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Electrophysiological Perspectives on the Therapeutic Use of Nicotinic Acetylcholine Receptor Partial Agonists[S]

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ABSTRACT

Partial agonist therapies rely variously on two hypotheses: the partial agonists have their effects through chronic low-level receptor activation or the partial agonists work by decreasing the effects of endogenous or exogenous full agonists. The relative significance of these activities probably depends on whether acute or chronic effects are considered. We studied nicotinic acetylcholine receptors (nAChRs) expressed in *Xenopus laevis* oocytes to test a model for the acute interactions between acetylcholine (ACh) and weak partial agonists. Data were best-fit to a basic competition model that included an additional factor for noncompetitive inhibition. Partial agonist effects were compared with the nAChR antagonist bupropion in prolonged bath application experiments that were designed to mimic prolonged drug exposure typical of therapeutic drug delivery. A primary effect of prolonged application of nicotine was to decrease the response of all nAChR subtypes to acute applications of ACh. In addition, nicotine, cytisine, and varenicline produced detectable steady-state activation of $\alpha4\beta2^*$ [$(\alpha4)_2(\beta2)_3$, $(\alpha4)_3(\beta2)_2$, and $(\alpha4)_2(\beta2)_2\alpha5$)] receptor subtypes that was not seen with other test compounds. Partial agonists produced no detectable steady-state activation of $\alpha7$ nAChR, but seemed to show small potentiation of ACh-evoked responses; however, "run-up" of $\alpha7$ ACh responses was also sometimes observed under control conditions. Potential off-target effects of the partial agonists therefore included the modulation of $\alpha7$ responses by $\alpha4\beta2$ partial agonists and decreases in $\alpha4\beta2^*$ responses by $\alpha7$-selective agonists. These data indicate the dual effects expected for $\alpha4\beta2^*$ partial agonists and provide models and insights for utility of partial agonists in therapeutic development.

Introduction

The armamentarium of pharmacotherapeutics includes agonists (receptor activators) and antagonists (inhibitors of receptor activation). However, many drugs fall in between these two categories and are classified as partial agonists. In therapeutic applications, a partial agonist can act as a sort of activity buffer for naturally occurring strong agonists, providing receptor stimulation if the natural activator is low or absent, and diminishing the effects of a natural strong activator if the strong activator's concentrations are high.

Partial agonists may also be used to buffer the effects of drugs of abuse; an area of current interest for the development of partial agonist therapies is for the treatment of nicotine addiction and dependence. A new drug in this area is the cytisine-related compound, varenicline (Chantix). Varenicline is a weak partial agonist for nicotinic acetylcholine receptor (nAChR) subtypes in the brain that contain α4 and β2 subunits, which have been shown in animal studies to be essential for nicotine to stimulate the brain's neurochemical reward systems (Picciotto et al., 1998; Champtiaux et al., 2003).

One important factor limiting the efficacy of nicotinic partial agonist therapies for smoking cessation or other indications is the likelihood of side effects associated with the activation or inhibition of other nAChR subtypes. Both varenicline and cytisine are likely to have such off-target effects because, along with partial agonism of α4β2*-nAChR, they are full agonists for the α3β4-nAChR of autonomic ganglia and homomeric α7-nAChR found in brain and other tissues (Luetje and Patrick, 1991; Papke and Porter Papke, 2002).

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; GTS-21, 3-(2,4-dimethoxy-benzylidene)anabaseine; 3-pyr-Cyt, 3-(pyridin-3′-yl)-cytisine; S 24795, 2-[2-(4-bromophenyl)-2-oxoethyl]-1-methyl pyridinium.

The α7-nAChRs are also proposed targets for several partial agonist therapies for such diverse indications as Alzheimer's disease, schizophrenia, and inflammatory disease (Kem, 2000; de Jonge and Ulloa, 2007), and α7 partial agonists may have off-target effects as antagonists of other nAChRs (de Fiebre et al., 1995).

The natural balance of receptor subtypes may be perturbed in drug-dependent or disease states. There is well documented up-regulation of α4β2-nAChR in smokers and animals exposed to chronic nicotine (Benowitz, 2009). Concomitant to nicotine dependence there may also be a shift in the predominant form of α4β2-nAChR in the brain from a low-sensitivity form (LSα4β2), believed to contain three α4 subunits and two β2 subunits [$(\alpha4)_3(\beta2)_2$], to a high-sensitivity form (HSα4β2), with the reverse subunit stoichiometry [$(\alpha4)_2(\beta2)_3$)] (Walsh et al., 2008).

Although there is up-regulation of the targeted receptor for smoking cessation, there is good evidence that there may be functional deficiencies in the targeted α7 receptor in schizophrenia (Leonard et al., 2000). Expression of the α7 receptor is also down-regulated by stress hormones, and decreased α7 receptor function may be associated with depression (Lai et al., 2001). The incidence of smoking is very high in the mentally ill, and nicotine dependence in this patient population may develop secondarily to self-medication with nicotine in compensation for decreased α7-nAChR receptor function (Leonard et al., 2000). Numerous adverse neuropsychiatry effects have been reported for varenicline therapies (McClure et al., 2009), leading the Food and Drug Administration to issue a black-box warning. It may be the case that these adverse effects were caused at least in part by off-target effects on α7 receptors in patients already suffering from disease-related decreases in α7 function.

In this article we present a comprehensive characterization of α4 and α7 partial agonists using defined nAChR subtypes [$(\alpha4)_3(\beta2)_2$, $(\alpha4)_2(\beta2)_3$, $(\alpha4)_2(\beta2)_2\alpha5$, and α7] expressed in *Xenopus laevis* oocytes. We propose and test a quantitative model for the acute buffering effects of α7 and α4β2 partial agonists on the activity of the endogenous full agonist acetylcholine (ACh). We make an important extension of those studies to place the partial agonist delivery into a context relevant for therapeutics, evaluating how activation by ACh will be perturbed by the chronic applications of the partial agonists for both target and off-target receptors.

The predominant effect of bath-applied partial agonists seemed to be predesensitization. However, within limited concentration ranges, nicotine and the α4β2 partial agonists cytisine and varenicline (provided by Targacept, Winston Salem, NC) also produced mecamylamine-sensitive steady-state activation. It is noteworthy that although steady-state activation of α7 receptors was not detected, low concentrations of the α7-selective partial agonists 3-(2,4-dimethoxybenzylidene)anabaseine (GTS-21; provided by Taiho, Tokyo, Japan) and 2-[2-(4-bromophenyl)-2-oxoethyl]-1-methyl pyridinium (S 24795; provided by Servier, Cedex, France) were seen to potentiate or prime the receptors for greater activation by ACh pulses. However "run-up" of ACh-evoked responses was also seen in some control experiments, suggesting that ACh may be self-priming. Such priming activity suggests a novel mechanism of action for α7-selective drugs in therapeutics and possibly the utility of combination therapies to protect α7 receptor function from the off-target effects of other drugs such as varenicline.

Materials and Methods

Heterologous Expression of nAChR in *Xenopus* Oocytes

cDNA. Human nAChR receptor clones and concatamers were obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). The human RIC-3 clone, obtained from Dr. Millet Treinin (Hebrew University, Jerusalem, Israel), was coinjected with α7 to improve the levels and speed of receptor expression.

Oocyte Preparation. Oocytes were surgically removed from mature *X. laevis* frogs and injected with 50 nl (5–20 ng) of appropriate nAChR subunit cRNAs as described previously (Horenstein et al., 2008).

Oocyte Recording and Data Analysis

Experiments were conducted using OpusXpress 6000A (Molecular Devices, Sunnyvale, CA) and analyzed as described previously (Papke and Porter Papke, 2002; Horenstein et al., 2008; Papke and Stokes, 2010).

Acute Coapplication Experiments. After two initial ACh control applications [300 μM for the α7 experiments and 100 μM for the $(\alpha4)_3(\beta2)_2$ experiments] cells were treated in alternation with mixtures of ACh and the partial agonist or ACh alone. Data were rejected if there was more than a 25% variation between the ACh controls before or subsequent to the experimental coapplication. The data used for the fitting procedures were average values from at least four cells given the same treatments, normalized to the respective ACh control responses recorded before the experimental coapplications. In the case of the α4β2 data, a further correction was made to adjust for the ratio of the ACh control and the ACh maximal response, because responses to the control concentration of ACh were only 46% the amplitude of the ACh maximal responses (see below).

