Insights Into the Differential Desensitization of α4β2 Nicotinic Acetylcholine Receptor Isoforms Obtained With Positive Allosteric Modulation of Mutant Receptors[S]

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ABSTRACT

The development of highly efficacious positive allosteric modulators (PAMs) of α7 nicotinic acetylcholine receptors (nAChR) has proven useful in defining the ligand dependence of the conformational dynamics of α7 receptors. No such effective modulators are known to exist for the α4β2 nAChR of the brain, limiting our ability to understand the importance of desensitization for the activity profile of specific ligands. In this study, we used mutant β2 subunits that allowed the use of the α7 PAM 3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3H-cyclopentan[c]quinoline-8-sulfonamide (TQS) to probe the desensitizing effects of nicotinic ligands on the two forms of α4β2 receptors; high sensitivity (HS) (two α4 and three β2 subunits) and low sensitivity (LS) (three α4 and two β2 subunits). A total of 28 different ligands of 8 different categories, based on activity and selectivity, were tested for their ability to induce TQS-sensitive desensitization of HS and LS α4β2 receptors. Results confirm that HS α4β2 receptor responses are strongly limited by desensitization, by at least an order of magnitude more so than the responses of LS receptors. The activation of α4β2 receptors by the smoking-cessation drugs cytisine and varenicline is strongly limited by desensitization, as is the activation

of LS receptors by the HS-selective agonists 6-[5-[(2S)-2-Azetidinylmethoxy]-3-pyridinyl]-5-hexyn-1-ol dihydrochloride and 4-(5-ethoxy-3-pyridinyl)-N-methyl-(3E)-3-buten-1-amine difumarate. The evaluation of drugs previously identified as α7-selective agonists revealed varying patterns of α4β2 cross-desensitization that were predictive of the effects of these drugs on the activation of wild-type α4β2 receptors by acetylcholine, supporting the utility of TQS-sensitive receptors for the development of focused therapeutics.

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

The two configurations of α4β2 nAChR differ greatly in their functional properties, with one notable difference being that

ABBREVIATIONS: ACh, acetylcholine; HS, high sensitivity; LS, low sensitivity; MLA, methyllycaconitine citrate; nAChR, nicotinic acetylcholine receptor; PAM, positive allosteric modulators.

receptors with the α4(2)β2(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 α4(3)β2(2) configuration, in general, generate larger currents across a wider range of concentration; these are known as the low sensitivity (LS) subtype (Nelson et al., 2003; López-Hernández et al., 2004; Eaton et al., 2014). 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 use 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 that reverses desensitization selectively increases the response of HS receptors compared with LS receptors.

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

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

Materials and Methods

Acetylcholine chloride, atropine, choline, methyllycaconitine citrate (MLA), dihydro-b-erythrodine hydrobromide, N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide (PNU-282987), cytisine, arecoline, nicotine, cotinine, and other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). TQS, 2-(3-Pyridinyl)-1-azabicyclo[3.2.2]nonane dihydrochloride (TC-1698), (3S)-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, varenicline, 6-[5-[(2S)-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

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-ethoxy-3-pyridinyl)-N-methyl-(3E)-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 α4β2 receptors independently of each other in Xenopus oocytes. One approach has been to inject the α4 and β2 RNA at ratios that would favor the assembly of LS or HS receptors (usually 10:1, α4 to β2 for LS receptors or 1:10 α4 to β2 for HS receptors) (Zwart et al., 2008). However, this approach generates a heterogeneous population of receptors and if applied to the present study would give an unequal number of mutant β subunits in the LS and HS biased population. The alternative approach is to use linked α4−β2 subunits (Zhou et al., 2003), which permits the coexpression of the concatamer with monomeric α4 or β2 subunit to yield pure populations of defined subunit composition; furthermore, 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 laboratory, indicate that the mutation did not disrupt the function of the concatamers. The human nAChR clones and the original β2−6−α4 concatamer were obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia, PA). The β2 L15'M mutant in the concatamer was made as 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 to 20 minutes 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 hours at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO3, 0.82 mM MgSO4, 15 mM HEPES, and 12 mg/l tetracycline, pH 7.6) to remove the follicular layer. Stage V oocytes were subsequently isolated and injected with 50 nl water containing 5 ng concatamer plus 5 ng α4 or β2 nAChR subunit cRNA. Recordings were carried out 2 to 7 days after injection.

Two-Electrode Voltage Clamp Electrophysiology. Experiments were conducted at room temperature (24°C) using OpusXpress 6000A

(Molecular Devices, Union City, CA) (Papke and Stokes, 2010). Both the voltage and current electrodes were filled with 3 M KCl. Oocytes were voltage-clamped at −60 mV. The oocytes were bath-perfused with Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 10 mM HEPES, and 1 μM atropine, pH 7.2) at 4 ml/min. Drug applications were 6 seconds in duration followed by 241-second 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 coapplication of the test compound with 30 μM racemic TQS, and a final control application of ACh. The control ACh concentrations were 10 μM for HS receptors and 100 μM for LS receptors. The concentrations of the test compounds are provided in Table 1. All experiments began with eight 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 ± S.D. 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 coapplied 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 ± S.D. 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

TABLE 1
Test compounds concentrations and n values

Drug	Concentration	n_{HS}	n_{LS}
Antagonists			
MLA	100 μM	8	4
DHβE	10 μM	8	7
α7 silent agonists			
NS6740	30 μM	8	8
n-MP	300 μM	7	8
triEMA	100 μM	7	8
α9 agonists			
pCN diEPP	100 μM	8	7
pCONH2-diEPP	100 μM	7	7
diMPiP[a]	100 μM	8	7
α4β2 modulators			
NS9283[b]	30 μM	8	8
dFBr	30 μM	8	7
Non-selective agonists			
Carbachol	100 μM	6	8
Epibatidine	3 μM	8	6
Anabaseine	100 μM	7	8
Nicotine	10 μM	7	8
Cotinine	100 μM	6	8
Nor-nicotine	10 μM	5	6
Anatabine	100 μM	4	8
HS selective agonists			
sazetidine-A	30 μM	7	7
TC-2559	30 μM	6	8
α4β2 partial agonists			
TC-2403	10 μM	8	8
cytisine	100 μM	6	6
varenicline	10 μM	7	7
arecoline	100 μM	8	7
α7-selective agonists			
GTS-21	100 μM	7	7
PNU-282987	10 μM	8	8
TC-1698	10 μM	8	8
AR-R17779	10 μM	7	5
choline	1 mM	7	8

[a]1,1-dimethylpiperidinium.
[b]3-[3-(3-Pyridinyl)-1,2,4-oxadiazol-5-yl]benzonitrile 3-[3-(3-Pyridinyl)-1,2,4-oxadiazol-5-yl]benzonitrile.
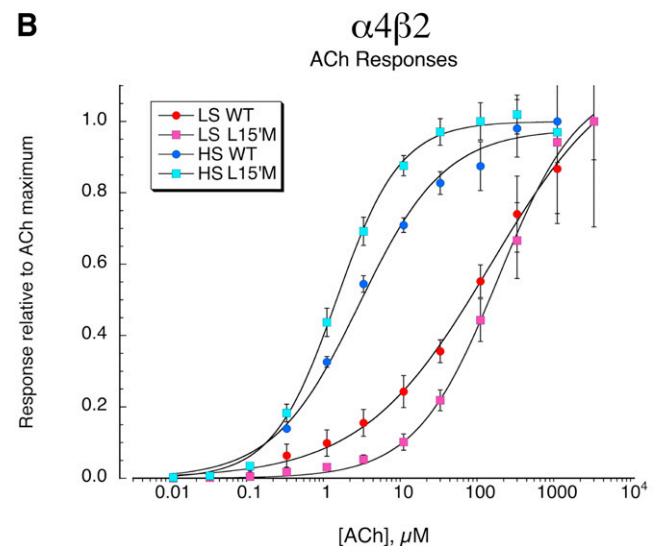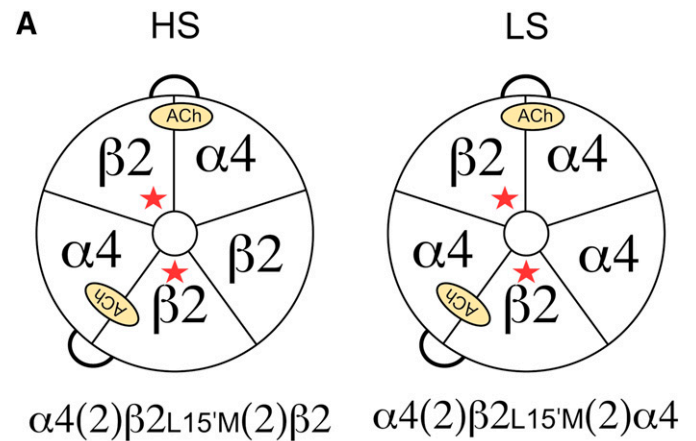
Fig. 1. TQS-sensitive HS and LS α4β2 receptors. (A) Subunit configuration of the receptors composed of α4−β2 concatamers with the β2L15'M mutation (represented by the star) in the β subunits. When coexpressed with wild-type β2 subunits, they yield HS α4(2)β2L15'M(2)β2 receptors (left). When coexpressed with wild-type α4 subunits, they yield LS α4(2)β2L15'M(2)α4 receptors (right). (B) ACh concentration-response data for wild-type (circles) and mutant (squares) receptors. Points represent the average of four to eight oocyte responses at each concentration (± S.D.), normalized to preceding control ACh responses obtained from the same cells. ACh controls were 10 μM for the HS receptors and 100 μM for the LS receptors. The Levenberg-Marquardt algorithm was used in Kaleidagraph to generate curves based on the Hill equation that best fit the data.

