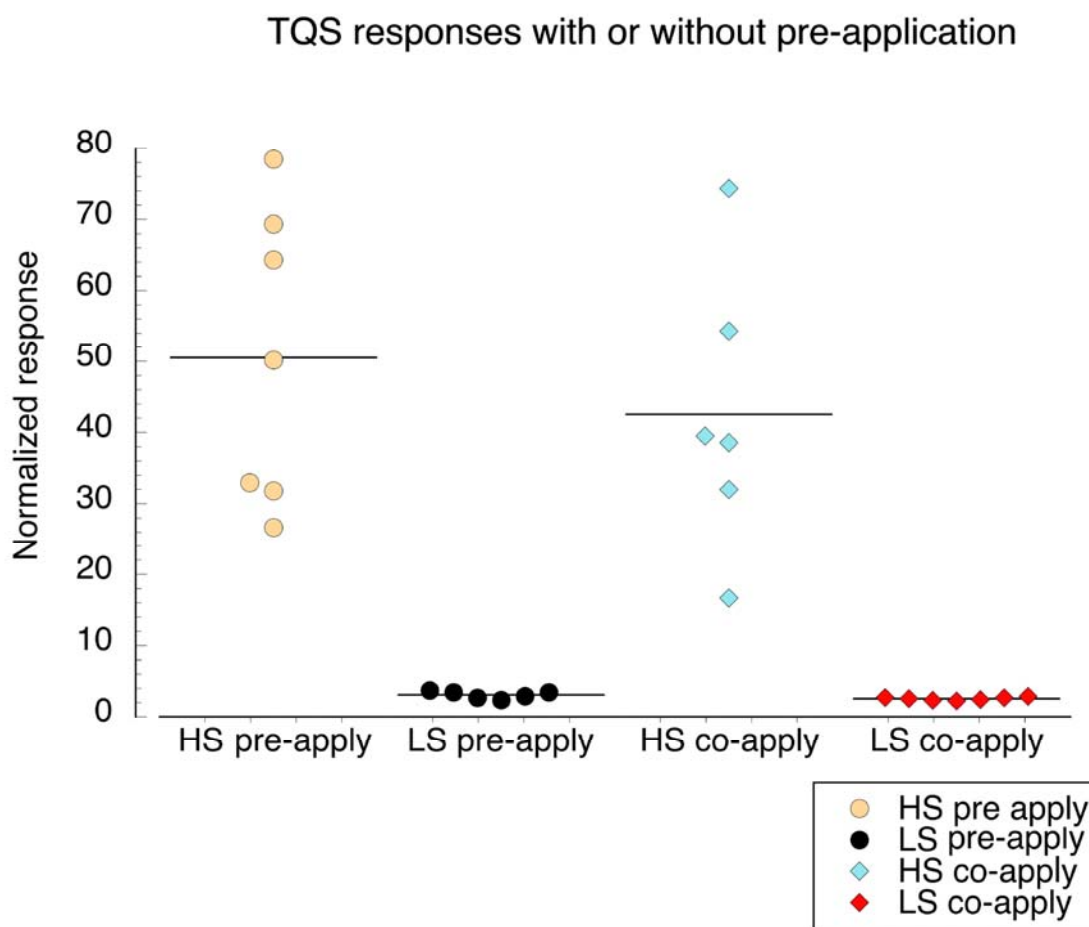
Insights into the differential desensitization of $\alpha 4\beta 2$ nicotinic acetylcholine receptor isoforms obtained with positive allosteric modulation of mutant receptors.

