


# Insights Into the Differential Desensitization of $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptor Isoforms Obtained With Positive Allosteric Modulation of Mutant Receptors<sup>§</sup>

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Received July 13, 2022; accepted November 3, 2022

## ABSTRACT

The development of highly efficacious positive allosteric modulators (PAMs) of  $\alpha 7$  nicotinic acetylcholine receptors (nAChR) has proven useful in defining the ligand dependence of the conformational dynamics of  $\alpha 7$  receptors. No such effective modulators are known to exist for the  $\alpha 4\beta 2$  nAChR of the brain, limiting our ability to understand the importance of desensitization for the activity profile of specific ligands. In this study, we used mutant  $\beta 2$  subunits that allowed the use of the  $\alpha 7$  PAM 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  $\alpha 4\beta 2$  receptors; high sensitivity (HS) (two  $\alpha 4$  and three  $\beta 2$  subunits) and low sensitivity (LS) (three  $\alpha 4$  and two  $\beta 2$  subunits). A total of 28 different ligands of 8 different categories, based on activity and selectivity, were tested for their ability to induce TQS-sensitive desensitization of HS and LS  $\alpha 4\beta 2$  receptors. Results confirm that HS  $\alpha 4\beta 2$  receptor responses are strongly limited by desensitization, by at least an order of magnitude more so than the responses of LS receptors. The activation of  $\alpha 4\beta 2$  receptors by the smoking-cessation drugs cytisine and varenicline is strongly limited by desensitization, as is the activation

of LS receptors by the HS-selective agonists 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  $\alpha 7$ -selective agonists revealed varying patterns of  $\alpha 4\beta 2$  cross-desensitization that were predictive of the effects of these drugs on the activation of wild-type  $\alpha 4\beta 2$  receptors by 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  $\alpha 4$  and  $\beta 2$  subunits (Millar and Gotti, 2009). Pentamers composed of just two different subunits necessarily can vary in subunit stoichiometry, such that while two agonist binding sites are configured at  $\alpha 4$ - $\beta 2$  interfaces, the fifth position can be occupied by either an  $\alpha 4$  or a  $\beta 2$  subunit (Nelson et al., 2003). While some expression systems may bias receptor expression toward the  $\alpha 4(3)\beta 2(2)$  configuration, both types are present in the brain (Fasoli et al., 2016), and chronic nicotine favors the expression of the  $\alpha 4(2)\beta 2(3)$  configuration due to nicotine's ability to selectively chaperone receptors of that configuration to the membrane (Nelson et al., 2003; Kuryatov et al., 2005; Srinivasan et al., 2011).

The two configurations of  $\alpha 4\beta 2$  nAChR differ greatly in their functional properties, with one notable difference being that

This work was supported by National Institutes of Health National Institute of General Medical Sciences [Grant R01-GM57481].

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

<https://doi.org/10.1124/molpharm.122.000591>

<sup>§</sup> This article has supplemental material available at [molpharm.aspetjournals.org](http://molpharm.aspetjournals.org).

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

receptors with the  $\alpha 4(2)\beta 2(3)$  configuration respond to low concentrations of ACh or nicotine but saturate their responses when agonist concentrations are raised to higher levels. They have therefore come to be referred to as a high sensitivity (HS) subtype. In contrast, receptors of  $\alpha 4(3)\beta 2(2)$  configuration, in general, generate larger currents across a wider range of concentration; these are known as the low sensitivity (LS) subtype (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  $\alpha 7$  nAChR (Uteshev et al., 2002), the second most abundant nAChR in brain (Millar and Gotti, 2009). Our understanding of  $\alpha 7$  receptor desensitization has been greatly enhanced by the discovery of the type II class of  $\alpha 7$ -selective PAMs (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  $\alpha 7$ -selectivity of PAMs like 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea (PNU-120596) and 3a,4,5,9b-tetrahydro-4-(1-naphthalenyl)-3H-cyclopentan[c]quinoline-8-sulfonamide (TQS) is due to the presence of a methionine, unique to  $\alpha 7$  among nAChR, in the 15' position of the pore-forming second transmembrane domain (Young et al., 2008). The transfer of that residue into  $\beta 2$  or  $\beta 4$  allows for the formation of heteromeric nAChR that are sensitive to potentiation by TQS (Stokes et al., 2019). In the present study, we used a concatamer (Zhou et al., 2003) of  $\alpha 4$  and  $\beta 2$ L15'M mutants, coexpressed in *Xenopus* oocytes with monomers of wild-type  $\alpha 4$  or  $\beta 2$  subunits, to selectively form TQS-sensitive LS or HS  $\alpha 4\beta 2$  receptors. Using TQS to reveal the extent of  $\alpha 4\beta 2$  receptor desensitization, we demonstrate the great degree to which desensitization limits HS receptor responses to determine the degree to which their activities on the two  $\alpha 4\beta 2$  isoforms are limited by TQS-sensitive desensitization. Finally, we tested the relevance of these observation to the functional activation of wild-type  $\alpha 4\beta 2$  receptors by ACh in the presence of putative  $\alpha 7$ -selective agonists.

## Materials and Methods

Acetylcholine chloride, atropine, choline, methyllycaconitine citrate (MLA), dihydro-b-erythroline hydrobromide, N-(3R)-1-Azabicyclo[2.2.2]oct-3-yl-4-chlorobenzamide (PNU-282987), cytosine, arecoline, nicotine, cotinine, and other chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). 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]benzotrile, 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). ( $\pm$ )-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-(3*E*)-3-buten-1-amine difumarate (TC-2559) from Targacept (Winston-Salem, NC); and 1,2,3,6-tetrahydro-2,3'-bipyridine (anatabine) from Cayman Chemical (Ann Arbor, MI).

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

**Heterologous Expression of nAChRs in *Xenopus* Laevis Oocytes.** Two approaches have been developed to study HS and LS  $\alpha 4\beta 2$  receptors independently of each other in *Xenopus* oocytes. One approach has been to inject the  $\alpha 4$  and  $\beta 2$  RNA at ratios that would favor the assembly of LS or HS receptors (usually 10:1,  $\alpha 4$  to  $\beta 2$  for LS receptors or 1:10  $\alpha 4$  to  $\beta 2$  for HS receptors) (Zwart et al., 2008). However, this approach generates a heterogeneous population of receptors and if applied to the present study would give an unequal number of mutant  $\beta$  subunits in the LS and HS biased population. The alternative approach is to use linked  $\alpha 4$ - $\beta 2$  subunits (Zhou et al., 2003), which permits the coexpression of the concatamer with monomeric  $\alpha 4$  or  $\beta 2$  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  $\beta 2$ -6- $\alpha 4$  concatamer were obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia, PA). The  $\beta 2$  L15'M mutant in the concatamer was made as previously described (Stokes et al., 2019). Subsequent to linearization and purification of the plasmid cDNAs, cRNAs were prepared using the mMessage mMachine in vitro RNA transfection kit (Ambion, Austin, TX).