Fig. 2. Averaged data from HS $\alpha4(2)\beta2L15'M(2)\beta2$ receptors (left) and LS $\alpha4(2)\beta2L15'M(2)\alpha4$ receptors (right). (A) Cells were treated with control applications of ACh (red bars) and then after washout ACh coapplied with 30 μM TQS (blue bars) and then another application of ACh. ACh controls were 10 μM for the HS receptors and 100 μM for the LS receptors. The data are the averages of seven cells for each receptor subtype. Scale bars are based on the average initial ACh controls that were used for normalization (see *Materials and Methods*). (B) Averaged responses of HS and LS receptors to ACh and then 30 μM TQS applied alone, followed by another ACh application. The data are the averages of eight cells for the HS configuration and seven cells for the LS configuration. (C) Superimposition of the ACh preapplication controls and the responses to TQS alone taken from the traces in (B).
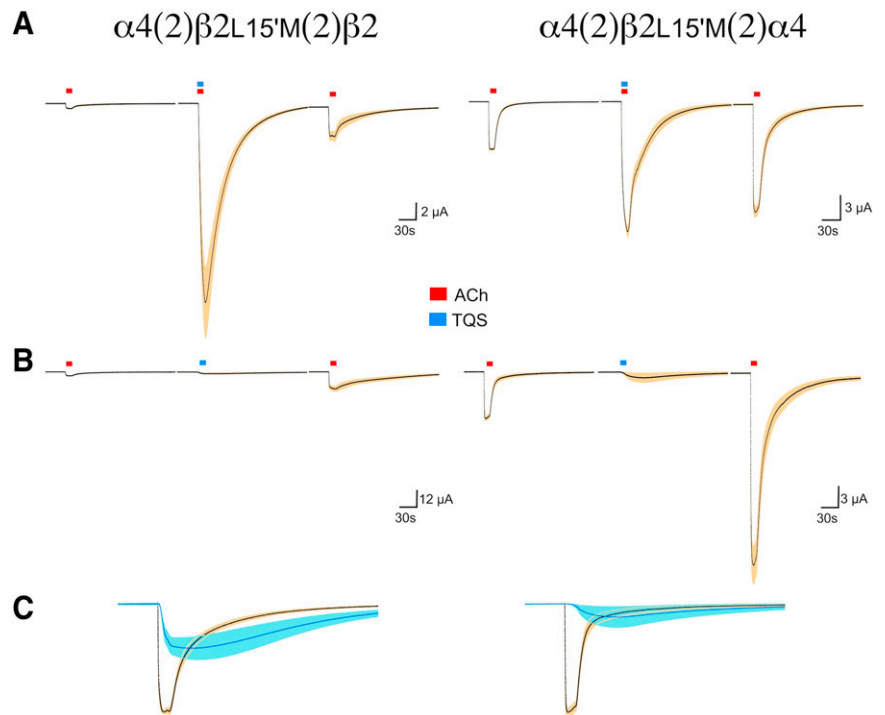
plotting program. The errors reported for the fit parameters are based on the goodness of fit.

We display multicell 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-second 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 multicell 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 $\alpha4\beta2$ nAChR. The L15'M mutation (Stokes et al., 2019) was made in the $\beta2$ subunit of the $\beta2 - 6 - \alpha4$ concatamer (Zhou et al., 2003), so that by coexpressing this concatamer with monomers of either $\beta2$ or $\alpha4$, we could obtain receptors with the subunit configuration shown in Fig. 1A. These receptors show the expected differences in ACh sensitivity previously reported for wild-type HS and LS receptors (Fig. 1B). For the HS receptors, the Log ACh EC_{50} values were 0.431 μM (Log error = $-.39$) and 0.11 μM (Log error = $-.1$) for the wild-type and mutant receptors, respectively. For the LS receptors, the ACh Log EC_{50} values were 2.13 μM (Log error = 1.39) and 2.27 μM (Log error = 1.27) for the wild-type and mutant receptors, respectively.

As expected, the ACh responses of oocytes expressing these constructs were strongly potentiated by coapplication of ACh with 30 μM TQS (Fig. 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). We compared responses obtained with our coapplication protocol to responses obtained when TQS was preapplied for 30 seconds prior to the coapplication of ACh and TQS. Responses were essentially identical with or without preapplications (Supplemental Fig. 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 $\alpha7$ ago-PAM GAT107 (Papke et al., 2014b) and was observed with the TQS-sensitive $\alpha4\beta2$ receptors, regardless of the test compound initially coapplied with

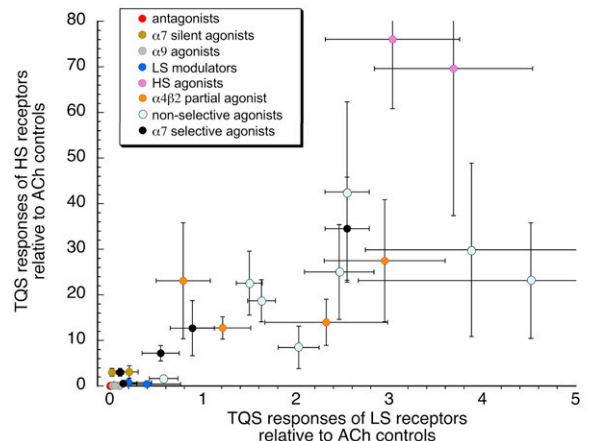**Fig. 3.** The TQS potentiated responses to all of the test compounds. The HS receptor responses, normalized to their ACh controls, are plotted relative to the scale on the *y*-axis. The LS receptor responses, normalized to their ACh controls, are plotted relative to the scale on the *x*-axis. All points are averages ($\pm$ S.D.). The *n* values are provided in Table 1. The various classes of drugs are color-coded as indicated.

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 (Fig. 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 (Fig. 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 coapplication 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 coapplied 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 (Fig. 3), consistent with TQS-sensitive desensitization as a factor limiting HS receptor responses.

Dynamic Conversion of Steady-State Desensitization to PAM-Potentiated Currents. To promote a progression toward steady-state desensitization, we preapplied 30 μM nicotine to HS and LS $\alpha 4\beta 2$L15'M receptors and then coapplied 30 μM nicotine and 30 μM TQS (Fig. 4A). The upper traces show the averaged responses (see *Materials and Methods*) of seven cells of each type, normalized to their initial ACh controls (not shown). The average peak amplitude of the HS 10 μM ACh controls was 2.47 μA (S.D. = 1.03), while the average peak amplitude of the LS 100 μM ACh controls was 15.9 μA (S.D. = 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 (S.D. = 67 nA), while the peak of the LS nicotine response was 982 nA (S.D. = 283 nA). A comparison of the normalized responses to the TQS coapplications (Fig. 4B) would indicate a larger TQS response for the HS receptors ($P < 0.01$). However, comparison of the responses without normalization (Fig. 4C) is consistent with TQS selectively increasing the HS response up to the level of the LS TQS responses (see Supplemental Material for statistics). Note that both experiments were conducted on the same day with cells from the same injection set.
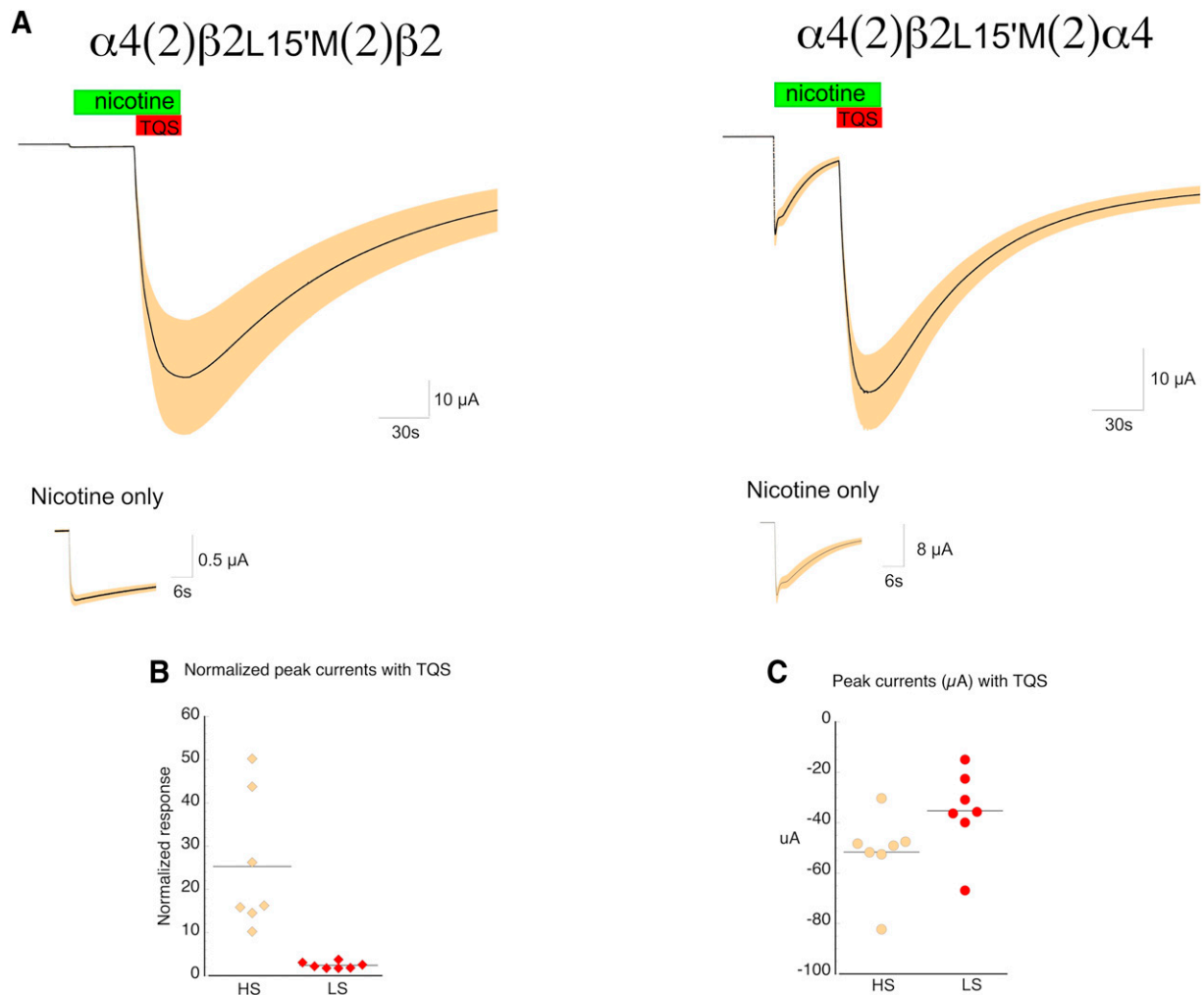
Fig. 4. (A) Averaged raw data traces ($n = 7$ in each case) of HS (on left) and LS (on right) $\alpha 4\beta 2$L15'M receptors to a 30-second preapplication of 30 μM nicotine followed by a coapplication of 30 μM nicotine and 30 μM TQS. The nicotine-only phases of the responses are shown as inserts below the main traces, scaled as indicated. (B) Peaks of TQS responses normalized to the initial ACh controls (see Supplemental Material for statistics). (C) Peaks of TQS responses measured in μAmps (see Supplemental Material for statistics).