Roger L. Papke and Clare Stokes

Molecular Pharmacology

MOLPHARM-AR-2022-000591

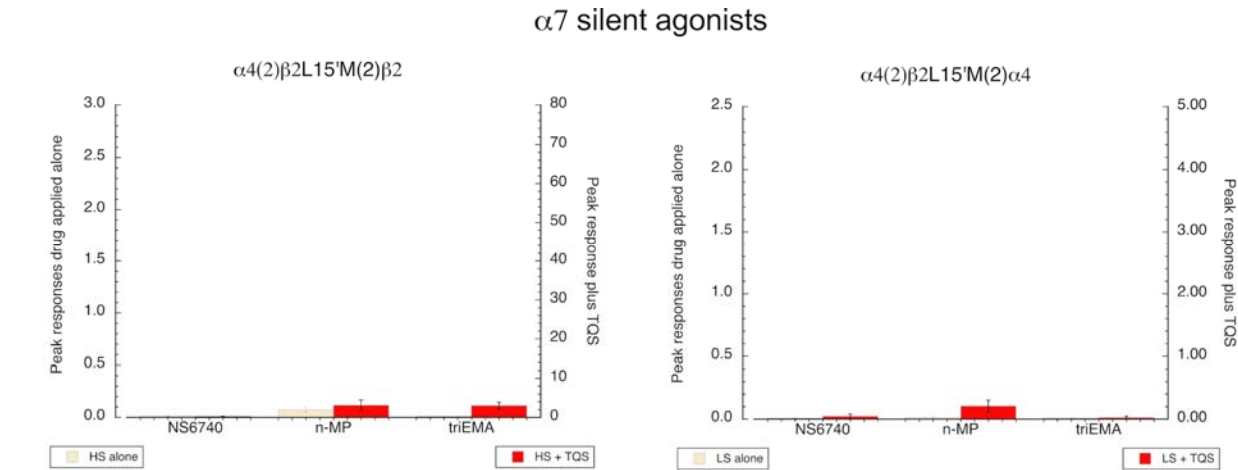
Supplemental Figure 1



Responses of TQS-sensitive $\alpha 4\beta 2$ receptors to control concentrations of ACh (10 μ M for HS receptors and 100 μ M for LS receptors) were compared under conditions where 30 μ M TQS was preapplied for 30 seconds before the co-application of ACh and TQS to simple co-application of TQS and ACh. Pre-applications had no significant effects. See below for ANOVA results.

The supplemental data figures 2-5 represent the normalized responses of the indicated TQS-sensitive $\alpha 4 \beta 2$ receptors, the HS form on the left and the LS form on the right. The tan bars represent the responses to the drugs applied alone, and the red bars are the responses to the test concentrations co-applied with 30 μ M TQS. Note that the right y-axis scales is set to match those in the main figures, for comparison. See main Table 1 for test concentrations and n values. The tables provide averages \pm standard deviations and the statistical analyses.

Supplemental Figure 1



Supplemental Data Figure 2. $\alpha 7$ silent agonists

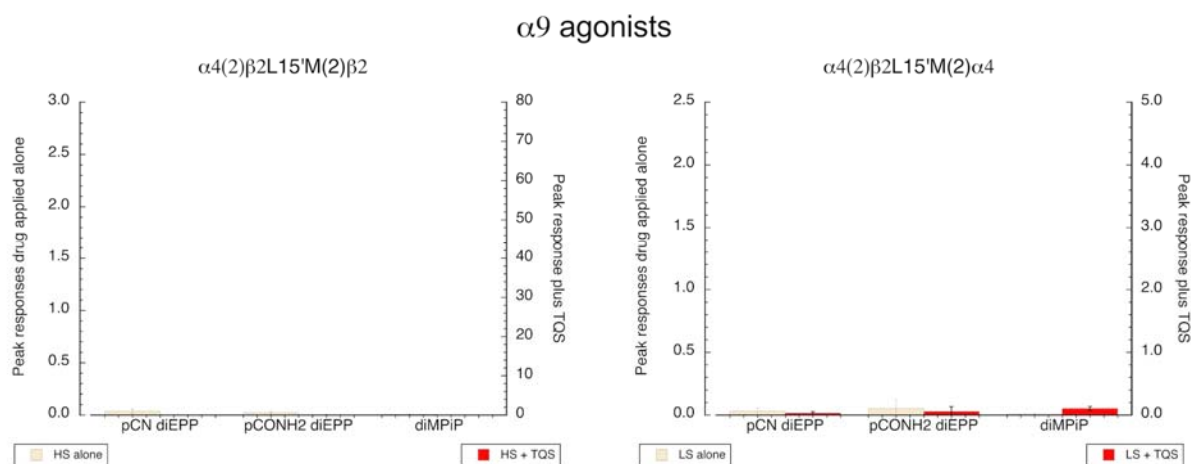
1a. Responses	HS activation	HS TQS	LS activation	LS TQS
	Average \pm SD	Average \pm SD	Average \pm SD	Average \pm SD
NS6740	0.013 \pm 0.004	0.222 \pm 0.128	0.006 \pm 0.003	0.043 \pm 0.037
n-MP	0.073 \pm 0.024	3.091 \pm 1.387	0.013 \pm 0.010	0.208 \pm 0.095
triEMA	0.008 \pm 0.006	3.017 \pm 0.847	0.007 \pm 0.007	0.023 \pm 0.024

1b. 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
NS6740	0.1975	0.0227*	0.7479	3.4665
n-MP	0.2234	0.2900	0.0013*	2.6369
triEMA	0.0279*	0.6943	0.0000 [†]	2.4169

*p<0.05 †P<0.001, p values less than 0.0001 are indicated as 0.0000

The type II $\alpha 7$ PAMs have been used to identify $\alpha 7$ -silent agonists which have been proposed to be useful activators of the cholinergic anti-inflammatory pathway (Horenstein and Papke, 2017)]. Three compounds in this class were tested for their ability to produce TQS-dependent activation of TQS-sensitive $\alpha 4 \beta 2$ receptors. One compound, NS6740 (Pismataro et al., 2020), is strongly desensitizing, inducing long-lived desensitization. In contrast, triethylmethyl ammonium (triEMA) is a structurally minimized silent agonist that produces only transient desensitization (Papke et al., 2014)]. A silent agonist produced during the roasting of coffee beans (Lang et al., 2013), 1-methylpyridinium (n-MP) (Papke and Horenstein, 2021), is used as a biomarker for coffee consumption. None of these compounds produced much activation of $\alpha 4 \beta 2$ receptors, and in co-application with TQS, only triEMA produced responses significantly larger than those produced by TQS alone, although these responses were still rather small.