Bath Application Experiments. Baseline conditions were defined by two ACh control applications made before the introduction of the test compounds into the bath. The ACh control concentrations were 30 μM for $(\alpha4)_3(\beta2)_2$, 10 μM for $(\alpha4)_2(\beta2)_3$, 3 μM for α4β2α5, and 60 μM for α7. These concentrations were selected because they gave robust responses that showed little or no rundown when applied repeatedly at 4-min intervals. They were determined in separate experiments to be the EC_{46}, EC_{90}, EC_{50}, and EC_{60} for the respective subtypes. Once the experimental solutions were introduced in the bath cells were repeatedly stimulated at intervals of 223 s (α7) or 277 s (non-α7) with ACh at the control concentration made up in the experimental bath solution. All data obtained after the application of test compounds to the bath were normalized relative to the average of the first two ACh controls recorded in regular bath solution. Initial experiments were run for 50 min after the introduction of the experimental compounds into the bath. However, because the maximal effects were achieved after only 25 min, subsequent bath application experiments were run for only 35 min. Data in the summary plots are the average of the data from the last three of these ACh probes.

Modeling of Acute Agonist/Partial Agonist Interactions

Acute responses to agonist/partial agonist applications were fit with a general nonlinear regression model, implemented as an option in a Windows data analyses and graphical presentation program (CAS Data Forge, Stephen A. Bloom, Ecological Data Consultants, Archer, FL). The terms n_a, n_p, n_i, $R_{max}p$, $In_{max}p$, $EC_{50}a$, $EC_{50}p$, and $IC_{50}p$ were given starting estimates based on the data obtained with the drugs applied separately. Tolerance factor (typically 1×10^{-5}) range limits for each parameter were specified. For S 24795, the observed values were represented by a matrix of 11 values of ACh (a) and 10 values of S 24795 (p) for a total of 110 observations, with each value being the mean of multiple measurements for the given a and p concentrations. For cytisine, the replicate values for each ACh (a)

and cytisine (p) concentration were used for a total across all cells of 1416 observations. A predicted matrix was then calculated for each cell of the matrix, based on the a and p concentrations, the set of parameters listed above, and the governing equation. The residuals across all cells were calculated, and an iterative process was used to revise the parameters to minimize the residuals and allow the estimates to be revised accordingly. When the difference of estimates for any given parameter for consecutive iterations fell below the tolerance factor, that parameter was fixed at the last estimate. When all parameters were fixed or no convergent solution was found, the process stopped, and the set of parameters representing the best fit along with the resulting matrix was provided.

Results

Modulation of α7 by the Selective Agonist S 24795. We have previously reported (Lopez-Hernandez et al., 2007) that when the weak α7-selective partial agonist S 24795 was coapplied with ACh to hippocampal interneurons expressing α7 receptors, the coapplication responses could be variously increased, decreased, or unchanged, relative to the responses evoked by ACh alone. By comparison of the data obtained from brain slice experiments to data obtained for α7 receptors expressed in *Xenopus* oocytes, under conditions when the actual concentration of the full and partial agonists were known, we were able to estimate the dilution factor of the pressure applications in the hippocampal slice experiments to be approximately 30-fold. We have extended those experiments of receptors expressed in oocytes to encompass full ranges of mixed concentrations and have evaluated the data in the context of the model presented in Fig. 1. The model is based largely on competitive interactions between the full and partial agonists. However, because it has been suggested that partial agonists may also have limiting inhibitory effects that are noncompetitive (Luetje and Patrick, 1991), we have also included a factor allowing for noncompetitive inhibition.

The competitive activity was modeled as the weighted sum of the response evoked by the full and partial agonists, adjusted for both receptor availability and the effective concentration of the drugs, allowing for separate EC_{50} and n values for the two drugs. The I_{max} for the full agonist was constrained to 1, whereas the I_{max} for the partial agonist was a free parameter <1.0.

The data shown as points in Fig. 2A are the means of the experimental (net charge) data obtained when ACh at concentrations ranging from 30 nM to 1 mM was coapplied with S 24795 through the same concentration range. Each point represents the average response of at least four cells normalized to the ACh maximum (net charge) response. Curve fit values for the drugs when applied alone were used as seeds for the competitive interactions. A least-squares analysis was then conducted (see *Materials and Methods*), and curves generated by the best fit of the complete model are shown as the lines in Fig. 2A (parameters of the fit are given in Table 1). For these data, the best fit was achieved with relatively minor weight ascribed to the factor for noncompetitive inhibition (i.e., the curves were not greatly altered if the factor was eliminated). The largest disparities between the observed and predicted values were for the range of high ACh concentration coapplied with relatively low concentrations of S 24795. This is not surprising because with the applications of high ACh concentration the receptor-mediated response is generated almost entirely before the solution exchange is complete (Papke and Porter Papke, 2002), so that S 24795 concentrations were low at the time of the responses and the predicted competitive effects of S 24795 could not be achieved.

Acute Modulation of ACh-Evoked Responses of Low Sensitivity $(\alpha4)_3(\beta2)_2$ nAChR by the α4β2 Partial Agonist Cytisine. We tested the effects of cytisine coapplication on the ACh-evoked responses of human α4β2 receptors expressed in *Xenopus* oocytes. By using an α4-β2 concatamer coexpressed with monomeric α4 (Zhou et al., 2003), we were able to obtain pure populations of the form of α4β2 believed to be most abundantly expressed in human cell lines (Nelson et al., 2003), containing three α4 and two β2 subunits. The data shown as points in Fig. 2B are the means of the experimental (peak current) data obtained when ACh at concentrations ranging from 30 nM to 1 mM was coapplied with cytisine through the same concentration range. Each point represents the average response of at least five cells normalized to the ACh maximum (peak current) response. Curve fit values for the drugs when applied alone were used as seeds for the competitive interactions. A least-squares analysis (see

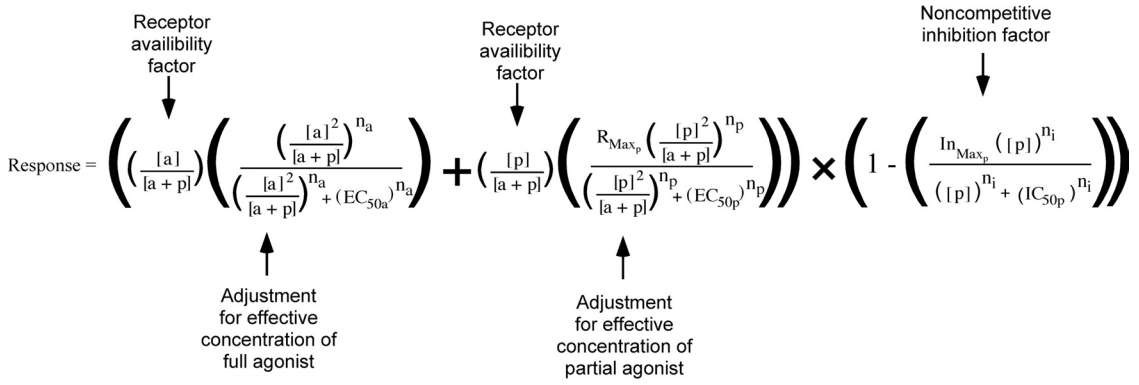


Fig. 1. Model applied to the activation of nAChR by simultaneous coapplications of the full agonist (a) and a partial agonist (p). The terms are defined as follows: [a], concentration of ACh, concentration of the partial agonist (either S 24795 or cytisine in Fig. 2, A or B, respectively); [a + p], the sum of the concentrations; n_a, the Hill coefficient for ACh agonist activity; n_p, the Hill coefficient for partial agonist activity; R_{maxP}, the maximal agonist activity for the partial agonist relative to ACh; EC_{50a}, the EC_{50} for ACh; EC_{50p}, the EC_{50} for the partial agonist; In_{maxP}, the maximal inhibitory activity of the partial agonist; IC_{50p}, the IC_{50} for the inhibitory effects of the partial agonist; n_i, the Hill coefficient for inhibition by the partial agonist. The I_{Max} of the full agonist is assigned a value of 1. The first and second terms of the equation represent the activation produced by the full and partial agonists, respectively, scaled by their potencies and weighted by receptor availability. The third term allows noncompetitive effects of the partial agonist. If such noncompetitive activity is inhibitory, then n_i is assigned a negative value.