Inactive Compounds. Since one of our goals was to probe compounds that would be equivalent to silent agonists (i.e., give currents only when coappled with the PAM), we tested three classes of compounds that were not expected to activate $\alpha4\beta2$ receptors when applied alone. These were the $\alpha7$ silent agonists NS6740, 1-methylpyridinium (Papke et al., 2022b), and triethylmethylammonium (Papke et al., 2014a) (Supplemental Fig. 2); the $\alpha9$-selective agonists pCN diEPP, pCONH2-diEPP, and 1,1-dimethylpiperidinium (Papke et al., 2022a) (Supplemental Fig. 3); and the LS $\alpha4\beta2$ modulators, 3-[3-(3-Pyridinyl)-1,2,4-oxadiazol-5-yl]benzonitrile (Wang et al., 2015) and dFBr (Weltzin and Schulte, 2010) (Supplemental Fig. 4). As expected, none of these compounds produced activation of either $\alpha4\beta2$ receptor when applied alone, and when coappled 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 $\alpha7$-selective antagonist methyllycaconitine (MLA) (Turek et al., 1995) and the $\alpha4\beta2$-selective antagonist dihydro-β-erythroidine (DHβE) (Damaj et al., 1995) were tested applied alone and in coapplication 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 coappled 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 Fig. 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 coapplication of TQS with the antagonists generated no responses, subsequent responses to ACh alone were primed by the coapplications (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., $> 10~\mu M$) 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, coapplications of TQS with ACh were conducted across a wide range of ACh concentrations (Fig. 5A). The TQS-potentiated ACh responses showed a concentration sensitivity that was similar to, or even greater than the responses to ACh alone, with an EC_{50} of 125 ± 22 nM for ACh plus TQS compared with $1.39 \pm 0.07~\mu M$ 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 $\alpha4(3)\beta2(2)$ configuration are characterized as low sensitivity, the L15'M receptors with this configuration were also tested with 11 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 7 of the 11 compounds tested at
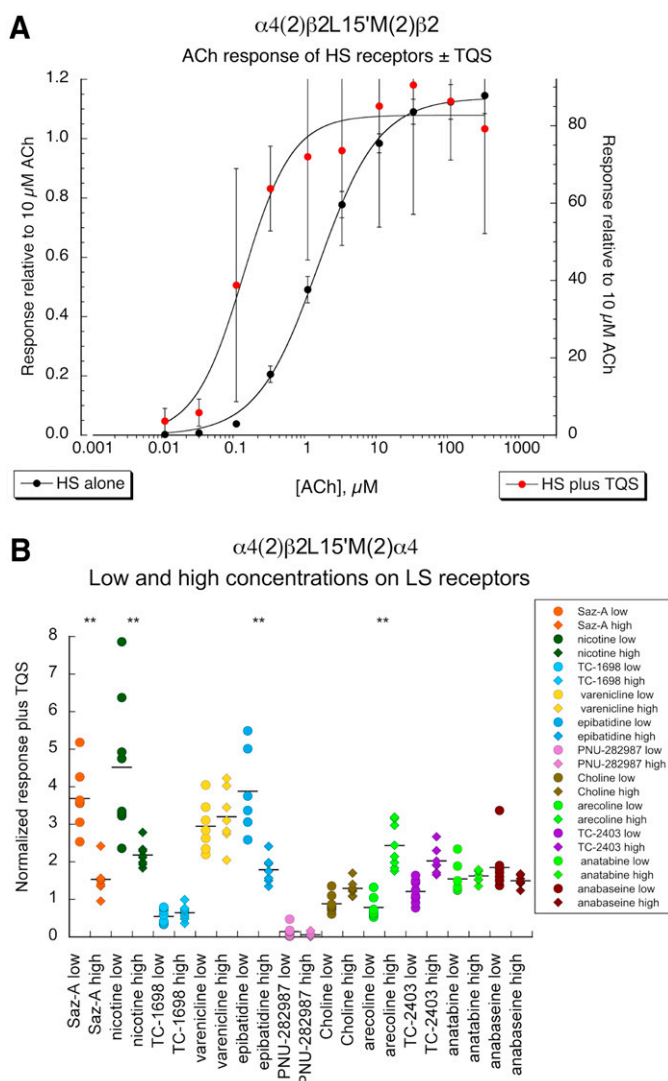
Fig. 5. Agonist concentration dependence of TQS-potentiated responses. (A) TQS potentiation of ACh HS receptor responses across a range of ACh concentrations. Plotted are the average peak current responses of HS receptors to coapplications of ACh and 30 μM TQS (red symbols, right y-axis) of five to eight cells ($\pm$ S.D.) at each concentration, compared with the responses to ACh alone (black symbols, left y-axis, data from Figure 1B). In both cases the responses were normalized and expressed relative to the initial peak currents of the 10 μM ACh controls from the same cells. The estimated I_{max} for ACh alone was only 1.14 ± 0.012 the ACh controls ($r = 0.999$), while for the TQS-potentiated current the Imax was 82.7 ± 2.6 ($r = 0.988$). (B) TQS potentiation of LS receptors at two different concentrations. Circles represent the average normalized peak current responses obtained with TQS coappled with the test compounds at the concentrations indicated in Table 1. Diamonds represent the average normalized peak current responses obtained with TQS coappled 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.

higher concentrations (see ANOVA results Table 2), there were no statistically significant differences in the TQS-potentiated responses at the two concentrations (Fig. 5B). However, responses to sazetidine-A, nicotine, and epibatidine coappled at the higher concentration with TQS were roughly 50% smaller ($P < 0.0001$) than the responses to the lower concentrations coappled with TQS. Only responses to 1 mM arecoline coappled with TQS were larger ($P < 0.001$, Table 2), by roughly a factor of 2 than when TQS was coappled with the 10-fold lower concentration of arecoline.

TABLE 2
Analysis of variance high versus low concentrations
Data were extracted from a larger ANOVA with Bonferroni correction for multiple comparisons and are not based on pairwise tests.

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	

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

When applied alone at the test concentration (Table 1), these compounds had varying levels of activity (Fig. 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 Material for statistical analysis). The TQS effects also differed somewhat for the HS and LS receptors. TQS did not potentiate the low responses to cotinine for either subtype.

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

Potentiation of HS α4β2 -Selective Agonists. Sazetidine-A and TC-2559, two agents that are potent activators of HS α4β2 receptors with little or no efficacy for activating LS receptors, were tested (Fig. 7B). Sazetidine-A was actually first published as a selective α4β2 desensitizer, since it was shown to primarily desensitize receptors in an expression system that was biased toward the formation of LS-type receptors (Xiao et al., 2006) and only later shown to be an HS-selective agonist when HS receptor formation was enhanced by injection of oocytes with a 10-fold excess of β2 relative to α4 RNA (Zwart et al., 2008). The same approach was also used to demonstrate the increased efficacy of TC-2559 for HS receptors (Zwart et al., 2006). As expected, when applied alone, these agents stimulated large responses for HS receptors with very little response in the LS receptors ($P < 0.0001$, Supplemental Material). However, when coapplied with TQS, both compounds were strong activators of both receptor types,

TABLE 3
Antagonists

3a. Responses	HS activation Average ± S.D.	HS TQS Average ± S.D.	LS activation Average ± S.D.	LS TQS Average ± S.D.
MLA	0.007 ± 0.003	0.016 ± 0.009[#]	0.003 ± 0.003	0.003 ± 0.003
DHβE	0.046 ± 0.032[#]	0.085 ± 0.046[#]	0.004 ± 0.002	0.004 ± 0.002
3b. Corrected *P* values				
	Drug versus Drug plus TQS		Drug plus TQS versus TQS alone	
	P value HS	*P* value LS	*P* value HS	*P* value LS
MLA	0.0492	0.1879	0.0053[#]	1.4235
DHβE	0.0201	2.0367	0.0209[#]	0.8528

