Use of an α3β4 nicotinic acetylcholine receptor subunit concatamer to characterize ganglionic receptor subtypes with specific subunit composition reveals species-specific pharmacologic properties

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ABSTRACT

Drug development for nicotinic acetylcholine receptors (nAChR) is challenged by subtype diversity arising from variations in subunit composition. On-target activity for neuronal heteromeric receptors is typically associated with CNS receptors that contain α4 and other subunits, while off-target activity could be associated with ganglionic-type receptors containing α3β4 binding sites and other subunits, including β3, β2, α5, or α3 as a structural subunit in the pentamer. Additional interest in α3β4 α5-containing receptors arises from genome-wide association studies linking these genes, and a single nucleotide polymorphism (SNP) in α5 in particular, to lung cancer and heavy smoking. While α3 and β4 readily form receptors in expression system such as the Xenopus oocyte, since α5 is not required for function, simple co-expression approaches may under-represent α5-containing receptors. We used a concatamer of human α3 and β4 subunits to form ligand-binding domains, and show that we can force the insertions of alternative structural subunits into the functional pentamers. These α3β4 variants differ in sensitivity to ACh, nicotine, varenicline, and cytisine. Our data indicated lower efficacy for varenicline and cytisine than expected for β4-containing receptors, based on previous studies of rodent receptors. We confirm that these therapeutically important α4 receptor partial agonists may present different autonomic-based side-effect profiles in humans than will be seen in rodent models, with varenicline being more potent for human than rat receptors and cytisine less potent. Our initial characterizations failed to find functional effects of the α5 SNP. However, our data validate this approach for further investigations.

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1. Introduction

Early single-channel studies of the nicotinic acetylcholine receptors of autonomic neurons revealed a rich diversity of channel subtypes (Papke, 1993). Although it is now appreciated that, at least in embryonic ganglia, rapidly desensitizing α7-containing nAChRs are of functional importance, blocking α7 receptors in adult animals generally does not impair ganglionic function, and it is likely that the early single-channel studies only detected an array of more slowly desensitizing heteromeric receptor subtypes. We now know, based on recent studies using knockout animals, that ganglionic receptors are primarily assembled as pentameric complexes containing varying arrangements of α3, β2, β4, and α5 subunits (David et al., 2010).

Although ganglionic blockers were the first drugs used clinically to target neuronal nAChR, most current drug development programs intended to target nAChR in the CNS, either for therapeutics or nicotine dependence, view ganglionic receptors containing α3 in various combinations with β2, β4, and α5 as potential sites for off-target side effects. The human α3β4-α5 genes are in a cluster at chromosomal location 15q24, and recent genome-wide association studies indicated strong correlations between single nucleotide polymorphisms in the α3-β4-α5 gene cluster and risk for both cancer and nicotine dependence (Chen et al., 2009; Stevens et al., 2008). Nicotine addiction and dependence has been clearly linked to α4* and α6* receptors (Wu and Lukas, 2011), and α5 co-assembly into α4* receptors also promotes high sensitivity to nicotine, suggesting a link between nicotine use and α4β2α5 receptors (Kuryatov et al., 2011). However, recent studies have also demonstrated a link between α3β4α5-containing receptors in the medial habenula and nicotine-related behavior, promoting...
receptors with combinations of these subunits as an alternative target for the development of smoking cessation drugs (Fowler et al., 2011; Frahm et al., 2011; Gallego et al., 2011; Salas et al., 2009).

It has been shown both in vivo (Grady et al., 2009) and in heterologous expression systems (Boulter et al., 1990; Gerzanich et al., 1998) that z3 will form receptors in various combinations with β2, β4, and z5 subunits. However, z3 and β4 subunits readily form functional receptors without additional subunits, and functional effects of z5 co-expression are much more easily detectable in β2-containing than in β4-containing receptors (Gerzanich et al., 1998). Therefore, since most effectively targeted drug development relies on the use of receptors with known subunit composition, we adopted a strategy previously shown to be useful for controlling the subunit composition of z4+ receptors (Zhou et al., 2003), by constructing a concatamer of β4 and z3 (β4–6–z3), suitable for co-expression with monomeric β3, β2, β4, or z5 subunits. The β4–6–z3 construct will provide ligand-binding domains with z3–4 interfaces, so that co-expressed subunit monomers will, with high likelihood, take the fifth position as a structural subunit in the assembled pentamer.