Oocytes were surgically removed from mature *Xenopus laevis* frogs (Nasco, Ft. Atkinson, WI) and injected with appropriate nAChR subunit cRNAs as described previously (Papke and Stokes, 2010). Frogs were maintained in the Animal Care Service facility of the University of Florida, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee (Approval #202002669). In brief, the frog was first anesthetized for 15 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 NaHCO<sub>3</sub>, 0.82 mM MgSO<sub>4</sub>, 15 mM HEPES, and 12 mg/l tetracycline, pH 7.6) to remove the follicular layer. Stage V oocytes were subsequently isolated and injected with 50 nl water containing 5 ng concatamer plus 5 ng  $\alpha 4$  or  $\beta 2$  nAChR subunit cRNA. Recordings were carried out 2 to 7 days after injection.

**Two-Electrode Voltage Clamp Electrophysiology.** Experiments were conducted at room temperature ( $24^{\circ}\text{C}$ ) 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 CaCl<sub>2</sub>, 10 mM HEPES, and 1  $\mu$ M 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  $\mu$ M racemic TQS, and a final control application of ACh. The control ACh concentrations were 10  $\mu$ M for HS receptors and 100  $\mu$ M 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  $\pm$  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.

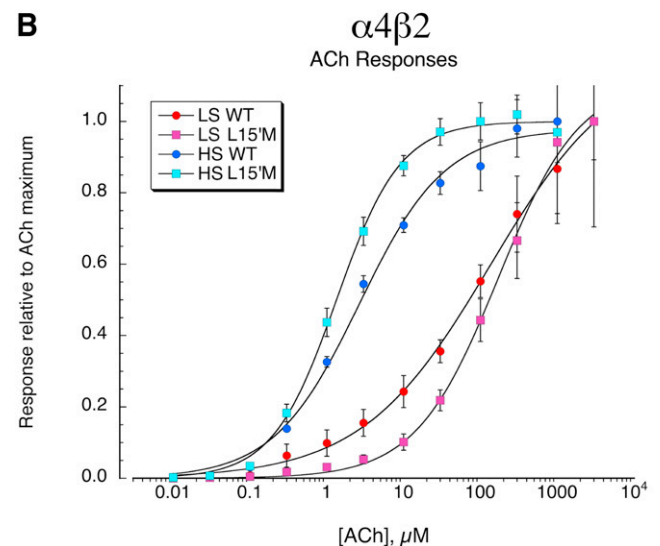
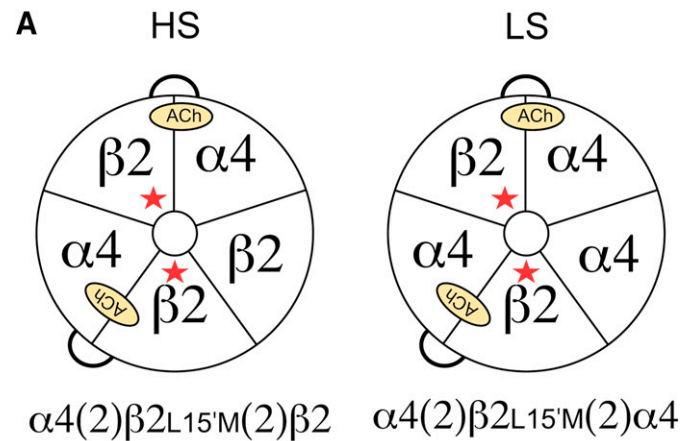
Data were collected at 50 Hz, filtered at 5 Hz, and analyzed by Clampfit 9.2 or 10.3 (Molecular Devices) and Excel (Microsoft, Redmond, WA). All experiments began with eight voltage-clamped oocytes set up for parallel analysis in the Opus-Xpress system. However, due to the fact that PAM-potentiated currents 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  $\pm$  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	$n_{\text{HS}}$	$n_{\text{LS}}$
<b>Antagonists</b>			
MLA	100 $\mu$ M	8	4
DH $\beta$ E	10 $\mu$ M	8	7
<b><math>\alpha 7</math> silent agonists</b>			
NS6740	30 $\mu$ M	8	8
n-MP	300 $\mu$ M	7	8
triEMA	100 $\mu$ M	7	8
<b><math>\alpha 9</math> agonists</b>			
$p$ CN diEPP	100 $\mu$ M	8	7
$p$ CONH2-diEPP	100 $\mu$ M	7	7
diMPiP <sup>2</sup>	100 $\mu$ M	8	7
<b><math>\alpha 4\beta 2</math> modulators</b>			
NS9283 <sup>b</sup>	30 $\mu$ M	8	8
dFBr	30 $\mu$ M	8	7
<b>Non-selective agonists</b>			
Carbachol	100 $\mu$ M	6	8
Epibatidine	3 $\mu$ M	8	6
Anabaseine	100 $\mu$ M	7	8
Nicotine	10 $\mu$ M	7	8
Cotinine	100 $\mu$ M	6	8
Nor-nicotine	10 $\mu$ M	5	6
Anatabine	100 $\mu$ M	4	8
<b>HS selective agonists</b>			
sazetidine-A	30 $\mu$ M	7	7
TC-2559	30 $\mu$ M	6	8
<b><math>\alpha 4\beta 2</math> partial agonists</b>			
TC-2403	10 $\mu$ M	8	8
cytisine	100 $\mu$ M	6	6
varenicline	10 $\mu$ M	7	7
arecoline	100 $\mu$ M	8	7
<b><math>\alpha 7</math>-selective agonists</b>			
GTS-21	100 $\mu$ M	7	7
PNU-282987	10 $\mu$ M	8	8
TC-1698	10 $\mu$ M	8	8
AR-R17779	10 $\mu$ M	7	5
choline	1 mM	7	8

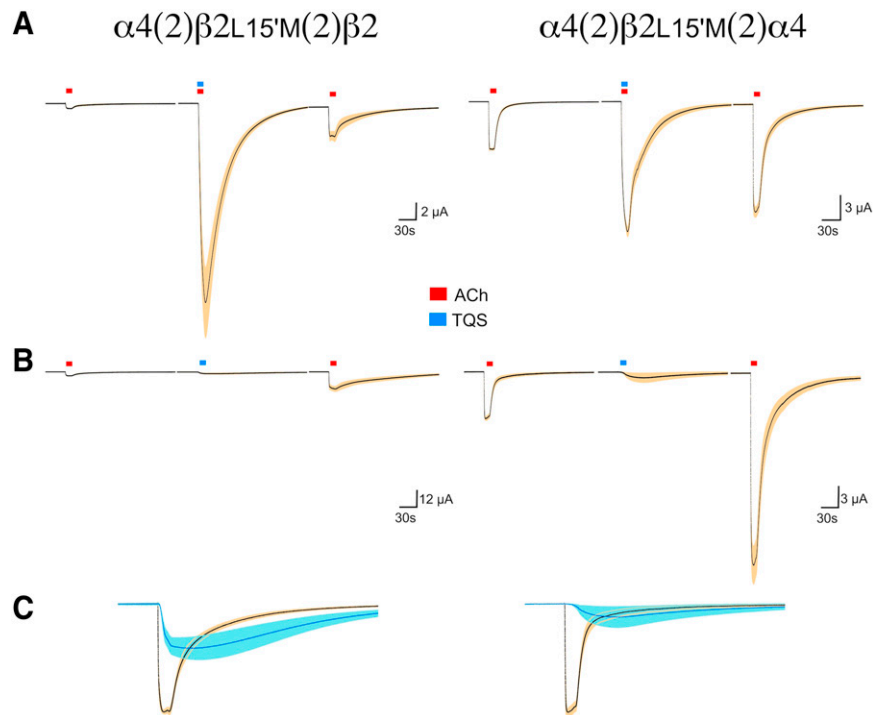
<sup>a</sup>1,1-dimethylpiperidinium.