[#]$P < 0.05$, drugs co-applied with TQS reduced response compared with TQS alone.
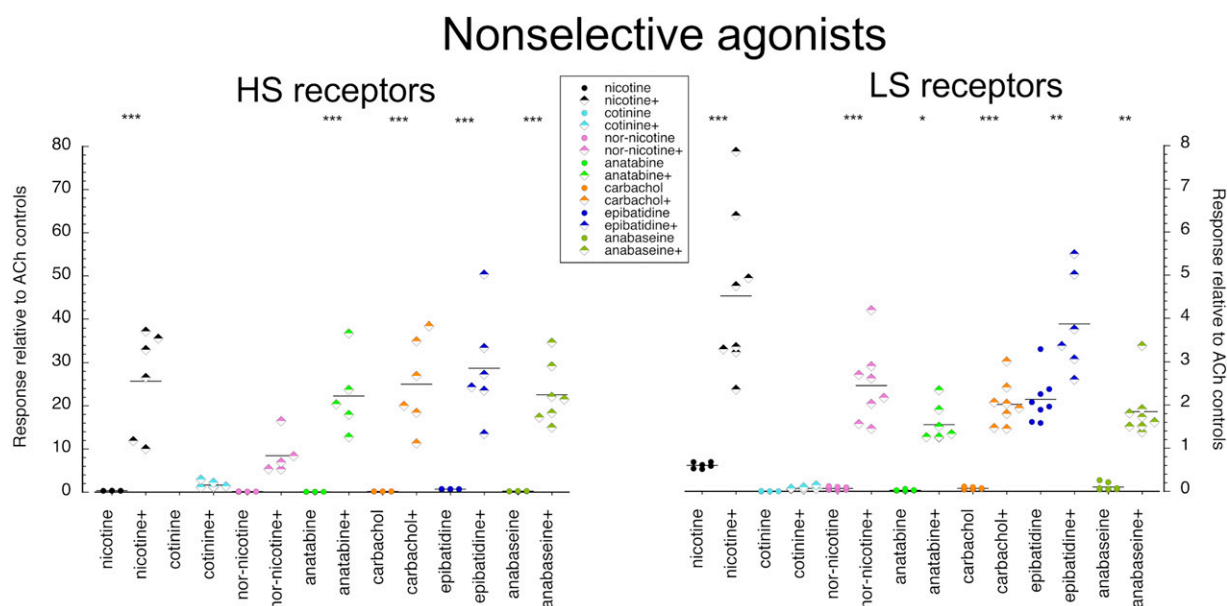
Fig. 6. Effects of nonselective agonists. (A) Dot plot of the peak current responses of HS (left) and LS receptors (right) to the nonselective agonists when applied alone (circles), compared with the responses to drugs coapplied with 30 μM TQS, indicated by the drug name with a plus sign and plotted as half-color diamonds.

confirming that they are indeed subtype-selective silent agonists (Fig. 7B).

α7-Selective Agonists. One of the most frequently sought-after goals in the preclinical developments on nicotinic drugs has been to identify drugs that will target α7 nAChR without affecting other subtypes like $\alpha4\beta2$ receptors (Papke and Horenstein, 2021), leading to the identification of several α7-selective agonists. Among the first α7-selective agonists to be published, and one of the most widely used, is GTS-21 (DMXB) (de Fiebre et al., 1995), although even in the first publication it was noted to also antagonize $\alpha4\beta2$ responses. Subsequently, several large pharmaceutical companies developed agents that were proposed to be more selective than GTS-21, including AR-R17779 (Levin et al., 1999), TC-1698 (Marrero et al., 2004), and PNU-282987 (Bodnar et al., 2005). The ACh precursor choline was also identified as an α7-selective agonist (Papke et al., 1996), although its low potency and ubiquitous presence in the brain and blood generally precludes its consideration as a therapeutic agent.

As expected, none of these drugs evoked much activation of the $\alpha4\beta2$ receptors, although there were small responses of HS receptors to GTS-21 (Table 4 and Fig. 8). This activity may have been missed in earlier studies that were based on the expression of $\alpha4$ and $\beta2$ 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 Material for ANOVA results) and were not larger than the responses to the other α7 agonists. When coapplied with TQS to HS receptors, 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 larger than responses to TQS alone. For the LS receptors, GTS-21 ($P < 0.0001$), TC-1698 ($P < 0.01$), and choline ($P < 0.0001$) coapplied with TQS gave responses larger than to TQS alone (see Supplemental Material).

Inhibition of Wild-Type $\alpha4\beta2$ ACh Responses by α7-Selective Agonists. The data in Fig. 9 suggest that some of the compounds proposed to activate α7 receptors would be effective desensitizing antagonists of $\alpha4\beta2$ receptors. To test this, cells expressing wild-type forms of HS and LS $\alpha4\beta2$ receptors were pre-exposed to the commercially developed α7-selective agonists for 30 seconds, and then ACh was coapplied at the control concentration along with the α7 agonists at the test concentration. The ACh responses were compared with the control ACh responses obtained prior to the application of the α7 agonists (Fig. 9). GTS-21 preapplication evoked a small response from the LS $\alpha4\beta2$ receptors and suppressed the ACh responses of both subtypes, with a greater effect on HS than on LS (Table 5). TC-1698 produced a nearly complete block of the ACh responses of both $\alpha4\beta2$ subtypes, while AR-R17779 produced a 50% block of the HS responses with no effect on the LS ACh response. PNU-282987 preapplication and coapplication caused no block of either $\alpha4\beta2$ receptor subtype (Fig. 9). These results with wild-type receptors are consistent with the TQS effects obtained on the receptors with L15'M mutant $\beta2$ subunits (Fig. 8).

Discussion

The results support the hypothesis that the responses of HS $\alpha4\beta2$ receptors are strongly limited by desensitization, even at low agonist concentrations. They also show that the desensitization is not specifically a factor limiting the response of the HS receptors to high agonist concentrations. The survey of the several classes of ligands identified some types of compounds, like the α7 silent agonist and the α9 agonists, that appear to be free of $\alpha4\beta2$ activating or desensitizing effects. They also indicate that desensitization is a factor limiting the efficacy of $\alpha4\beta2$ 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
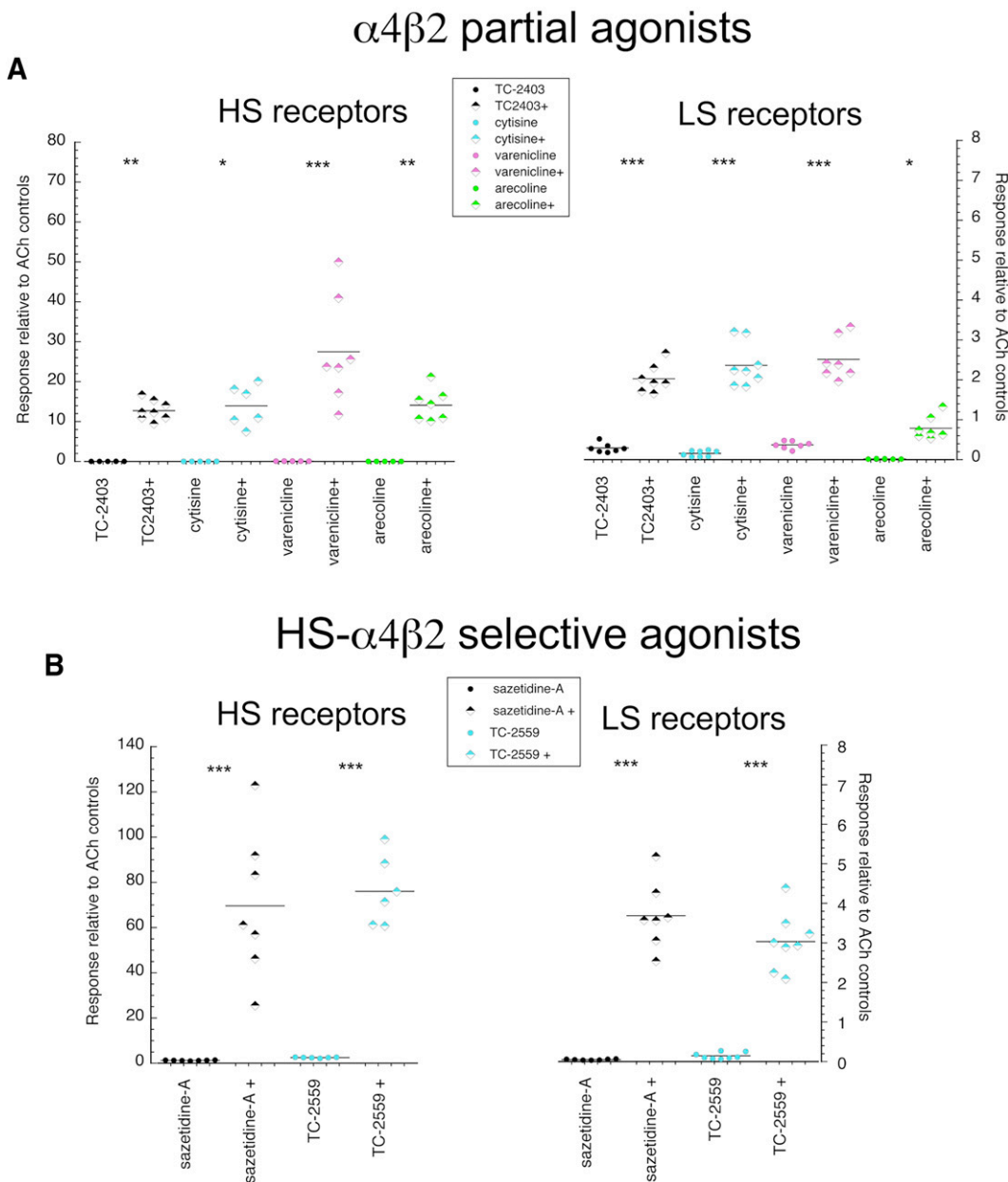
Fig. 7. Effects of selective agonists. (A) Dot plot of the peak current responses of HS (left) and LS receptors (right) to the α4β2 partial agonists when applied alone (circles), compared with the responses to drugs coapplied with 30 μM TQS, indicated by the drug name with a plus sign and plotted as half-color diamonds. (B) Dot plot of the peak current responses of HS (left) and LS receptors (right) to the HS α4β2 selective agonists when applied alone (circles), compared with the responses to drugs coapplied with 30 μM TQS, indicated by the drug name with a plus sign and plotted as half-color diamonds.

functionally HS receptor selective agonists and functionally LS receptor desensitizers.