We provide pharmacological validation of hypothesized subunit compositions and characterize the agonist and partial-agonist profiles of the z3β4 receptor subtypes for ACh, nicotine, and the smoking cessation agents, cytisine and varenicline. Cytisine and varenicline have been proposed to have therapeutic utility through potent partial agonist effects on CNS z4-containing receptors. However, it has been a concern that the reportedly high efficacy of these agents on ganglionic z3-containing receptors might be a source of autonomic side effects. We reevaluate those data and show significant differences from the previously reported data based on the use of rodent receptor subtypes and our current studies based on the use of human receptor clones. Additionally, we used the β4–6–z3 construct to study the D376N variant of z5, specifically associated with smoking and cancer risks.

2. Methods and materials

2.1. ACh receptor clones

Human nAChR clones were obtained from Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia PA). Alpha3 and β4 were subcloned into the pSGEM vector, obtained from Dr. Michael Hollmann (Ruhr University, Bochum, Germany), which contains Xenopus β-globin untranslated regions to aid Xenopus oocyte expression. Rat nAChR clones were obtained from Dr. Jim Boulter (University of California, Los Angeles).

2.2. Concatamer construction

As the C terminus of β4 is of similar length as that of β2, we followed the scheme of Zhou et al. (2003) (Zhou et al., 2003), and prepared the concatamer with, in sequence: β4 signal, mature [β4, 6(AGS) linker], then z3 mature (without signal sequence), all in frame, which should assemble with the z3–β4 binding pocket intact (Zhou et al., 2003). With this approach, co-injected subunits should co-assemble into the structural, non-ligand-binding-domain position.

Specifically, β4 was mutated silently to introduce a DraII restriction recognition site just before the stop codon. The site-directed mutagenesis was performed using the QuikChange kit (Agilent Technologies, Santa Clara CA); Long (100 bp) complementary oligos (sense strand:

GCTGGAGGAGCACAACATCGCATGAGAATCTCGAAATCCTGACCTGCACCTGACAGCTGAGTCCGGAGGAGCACAACATCGCATGAGAATCTCGAAATCCTGACCTGCACCTGACAGCTGAGTCCGGAG

and antisense strand:

GCTGGAGGAGCACAACATCGCATGAGAATCTCGAAATCCTGACCTGCACCTGACAGCTGAGTCCGGAGGAGCACAACATCGCATGAGAATCTCGAAATCCTGACCTGCACCTGACAGCTGAGTCCGGAG

incorporating the DraII recognition sequence at the end of β4 and before the stop codon, 6(AGS), the first 13 bases of mature z3 coding region including the unique βpBl site, and an Xhol recognition site were annealed following the protocol of Integrated DNA Technologies:

2.3. Expression in Xenopus laevis oocytes

X. laevis oocytes were surgically removed from frogs (Nasco, Ft. Atkinson WI) and treated with Type I collagenase (Worthington Biochemical Corporation, Freehold NJ) in calcium-free Barth’s solution (88 mM NaCl, 1 mM KCl, 2.38 mM NaHCO3, 0.82 mM MgSO4, 15 mM HEPES (pH 7.6), 12 mg/l tetracycline) in order to remove the follicular layer. Stage-5 oocytes were isolated and injected with 50 nl (3–20 ng) of each cRNA. After linearization and purification of cloned cDNAs, RNA transcripts were prepared in vitro using the appropriate mMessage mMachin kit (Ambion, Austin TX). Recordings were conducted 2–10 days post-injection.

2.4. Electrophysiology

Experiments were conducted using OpusXpress6000A ( Molecular Devices, Union City, CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the voltage and current electrodes were filled with 3 M KCl. The oocytes were clamped at a holding potential of ~60 mV. Data were collected at 50 Hz and filtered at 5 Hz. The oocytes were bath-perfused with Ringer’s solution (115 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 1.8 mM CaCl2) containing 1 μM atropine to block muscarinic acetylcholine receptors which may be native in the oocytes. Agonist solutions were delivered from 96-deepwell plates using disposable tips. Flow rates were set at 4 ml/min.