Supplemental Data Figure 3. α_9 agonists

2a. Responses	HS activation	HS TQS	LS activation	LS TQS
	Average \pm SD	Average \pm SD	Average \pm SD	Average \pm SD
<i>p</i> CN diEPP	0.036 \pm 0.022	0.054 \pm 0.016	0.031 \pm 0.026	0.029 \pm 0.025
<i>p</i> CONH2 diEPP	0.023 \pm 0.010	0.064 \pm 0.020	0.052 \pm 0.069	0.056 \pm 0.079
diMPIP	0.007 \pm 0.003	0.016 \pm 0.009	0.008 \pm 0.005	0.104 \pm 0.032

2b. Corrected p values

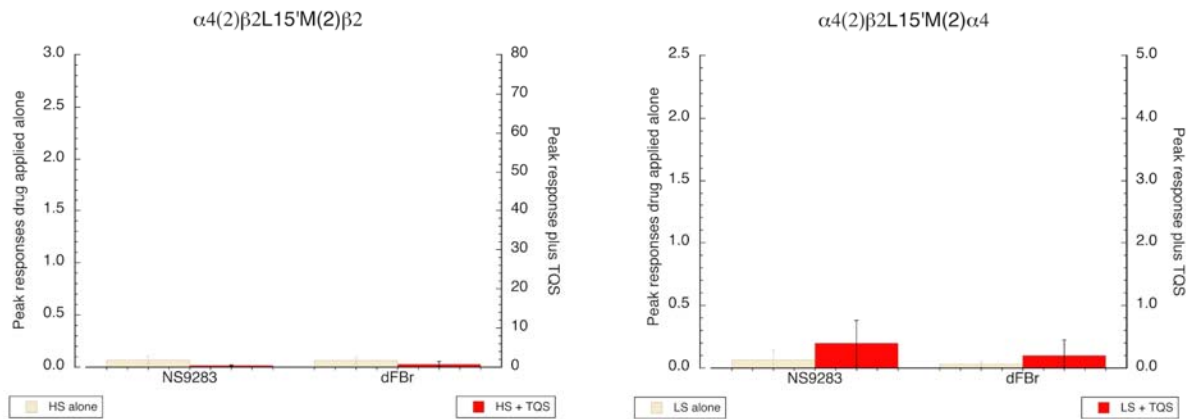
	Drug vs Drug plus TQS		Drug plus TQS vs TQS alone	
	p value HS	p value LS	p value HS	p value LS
<i>p</i> CN diEPP	0.2477	7.1317	0.0247 [#]	3.0222
<i>p</i> CONH2 diEPP	0.0150*	2.2441	0.0532	4.7463
diMPIP	0.1107	0.1350	0.0119 [#]	8.1796

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

* $p < 0.05$, however although drug plus TQS was greater than drug alone this was not greater than TQS alone.

Representative compounds from a structurally related group of compounds developed originally from the ganglionic agonist dimethyphenylpiperazinium (Manetti et al., 1999) but having a larger diethylphenylpiperazinium core and originally identified as α_7 -silent agonists (Quadri et al., 2016) were evaluated. Recently it has been shown that 4-(4-cyanophenyl)-1,1-diethylpiperazin-1-ium (*p*CN diEPP), 4-(4-carbamoylphenyl)-1,1-diethylpiperazin-1-ium (*p*CONH2 diEPP), and 1,1-diethyl-4-phenylpiperazin-1-ium (diMPIP) are α_9 -selective agonists (Papke et al. 2022). None of these compounds produced significant activation of the $\alpha_4\beta_2$ receptor either with or without TQS.

LS $\alpha 4\beta 2$ modulators



Supplemental Data Figure 4. $\alpha 4\beta 2$ modulators

3a. Responses	HS activation	HS TQS	LS activation	LS TQS
	Average \pm SD	Average \pm SD	Average \pm SD	Average \pm SD
NS9283	0.068 \pm 0.034	0.439 \pm 0.183	0.064 \pm 0.079	0.397 \pm 0.361
dFBr	0.064 \pm 0.030	0.779 \pm 0.655	0.031 \pm 0.023	0.199 \pm 0.248