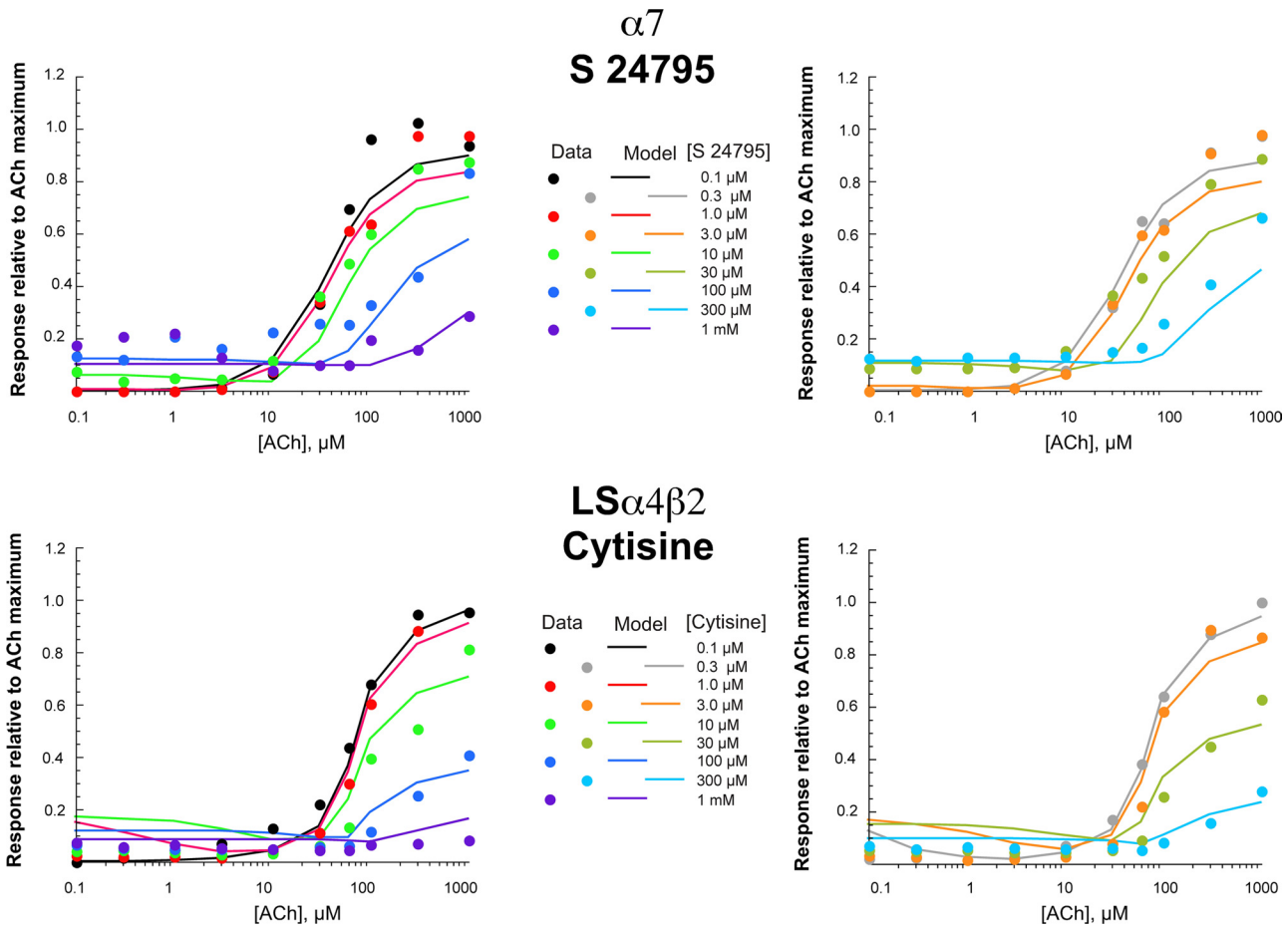


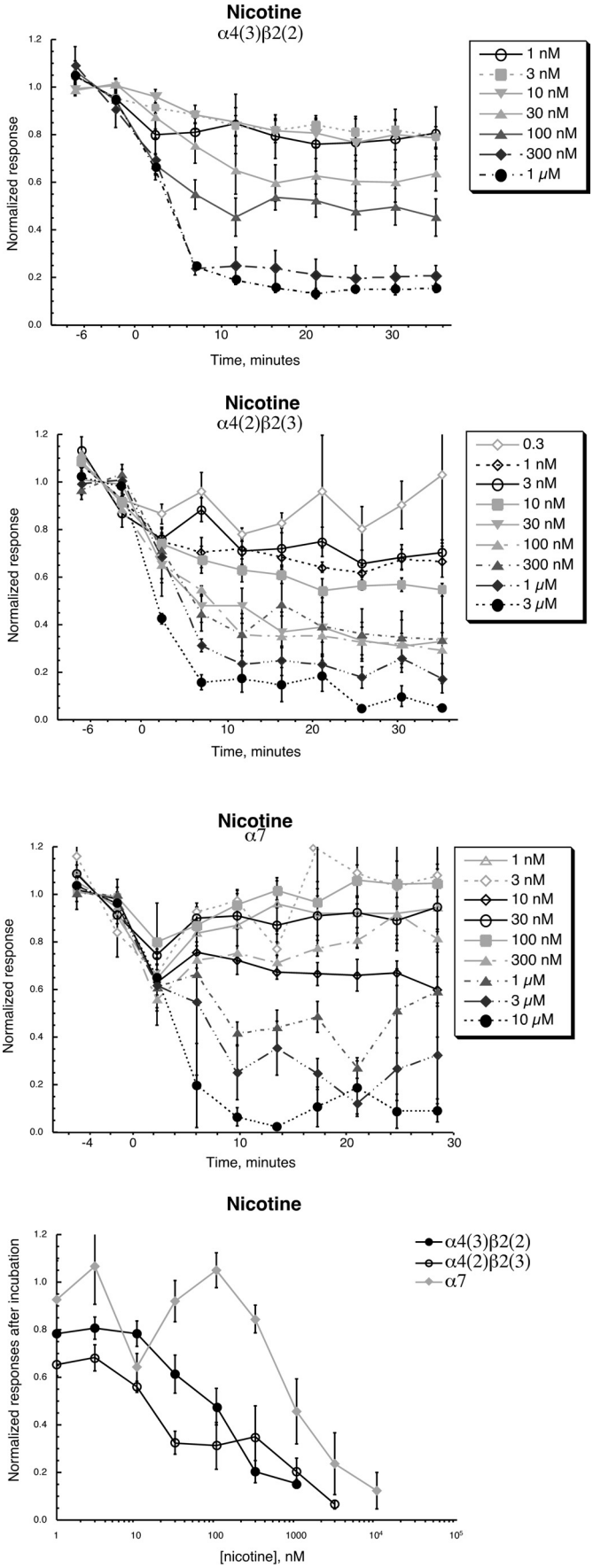
Fig. 2. Experimental (points) and predicted (lines) data for full and partial agonist interactions. A, the observed experimental data for the net charge responses of α7-expressing cells to the coapplications of ACh and S 24795 across a range of concentrations are compared with the responses predicted by the model at the same concentrations. For clarity of presentation, half of the data representing responses obtained in progressively increasing concentration of S 24795 is shown on the left (from 0.1 μM to 1 mM, by factors of 10), and the other half of the data is shown on the right (from 0.3 to 300 μM, by factors of 10). B, the experimental data for the peak current responses of cells expressing the $(\alpha4)_3(\beta2)_2$ form of α4β2 to coapplications of ACh and cytisine across a range of concentrations are compared with the responses predicted by the model at the same concentrations. For clarity of presentation, half of the data representing responses obtained in progressively increasing concentration of cytisine is shown on the left (from 0.1 μM to 1 mM, by factors of 10), and the other half of the data set is shown on the right (from 0.3 to 300 μM, by factors of 10). For an alternative presentation of these data as pseudo three-dimensional plots, see Supplemental Fig. 1.

TABLE 1
Best fit values for Fig. 2

	ACh and S 24795 on α7					ACh and Cytisine on $(\alpha4)_3(\beta2)_2$				
	R_{max}	n	EC_{50}	In_{max}	IC_{50}	R_{max}	n	EC_{50}	In_{max}	IC_{50}
			μM		mM			μM		μM
Activation										
ACh	1.0	1.45	37			1.0	1.5	148		
S 24795	0.20	0.85	14							
Cytisine						0.35	0.61	88		
Inhibition		0.26		1.0	1		1.7		0.7	32

Materials and Methods) was then conducted using data for all of the replicate measurements, and curves generated by the best fit of the complete model are shown as lines in Fig. 2B (parameters of the fit are given in Table 1). It is interesting to note that, although for the fit of the α7 data with ACh and S 24795 the inhibitory factor for the partial agonist was relatively minor (the best fit for the S 24795 IC_{50} was 70-fold higher than the EC_{50}), for the cytisine/ACh interaction the best fit for the cytisine IC_{50} was 3-fold lower than the EC_{50}. It is unlikely that the difference in the noncompetitive component's importance for α4β2-nAChR data versus the α7-nAChR data reflect the properties of the activation of the α7-nAChR, because many α7 partial agonists also manifest strong noncompetitive antagonist activity (Papke et al., 2009).