2.5. Experimental protocols and data analysis

Responses were calculated as both net charge and peak currents. Each oocyte received initial control applications of 100 μM acetylcholine (ACh), then experimental drug applications, and follow-up control applications of ACh. Responses to experimental drug applications were calculated relative to the preceding ACh control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. A second normalization step was applied to adjust for the empirically determined difference between the 100 μM ACh control responses and the observed ACh maximum for each receptor subtype. Average values and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental condition. For concentration–response relations, data were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated from the Hill equation:

\[
\text{Response} = \frac{I_{\text{max}} [\text{agonist}]^n}{[\text{agonist}]^n + (EC_{50})^n}
\]

\(I_{\text{max}}\) denotes the maximal response for a particular agonist/subunit combination, and \(n\) represents the Hill coefficient. \(I_{\text{max}}\), n, and the EC50 were all unconstrained for the fitting procedures, except in the case of the ACh receptor constructs. Since ACh is our reference full agonist, for the ACh concentration–response curves the data were normalized to the observed ACh maximum, and the \(I_{\text{max}}\) of the ACh curve fits were constrained to equal one.

3. Results

3.1. Experiments confirming the incorporation of specific structural subunits

When β4–6–z3 was expressed alone it was capable of forming functional receptors with properties similar to those formed when β4–6–z3 co-expressed with β4, suggesting the assembly of z3(2)[β4(3)] receptors with tethered supernumerary z3 subunits. That is, the ACh concentration–response curves and the recoveries from TMIP and BTMPS responses were similar (data not shown). In order to obtain better control of structural subunit identity, the RNAs for the monomeric constructs were co-expressed at a five-fold excess to the concatamer. We confirmed pharmacologically that this approach was successful, as shown in Fig. 1.

When mutations known to increase the reversibility of inhibition by the non-competitive antagonist BTMPS (Francis et al., 1998) were present in the β2 or β4 subunits expressed as monomers, the

The z5 single nucleotide polymorphism affecting amino acid translation, e50D376N, was constructed using the QuickChange kit.

4. Discussion

It has been shown that the native nicotinic receptors on autonomic ganglia are composed of 1 β2 and 3 z3 subunits, with those from other brain regions containing 1 β3 and 4 β4 subunits, suggesting that the z3 subunit is particularly important in autonomic function (Zhou et al., 2003). The z3 subunit has been shown to regulate both intrinsic and extrinsic nicotinic receptors in autonomic ganglia, including for example, those for ACh, nicotine, and the smoking cessation agents, cytisine and varenicline, in vivo (Grady et al., 2009). It has been shown both in vivo (Grady et al., 2009) and in heterologous expression systems (Boulter et al., 1990; Gerzanich et al., 1998) that z3 will form receptors in various combinations with β2, β4, and z5 subunits. However, z3 and β4 subunits readily form functional receptors without additional subunits, and functional effects of z5 co-expression are much more easily detectable in β2-containing than in β4-containing receptors (Gerzanich et al., 1998). Therefore, since most effectively targeted drug development relies on the use of receptors with known subunit composition, we adopted a strategy previously shown to be useful for controlling the subunit composition of z4+ receptors (Zhou et al., 2003), by constructing a concatamer of β4 and z3 (β4–6–z3), suitable for co-expression with monomeric β3, β2, β4, or z5 subunits. The β4–6–z3 construct will provide ligand-binding domains with z3–4 interfaces, so that co-expressed subunit monomers will, with high likelihood, take the fifth position as a structural subunit in the assembled pentamer.

We provide pharmacological validation of hypothesized subunit compositions and characterize the agonist and partial-agonist profiles of the z3β4 receptor subtypes for ACh, nicotine, and the smoking cessation agents, cytisine and varenicline. Cytisine and varenicline have been proposed to have therapeutic utility through potent partial agonist effects on CNS z4-containing receptors. However, it has been a concern that the reportedly high efficacy of these agents on ganglionic z3-containing receptors might be a source of autonomic side effects. We reevaluate those data and show significant differences from the previously reported data based on the use of rodent receptor subtypes and our current studies based on the use of human receptor clones. Additionally, we used the β4–6–z3 construct to study the D376N variant of z5, specifically associated with smoking and cancer risks.
receptors assembled with the concatamers showed more rapid recovery from inhibition by BTMPS (Fig. 1A), confirming the functional incorporation of the mutant subunits in the accessory subunit position with the concatamer providing the ligand-binding domains.

We have previously shown that receptors containing α5 subunits have reduced sensitivity to prolonged inhibition by the antagonist TMPH (Papke et al., 2005), and the co-expression of α5 with the β4–6–α3 concatamer yielded reduced sensitivity compared to receptors formed with other subunit compositions (Fig. 1B). We also confirmed that the β4–6–α3 concatamer could be used to generate functional receptors containing the potentially important α5 single nucleotide polymorphism (SNP) which generates a D376N mutation.