<sup>b</sup>3-[3-(3-Pyridinyl)-1,2,4-oxadiazol-5-yl]benzotrile 3-[3-(3-Pyridinyl)-1,2,4-oxadiazol-5-yl]benzotrile.



**Fig. 1.** TQS-sensitive HS and LS  $\alpha 4\beta 2$  receptors. (A) Subunit configuration of the receptors composed of  $\alpha 4$ - $\beta 2$  concatamers with the  $\beta 2L15'M$  mutation (represented by the star) in the  $\beta$  subunits. When coexpressed with wild-type  $\beta 2$  subunits, they yield HS  $\alpha 4(2)\beta 2L15'M(2)\beta 2$  receptors (left). When coexpressed with wild-type  $\alpha 4$  subunits, they yield LS  $\alpha 4(2)\beta 2L15'M(2)\alpha 4$  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 ( $\pm$  S.D.), normalized to preceding control ACh responses obtained from the same cells. ACh controls were 10  $\mu$ M for the HS receptors and 100  $\mu$ M for the LS receptors. The Levenberg-Marquardt algorithm was used in Kaleidagraph to generate curves based on the Hill equation that best fit the data.

**Fig. 2.** Averaged data from HS  $\alpha 4(2)\beta 2L15'M(2)\beta 2$  receptors (left) and LS  $\alpha 4(2)\beta 2L15'M(2)\alpha 4$  receptors (right). (A) Cells were treated with control applications of ACh (red bars) and then after washout ACh coapplied with 30  $\mu M$  TQS (blue bars) and then another application of ACh. ACh controls were 10  $\mu M$  for the HS receptors and 100  $\mu M$  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  $\mu M$  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

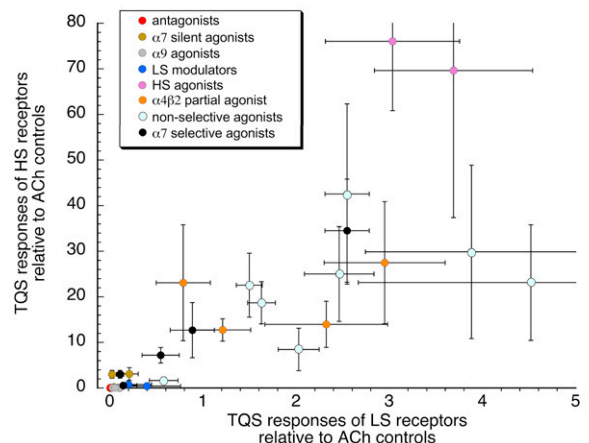
**The Generation of TQS-Sensitive HS and LS  $\alpha 4\beta 2$  nAChR.** The L15'M mutation (Stokes et al., 2019) was made in the  $\beta 2$  subunit of the  $\beta 2-6-\alpha 4$  concatamer (Zhou et al., 2003), so that by coexpressing this concatamer with monomers of either  $\beta 2$  or  $\alpha 4$ , 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  $\mu M$  (Log error =  $-0.39$ ) and 0.11  $\mu M$  (Log error =  $-0.1$ ) for the wild-type and mutant receptors, respectively. For the LS receptors, the ACh Log  $EC_{50}$  values were 2.13  $\mu M$  (Log error = 1.39) and 2.27  $\mu M$  (Log error = 1.27) for the wild-type and mutant receptors, respectively.

As expected, the ACh responses of oocytes expressing these constructs were strongly potentiated by coapplication of ACh with 30  $\mu M$  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  $\alpha 7$  ago-PAM GAT107 (Papke et al., 2014b) and was observed with the TQS-sensitive  $\alpha 4\beta 2$  receptors, regardless of the test compound initially coapplied with

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



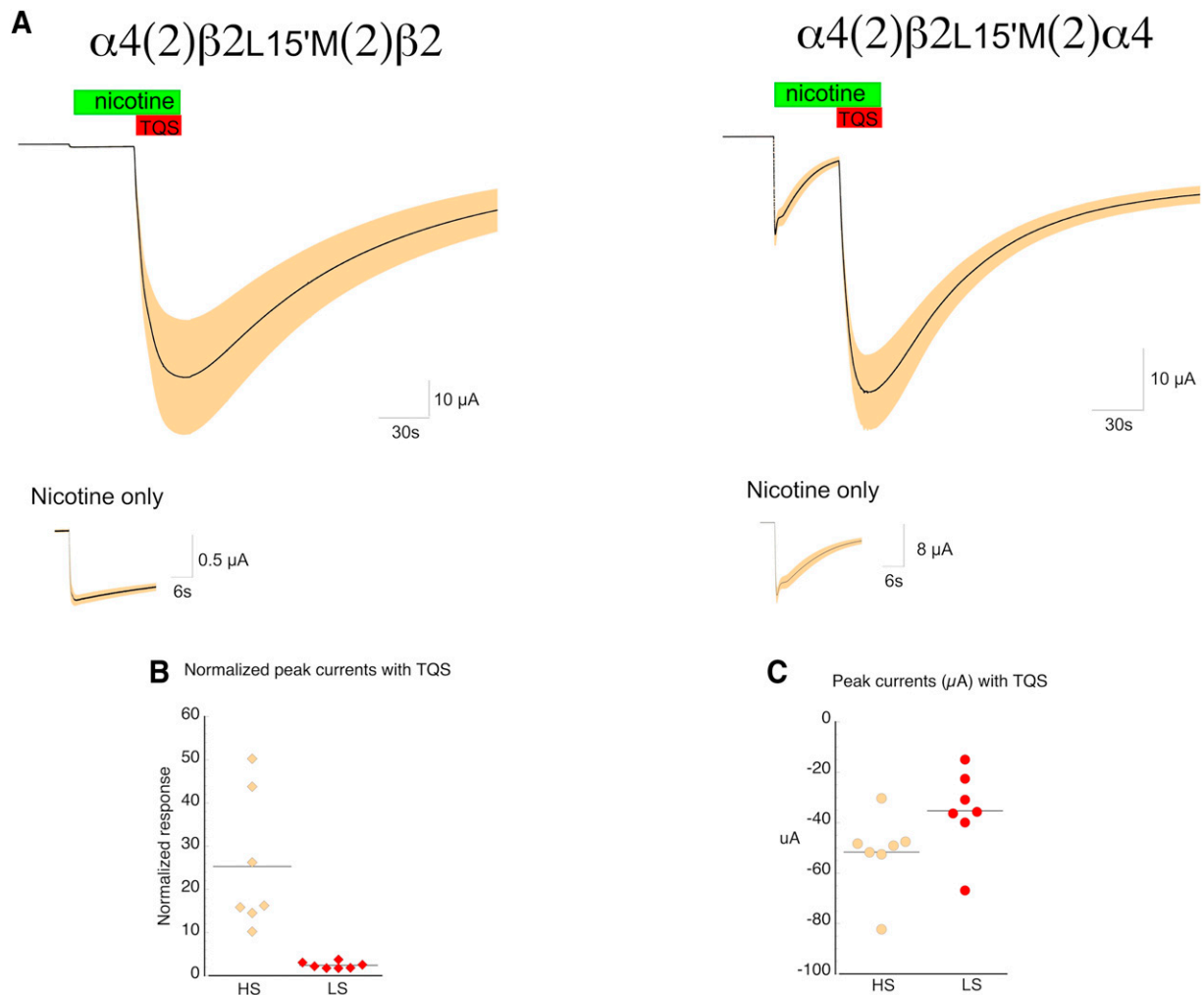
**Fig. 3.** The TQS potentiated responses to all of the test compounds. The HS receptor responses, normalized to their ACh controls, are plotted relative to the scale on the y-axis. The LS receptor responses, normalized to their ACh controls, are plotted relative to the scale on the x-axis. All points 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  $30 \mu\text{M}$  nicotine to HS and LS  $\alpha 4\beta 2\text{L}15'\text{M}$  receptors and then coapplied  $30 \mu\text{M}$  nicotine and  $30 \mu\text{M}$  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  $10 \mu\text{M}$  ACh controls was  $2.47 \mu\text{A}$  (S.D. = 1.03), while the average peak amplitude of the LS  $100 \mu\text{M}$  ACh controls was  $15.9 \mu\text{A}$  (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  $330 \text{ nA}$  (S.D. = 67 nA), while the peak of the LS nicotine response was  $982 \text{ nA}$  (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.