Modulation of ACh-Evoked Responses of α7 and α4β2 Receptors Expressed in *Xenopus* Oocytes by Bath-Applied Nicotine. Although an acute coapplication protocol was appropriate to test the basic model of full and partial agonist competition shown in Fig. 1, such a protocol is not readily applicable to either therapeutics or drug self-administration when drug delivery to the brain is relatively



slow and prolonged. Under such circumstances effects of the drugs will pre-equilibrate before the presentation of phasic stimulations by endogenous ACh. In the case of nicotine self-administration and nicotine replacement therapies, there is controversy about the degree to which chronic low levels of nicotine activate or simply desensitize nAChR in the brain (Picciotto et al., 2008). To address this question, we determined the degree to which nAChR function was able to be modulated by bath-applied nicotine. The results in Fig. 3 show that nicotine is a potent agent for producing predesensitization of α4β2* nAChR with relatively little effect on ACh-evoked activation of α7, except at high concentrations.

Modulation of ACh-Evoked Responses of α7 and α4β2 Receptors Expressed in *Xenopus* Oocytes by Partial Agonists Used as Smoking Cessation Drugs. Drugs that are partial agonists for α4β2* receptors have become important candidates for smoking cessation therapies and alternatives to simple nicotine replacement approaches (i.e., nicotine patches or gum). However, it is unclear to what degree drugs such as cytisine, used as a smoking cessation agent in Europe, or varenicline, a cytisine-related compound that has been approved as a smoking cessation aid in both the United States and abroad, really differ from nicotine in their modulation of brain nAChR ACh-mediated activity. Therefore, we investigated the ability of these two agents to decrease the ACh-evoked responses of both the high-sensitivity $[(\alpha4)_2(\beta2)_3]$ and low-sensitivity $[(\alpha4)_3(\beta2)_2]$ forms of human α4β2-nAChR and compared the effects with their ability to modulate the function of α7 nAChR. In acute application experiments both of these agents were strong activators of α7, and in a therapeutic context effects on α7 receptor function would most likely be a negative and certainly considered "off target."

As shown in Fig. 4, when bath-applied, within 30 min both of these compounds produced progressive concentration-dependent decreases of α4β2 ACh-evoked responses. To summarize these data, we calculated the average of the ACh responses obtained after 30 ± 4 min of bath application relative to the pretreatment control responses. The lower plots in Fig. 4 show those data as a function of the bath concentrations applied and were used to calculate the IC_{50} values in Table 2.

Note that varenicline was more potent than cytisine at decreasing α4β2 ACh-evoked responses, and $(\alpha4)_2(\beta2)_3$ receptors seemed somewhat more sensitive than $(\alpha4)_3(\beta2)_2$ receptors, although the differences were not statistically significant. The ACh-evoked responses of human α7 receptors were also decreased by the bath applications of these drugs at higher concentrations.

Modulation of ACh-Evoked Responses of α4β2α5 Receptors Expressed in *Xenopus* Oocytes by Bath-Applied Nicotine or Cytisine-Related Compounds. Attention has been drawn to α5 as a member of a gene cluster

Fig. 3. Effects of bath-applied nicotine on the ACh-evoked responses of α4β2 and α7 nAChR. Pure populations of $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ nAChRs were obtained by coexpressing the β2–6-α4 concatamer with monomeric α4 or β2, respectively. After measuring two baseline ACh-evoked responses, the α4β2 partial agonists were added to the bath solution, and the cells were repeatedly probed for their ACh responses. The ranges of nicotine concentrations tested were 1 nM to 3 μM and 0.3 nM to 3 μM for $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$, respectively, and 1 nM to 10 μM for α7. All points represent an average of at least four oocytes (±S.E.M.) for each condition. The summary at the bottom displays the average of the last three responses in the upper graphs, plotted as functions of the bath concentrations applied.

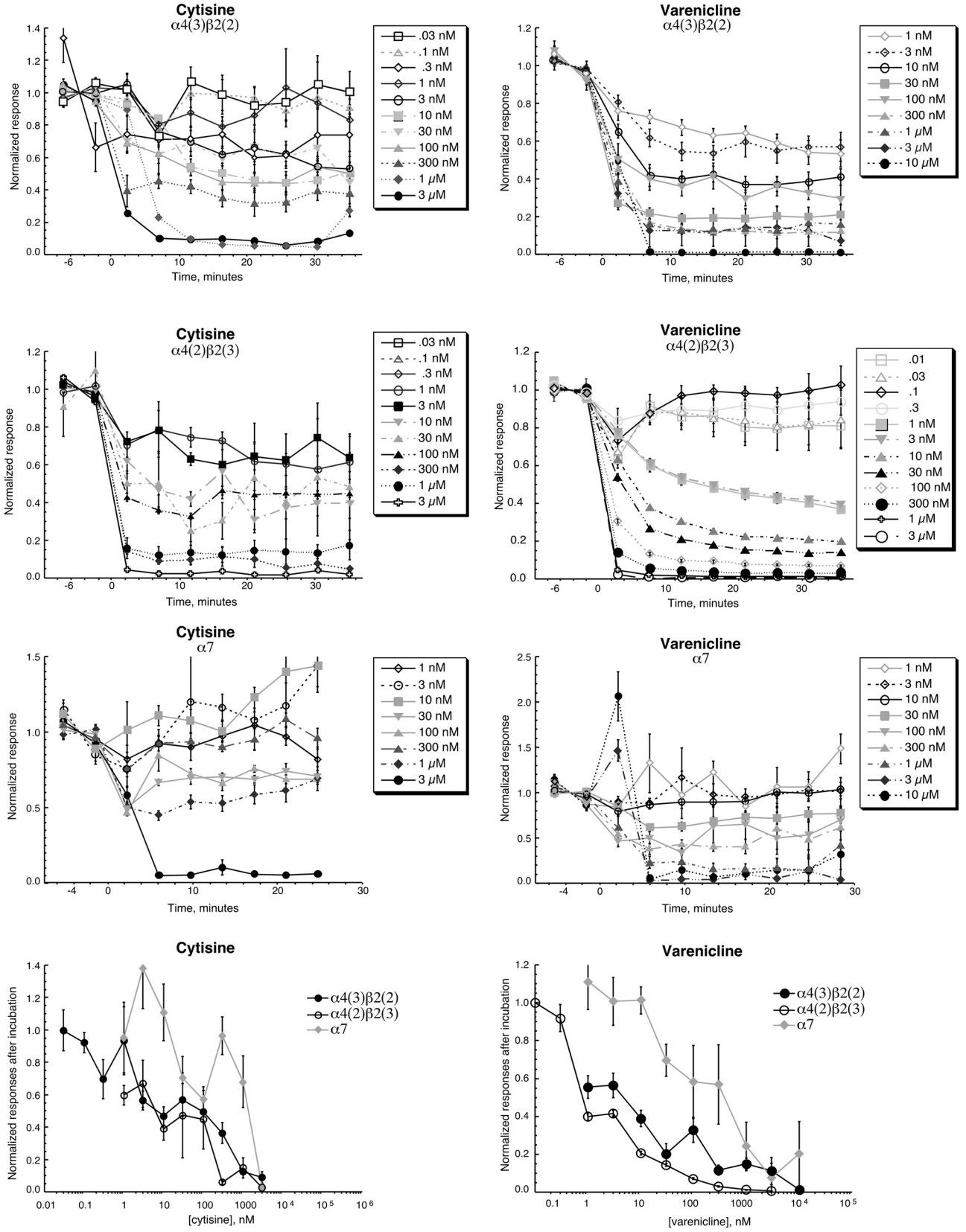


Fig. 4. Effects of bath-applied cytisine and varenicline on the ACh-evoked responses of α4β2 and α7 nAChR. Pure populations of $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ nAChR were obtained by coexpressing the β2–6-α4 concatamer (Zhou et al., 2003) with monomeric α4 or β2, respectively. After measuring two baseline ACh-evoked responses, the α4β2 partial agonists were added to the bath solution, and the cells were repeatedly probed for their ACh responses at intervals of 223 s (α7) or 277 s (non-α7). The ranges of cytisine concentrations tested were: 0.03 nM to 3 μM for both $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ and 1 nM to 1 μM for α7. The ranges of varenicline concentrations tested were 1 nM to 19 μM and 0.01 nM to 3 μM for $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$, respectively, and 1 nM to 10 μM for α7. All points represent an average of at least four oocytes (±S.E.M.) for each condition. The summaries at the bottom display the average of the last three responses in the upper graphs, plotted as functions of the bath concentrations applied.

TABLE 2
IC_{50} values (nM) for the inhibition of ACh-evoked responses by bath applications of agonists and partial agonists

	$(\alpha4)_3(\beta2)_2$	$(\alpha4)_2(\beta2)_3$	$(\alpha4)_2(\beta2)_2\alpha5$	α7
		nM		
Nicotine	56 ± 16	15 ± 5	17 ± 7	1000 ± 380
Cytisine	27 ± 14	8 ± 5	3.2 ± 1.6	1200 ± 570
Varenicline	2.8 ± 1.3	1.6 ± 0.5	3.3 ± 0.9	334 ± 140
3-pyr-Cyt	16,000 ± 12,000	600 ± 500	2300 ± 1000	N.A.
GTS-21	1000 ± 400	170 ± 100		1300 ± 475*
S 24795	36,000 ± 7000	53,000 ± 6000		15,000 ± 4000*

N.A., not available.
*Inhibition after a phase of potentiation.