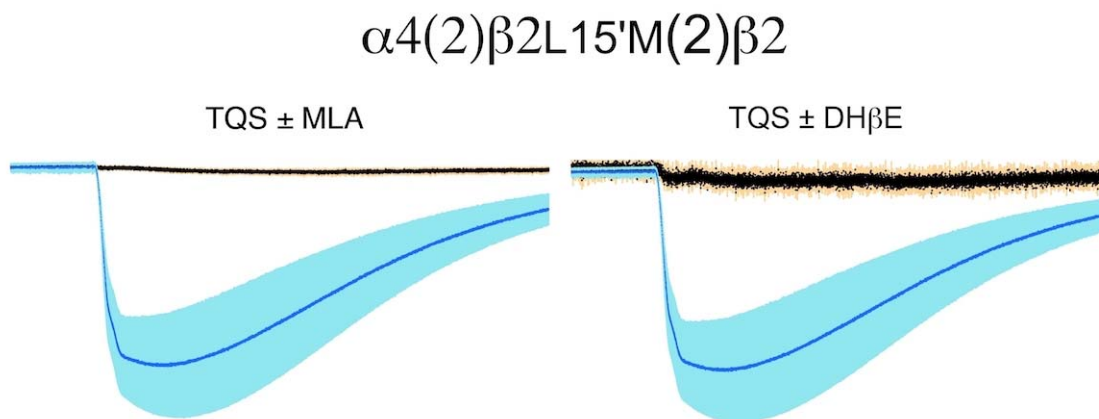
3b. Corrected p values

	Drug vs Drug plus TQS		Drug plus TQS vs TQS alone	
	p value HS	p value LS	p value HS	p value LS
NS9283	0.0159*	0.0759	3.9030	0.3840
dFBr	0.4264	0.3124	0.7878	2.0412

* $p < 0.05$, however although drug plus TQS was greater than drug alone this was not greater than TQS alone.

Another factor distinguishing HS and LS $\alpha 4\beta 2$ receptors is that there is a unique low affinity binding site at the $\alpha 4$ - $\alpha 4$ interface in LS receptors (Lucero et al., 2016). This site can be targeted by LS receptor-selective allosteric modulators including NS9283 (Timmermann et al., 2012; Wang and Lindstrom, 2017) and desformylflustrabromine (dFBr) (Kim et al., 2007). We tested whether either of the ligands could produce TQS-dependent activation of $\alpha 4\beta 2$ receptors with the $\beta 2L15'M$ mutations. No significant activation was observed when applied on their own; and when co-applied with TQS, responses were no larger than when TQS was applied alone.

Supplemental Figure 5 inhibition of responses to TQS alone with antagonists.



Effects on nAChR antagonists co-applied with TQS (black) to HS receptors compared to the averaged response to TQS alone (blue, from Figure 2).

ANOVA results and statistical analyses

Supplemental Figure 1

One Way ANOVA

Data Table: co-pre apply dots

Factor A: 4 Groups

HS pre apply, LS pre-apply, HS co-apply, LS co-apply

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	25	17328.882	693.1553		
A	3	12822.599	4274.1996	20.86695	< .0001
Error	22	4506.2835	204.83107		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
HS pre apply vs LS pre-apply	47.4364	5.9575	< .0001	24.357 to 70.516
HS pre apply vs HS co-apply	7.97196	1.0012	1	-15.107 to 31.051
HS pre apply vs LS co-apply	47.9923	6.2735	< .0001	25.818 to 70.166
LS pre-apply vs HS co-apply	-39.4645	4.776	0.0005	-63.415 to -15.514
LS pre-apply vs LS co-apply	0.555926	0.0698	1	-22.523 to 23.635
HS co-apply vs LS co-apply	40.0204	5.0262	0.0003	16.941 to 63.1

Figure 5A Normalized responses to TQS

Student t Test for unpaired data with equal variance

Group 1: HS normed

Group 2: LS normed

	Group 1	Group 2
Count	7	7
Mean	25.3005	2.403
Variance	246.75	0.572504
Std. Dev.	15.7083	0.75664
Std. Err	5.93717	0.285983

Mean Difference 22.8975
 Degrees of Freedom 12
 t Value 3.8522
 t Probability 0.002301

Figure 5B Responses to TQS without normalization

Student t Test for unpaired data with equal variance

Group 1: HS peak

Group 2: LS peak

	Group 1	Group 2
Count	7	7
Mean	-51.6683	-35.2803
Variance	238.404	269.346
Std. Dev.	15.4403	16.4118
Std. Err	5.8359	6.20307

Mean Difference -16.3881
 Degrees of Freedom 12
 t Value -1.9242
 t Probability 0.07837

Figure 7

Non-selective agonists

One Way ANOVA

Data Table: Comparisons of drugs applied alone normalized to ACh controls

One Way ANOVA

Data Table: New Dot plot data V2

Factor A: 14 Groups

nicotine hs, cotinine hs, nor-nicotine hs, anatabine hs, carbachol hs, epibatidine hs, anabaseine hs, nicotine ls,

cotinine ls, nor-nicotine ls, anatabine ls, carbachol ls, epibatidine ls, anabaseine ls