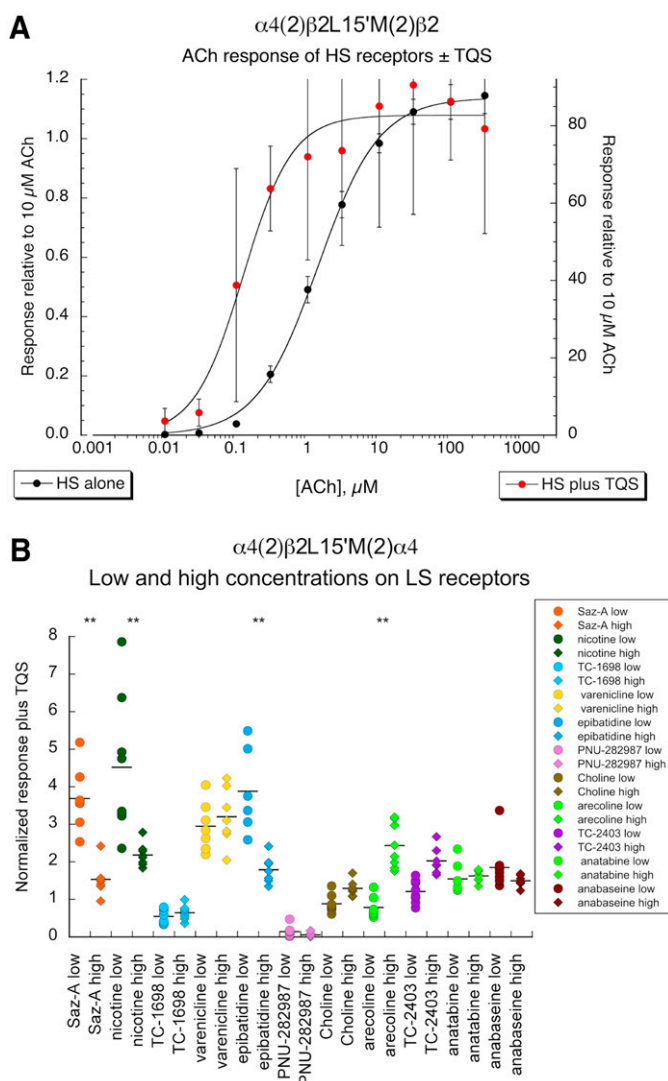
**Fig. 4.** (A) Averaged raw data traces ( $n = 7$  in each case) of HS (on left) and LS (on right)  $\alpha 4\beta 2\text{L}15'\text{M}$  receptors to a 30-second preapplication of  $30 \mu\text{M}$  nicotine followed by a coapplication of  $30 \mu\text{M}$  nicotine and  $30 \mu\text{M}$  TQS. The nicotine-only phases of the responses are shown as inserts below the main traces, scaled as indicated. (B) Peaks of TQS responses normalized to the initial ACh controls (see Supplemental Material for statistics). (C) Peaks of TQS responses measured in  $\mu\text{Amps}$  (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 coapplied with the PAM), we tested three classes of compounds that were not expected to activate  $\alpha 4\beta 2$  receptors when applied alone. These were the  $\alpha 7$  silent agonists NS6740, 1-methylpyridinium (Papke et al., 2022b), and triethylmethylammonium (Papke et al., 2014a) (Supplemental Fig. 2); the  $\alpha 9$ -selective agonists *p*CN diEPP, *p*CONH2-diEPP, and 1,1-dimethylpiperidinium (Papke et al., 2022a) (Supplemental Fig. 3); and the LS  $\alpha 4\beta 2$  modulators, 3-[3-(3-Pyridinyl)-1,2,4-oxadiazol-5-yl]benzotrile (Wang et al., 2015) and dFBr (Weltzin and Schulte, 2010) (Supplemental Fig. 4). As expected, none of these compounds produced activation of either  $\alpha 4\beta 2$  receptor when applied alone, and when coapplied with TQS using our standard protocol, none of these compounds produced responses greater than those seen to TQS applied alone.

**Effects of nAChR Antagonists.** The  $\alpha 7$ -selective antagonist methyllycaconitine (MLA) (Turek et al., 1995) and the  $\alpha 4\beta 2$ -selective antagonist dihydro- $\beta$ -erythroindine (DH $\beta$ E) (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 coapplied 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.,  $> 10 \mu\text{M}$ ) where applications of ACh alone show little further increase, effectively causing a rightward shift in the ACh response curve, making them more LS-like in that regard. Therefore, to determine whether the inability of HS receptors to show increased responses to higher concentrations of ACh was due to progressively larger amounts of TQS-sensitive desensitization, 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  $\text{EC}_{50}$  of  $125 \pm 22 \text{ nM}$  for ACh plus TQS compared with  $1.39 \pm 0.07 \mu\text{M}$  for ACh alone. These data suggest that TQS-sensitive desensitization is a limiting factor even at the lowest ACh concentrations and not a factor especially limiting HS responses to higher concentrations of agonist.