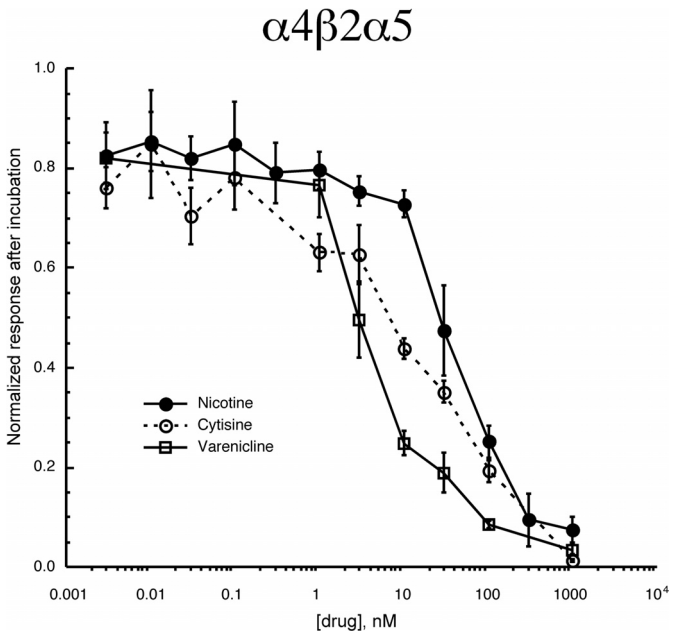


Fig. 5. Summary of effects obtained with α4β2α5 nAChR. Pure populations of α4β2α5 nAChR were obtained by coexpressing the β2–6-α4 concatamer with monomeric α5 (Kuryatov et al., 2008). Bath application experiments were conducted as described for the $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ receptors (Fig. 4). This summary shows the average of the three responses obtained after nicotine or the cytisine-related compounds were in the bath for 30 ± 4 min.

associated with high risk for cancer and heavy smoking (Saccone et al., 2007). In the periphery, α5 is most likely expressed in association with the other members of its gene cluster, α3 and β4. In the brain, α5 may be associated with α4 and β2, generating another high-sensitivity receptor subtype that could be related to nicotine dependence or reward (Grady et al., 2010). We therefore investigated the effects of bath-applied nicotine, cytisine, and varenicline on α4β2α5 receptors formed by coexpressed α5 with the β2–6-α4 concatamer (Kuryatov et al., 2008). It has been previously reported that such α4β2α5 receptors also show relatively high sensitivity to a variety of agonists (Kuryatov et al., 2008). In our experiments the EC_{50} values of α4β2α5 responses to nicotine, cytisine, and varenicline were 180 ± 20, 18 ± 9, and 35 ± 3 nM, respectively (data not shown). The efficacy of nicotine for α4β2α5 receptors was approximately 35% that of ACh, whereas cytisine and varenicline had efficacies of 6 ± 1 and 9 ± 1% that of ACh, respectively. In bath application experiments the sensitivity of α4β2α5 nAChR was similar to that of the $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ receptors for all of the agents tested (Fig. 5 and Table 2).

Steady-State Activation of α4β2* Receptors. One conundrum related to the therapeutic applications of partial agonists is the question of whether the drugs themselves will produce significant amounts of receptor activation or merely function as time-averaged antagonists of the endogenous activators. Most of the data obtained with the α4β2*-nAChR would support the latter hypothesis (Picciotto et al., 2008). However, it has been proposed that, even when the predominant effect is to induce desensitization, there may nonetheless be sufficient equilibration between activation and desensitization to produce something equivalent to low levels of steady-state activation within a narrow range (window) of drug concentration. Such window currents have been described for the relatively slow desensitizing β4*-nAChR of neurons in the medial habenula (Lester, 2004).

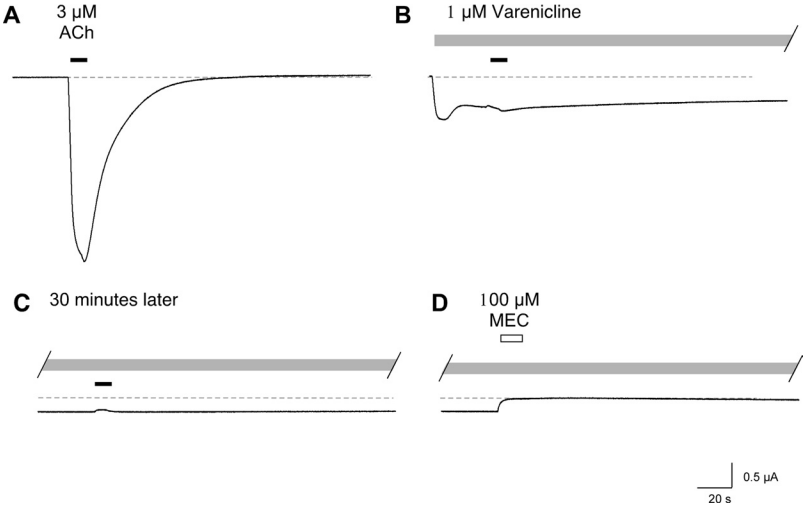


Fig. 6. Effects of mecamylamine on steady-state baseline current of α4β2α5 receptors stimulated by bath-applied varenicline. A, before the addition of varenicline to the bath, application of 3 μM ACh (black bar) produced a large transient current, as illustrated by the representative response. B, the bath application of 1 μM varenicline (gray bar) stimulated a response that appeared as a sustained shift in baseline current. The presence of 1 μM varenicline in the bath also had the effects of suppressing the transient response to an application of 3 μM ACh (black bar). C, after 30 min of continuous varenicline bath application, the baseline current remained elevated and ACh-evoked responses were suppressed. D, after 30 min of continuous varenicline bath application, 100 μM mecamylamine (Mec) was applied (open bar), which reduced the baseline current to the original control level, indicating that the baseline shift was caused by steady-state activation of α4β2α5 nAChR.

TABLE 3
Mecamylamine-sensitive steady-state activation of α4β2* nAChR, relative to ACh maximum-evoked transient responses recorded in oocytes

	$(\alpha4)_3(\beta2)_2$	$(\alpha4)_2(\beta2)_3$	$(\alpha4)_2(\beta2)_2\alpha5$
		%	
Nicotine	1.8 ± 0.1 (300 nM)	7 ± 1 (300 nM)	2.5 ± 1 (100 nM)
Cytisine	1.1 ± 0.2 (300 nM)	1.4 ± 0.1 (100 nM)	2.5 ± 0.5 (1 μM)
Varenicline	1.7 ± 0.2 (1 μM)	2.1 ± 0.4 (30 nM)	2.5 ± 0.5 (100 nM)

In our bath application experiments we frequently saw small responses at the beginning of the bath applications, and sometimes there were significant shifts in baseline holding current that occurred over long periods of recording. Although increases in baseline current would be consistent with the stimulation of steady-state currents, baseline shifts can occur for other reasons as well, especially if the quality of the voltage clamp diminished over a long period of recording. Therefore we repeated the experiments with the drug-receptor combinations that seemed to have possible increases in steady-state (baseline) current, substituting the ACh probe application 30 min after the start of the bath application with a 100-μM pulse of mecamylamine, to definitively test whether there was a receptor-mediated steady-state current contributing to the change in baseline.