The desensitization of nAChR is a complex and multiphasic process (Feltz and Trautmann, 1982; Simasko et al., 1986; Boyd, 1987; Sine and Steinbach, 1987; Forman and Miller, 1988; Dilger and Liu, 1992; Quick and Lester, 2002; Lester, 2004; Papke et al., 2009), and the effect of type II PAMs on α7 receptors is selective for only some form(s) of desensitization (Williams et al., 2011). Although the effects of some type II PAMs can be quite large, they do not reverse all the receptor desensitization or affect all receptors equally. Indeed, the effects of the α7 PAM PNU-120596 are to enormously increase

TABLE 4
α7-selective agonists

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

See Supplemental Material for ANOVA results.

Effects of α7-selective agonists on α4β2L15'M receptors
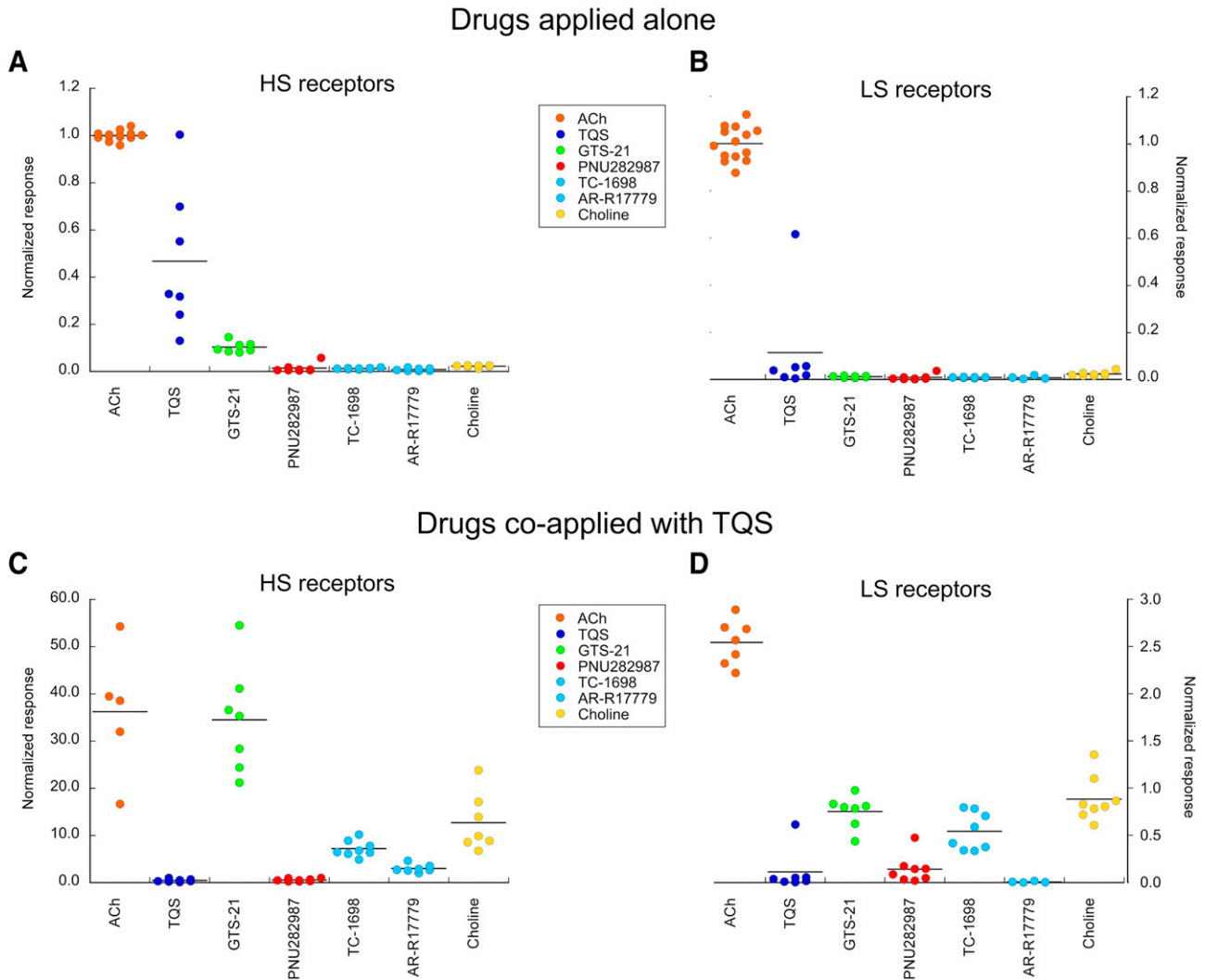
Drugs applied alone

Drugs co-applied with TQS

Fig. 8. Effects of α7-selective agonists (Table 4). (A) Dot plot of the peak current responses of TQS-sensitive HS receptors to the α7-selective agonists when applied alone, compared with ACh control responses and the responses to 30 μM TQS applied alone. Although GTS-21 appeared to give detectable responses, these were not statistically significant compared with the other α7-selective agonists (see Supplemental Material for ANOVA results). (B) Responses of TQS-sensitive HS α4β2 receptors to α7-selective agonists coapplied 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 Material for ANOVA results). (C) The lack of responses of TQS-sensitive LS α4β2 receptors to the α7-selective agonists when applied alone, compared with ACh control responses and the responses to 30 μM TQS applied alone. (D) Responses of TQS-sensitive LS α4β2 receptors to α7-selective agonists coapplied with TQS. GTS-21 ($P < 0.0001$), TC-1698 ($P < 0.01$), and choline ($P < 0.0001$) coapplied with TQS gave responses greater than to TQS alone (see Supplemental Material for ANOVA results).

the activation of a small fraction of receptors while the majority of receptors remain in desensitized states (Williams et al., 2011; Andersen et al., 2016). The single-channel effects of the ago-PAM 4BP-TQS (GAT107) on α7 receptors are similar to those of PNU-120596, (Pałczyńska et al., 2012; Quadri et al., 2019), while the effects of TQS on α7 ACh responses are somewhat less (Pałczyńska et al., 2012). However, the basic mechanisms of α7 desensitization are fundamentally different from those of α4β2 receptors, so likewise the TQS effects on the TQS-sensitive α4β2 receptors might be very different on the molecular level from the effects on α7 receptors. While α7 receptors show no activation at all with high agonist concentrations (Williams et al., 2011), α4β2 receptors can smolder (Campling et al., 2013), occasionally opening under predominantly desensitizing conditions.

While TQS is considered strictly a PAM for α7, since we observed it to activate the TQS-sensitive α4β2 receptors (particularly the HS receptors) when applied alone, it might be classified as a weak ago-PAM or allosteric agonist for these receptors, behaving like the TQS analog (+)4BP-TQS (GAT107) on α7 receptors. By definition, "ago-PAMs" potentiate the responses evoked by agonists but also produce activation on their own and may also prime the potentiation of subsequent agonist application. The direct allosteric activation of HS α4β2L15'M receptors by TQS was blocked by 10 μM of the α4β2-selective antagonist DHβE as well as by 100 μM MLA, a concentration at which the drug is no longer selective for α7 (Buisson et al., 1996). While the allosteric activation of α7 by GAT107 can be blocked by 10 μM of the α7 selective antagonist MLA (Papke et al., 2014b), it is insensitive to 100 μM DHβE (R.L. Papke, unpublished

Wild-type receptors

HS
α4(2)β2(3)