**LS Receptor Potentiation at Higher Drug Concentrations.** Given that the receptors with the  $\alpha 4(3)\beta 2(2)$  configuration are characterized as low sensitivity, the L15'M receptors with this configuration were also tested with 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  $30 \mu\text{M}$  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  $10 \mu\text{M}$  ACh controls from the same cells. The estimated  $I_{\text{max}}$  for ACh alone was only  $1.14 \pm 0.012$  the ACh controls ( $r = 0.999$ ), while for the TQS-potentiated current the  $I_{\text{max}}$  was  $82.7 \pm 2.6$  ( $r = 0.988$ ). (B) TQS potentiation of LS receptors at two different concentrations. Circles represent the average normalized peak current responses obtained with TQS coapplied with the test compounds at the concentrations indicated in Table 1. Diamonds represent the average normalized peak current responses obtained with TQS coapplied 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 coapplied at the higher concentration with TQS were roughly 50% smaller ( $P < 0.0001$ ) than the responses to the lower concentrations coapplied with TQS. Only responses to 1 mM arecoline coapplied with TQS were larger ( $P < 0.001$ , Table 2), by roughly a factor of 2 than when TQS was coapplied 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	

**Potential 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  $\alpha 4\beta 2$  agonist (Papke et al., 2007), cotinine is generally thought of as primarily being a biomarker for nicotine use with very low potency as an agonist (Tan et al., 2021). We also tested the minor tobacco alkaloid anatabine (Wu et al., 2002), previously reported to be an  $\alpha 4\beta 2$  agonist (Alijevic et al., 2020). We also tested carbachol (Parker et al., 1998), an agonist for both nicotinic and muscarinic AChR, along with epibatidine (Badio and Daly, 1994), a toxin isolated from frogs that is 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  $\alpha 4\beta 2$  Partial Agonists.** Four  $\alpha 4\beta 2$  partial agonists, including the smoking-cessation drugs cytisine (Etter et al., 2008) and varenicline (Coe et al., 2005), as well as arecoline (Papke et al., 2015), an active agent in areca associated with betel quid addiction (Gupta and

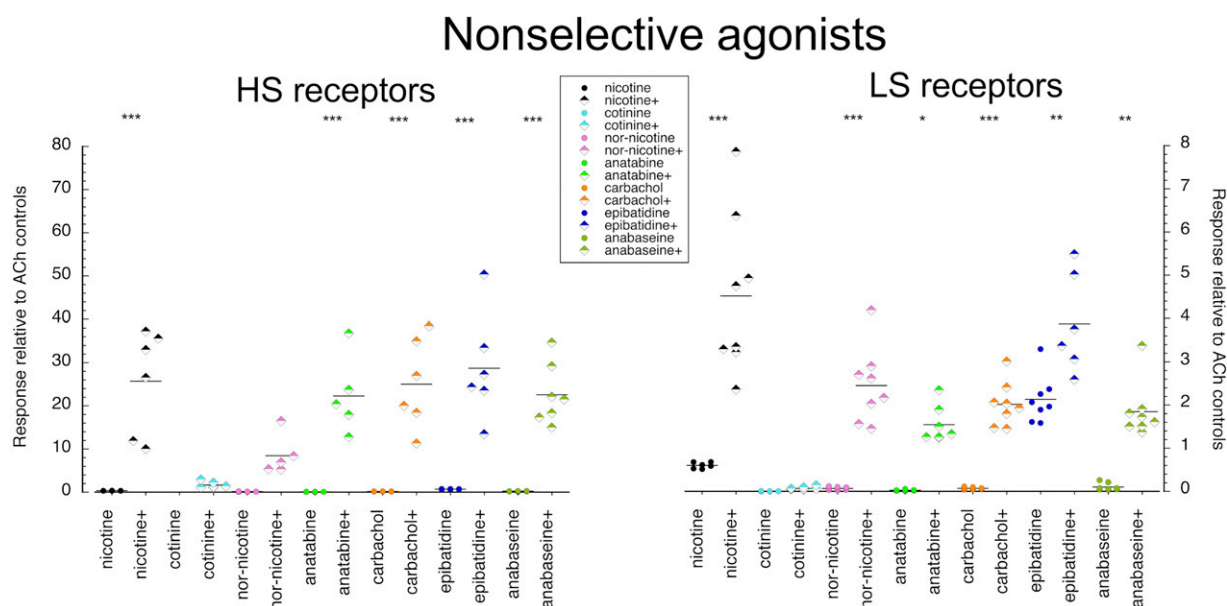
Warnakulasuriya, 2002), and TC-2403 (Papke, 2002) were tested. As expected, responses to these agents were low when applied alone, especially for the HS receptors. Normalized to their respective ACh controls, the responses of LS receptors to varenicline and TC-2403 were greater compared to those of HS receptors ( $P < 0.001$ , see Supplemental 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  $\alpha 4\beta 2$ \* 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  $\alpha 4\beta 2$ -Selective Agonists.** Sazetidine-A and TC-2559, two agents that are potent activators of HS  $\alpha 4\beta 2$  receptors with little or no efficacy for activating LS receptors, were tested (Fig. 7B). Sazetidine-A was actually first published as a selective  $\alpha 4\beta 2$  desensitizer, since it was shown to primarily desensitize receptors in an expression system that was biased toward the formation of LS-type receptors (Xiao et al., 2006) and only later shown to be an HS-selective agonist when HS receptor formation was enhanced by injection of oocytes with a 10-fold excess of  $\beta 2$  relative to  $\alpha 4$  RNA (Zwart et al., 2008). The same approach was also used to demonstrate the increased efficacy of TC-2559 for HS receptors (Zwart et al., 2006). As expected, when applied alone, these agents stimulated large responses for HS receptors with very little response in the LS receptors ( $P < 0.0001$ , Supplemental Material). However, when coapplied with TQS, both compounds were strong activators of both receptor types,

TABLE 3  
Antagonists

3a. Responses	HS activation Average $\pm$ S.D.	HS TQS Average $\pm$ S.D.	LS activation Average $\pm$ S.D.	LS TQS Average $\pm$ S.D.
MLA	0.007 $\pm$ 0.003	0.016 $\pm$ 0.009 <sup>#</sup>	0.003 $\pm$ 0.003	0.003 $\pm$ 0.003
DH $\beta$ E	0.046 $\pm$ 0.032 <sup>#</sup>	0.085 $\pm$ 0.046 <sup>#</sup>	0.004 $\pm$ 0.002	0.004 $\pm$ 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 <sup>#</sup>	1.4235
DH $\beta$ E	0.0201	2.0367	0.0209 <sup>#</sup>	0.8528

<sup>#</sup> $P < 0.05$ , 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  $\mu$ M 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).

**$\alpha$ 7-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  $\alpha$ 7 nAChR without affecting other subtypes like  $\alpha$ 4 $\beta$ 2 receptors (Papke and Horenstein, 2021), leading to the identification of several  $\alpha$ 7-selective agonists. Among the first  $\alpha$ 7-selective agonists to be published, and one of the most widely used, is GTS-21 (DMXB) (de Fiebre et al., 1995), although even in the first publication it was noted to also antagonize  $\alpha$ 4 $\beta$ 2 responses. Subsequently, several large pharmaceutical companies developed agents that were proposed to be more selective than GTS-21, including AR-R17779 (Levin et al., 1999), TC-1698 (Marrero et al., 2004), and PNU-282987 (Bodnar et al., 2005). The ACh precursor choline was also identified as an  $\alpha$ 7-selective agonist (Papke et al., 1996), although its low potency and ubiquitous presence in the brain and blood generally precludes its consideration as a therapeutic agent.