A representative experiment is shown in Fig. 6. The peak current associated with the bath application of 1 μM varenicline to the α4β2α5 receptors was small compared with the initial 3 μM ACh-evoked response; however, the current was sustained. Even after just 30 min of continual bath application, a large shift in baseline was apparent. At that time the cells showed 100% inhibition of transient ACh-evoked responses, and the application of 100 μM mecamylamine caused a return to the original baseline. In this experiment the magnitude of the mecamylamine-sensitive steady-state current was 2.5 ± 5% that of the maximum peak transient current that could be activated by ACh. Table 3 provides a summary of the mecamylamine-sensitive steady-state currents measured for each of the drug-receptor combinations associated with drifting baselines. The greatest steady-state current observed was with $(\alpha4)_2(\beta2)_3$ receptors in the pres-

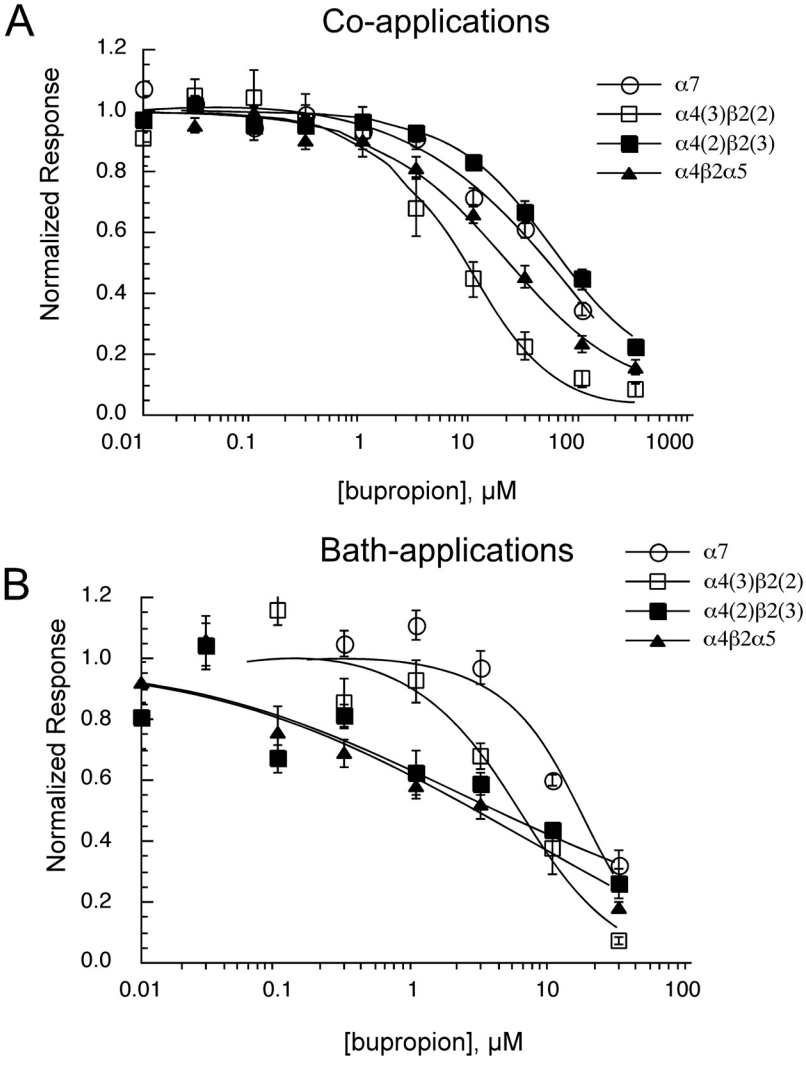


Fig. 7. Effects of bupropion on the ACh-evoked responses of nAChR. A, bupropion was coapplied with ACh, and the evoked responses were calculated relative to the responses to ACh applied alone. B, the effects of bath-applied bupropion on the ACh-evoked responses of α4β2 and α7 nAChR. Pure populations of $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ and α4β2α5 nAChR were obtained by coexpressing the β2–6-α4 concatamer (Zhou et al., 2003) with monomeric α4, β2, or α5, respectively. After measuring two baseline ACh-evoked responses, bupropion was added to the bath solution, and the cells were repeatedly probed for their ACh responses. The plot displays the average of three responses obtained after bupropion had been in the bath for 30 min, plotted as functions of the bath concentrations applied. All points represent an average of at least four oocytes (±S.E.M.) for each condition.

ence of 300 nM nicotine. There were no baseline shifts or steady-state currents in α7-expressing cells under any of the drug conditions.

Modulation of ACh-Evoked Responses by the Alternative Smoking Cessation Drug Bupropion. In addition to nicotine replacement and partial agonist therapies, the antidepressant bupropion has been used to improve smoking cessation efforts (Hurt et al., 1997). The basis of this therapeutic approach has been suggested, at least in part, to relate to bupropion's activity as an nAChR antagonist (Slemmer et al., 2000). We applied bupropion to α4-containing nAChR [i.e., $(\alpha4)_3(\beta2)_2$, $(\alpha4)_2(\beta2)_3$, and $(\alpha4)_2(\beta2)_2\alpha5$] and α7 nAChR at concentrations from 300 nM to 300 μM and saw no activation above our level of detection (not shown). We applied two approaches for measuring the antagonist activity of bupropion: coapplications and prolonged bath application. As shown in Fig. 7A, when bupropion was coapplied with control concentrations of ACh (see *Materials and Methods*), all of the nAChR subtypes tested were inhibited in a dose-dependent manner. The most potent inhibition was for the $(\alpha4)_3(\beta2)_2$ receptors (Table 4). We also tested bupropion with the same bath-application protocol used for the evaluation of the partial agonists. Shown in Fig. 7B are the averages of three ACh-evoked responses after bupropion had been present in the bath for 30 min. Although the potency of bupropion for the inhibition of α7 and $(\alpha4)_3(\beta2)_2$ receptors was relatively unchanged with this protocol (Table 4), the $(\alpha4)_2(\beta2)_3$ and α4β2α5 receptors showed partial blockade at relatively low bath concentrations of bupropion, resulting in an inhibition curve with a very low Hill slope. This may have been caused by slow equilibration of inhibition for these subtypes or alternatively might represent multiple mechanisms of inhibition. Whereas these experiments support the potential mechanism of bupropion as an nAChR antagonist, they also indicate a much lower potency for this effect than was seen with nicotine, varenicline, or cytisine (Table 2).

Modulation of ACh-Evoked Responses by a Novel Cytisine-Related Drug. The success of varenicline, albeit modest, as a smoking cessation agent has led to the development of additional cytisine analogs for smoking cessation and potentially therapeutic agents for treating depression. One such compound is 3-(pyridin-3′-yl)-cytisine (3-pyr-Cyt), an agent proposed to have very little likelihood of off-target α7 activity (Mineur et al., 2009). As shown in Fig. 8, 3-pyr-Cyt can decrease the ACh-evoked responses of α4β2 receptors, producing partial inhibition of the $(\alpha4)_2(\beta2)_3$ form even at relatively low (30 nM) concentration, although high concentrations are required to achieve strong inhibition (Table 3). Significant inhibition of α7-mediated responses was achieved only with 100 μM, the highest concentration tested. There were no baseline shifts or steady-state currents in the presence of 3-pyr-Cyt.

TABLE 4
Inhibition of α7- and α4-containing receptors by bupropion

	Coapplication		Bath Application	
	n	IC_{50}	*n*	IC_{50}
		μ*M*		μ*M*
α7	−0.74 ± 0.09	46 ± 7	−1.5 ± 0.5	16 ± 3
$(\alpha4)_3(\beta2)_2$	−0.93 ± 0.11	8.3 ± 1.2	−1.2 ± 0.4	5.9 ± 1.5
$(\alpha4)_2(\beta2)_3$	−0.80 ± 0.05	72 ± 5	−0.40 ± 0.10	4.4 ± 2.3
α4β2α5	−0.70 ± 0.04	23 ± 2	−0.47 ± 0.09	2.8 ± 1.0