3.2. Pharmacological characterization of α3β4 receptor subtypes

3.2.1. ACh responses

We conducted studies of the ACh concentration–response relationships of the various α3β4 subtypes. The data indicated that substitution of α3, but not α5, for the β4 subunit in the accessory position produced a decrease in ACh potency, while the substitution of a β2 subunit produced receptors with increased ACh sensitivity (Fig. 2 and Table 1). For all of the combinations studied, the kinetics of the ACh-evoked responses were similar with both high and low concentrations of ACh (Fig. 3), so the results based on either peak currents or net charge were equivalent (not shown). However, as shown in Fig. 3, the currents of α3 and α5 containing receptors evoked by 1 mM ACh showed decay during the agonist application pulse. This was most likely due to channel block by ACh, consistent with the appearance of rebound current when the ACh began to be washed out of the chamber.

The curves for α3-containing and β2-containing receptors were fit with Hill slopes that were distinctly different than the curves for the β4 and α5 containing receptors. It is difficult to interpret the Hill slopes for macroscopic current responses since multiple factors can
affect the amplitude and kinetics of the responses (Papke, 2009). For example, a steep Hill slope, as seen for the \(a_3(3)b_4(2)\) receptors, could be produced if channel block by agonist became a limiting factor to the ACh-evoked responses, thereby creating a narrow range for effectively increasing ACh-evoked current. In this case of the \(a_3(3)b_4(2)\) receptors the potency for activation is low and does approach the expected potency of ACh for channel block (Lape et al., 2008). The shallow Hill slope of \(b_2\)-containing receptors could be due to a mixed population of receptors or possibly alternative subunit interfaces functioning as low potency agonist binding sites.

### 3.2.2. Nicotine responses

For each of the receptors studied, the waveforms of the nicotine-evoked responses were different from the ACh-evoked responses (Fig. 3), so that concentration–response data based on peak currents and net charge were significantly different (Fig. 4). Analysis of peak currents suggested that nicotine was a full agonist for \(a_3(2)b_4(2)b_2\) receptors, a 30% partial agonist for \(a_3(2)b_4(2)a_5\) and \(a_3(3)b_4(3)\) receptors, and with intermediate efficacy for \(a_3(3)b_4(2)\) receptors (Table 2). At high concentrations of nicotine, evoked responses were protracted for all subunit combinations. For the \(a_3(2)b_4(2)b_2\) receptor net charge data, this effect had the appearance of making nicotine appear more efficacious than ACh, possibly because nicotine was retained at the binding site of these receptors and remained continuously active when the free drug concentrations were being reduced.

It should also be noted that from the appearance of rapid peak currents and subsequent rebounds for several receptors, channel block by nicotine may have also played a role in shaping the unique waveforms of the responses and limiting the apparent efficacy for all subtypes other than the \(a_3(2)b_4(2)b_2\) receptors.

### 3.2.3. Responses to cytisine and varenicline

Cytisine and varenicline are agents presently in use as smoking cessation aids (Rollema et al., 2010). Their efficacy for this indication is believed to be related to a potent but weak partial agonism of the \(a_4b_2\) nAChR subtype(s) (Mihalak et al., 2006; Papke and Heinemann, 1994; Papke et al., 2011, 2010) which are highly expressed in the CNS. However, these agents have also been reported to be strong activators of ganglionic \(a_3b_4\) nAChR (Mihalak et al., 2006; Papke and Heinemann, 1994), a hypothetically off-target effect assumed to be a potential source of autonomic side effects. We evaluated the activity of these agents on the \(a_3b_4\) receptors formed with different accessory subunits (Fig. 5). Contrary to expectations based on prior literature, we found these agents to be only partial agonists of the \(a_3b_4\) receptor subtypes.

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**Table 1**

<table>
<thead>
<tr>
<th>Accessory subunit</th>
<th>EC50, (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a_3)</td>
<td>349.4 ± 22.0</td>
</tr>
<tr>
<td>(b_4)</td>
<td>153.9 ± 9.9</td>
</tr>
<tr>
<td>(b_2)</td>
<td>34.1 ± 3.9</td>
</tr>
<tr>
<td>(a_5)</td>
<td>157.7 ± 16.4</td>
</tr>
<tr>
<td>(a_5D376N)</td>
<td>126.8 ± 8.0</td>
</tr>
</tbody>
</table>