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	96	33.866232	0.35277325		
A	13	31.636041	2.4335416	90.568012	< .0001
Error	83	2.2301909	0.02686977		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
nicotine hs vs nicotine ls	-0.264985	2.9056	0.4274	-0.59283 to 0.062863
cotinine hs vs cotinine ls	0.004371	0.0494	1	-0.31388 to 0.32262
nor-nicotine hs vs nor-nicotine ls	0.0843834	0.903	1	-0.25156 to 0.42033
anatabine hs vs anatabine ls	0.0765322	0.7974	1	-0.26852 to 0.42158
carbachol hs vs carbachol ls	0.123573	1.3959	1	-0.19468 to 0.44182
epibatidine hs vs epibatidine ls	-1.43324	17.4871	< .0001	-1.7279 to -1.1386
anabaseine hs vs anabaseine ls	0.138836	1.6365	1	-0.16615 to 0.44382

T-test epibatidine

Student t Test for unpaired data with equal variance

Group 1: epibatidine hs

Group 2: epibatidine ls

	Group 1	Group 2
Count	8	8
Mean	0.699389	2.13263
Variance	0.00375492	0.299057
Std. Dev.	0.0612774	0.546861
Std. Err	0.0216648	0.193344

Mean Difference	-1.43324
Degrees of Freedom	14
t Value	-7.3668
t Probability	< .0001

TQS effects

One Way ANOVA

Data Table: HS receptors non-selective agonists TQS effects

Factor A: 14 Groups

nicotine, nicotine+, cotinine, cotinine+, nor-nicotine, nor-nicotine+, anatabine, anatabine+, carbachol, carbachol+

, epibatidine, epibatidine+, anabaseine, anabaseine+

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	84	14123.836	168.14091		
A	13	11379.711	875.36241	22.648653	< .0001
Error	71	2744.1249	38.649646		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
nicotine vs nicotine+	-25.3815	7.3383	< .0001	-37.901 to -12.862
cotinine vs cotinine+	-1.64163	0.4574	1	-14.633 to 11.35
nor-nicotine vs nor-nicotine+	-8.30685	2.1127	1	-22.538 to 5.9248
anatabine vs anatabine+	-22.2065	5.6478	< .0001	-36.438 to -7.9749
carbachol vs carbachol+	-24.8206	6.9151	< .0001	-37.812 to -11.829
epibatidine vs epibatidine+	-28.0311	8.3488	< .0001	-40.184 to -15.878
anabaseine vs anabaseine+	-22.3352	6.7213	< .0001	-34.363 to -10.307

One Way ANOVA

Data Table: Non selective agonists LS receptors TQS effects

Factor A: 14 Groups

nicotine, nicotine+, cotinine, cotinine+, nor-nicotine, nor-nicotine+, anatabine, anatabine+, carbachol, carbachol+

, epibatidine, epibatidine+, anabaseine, anabaseine+

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	105	264.90003	2.5228575		
A	13	221.07531	17.005793	35.699779	< .0001
Error	92	43.824724	0.47635569		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
------------	-----------------	---	---	--------

nicotine vs nicotine+	-3.91539	10.5043	< .0001	-5.2501 to -2.5807
cotinine vs cotinine+	-0.066673	0.1932	1	-1.3024 to 1.1691
nor-nicotine vs nor-nicotine+	-2.38236	6.9036	< .0001	-3.6181 to -1.1466
anatabine vs anatabine+	-1.51925	4.1181	0.0076	-2.8403 to -0.1982
carbachol vs carbachol+	-1.94806	5.645	< .0001	-3.1838 to -0.71233
epibatidine vs epibatidine+	-1.74793	4.6894	0.0009	-3.0827 to -0.41318
anabaseine vs anabaseine+	-1.74758	5.0641	0.0002	-2.9833 to -0.51185

Figure 8A

Activation by drug alone

One Way ANOVA

Data Table: New Dot plot data V2

Factor A: 8 Groups

TC2403 hs, cytisine hs, varenicline hs, arecoline hs, TC-2403 ls, cytisine ls, varenicline ls, arecoline ls

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	56	1.0112826	0.018058617		
A	7	0.83911568	0.11987367	34.116956	< .0001
Error	49	0.17216688	0.0035136098		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
TC2403 hs vs TC-2403 ls	-0.248784	8.1095	< .0001	-0.35015 to -0.14742
cytisine hs vs cytisine ls	-0.114117	3.5647	0.0231	-0.21989 to -0.008343
varenicline hs vs varenicline ls	-0.262664	8.2901	< .0001	-0.36735 to -0.15798
arecoline hs vs arecoline ls	0.0327062	1.0323	1	-0.071983 to 0.13739

t-tests

Student t Test for unpaired data with equal variance

Group 1: TC2403 hs

Group 2: TC-2403 ls

	Group 1	Group 2
Count	8	7
Mean	0.0450739	0.293858
Variance	0.000107237	0.0133611
Std. Dev.	0.0103555	0.11559
Std. Err	0.00366124	0.0436889