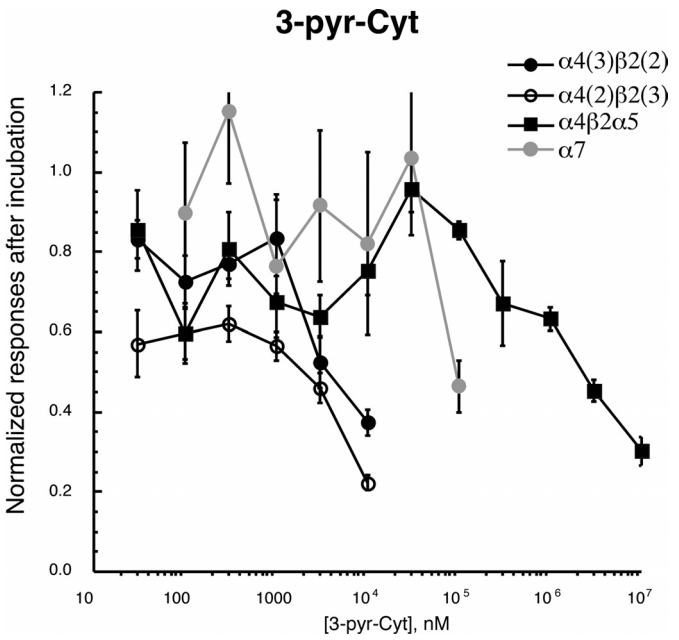
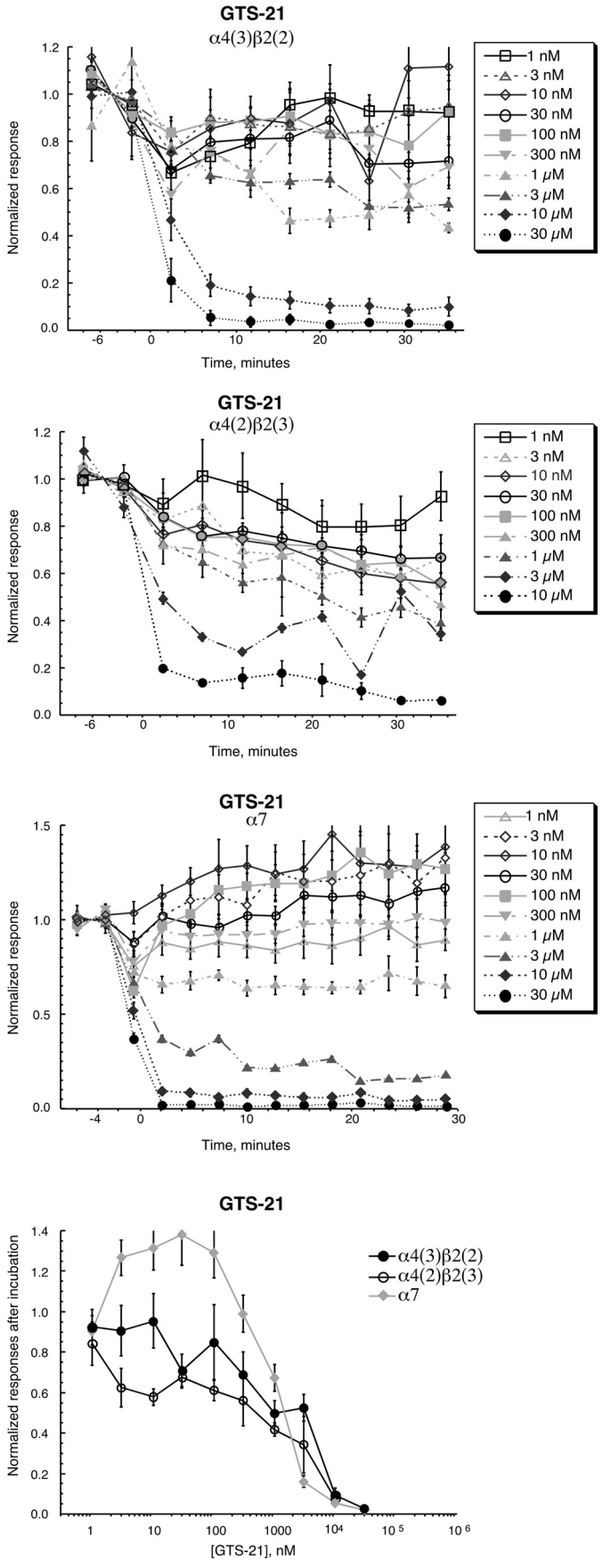


Fig. 8. Summary of the effects of bath-applied 3-pyr-Cyt on $(\alpha4)_3(\beta2)_2$, $(\alpha4)_2(\beta2)_3$, and α7 nAChR. Bath application experiments were conducted as described for cytisine, varenicline, and nicotine. This summary shows the average of the three response obtained after 3-pyr-Cyt was in the bath for 30 ± 4 min.

Modulation of ACh-Evoked Responses of α7 and α4β2* Receptors Expressed in *Xenopus* Oocytes by Bath Application of α7-Selective Compounds. Although α4β2 partial agonists are being developed for smoking cessation and possibly depression, α7-selective partial agonists have been proposed as therapeutics for a wide range of indications from Alzheimer's disease to arthritis (Kem, 2000; de Jonge and Ulloa, 2007). For such drugs, strong inhibition of α4β2* receptors might be considered an undesirable off-target activity. Therefore we investigated the modulation of $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ receptors and α7 by bath applications of the prototypical α7-selective partial agonist GTS-21 (also called DMXBA or diMeOBA). The results shown in Fig. 9 indicate that, somewhat unexpectedly, whereas the responses of α4β2 receptors were decreased in the presence of bath-applied GTS-21, α7 ACh-evoked responses showed significant ($p < 0.05$) potentiation at concentrations (1–100 nM) lower than those that would normally be used to produce transient receptor activation (EC_{50} ≈6 μM; Papke and Porter Papke, 2002). The efficacy of GTS-21 for human α7 receptors is relatively low, only approximately 20% that of ACh, whereas the responses recorded with bath applications of 30 nM GTS-21 were 37 ± 15% above the baseline controls. We tested whether similar effects might be seen with the alternative low-efficacy α7-selective partial agonist S 24795 (Fig. 10). These results suggest that low concentrations of an α7-activating drug might have potentiating or priming effects; however, similar run-up was also sometimes (although not consistently) observed in control experiments with just repeated applications of ACh to α7-expressing cells (not shown). The washing in and out of ACh in the control experiments might have had a priming effect, similar to that of low concentrations of GTS-21 or S 24795, or, alternatively, run-up of α7 responses with repeated stimulations might be a property of the oocyte expression system.



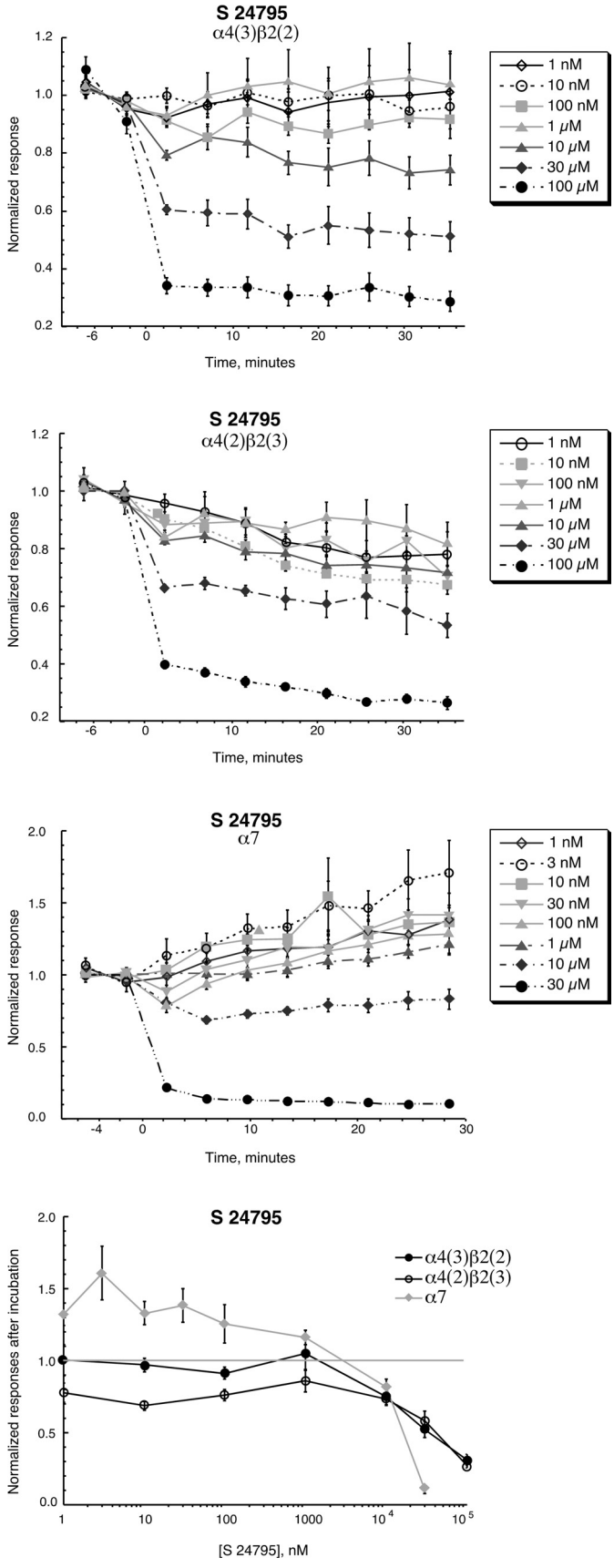
Discussion

Our experiments with acute coapplications of partial agonists with ACh largely support the traditional view that the combined effects of the drugs can be predicted based on competitive interactions. However, for specific drug-receptor combinations additional factors may be limiting, as was the case with cytisine, which seemed to have additional noncompetitive antagonist activity. For other drug-receptor combinations, such as GTS-21 and α7 receptors, at high partial agonist concentrations the induction of stable desensitization will also be a limiting factor (Papke et al., 2010). Although the induction of stable desensitization seems to be a factor limiting the efficacy of GTS-21 in acute applications, it does not seem to be an important factor in defining the activity profile of other benzylidene anabaseines or S 24795 (Lopez-Hernandez et al., 2007).