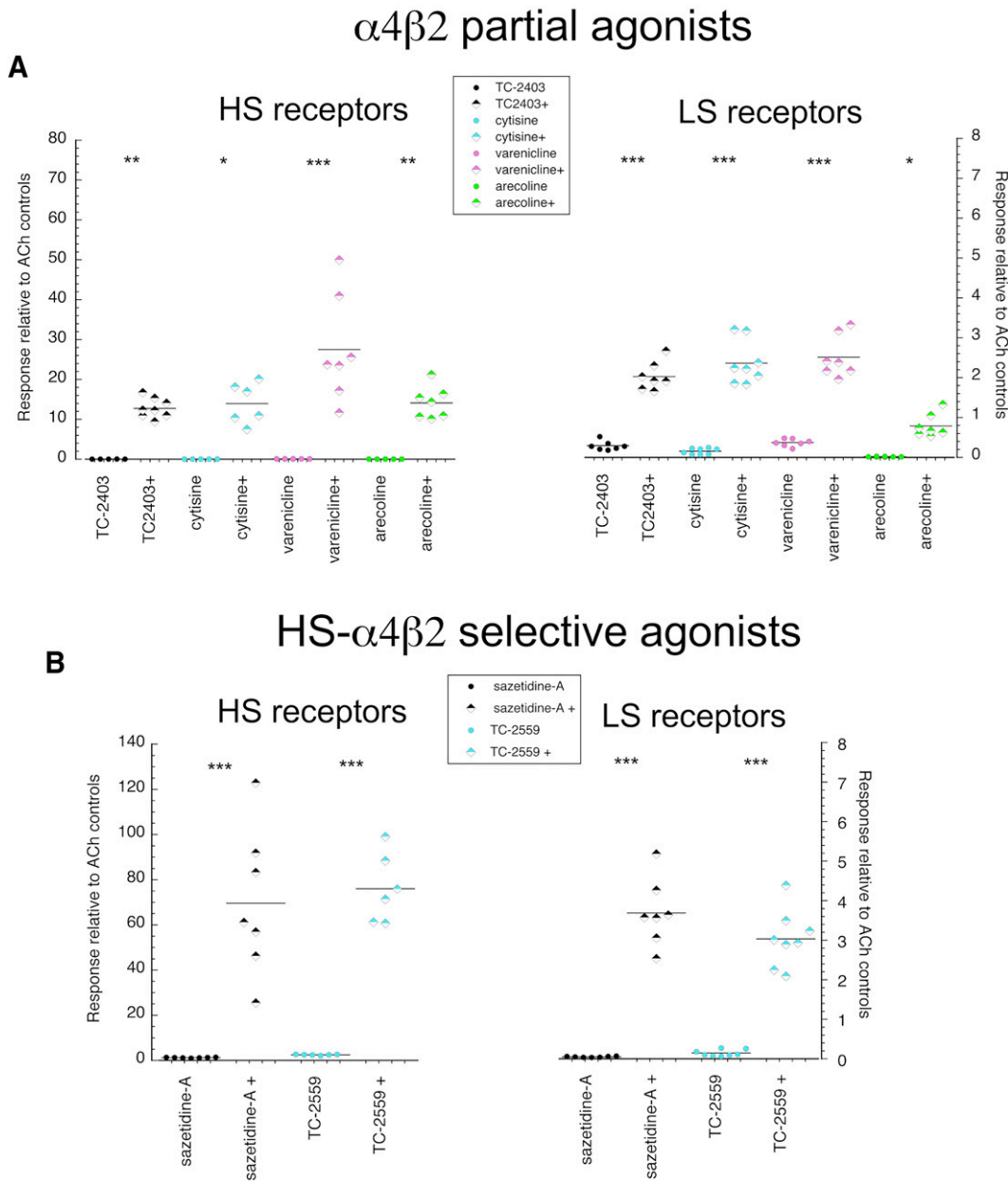
As expected, none of these drugs evoked much activation of the  $\alpha$ 4 $\beta$ 2 receptors, although there were small responses of HS receptors to GTS-21 (Table 4 and Fig. 8). This activity may have been missed in earlier studies that were based on the expression of  $\alpha$ 4 and  $\beta$ 2 injected at equal ratios in *Xenopus* oocytes and might have biased expression toward the LS form. In any case, these responses were smaller than responses to ACh or TQS alone ( $P < 0.0001$ , see Supplemental Material for ANOVA results) and were not larger than the responses to the other  $\alpha$ 7 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  $\alpha$ 4 $\beta$ 2 ACh Responses by  $\alpha$ 7-Selective Agonists.** The data in Fig. 9 suggest that some of the compounds proposed to activate  $\alpha$ 7 receptors would be effective desensitizing antagonists of  $\alpha$ 4 $\beta$ 2 receptors. To test this, cells expressing wild-type forms of HS and LS  $\alpha$ 4 $\beta$ 2 receptors were pre-exposed to the commercially developed  $\alpha$ 7-selective agonists for 30 seconds, and then ACh was coapplied at the control concentration along with the  $\alpha$ 7 agonists at the test concentration. The ACh responses were compared with the control ACh responses obtained prior to the application of the  $\alpha$ 7 agonists (Fig. 9). GTS-21 preapplication evoked a small response from the LS  $\alpha$ 4 $\beta$ 2 receptors and suppressed the ACh responses of both subtypes, with a greater effect on HS than on LS (Table 5). TC-1698 produced a nearly complete block of the ACh responses of both  $\alpha$ 4 $\beta$ 2 subtypes, while AR-R17779 produced a 50% block of the HS responses with no effect on the LS ACh response. PNU-282987 preapplication and coapplication caused no block of either  $\alpha$ 4 $\beta$ 2 receptor subtype (Fig. 9). These results with wild-type receptors are consistent with the TQS effects obtained on the receptors with L157M mutant  $\beta$ 2 subunits (Fig. 8).

## Discussion

The results support the hypothesis that the responses of HS  $\alpha$ 4 $\beta$ 2 receptors are strongly limited by desensitization, even at low agonist concentrations. They also show that the desensitization is not specifically a factor limiting the response of the HS receptors to high agonist concentrations. The survey of the several classes of ligands identified some types of compounds, like the  $\alpha$ 7 silent agonist and the  $\alpha$ 9 agonists, that appear to be free of  $\alpha$ 4 $\beta$ 2 activating or desensitizing effects. They also indicate that desensitization is a factor limiting the efficacy of  $\alpha$ 4 $\beta$ 2 partial agonists like cytisine and varenicline and that selective desensitization of LS receptors may tune the efficacy of agents like sazetidine-A and TC-2559 so that they are





**Fig. 7.** Effects of selective agonists. (A) Dot plot of the peak current responses of HS (left) and LS receptors (right) to the  $\alpha 4\beta 2$  partial agonists when applied alone (circles), compared with the responses to drugs coapplied with 30  $\mu\text{M}$  TQS, indicated by the drug name with a plus sign and plotted as half-color diamonds. (B) Dot plot of the peak current responses of HS (left) and LS receptors (right) to the HS  $\alpha 4\beta 2$  selective agonists when applied alone (circles), compared with the responses to drugs coapplied with 30  $\mu\text{M}$  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.