Mean Difference	-0.248784
Degrees of Freedom	13
t Value	-6.0929
t Probability	< .0001

Student t Test for unpaired data with equal variance

Group 1: varenicline hs

Group 2: varenicline ls

	Group 1	Group 2
Count	7	7
Mean	0.109412	0.372076
Variance	0.000180956	0.00869126
Std. Dev.	0.013452	0.0932269
Std. Err	0.00508438	0.0352365

Mean Difference	-0.262664
Degrees of Freedom	12

t Value -7.3779
t Probability < .0001

One Way ANOVA of TQS effects

Data Table: Partial agonists **HS receptors TQS effects**

Factor A: 8 Groups

TC-2403, TC2403+, cytisine, cytisine+, varenicline, varenicline+, arecoline, arecoline+

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	55	6407.5636	116.50116		
A	7	5063.1105	723.3015	25.82349	< .0001
Error	48	1344.4531	28.00944		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
TC-2403 vs TC2403+	-12.7025	4.8003	0.0004	-21.456 to -3.9486
cytisine vs cytisine+	-13.9386	4.5617	0.001	-24.047 to -3.8306
varenicline vs varenicline+	-27.3777	9.6779	< .0001	-36.736 to -18.019
arecoline vs arecoline+	-14.074	4.9751	0.0002	-23.432 to -4.7158

One Way ANOVA

Data Table: Partial agonists **LS receptors TQS effects**

Factor A: 8 Groups

TC-2403, TC2403+, cytisine, cytisine+, varenicline, varenicline+, arecoline, arecoline+

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	57	62.189463	1.0910432		
A	7	57.043089	8.1490127	79.17238	< .0001
Error	50	5.1463735	0.10292747		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
TC-2403 vs TC2403+	-1.73332	10.1076	< .0001	-2.2993 to -1.1674
cytisine vs cytisine+	-2.21021	13.7784	< .0001	-2.7396 to -1.6808
varenicline vs varenicline+	-2.1412	12.4861	< .0001	-2.7072 to -1.5752
arecoline vs arecoline+	-0.768306	4.4803	0.0012	-1.3343 to -0.20234

Figure 8B

One Way ANOVA

Data Table: **Comparison of activation by HS-selective agonists**

Factor A: 4 Groups

sazetidine-A hs, TC-2559 hs, sazetidine-A ls, TC-2559 ls

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	27	25.977559	0.9621318		
A	3	25.609068	8.5363562	555.97845	< .0001
Error	24	0.36849009	0.015353754		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
sazetidine-A hs vs sazetidine-A ls	1.20093	18.132	< .0001	1.0105 to 1.3914
TC-2559 hs vs TC-2559 ls	2.34347	35.0194	< .0001	2.1511 to 2.5359

T-tests

Student t Test for unpaired data with equal variance

Group 1: sazetidine-A hs

Group 2: sazetidine-A ls

	Group 1	Group 2
Count	7	7
Mean	1.2528	0.0518704
Variance	0.0231387	0.000188514
Std. Dev.	0.152114	0.01373
Std. Err	0.0574937	0.00518946
Mean Difference	1.20093	
Degrees of Freedom	12	
t Value	20.803	
t Probability	< .0001	

Student t Test for unpaired data with equal variance

Group 1: TC-2559 hs

Group 2: TC-2559 ls

	Group 1	Group 2
Count	6	8
Mean	2.48889	0.145427
Variance	0.0363593	0.00667576
Std. Dev.	0.190681	0.0817053
Std. Err	0.0778453	0.0288872
Mean Difference	2.34347	
Degrees of Freedom	12	
t Value	31.444	
t Probability	< .0001	

One Way ANOVA for TQS effects

Data Table: HS selective HS receptors TQS effects

Factor A: 4 Groups

sazetidine-A, sazetidine-A +, TC-2559, TC-2559 +

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	25	40102.538	1604.1015		
A	3	32702.814	10900.938	32.409403	< .0001
Error	22	7399.724	336.35109		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
sazetidine-A vs sazetidine-A +	-68.3893	6.9763	< .0001	-96.804 to -39.975
TC-2559 vs TC-2559 +	-73.5716	6.9482	< .0001	-104.26 to -42.88

One Way ANOVA

Data Table: HS selective LS receptors TQS effects

Factor A: 4 Groups

sazetidine-A, sazetidine-A +, TC-2559, TC-2559 +

Analysis of Variance Results

Source	DF	SS	MS	F	P
--------	----	----	----	---	---

Total	29	88.221166	3.0421092		
A	3	80.22215	26.740717	86.918017	< .0001
Error	26	7.9990163	0.30765447		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
sazetidine-A vs sazetidine-A +	-3.63613	12.2643	< .0001	-4.4827 to -2.7895
TC-2559 vs TC-2559 +	-2.88787	10.413	< .0001	-3.6798 to -2.0959