LS
α4(3)β2(2)
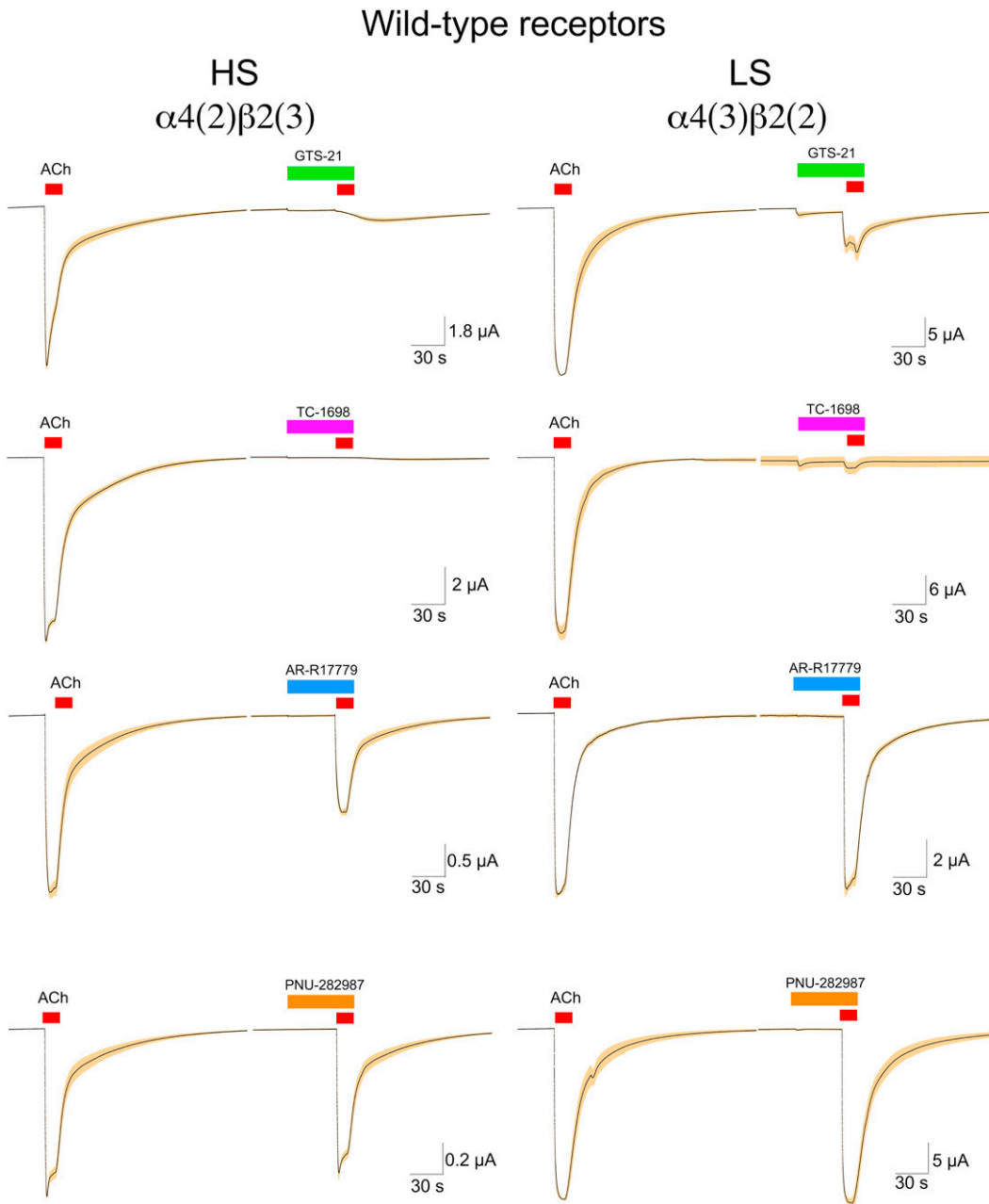
Fig. 9. Effects of α7-selective agonists on the ACh responses of wild-type HS and LS α4β2 receptors. Averaged data were prepared as described (*Materials and Methods*). Following the ACh control responses (red bars), the α7 agonists were preapplied for 30 seconds (colored bars). Then without washout the α7 agonists were coapplied with ACh at the control concentration (10 μM for HS and 100 μM for LS). The *n* values for the GTS-21 experiments were 8 for the HS receptors and 7 for the LS receptors. The *n* values for the TC-1698 experiments were 7 for the HS receptors and 7 for the LS receptors. The *n* values for the AR-R17779 experiments were 7 for the HS receptors and 4 for the LS receptors. The *n* values for the PNU-282987 experiments were 8 for the HS receptors and 6 for the LS receptors.

manuscript; data not shown). It seems unlikely that TQS itself is a suitable ligand for the ACh binding sites, since it lacks a positively charged nitrogen common to most nicotinic agonists, so it is possible that, especially for the HS receptors, there was an incomplete washout of ACh from the previous control application of ACh and that residual ACh facilitated the effects of

TQS when nominally applied alone. Alternatively, TQS may actually function as a weak ago-PAM for these receptors, and occupancy of the ACh sites by the competitive antagonists might be sufficient to inhibit the allosteric activation by TQS.

The therapeutic development of α7-selective agonists for indications such as schizophrenia (Hajós and Rogers, 2010;

TABLE 5
Preapplications of α7 agonists to wild-type receptors

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

Haydar and Dunlop, 2010; Cannon et al., 2013; 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 $\alpha4\beta2$ receptors in the brain. This is a particular concern in the case of Alzheimer's disease, since evidence suggests that $\alpha4\beta2$ receptor function is specifically impaired in this patient population (Court et al., 2001; Gotti et al., 2006). The results with the $\alpha7$-selective agonists tested indicate that, depending on the specific agent, a number of differing profiles of $\alpha7$ agonism and $\alpha4\beta2$ antagonism may be available, with PNU-282987 being the least likely to affect $\alpha4\beta2$ function. As noted earlier, GTS-21 was the first synthetic $\alpha7$-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 $\alpha7$ 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., 2021; Zhou et al., 2021). However, it is important to note that, from an electrophysiological perspective, these compounds have very different activity profiles for $\alpha7$ receptors. PNU-282987 is relatively potent and nearly a full agonist (Hajós et al., 2005), while GTS-21 has lower potency and efficacy and additionally produces residual $\alpha7$-receptor desensitization (Papke et al., 2009). Interestingly, the $\alpha7$ desensitizing activity of GTS-21 may be important for its CAP activity (Thomsen and Mikkelsen, 2012; Horenstein and Papke, 2017). 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 $\alpha4\beta2^*$ and $\alpha7$ receptors, with GTS-21 capable of decreasing $\alpha4\beta2$ function as well as working on $\alpha7$ and PNU-282987 affecting $\alpha7$ 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 $\alpha7$ nAChR, especially in the context of the CAP mediated by $\alpha7$, and possibly $\alpha9\alpha10$, receptors in immune cells (Tracey, 2007; Rosas-Ballina and Tracey, 2009) 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; King et al., 2017; Kabbani and Nichols, 2018; King et al., 2018). This has led to the proposed development of weak $\alpha7$ agonists like GTS-21 (Kong et al., 2018; Wang et al., 2020) and even silent agonists (; Richter et al., 2016; Horenstein and Papke, 2017; Bagdas et al., 2018; Godin

et al., 2020; Papke and Horenstein, 2021) 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 (Stokes et al., 2015), and interestingly the intracellular domain of $\alpha4$ is the largest of any nAChR subunit, by a factor of 3 or more. Although not so well studied as $\alpha7$ in this regard, there have been reports that $\alpha4$-containing nAChR also play roles in the regulation of inflammatory and neuropathic pain (Nordman et al., 2014; Acharya et al., 2020) 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 $\alpha4\beta2$ receptors and may prove useful for the development of more focused therapeutics.

Authorship Contributions

Participated in research design: Papke.
Performed data analysis: Papke, Stokes.
Wrote or contributed to the writing of the manuscript: Papke, Stokes.

References

Acharya S, Kundu D, Choi HJ, and Kim KM (2020) Metabotropic signaling cascade involved in $\alpha4\beta2$ nicotinic acetylcholine receptor-mediated PKCβII activation. *Biochim Biophys Acta Mol Cell Res* **1867:**118721.
Aickin M and Gensler H (1996) Adjusting for multiple testing when reporting research results: the Bonferroni vs Holm methods. *Am J Public Health* **86:**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 $\alpha4\beta2$ 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:**415–425.
Bodnar AL, Cortes-Burgos LA, Cook KK, Dinh DM, Groppi VE, Hajós M, Higdon NR, Hoffmann WE, Hurst RS, Myers JK, et al. (2005) Discovery and structure-activity relationship of quinuclidine benzamides as agonists of alpha7 nicotinic acetylcholine receptors. *J Med Chem* **48:**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:**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:**7880–7891.
Campling BG, Kuryatov A, and Lindstrom J (2013) Acute activation, desensitization and smoldering activation of human acetylcholine receptors. *PLoS One* **8:**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:**254–268.
Coe JW, Brooks PR, Vetelino MG, Wirtz MC, Arnold EP, Huang J, Sands SB, Davis TI, Lebel LA, Fox CB, et al. (2005) Varenicline: an alpha4beta2 nicotinic receptor partial agonist for smoking cessation. *J Med Chem* **48:**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:**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:**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:**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:**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:**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)-dimethylaminocinnamylidine derivative (DMAC) is a selective agonist at neuronal nicotinic α7/[^{125}I]α-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:**915–929.

Descarries L, Gisiger V, and Steriade M (1997) Diffuse transmission by acetylcholine in the CNS. *Prog Neurobiol* **53:**603–625.

Dilger JP and Liu Y (1992) Desensitization of acetylcholine receptors in BC3H-1 cells. *Pflugers Arch* **420:**479–485.

Eaton JB, Lucero LM, Stratton H, Chang Y, Cooper JF, Lindstrom JM, Lukas RJ, and Whiteaker P (2014) The unique α4+/-α4 agonist binding site in (α4)3(β2)2 subtype nicotinic acetylcholine receptors permits differential agonist desensitization pharmacology versus the (α4)2(β2)3 subtype. *J Pharmacol Exp Ther* **348:**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:**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 α4β2 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 from Torpedo. *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 Pharmacol* **48:**774–782.

Godin JR, Roy P, Quadri M, Bagdas D, Toma W, Narendrula-Kotha R, Kishta OA, Damaj MI, Horenstein NA, Papke RL, et al. (2020) A silent agonist of α7 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, et al. (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:**481–489.

Grønlien JH, Håkerud 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:**715–724.

Gupta PC and Warnakulasuriya S (2002) Global epidemiology of areca nut usage. *Addict Biol* **7:**77–83.

Hajós 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:**1213–1222.

Hajós M and Rogers BN (2010) Targeting alpha7 nicotinic acetylcholine receptors in the treatment of schizophrenia. *Curr Pharm Des* **16:**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:**144–152.

Horenstein NA and Papke RL (2017) Anti-inflammatory silent agonists. *ACS Med Chem Lett* **8:**989–991.

Kabbani N and Nichols RA (2018) Beyond the channel: metabotropic signaling by nicotinic receptors. *Trends Pharmacol Sci* **39:**354–366.

Katz B and Thesleff S (1957) A study of the desensitization produced by acetylcholine at the motor end-plate. *J Physiol* **138:**63–80.

Kem WR, Mahnir VM, Papke RL, and Lingle CJ (1997) Anabaseine is a potent agonist on muscle and neuronal alpha-bungarotoxin-sensitive nicotinic receptors. *J Pharmacol Exp Ther* **283:**979–992.

King JR, Gillevet TC, and Kabbani N (2017) A G protein-coupled α7 nicotinic receptor regulates signaling and TNF-α release in microglia. *FEBS Open Bio* **7:**1350–1361.