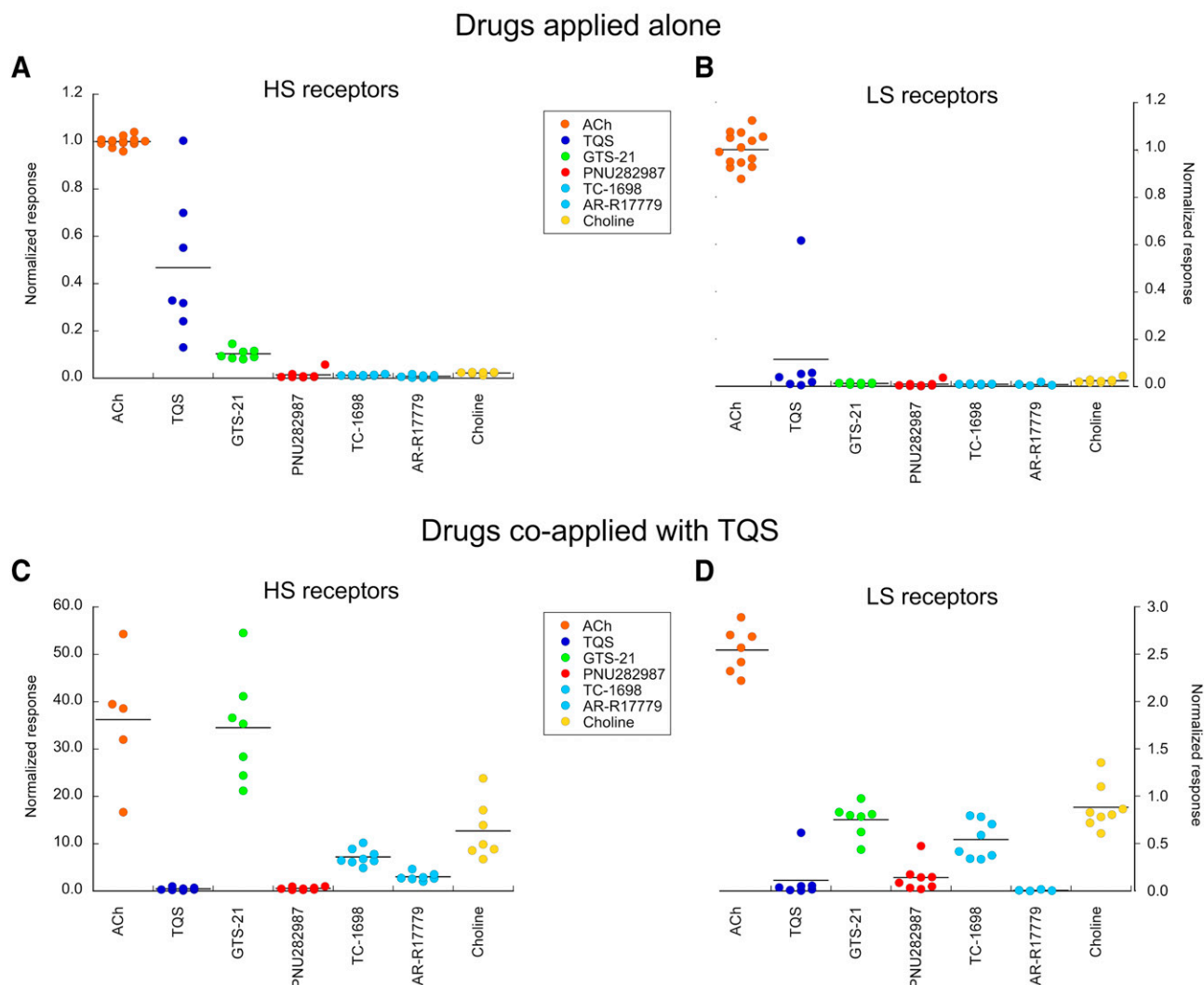
The desensitization of nAChR is a complex and multiphasic process (Feltz and Trautmann, 1982; Simasko et al., 1986; Boyd, 1987; Sine and Steinbach, 1987; Forman and Miller, 1988; Dilger and Liu, 1992; Quick and Lester, 2002; Lester,

2004; Papke et al., 2009), and the effect of type II PAMs on  $\alpha 7$  receptors is selective for only some form(s) of desensitization (Williams et al., 2011). Although the effects of some type II PAMs can be quite large, they do not reverse all the receptor desensitization or affect all receptors equally. Indeed, the effects of the  $\alpha 7$  PAM PNU-120596 are to enormously increase

TABLE 4  
 $\alpha 7$ -selective agonists

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

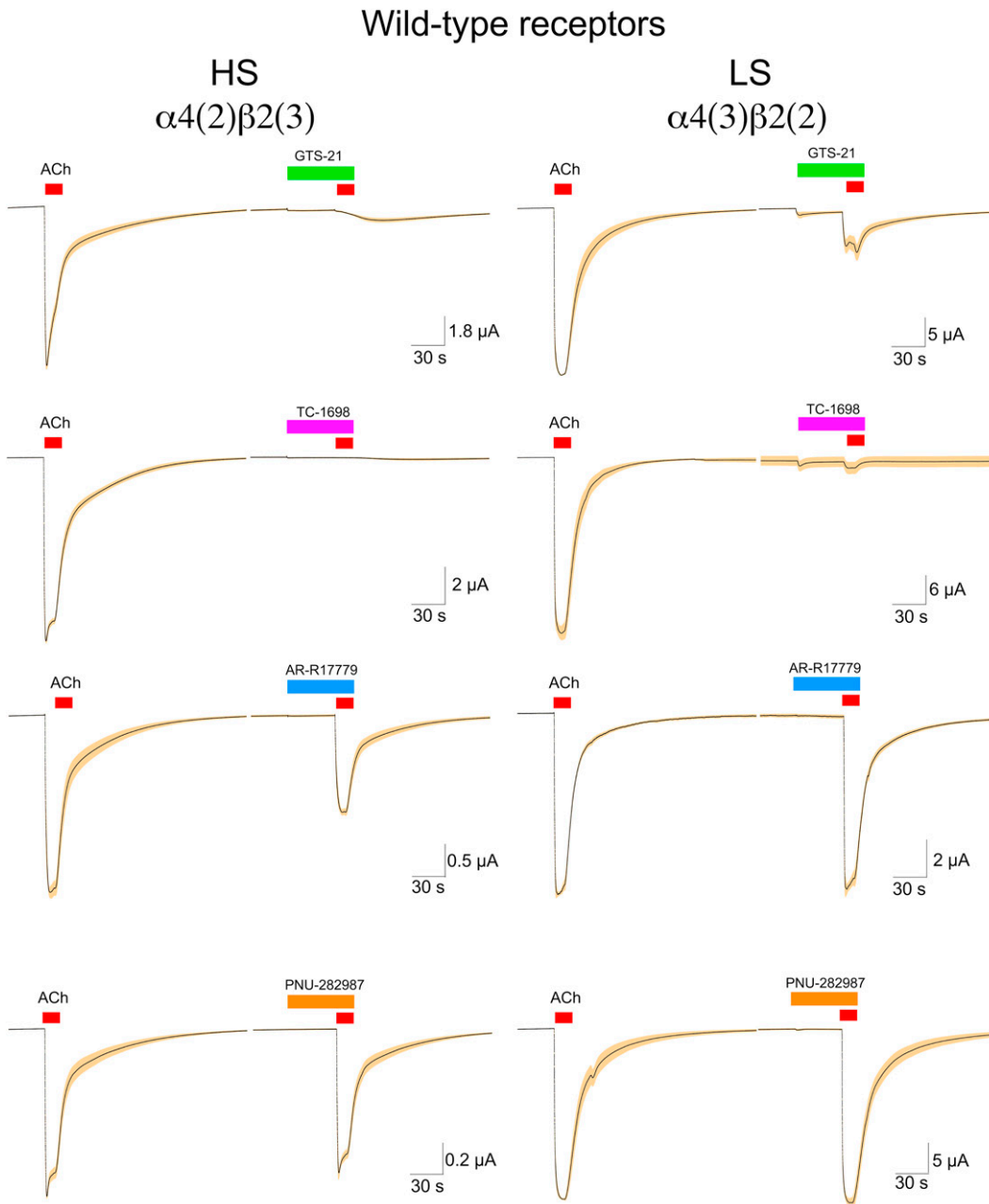
See Supplemental Material for ANOVA results.