Figure 9

α 7-selective agonists ANOVA results

HS drugs alone

One Way ANOVA

Data Table: □□ agonist dot-plot data

Factor A: 7 Groups

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

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	57	10.322772		0.18110127	
A	6	9.757436		1.6262393	146.70596 < .0001
Error	51	0.56533632		0.011085026	

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
ACh vs TQS	0.532336	10.9225	< .0001	0.37649 to 0.68818
ACh vs GTS-21	0.896546	18.3953	< .0001	0.7407 to 1.0524
ACh vs PNU282987	0.985603	21.1218	< .0001	0.83639 to 1.1348
ACh vs TC-1698	0.987236	21.1568	< .0001	0.83802 to 1.1364
ACh vs AR-R17779	0.991877	20.3513	< .0001	0.83603 to 1.1477
ACh vs Choline	0.977632	20.059	< .0001	0.82178 to 1.1335
TQS vs GTS-21	0.36421	6.4717	< .0001	0.18425 to 0.54417
TQS vs PNU282987	0.453266	8.3183	< .0001	0.27902 to 0.62751
TQS vs TC-1698	0.454899	8.3482	< .0001	0.28066 to 0.62914
TQS vs AR-R17779	0.45954	8.1656	< .0001	0.27958 to 0.6395
TQS vs Choline	0.445295	7.9125	< .0001	0.26534 to 0.62525
GTS-21 vs PNU282987	0.0890563	1.6343	1	-0.085187 to 0.2633
GTS-21 vs TC-1698	0.0906895	1.6643	1	-0.083554 to 0.26493
GTS-21 vs AR-R17779	0.0953301	1.6939	1	-0.084628 to 0.27529
GTS-21 vs Choline	0.0810851	1.4408	1	-0.098873 to 0.26104
PNU282987 vs TC-1698	0.00163321	0.031	1	-0.1667 to 0.16997
PNU282987 vs AR-R17779	0.00627389	0.1151	1	-0.16797 to 0.18052
PNU282987 vs Choline	-0.00797114	0.1463	1	-0.18221 to 0.16627
TC-1698 vs AR-R17779	0.00464068	0.0852	1	-0.1696 to 0.17888
TC-1698 vs Choline	-0.00960435	0.1763	1	-0.18385 to 0.16464
AR-R17779 vs Choline	-0.014245	0.2531	1	-0.1942 to 0.16571

HS receptors, drugs co-applied with TQS

One Way ANOVA

Data Table: $\alpha 7$ agonist dot-plot data

Factor A: 7 Groups

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

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	48	10962.162		228.37837	
A	6	9207.9551		1534.6592	36.743495 < .0001
Error	42	1754.2067	41.766826		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
ACh vs TQS	35.7489	9.4469	< .0001	23.511 to 47.986
ACh vs GTS-21	1.70277	0.45	1	-10.535 to 13.94
ACh vs PNU282987	35.6508	9.6763	< .0001	23.736 to 47.565
ACh vs TC-1698	29.0194	7.8765	< .0001	17.105 to 40.934
ACh vs AR-R17779	33.1994	8.7732	< .0001	20.962 to 45.437
ACh vs Choline	23.5145	6.2139	< .0001	11.277 to 35.752
TQS vs GTS-21	-34.0462	9.8557	< .0001	-45.217 to -22.875
TQS vs PNU282987	-0.0981732	0.0294	1	-10.915 to 10.718
TQS vs TC-1698	-6.72948	2.0119	1	-17.546 to 4.0871
TQS vs AR-R17779	-2.5495	0.738	1	-13.721 to 8.6218
TQS vs Choline	-12.2345	3.5416	0.0208	-23.406 to -1.0632
GTS-21 vs PNU282987	33.948	10.1495	< .0001	23.131 to 44.765
GTS-21 vs TC-1698	27.3167	8.167	< .0001	16.5 to 38.133
GTS-21 vs AR-R17779	31.4967	9.1176	< .0001	20.325 to 42.668
GTS-21 vs Choline	21.8117	6.314	< .0001	10.64 to 32.983
PNU282987 vs TC-1698	-6.63131	2.0522	0.9748	-17.081 to 3.8185
PNU282987 vs AR-R17779	-2.45132	0.7329	1	-13.268 to 8.3652
PNU282987 vs Choline	-12.1363	3.6284	0.0161	-22.953 to -1.3197
TC-1698 vs AR-R17779	4.17998	1.2497	1	-6.6366 to 14.997
TC-1698 vs Choline	-5.50499	1.6458	1	-16.322 to 5.3116
AR-R17779 vs Choline	-9.68497	2.8036	0.16	-20.856 to 1.4863