Responses measured in the acute application experiments result from the simultaneous integration of the activation, and desensitization produced the drugs in combination. On the time scale of the oocyte experiments, the peak currents are likely to represent the net result of the convolution of these processes and solution exchange (Papke, 2010). In contrast, the bath application experiments allowed for the effects of the partial agonists to approach equilibrium, so that the applications of the full agonist served as a probe of the predesensitization endpoints. For the α4-containing receptors used in these studies we were also able to detect and measure steady-state activations that for cytisine and varenicline were rather high, considering the apparently low efficacy of these compounds in acute application experiments.

In the search for candidate drugs to treat nicotine dependence, of equal importance to understanding the functional properties of the specific nAChR in the brain responsible for addiction and dependence is an understanding of how the candidate drugs function to either blunt or substitute for the nicotine reward. The concept that an α4β2 partial agonist would have utility as a smoking cessation agent has relied variously on two hypotheses that have not previously been rigorously tested. One hypothesis was that a partial agonist stimulates the same reward circuit that nicotine does, albeit weakly, and that this activity would reduce craving. The second hypothesis is that partial agonist therapies work by decreasing the effects of a self-administered full agonist (e.g., nicotine). This second mechanism seems especially likely if the contrasting pharmacokinetic modes are considered, with the therapeutic agent preadministered at a low dose, producing predesensitization and thereby blunting the peaks of receptor activation that would be produced by the more pulsatile administration of nicotine via cigarette smoking. This latter mechanism might also be applied to the patch form of

Fig. 9. Effects of bath-applied GTS-21 on the ACh-evoked responses of α4β2 and α7 nAChR. Pure populations of $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ nAChR were obtained by coexpressing the β2–6-α4 concatamer (Zhou et al., 2003) with monomeric α4 or β2, respectively. After measuring two baseline ACh-evoked responses, the α7-selective partial agonist was added to the bath solution, and the cells were repeatedly probed for their ACh responses. The ranges of GTS-21 concentrations tested were 1 nM to 30 μM and 1 nM to 10 μM for $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$, respectively, and 1 nM to 30 μM for α7. All points represent an average of at least four oocytes (±S.E.M.) for each condition. The summary at the bottom displays the average of the last three responses in the upper graphs, plotted as functions of the bath concentrations applied.



nicotine replacement therapy, although it remains controversial to what degree receptor desensitization plays a role in nicotine self-administration and dependence (Picciotto et al., 2008). Our data support the concept that partial agonist therapies may rely on both of these mechanisms, although the concentration window for tonic activation seemed to be rather narrow. The in vitro effects we observed occur on physiologically relevant time scales and within concentration ranges for nicotine self-administration and smoking cessation-related therapeutics and are consistent with recent in vivo studies (Marks et al., 2010).

Our data support the plausibility of the hypothesis that at least part of the utility of bupropion as a smoking cessation agent may be caused by blockade of nicotine's reinforcing effects mediated by α4* receptors (Slemmer et al., 2000). However, this activity alone would not be equivalent to the effects of a nicotine patch or a partial agonist, because it would not provide a similar factor of tonic activation. Perhaps bupropion's activity as a blocker of dopamine reuptake (Miller et al., 2002) compensates for a decrease in nicotine-mediated dopamine-dependent reward.

In addition to working through these expected competitive mechanisms, our data support an earlier report that suggested that cytisine may antagonize the function of α4β2 receptors through noncompetitive effects (Luetje and Patrick, 1991). This result is of particular interest because there have been several recent studies suggesting that nicotinic antagonists may have efficacy as antidepressants (Mineur and Picciotto, 2009). It has been shown that in the mouse forced-swim model cytisine is equipotent as the pure antagonist mecamylamine at reducing immobility (Mineur et al., 2009).

Although our data did not indicate that weak α7-selective partial agonists will produce tonic activation of the receptors when present for long periods of time, we observed that under such conditions they did not desensitize and may even potentiate ACh-evoked responses. It may be the case that partial or intermittent occupancy of the α7 binding sites has priming effects for subsequent stimulation, although allosteric modulation based on binding to other sites cannot be ruled out by the available data. However, it has also been proposed that high levels of agonist site occupancy by a strong agonist is intrinsically desensitizing for α7 receptors (Papke et al., 2000). In addition, although the steady-state applications of α7 agonists at low concentrations did not seem to lessen the acute activation by ACh, it is possible that they could have perturbed the equilibrium among the various ligand-bound nonconducting states, such as those that are competent for activation by allosteric modulators (Papke et al., 2009).

As noted above, one complication to a partial agonist therapy for the treatment of nicotine dependence is that, al-

Fig. 10. Effects of bath-applied S 24795 on the ACh-evoked responses of α4β2 and α7 nAChRs. Pure populations of $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ nAChRs were obtained by coexpressing the β2–6-α4 concatamer (Zhou et al., 2003) with monomeric α4 or β2, respectively. After measuring two baseline ACh-evoked responses, the α7-selective partial agonist was added to the bath solution, and the cells were repeatedly probed for their ACh responses. The ranges of S 24795 concentrations tested were 1 nM to 100 μM for $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ and 1 nM to 30 μM for α7. All points represent an average of at least four oocytes (±S.E.M.) for each condition. The summary at the bottom displays the average of the last three responses in the upper graphs, plotted as functions of the bath concentrations applied.

though a candidate drug may have only weak agonist activity at the target receptors (i.e., brain α4β2* nAChR), a drug such as varenicline may have serious side effects caused by activity at other receptor subtypes, such as α3β4*-nAChR found in the peripheral nervous system (Papke and Heinemann, 1994) and homomeric α7 receptors in the brain (Papke and Porter Papke, 2002). Therefore it must be considered that an important sequela to nicotinic partial agonist therapies is that, along with blunting drug reward signals, endogenous cholinergic function is likely to be seriously compromised for both on-target and off-target nAChR. Although varenicline has been shown to improve smoking cessation outcomes, there have been reports of adverse effects with varenicline, including the exacerbation of neuropsychiatric conditions (McClure et al., 2009). These symptoms are likely to be associated with a functional down-regulation of α7-type nAChR receptors in schizophrenia and depression (Leonard et al., 2000) and the effects of varenicline to worsen that condition.

There is strong support for the hypothesis that the comorbidity of smoking and depression may relate to an induced imbalance between the activity of homomeric and heteromeric nAChR in the brain (Mineur and Picciotto, 2009). Smoking behavior itself produces increases in α4β2-type receptors relative to α7-type receptors (Lester et al., 2009), and in circuits mediating nicotine reward these receptors can play opposing functions (Mansvelder et al., 2002). In addition, depression is strongly associated with stress and stress-related increases in glucocorticoids (Pittenger and Duman, 2008), and chronic elevations in corticoids have been shown to reduce the expression and function of homomeric α7-type receptors in the brain (Bullock et al., 1997).

It is well documented that the incidence of smoking is very high in neuropsychiatric patients, especially those with schizophrenia or attention-deficit hyperactivity disorder (Benowitz, 2009). The incidence of smoking in schizophrenics is 80% compared with 25% in the general population (Leonard et al., 2007). There is good evidence that a deficiency in α7 nAChR function may be an underlying cause, or at least an important contributing factor, to the etiology of these diseases, and several genetic links have been found between α7-related genes and polymorphisms and schizophrenia (Olincy et al., 2006). These data support the widely held hypothesis that the smoking behavior in both schizophrenics and patients with attention-deficit hyperactivity disorder is a form of self-medication to make up for deficiencies in α7 receptor function. This hypothesis is further supported by studies of auditory gating in schizophrenics and normal individuals. Consistent with the hypothesis that this effect of nicotine is mediated by α7 receptors, impairment in auditory sensory gating has been linked to the α7 nicotinic receptor gene (chromosomal locus15q14) (Martin and Freedman, 2007), and α7-selective agonists have been shown to reduce the gating deficits in animal models (Olincy and Stevens, 2007).

Varenicline and GTS-21 both have crossed the threshold into safe use in human trials, so combination therapy with these two drugs is plausible. However, it is not clear that either of these drugs are optimized; additional preclinical studies are warranted. For example, if the α7 activity of varenicline produces neuropsychiatric side effects, then drug development should be targeted toward other cytisine derivatives with reduced effects on α7, such as the recently identified candidate 3-pyr-Cyt (Mineur et al., 2009). Likewise, since the first publications on GTS-21 (de Fiebre et al., 1995), many alternative α7-selective full and partial agonists such as S 24795 have been identified (Papke et al., 2009). The successful development of such drug candidates into useful therapeutics will benefit the use of protocols such as those described in this work for the detection of both on-target and off-target activities.

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Authorship Contributions

Participated in research design: Papke and Trocmé-Thibierge.

Conducted experiments: Al Rubaiy.

Contributed new reagents or analytic tools: Trocmé-Thibierge, Guendisch, and Bloom.

Performed data analysis: Papke, Al Rubaiy, and Bloom.

Wrote or contributed to the writing of the manuscript: Papke and Trocmé-Thibierge.

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