Effects of  $\alpha 7$ -selective agonists on  $\alpha 4\beta 2$ L15M receptors

**Fig. 8.** Effects of  $\alpha 7$ -selective agonists (Table 4). (A) Dot plot of the peak current responses of TQS-sensitive HS receptors to the  $\alpha 7$ -selective agonists when applied alone, compared with ACh control responses and the responses to  $30 \mu\text{M}$  TQS applied alone. Although GTS-21 appeared to give detectable responses, these were not statistically significant compared with the other  $\alpha 7$ -selective agonists (see Supplemental Material for ANOVA results). (B) Responses of TQS-sensitive HS  $\alpha 4\beta 2$  receptors to  $\alpha 7$ -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  $\alpha 4\beta 2$  receptors to the  $\alpha 7$ -selective agonists when applied alone, compared with ACh control responses and the responses to  $30 \mu\text{M}$  TQS applied alone. (D) Responses of TQS-sensitive LS  $\alpha 4\beta 2$  receptors to  $\alpha 7$ -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  $\alpha 7$  receptors are similar to those of PNU-120596, (Pałczyńska et al., 2012; Quadri et al., 2019), while the effects of TQS on  $\alpha 7$  ACh responses are somewhat less (Pałczyńska et al., 2012). However, the basic mechanisms of  $\alpha 7$  desensitization are fundamentally different from those of  $\alpha 4\beta 2$  receptors, so likewise the TQS effects on the TQS-sensitive  $\alpha 4\beta 2$  receptors might be very different on the molecular level from the effects on  $\alpha 7$  receptors. While  $\alpha 7$  receptors show no activation at all with high agonist concentrations (Williams et al., 2011),  $\alpha 4\beta 2$  receptors can smolder (Campling et al., 2013), occasionally opening under predominantly desensitizing conditions.

While TQS is considered strictly a PAM for  $\alpha 7$ , since we observed it to activate the TQS-sensitive  $\alpha 4\beta 2$  receptors (particularly the HS receptors) when applied alone, it might be classified as a weak ago-PAM or allosteric agonist for these receptors, behaving like the TQS analog (+)4BP-TQS (GAT107) on  $\alpha 7$  receptors. By definition, “ago-PAMs” potentiate the responses evoked by agonists but also produce activation on their own and may also prime the potentiation of subsequent agonist application. The direct allosteric activation of HS  $\alpha 4\beta 2$ L15M receptors by TQS was blocked by  $10 \mu\text{M}$  of the  $\alpha 4\beta 2$ -selective antagonist DH $\beta$ E as well as by  $100 \mu\text{M}$  MLA, a concentration at which the drug is no longer selective for  $\alpha 7$  (Buisson et al., 1996). While the allosteric activation of  $\alpha 7$  by GAT107 can be blocked by  $10 \mu\text{M}$  of the  $\alpha 7$  selective antagonist MLA (Papke et al., 2014b), it is insensitive to  $100 \mu\text{M}$  DH $\beta$ E (R.L. Papke, unpublished



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

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  $\alpha 7$ -selective agonists for indications such as schizophrenia (Hajós and Rogers, 2010;

TABLE 5  
Preapplications of  $\alpha 7$  agonists to wild-type receptors

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

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  $\alpha 4\beta 2$  receptors in the brain. This is a particular concern in the case of Alzheimer's disease, since evidence suggests that  $\alpha 4\beta 2$  receptor function is specifically impaired in this patient population (Court et al., 2001; Gotti et al., 2006). The results with the  $\alpha 7$ -selective agonists tested indicate that, depending on the specific agent, a number of differing profiles of  $\alpha 7$  agonism and  $\alpha 4\beta 2$  antagonism may be available, with PNU-282987 being the least likely to affect  $\alpha 4\beta 2$  function. As noted earlier, GTS-21 was the first synthetic  $\alpha 7$ -selective agonist identified, and since 1994 it has been cited in 248 PubMed publications, including 41 since 2019. PNU-282987, has been cited in 202 PubMed publications since it was first reported in 2005, and it is also in current use, with 39 citations since 2019. The  $\alpha 7$  agonists TC-1698 and AR-R17779 are far less commonly used, with only 3 and 50 total PubMed citations, respectively. Two studies related to CAP activity reported comparable effects with GTS-21 and PNU-282987 (Yuan et al., 2021; Zhou et al., 2021). However, it is important to note that, from an electrophysiological perspective, these compounds have very different activity profiles for  $\alpha 7$  receptors. PNU-282987 is relatively potent and nearly a full agonist (Hajós et al., 2005), while GTS-21 has lower potency and efficacy and additionally produces residual  $\alpha 7$ -receptor desensitization (Papke et al., 2009). Interestingly, the  $\alpha 7$  desensitizing activity of GTS-21 may be important for its CAP activity (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  $\alpha 4\beta 2^*$  and  $\alpha 7$  receptors, with GTS-21 capable of decreasing  $\alpha 4\beta 2$  function as well as working on  $\alpha 7$  and PNU-282987 affecting  $\alpha 7$  exclusively.

The perspective of nAChR as mediators of fast synaptic transmission, which is their function at neuromuscular junctions and autonomic ganglia, relegates desensitization to the background as perhaps nothing more than a safety valve to prevent overstimulation. However, as modulators of neurotransmission in the brain, often as presynaptic receptors on neurons that release other neurotransmitters, desensitization must be accounted for in the cholinergic control of brain function. Consideration of desensitization is even more important when considering the effects of self-administered nicotine, especially after a smoker's very first cigarette puff of the day (Picciotto et al., 2008). However, does desensitization just move receptors to the sidelines, or is it possible that desensitized receptors serve other functions, independent of ion channel activity? Recent studies of  $\alpha 7$  nAChR, especially in the context of the CAP mediated by  $\alpha 7$ , and possibly  $\alpha 9\alpha 10$ , receptors in immune cells (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  $\alpha 7$  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  $\alpha 4$  is the largest of any nAChR subunit, by a factor of 3 or more. Although not so well studied as  $\alpha 7$  in this regard, there have been reports that  $\alpha 4$ -containing nAChR also play roles in the regulation of inflammatory and neuropathic pain (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  $\alpha 4\beta 2$  receptors and may prove useful for the development of more focused therapeutics.

#### Acknowledgments

Oocyte recordings were conducted by Lu Wenchi Corrie.

#### Authorship Contributions

Participated in research design: Papke.

Performed data analysis: Papke, Stokes.

Wrote or contributed to the writing of the manuscript: Papke, Stokes.

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