LS receptors drugs applied alone

One Way ANOVA

Data Table: $\alpha 7$ agonist dot-plot data

Factor A: 7 Groups

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

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	55	10.314108		0.18752923	
A	6	9.9491385		1.6581897	222.62514 < .0001
Error	49	0.36496911		0.0074483491	

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
ACh vs TQS	0.885976	22.1766	< .0001	0.75795 to 1.014
ACh vs GTS-21	0.988008	24.7306	< .0001	0.85998 to 1.116
ACh vs PNU282987	0.991018	25.9089	< .0001	0.86844 to 1.1136
ACh vs TC-1698	0.991181	24.81	< .0001	0.86316 to 1.1192
ACh vs AR-R17779	0.992833	22.081	< .0001	0.84875 to 1.1369
ACh vs Choline	0.976113	25.5192	< .0001	0.85354 to 1.0987
TQS vs GTS-21	0.102032	2.2118	0.6652	-0.045797 to 0.24986
TQS vs PNU282987	0.105042	2.3517	0.4778	-0.038093 to 0.24818
TQS vs TC-1698	0.105205	2.2806	0.5662	-0.042624 to 0.25303
TQS vs AR-R17779	0.106857	2.1145	0.8312	-0.055082 to 0.2688
TQS vs Choline	0.0901374	2.018	1	-0.052998 to 0.23327
GTS-21 vs PNU282987	0.00300982	0.0674	1	-0.14013 to 0.14614
GTS-21 vs TC-1698	0.00317303	0.0688	1	-0.14466 to 0.151
GTS-21 vs AR-R17779	0.00482441	0.0955	1	-0.15711 to 0.16676
GTS-21 vs Choline	-0.0118948	0.2663	1	-0.15503 to 0.13124
PNU282987 vs TC-1698	0.000163218	0.0037	1	-0.14297 to 0.1433
PNU282987 vs AR-R17779	0.00181459	0.0369	1	-0.15585 to 0.15948
PNU282987 vs Choline	-0.0149047	0.3454	1	-0.15319 to 0.12338
TC-1698 vs AR-R17779	0.00165137	0.0327	1	-0.16029 to 0.16359
TC-1698 vs Choline	-0.0150679	0.3373	1	-0.1582 to 0.12807
AR-R17779 vs Choline	-0.0167193	0.3398	1	-0.17438 to 0.14095

LS receptors drugs co-applied with TQS

One Way ANOVA

Data Table: $\alpha 7$ agonist dot plot data

Factor A: 7 Groups

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

Analysis of Variance Results

Source	DF	SS	MS	F	P
Total	49	33.227677		0.67811585	
A	6	31.594456	5.2657427	138.63834	< .0001
Error	43	1.6332202	0.037981866		

Bonferroni's All Pairs Comparison

Comparison	Mean Difference	t	P	95% CL
ACh vs TQS	2.43219	23.3477	< .0001	2.0958 to 2.7686
ACh vs GTS-21	1.79287	17.2105	< .0001	1.4565 to 2.1292
ACh vs PNU282987	2.40405	23.8343	< .0001	2.0783 to 2.7297
ACh vs TC-1698	2.00158	19.8442	< .0001	1.6759 to 2.3273
ACh vs AR-R17779	2.53905	22.2498	< .0001	2.1706 to 2.9075
ACh vs Choline	1.66108	16.4683	< .0001	1.3354 to 1.9868
TQS vs GTS-21	-0.639322	6.1371	< .0001	-0.9757 to -0.30294
TQS vs PNU282987	-0.0281432	0.279	1	-0.35384 to 0.29756
TQS vs TC-1698	-0.430609	4.2692	0.0022	-0.75631 to -0.10491
TQS vs AR-R17779	0.106857	0.9364	1	-0.26163 to 0.47534
TQS vs Choline	-0.771114	7.645	< .0001	-1.0968 to -0.44542
GTS-21 vs PNU282987	0.611179	6.0594	< .0001	0.28548 to 0.93688
GTS-21 vs TC-1698	0.208713	2.0692	0.9359	-0.11699 to 0.53441
GTS-21 vs AR-R17779	0.746179	6.5388	< .0001	0.37769 to 1.1147
GTS-21 vs Choline	-0.131792	1.3066	1	-0.45749 to 0.19391
PNU282987 vs TC-1698	-0.402466	4.1302	0.0034	-0.71712 to -0.087811
PNU282987 vs AR-R17779	0.135	1.2151	1	-0.22376 to 0.49376
PNU282987 vs Choline	-0.742971	7.6245	< .0001	-1.0576 to -0.42832
TC-1698 vs AR-R17779	0.537466	4.8375	0.0004	0.1787 to 0.89623
TC-1698 vs Choline	-0.340505	3.4943	0.0234	-0.65516 to -0.02585
AR-R17779 vs Choline	-0.877971	7.9022	< .0001	-1.2367 to -0.51921