King JR, Ullah A, Bak E, Jafri MS, and Kabbani N (2018) Ionotropic and metabotropic mechanisms of allosteric modulation of α7 nicotinic receptor intracellular calcium. *Mol Pharmacol* **93:**601–611.

Kong W, Kang K, Gao Y, Liu H, Meng X, Cao Y, Yang S, Liu W, Zhang J, Yu K, et al. (2018) GTS-21 protected against LPS-induced sepsis myocardial injury in mice through α7nAChR. *Inflammation* **41:**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:**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:**7200–7204.

Leiser SC, Bowlby MR, Comery TA, and Dunlop J (2009) A cog in cognition: how the alpha 7 nicotinic acetylcholine receptor is geared towards improving cognitive deficits. *Pharmacol Ther* **122:**302–311.

Lester RA (2004) Activation and desensitization of heteromeric neuronal nicotinic receptors: implications for non-synaptic transmission. *Bioorg Med Chem Lett* **14:**1897–1900.

Levin ED, Bettegowda C, Blosser J, and Gordon J (1999) AR-R17779, and alpha7 nicotinic agonist, improves learning and memory in rats. *Behav Pharmacol* **10:**675–680.

López-Hernández GY, Sánchez-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:**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:**16–27.

Millar NS and Gotti C (2009) Diversity of vertebrate nicotinic acetylcholine receptors. *Neuropharmacology* **56:**237–246.

Nelson ME, Kuryatov A, Choi CH, Zhou Y, and Lindstrom J (2003) Alternate stoichiometries of alpha4beta2 nicotinic acetylcholine receptors. *Mol Pharmacol* **63:**332–341.

Nordman JC, Muldoon P, Clark S, Damaj MI, and Kabbani N (2014) The α4 nicotinic receptor promotes CD4+ T-cell proliferation and a helper T-cell immune response. *Mol Pharmacol* **85:**50–61.

Pałczyńska MM, Jindrichova M, Gibb AJ, and Millar NS (2012) Activation of α7 nicotinic receptors by orthosteric and allosteric agonists: influence on single-channel kinetics and conductance. *Mol Pharmacol* **82:**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:**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. *J Mol Neurosci* **40:**77–86.

Papke RL, Andleeb H, Stokes C, Quadri M, and Horenstein NA (2022a) Selective agonists and antagonists of α9 versus α7 nicotinic acetylcholine receptors. *ACS Chem Neurosci* **13:**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 alpha 7 subtype. *Neurosci Lett* **213:**201–204.

Papke RL, Chojnacka K, and Horenstein NA (2014a) The minimal pharmacophore for silent agonism of alpha7 nAChR. *J Pharmacol Exp Ther* **350:**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:**160–167.

Papke RL and Horenstein NA (2021) Therapeutic targeting of alpha7 nicotinic acetylcholine receptors. *Pharmacol Rev* **73:**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 α7 nicotinic acetylcholine receptors (nAChR), is regulated by aromatic amino acids that span the subunit interface. *J Biol Chem* **289:**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:**e0140907.

Papke RL, Karaffa M, Horenstein NA, and Stokes C (2022b) Coffee and cigarettes: modulation of high and low sensitivity α4β2 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 benzylidene anabaseines and nicotine. *J Pharmacol Exp Ther* **329:**791–807.

Papke RL and Stokes C (2010) Working with OpusXpress: methods for high volume oocyte experiments. *Methods* **51:**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:**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:**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:**43–61.

Quadri M, Papke RL, and Horenstein NA (2016) Dissection of N,N-diethyl-N'-phenyl-piperazines as α7 nicotinic receptor silent agonists. *Bioorg Med Chem* **24:**286–293.

Quick MW and Lester RA (2002) Desensitization of neuronal nicotinic receptors. *J Neurobiol* **53:**457–478.

Richter K, Mathes V, Fronius M, Althaus M, Hecker A, Krasteva-Christ G, Padberg W, Hone AJ, McIntosh JM, Zakrzewicz A, et al. (2016) Phosphocholine—an agonist of metabotropic but not of ionotropic functions of α9-containing nicotinic acetylcholine receptors. *Sci Rep* **6:**28660.

Rosas-Ballina M and Tracey KJ (2009) Cholinergic control of inflammation. *J Intern Med* **265:**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:**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:**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 β subunits acquire sensitivity to α7-selective positive allosteric modulators. *J Pharmacol Exp Ther* **370:**252–268.

Stokes C, Treinin M, and Papke RL (2015) Looking below the surface of nicotinic acetylcholine receptors. *Trends Pharmacol Sci* **36:**514–523.

Swango JH, Bhatti BS, Qureshi MM, and Crooks PA (1999) A novel enantioselective synthesis of (S)-(?)- and (R)-(+)-nornicotine via alkylation of a chiral 2-hydroxy-3-pinanone ketimine template. *Chirality* **11:**316–318.

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 α7 nicotinic acetylcholine receptor ligands methyllycaconitine, NS6740 and GTS-21 reduce lipopolysaccharide-induced TNF-α release from microglia. *J Neuroimmunol* **251:**65–72.

Tracey KJ (2007) Physiology and immunology of the cholinergic antiinflammatory pathway. *J Clin Invest* **117:**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:**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:**33–46.

Walling D, Marder SR, Kane J, Fleischhacker WW, Keefe RS, Hosford DA, Dvergsten C, Segreti AC, Beaver JS, Toler SM, et al. (2016) Phase 2 trial of an alpha-7 nicotinic receptor agonist (TC-5619) in negative and cognitive symptoms of schizophrenia. *Schizophr Bull* **42:**335–343.

Wang H, Cai D, Chen Z, and Wang Y (2020) GTS-21 promotes α7 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 α4β2* nicotinic acetylcholine receptors. *J Biol Chem* **290:**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:**917–926.

Wheeler JW, Olubajo O, Storm CB, and Duffield RM (1981) Anabaseine: venom alkaloid of aphaenogaster ants. *Science* **211:**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:**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:**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:**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 USA* **105:**14686–14691.

Yuan F, Jiang L, Li Q, Sokulsky L, Wanyan Y, Wang L, Liu X, Zhou L, Tay HL, Zhang G, et al. (2021) A selective α7 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, et al. (2012) Analgesic effects mediated by neuronal nicotinic acetylcholine receptor agonists: correlation with desensitization of α4β2* receptors. *Eur J Pharm Sci* **47:**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 α7nAChR protects against gastric inflammation and dysmotility in Parkinson's disease rats. *Front Pharmacol* **12:**793374.

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:**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:**10–17.

Zwart R, Carbone AL, Moroni M, Bermudez I, Mogg AJ, Folly EA, Broad LM, Williams AC, Zhang D, Ding C, et al. (2008) Sazetidine-A is a potent and selective agonist at native and recombinant alpha 4 beta 2 nicotinic acetylcholine receptors. *Mol Pharmacol* **73:**1838–1843.

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

Roger L. Papke and Clare Stokes

Supplemental Figure 1
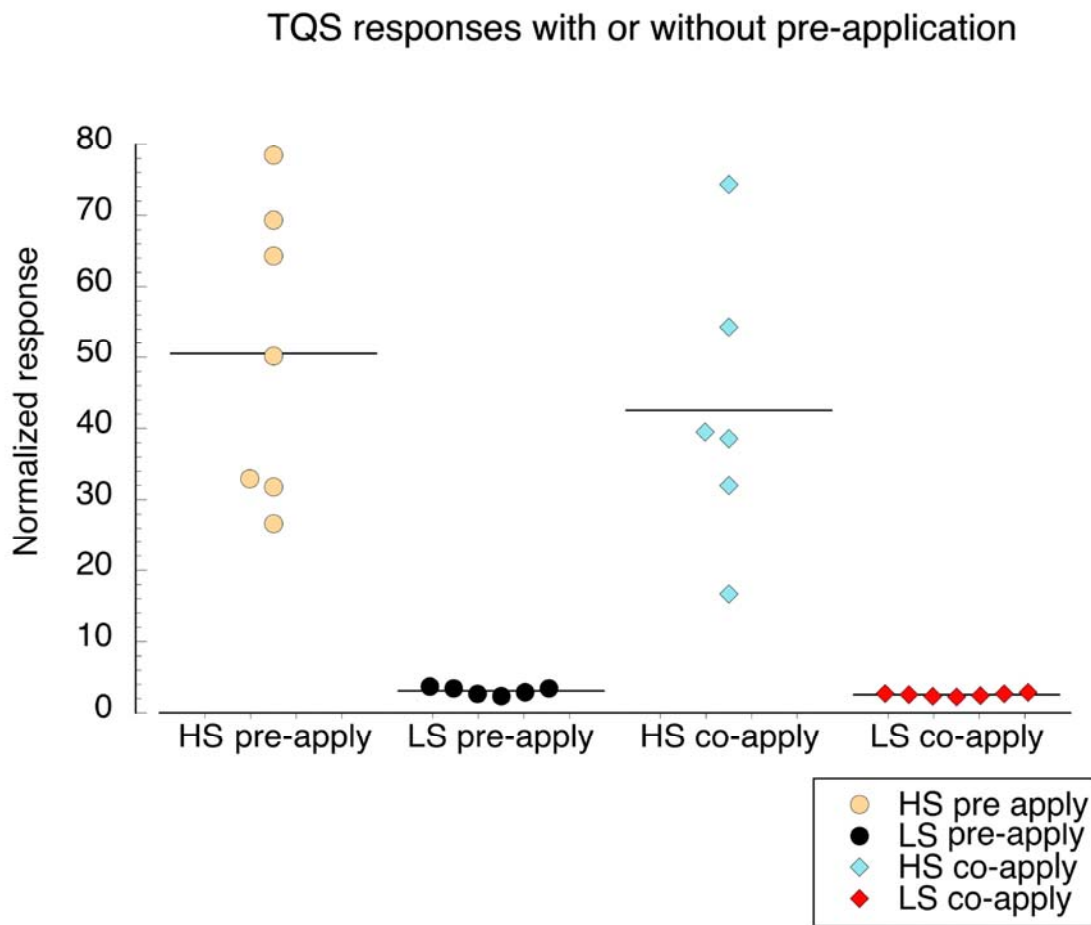
Responses of TQS-sensitive α4β2 receptors to control concentrations of ACh (10 µM for HS receptors and 100 µM for LS receptors) were compared under conditions where 30 µM TQS was preapplied for 30 seconds before the co-application of ACh and TQS to simple co-application of TQS and ACh. Pre-applications had no significant effects. See below for ANOVA results.

The supplemental data figures 2-5 represent the normalized responses of the indicated TQS-sensitive α4β2 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 µM 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 ± standard deviations and the statistical analyses.

Supplemental Figure 1

α7 silent agonists

α4(2)β2L15'M(2)β2   α4(2)β2L15'M(2)α4
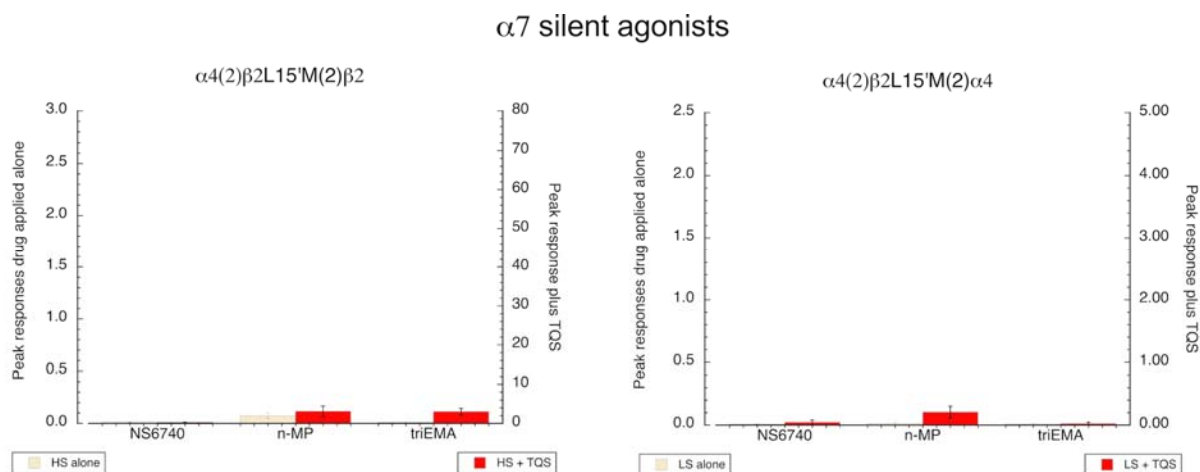
Supplemental Data Figure 2. α7 silent agonists

1a. Responses

	HS activation Average ± SD	HS TQS Average ± SD	LS activation Average ± SD	LS TQS Average ± SD
NS6740	0.013 ± 0.004	0.222 ± 0.128	0.006 ± 0.003	0.043 ± 0.037
n-MP	0.073 ± 0.024	3.091 ± 1.387	0.013 ± 0.010	0.208 ± 0.095
triEMA	0.008 ± 0.006	3.017 ± 0.847	0.007 ± 0.007	0.023 ± 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 α7 PAMs have been used to identify α7-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 α4β2 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 α4β2 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.

α9 agonists

α4(2)β2L15'M(2)β2   α4(2)β2L15'M(2)α4
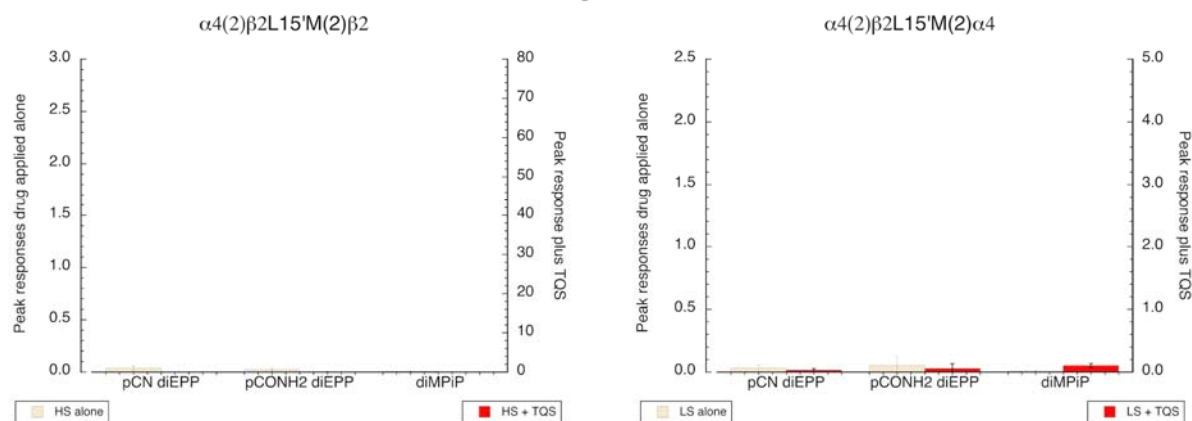
Supplemental Data Figure 3. α9 agonists

2a. Responses

	HS activation Average ± SD	HS TQS Average ± SD	LS activation Average ± SD	LS TQS Average ± SD
*p*CN diEPP	0.036 ± 0.022	0.054 ± 0.016	0.031 ± 0.026	0.029 ± 0.025
*p*CONH2 diEPP	0.023 ± 0.010	0.064 ± 0.020	0.052 ± 0.069	0.056 ± 0.079
diMPiP	0.007 ± 0.003	0.016 ± 0.009	0.008 ± 0.005	0.104 ± 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
*p*CN diEPP	0.2477	7.1317	0.0247[#]	3.0222
*p*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 α7-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 α9-selective agonists (Papke et al. 2022). None of these compounds produced significant activation of the α4β2 receptor either with or without TQS.
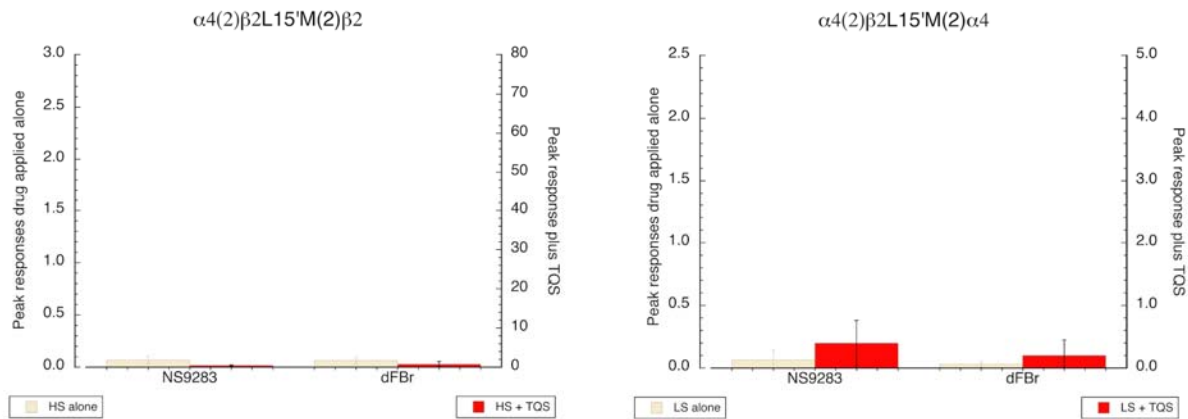
Supplemental Data Figure 4. α4β2 modulators

3a. Responses

	HS activation Average ± SD	HS TQS Average ± SD	LS activation Average ± SD	LS TQS Average ± SD
NS9283	0.068 ± 0.034	0.439 ± 0.183	0.064 ± 0.079	0.397 ± 0.361
dFBr	0.064 ± 0.030	0.779 ± 0.655	0.031 ± 0.023	0.199 ± 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 α4β2 receptors is that there is a unique low affinity binding site at the α4–α4 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 α4β2 receptors with the β2L15'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.

$$\alpha 4(2)\beta 2L15'M(2)\beta 2$$
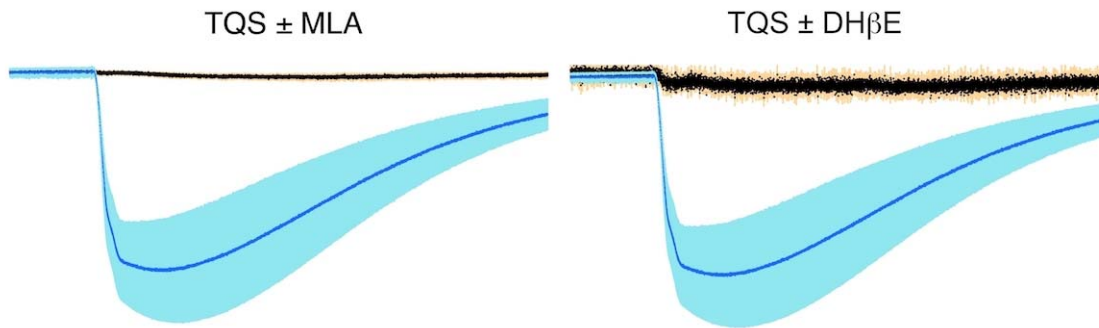
Effects on nAChR antagonists co-applied with TQS (black) to HS receptors compared to the averaged response to TQS alone (blue, from Figure 2).

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 effect**s
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
α7-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: α7 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: α7 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: α7 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