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![Sample traces of high ACh and nicotine responses of the receptors formed by concatamer and monomer co-expression, compared to 100 \(\mu M\) ACh controls obtained from the same cells. Note that the waveforms of the 1 mM ACh-evoked responses are relatively similar to the 100 \(\mu M\) ACh control responses, with only small indications of channel block for the \(a_3(3)b_4(2)\) (A) and \(a_3(2)b_4(2)a_5\) (C) receptors. In contrast, there is strong indication of channel block and rebound for the nicotine-evoked responses of all the subtypes.](image-url)
Cytisine was most efficacious but least potent for α3(3)β4(2) receptors and had an efficacy of no more than 20% that of ACh for the other subtypes tested. The pattern was similar for varenicline except that in all cases it was at least 30-fold more potent than cytisine (Table 3). Additionally we noted that at concentrations higher than 100 μM varenicline, but not cytisine had effects on the response waveforms (not shown) similar to those of nicotine (Fig. 3), (Shytle et al., 2011) suggesting limiting effects of channel block.

3.3. Species-specific features of cytisine and varenicline activation of α3β4 receptors

The initial studies (Mihalak et al., 2006; Papke and Heinemann, 1994) which reported high efficacy of cytisine and varenicline for α3β4 receptors utilized the standard procedure of injecting equal ratios of α3 and β4 RNAs and were conducted using rat cDNA clones. More recently, we reported that for mouse α3β4 receptors, cytisine was a full agonist, while varenicline had an efficacy approximately 50% that of ACh (Papke et al., 2010). In order to confirm that the low efficacy we observed for cytisine and varenicline was a characteristic of human α3β4, we conducted direct comparisons between human and rat α3β4 receptors with the standard procedure of monomer co-expression.

3.3.1. ACh responses

As shown in Fig. 6A, the ACh concentration–response curves were nearly identical for rat and human α3β4 receptors, with an ACh potency like that of the α3(2)β4(3) receptors generated with controlled accessory subunit (Table 4).

3.3.2. Responses to cytisine and varenicline

Consistent with previous data on rat α3β4 (Papke and Heinemann, 1994) we found (Fig. 6B) cytisine to be a full agonist for rat α3β4 receptors, although the potency was rather low.

Table 2
Nicotine potency and efficacy.

<table>
<thead>
<tr>
<th>Accessory subunit</th>
<th>Peak EC50 μM</th>
<th>Peak Imax relative to AChmax</th>
<th>Net charge EC50 μM</th>
<th>Net charge Imax relative to AChmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3</td>
<td>62.0 ± 15.6</td>
<td>0.69 ± 0.06</td>
<td>95.3 ± 8.36</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>β4</td>
<td>28.3 ± 6.7</td>
<td>0.32 ± 0.02</td>
<td>488.8 ± 77.2</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>β2</td>
<td>121.8 ± 21.6</td>
<td>1.4 ± 0.07</td>
<td>685.4 ± 184.9</td>
<td>2.21 ± 0.21</td>
</tr>
<tr>
<td>α5</td>
<td>35.6 ± 4.5</td>
<td>0.27 ± 0.01</td>
<td>1996 ± 2019</td>
<td>0.99 ± 0.31</td>
</tr>
<tr>
<td>α5D376N</td>
<td>24.9 ± 1.9</td>
<td>0.28 ± 0.01</td>
<td>366.41 ± 96.379</td>
<td>0.55 ± 0.04</td>
</tr>
</tbody>
</table>

Fig. 4. Nicotine peak current and net charge responses. A) Nicotine peak current concentration–response curves. The [4–6–α3] concatamer was co-expressed at a 1:5 RNA ratio with either α3, β4, β2, or α5. Data were initially normalized to 100 μM ACh control responses obtained immediately prior to the test responses and subsequently adjusted to the empirically determined ACh maximum responses. B) Nicotine net-charge concentration–response curves. The [4–6–α3] concatamer was co-expressed at a 1:5 RNA ratio with either α3, β4, β2, or α5. Data were initially normalized to 100 μM ACh control responses obtained immediately prior to the test responses and subsequently adjusted to the empirically determined ACh maximum responses. C) The ratio of net charge to peak current (relative to 100 μM ACh controls) for nicotine-evoked responses. A–C) Data for progressive increases in nicotine concentration were used from a single experiment only under conditions when ACh controls remained of stable amplitude throughout each experiment. In some cases, nicotine applications prevented the full recovery of ACh controls; in those cases, responses evoked by nicotine relative to ACh were determined in a series of single concentration experiments using new sets of cells for each nicotine concentration. All points represent the average data (+ SEM) for at least four cells.
evoked responses at concentrations from 3 μM to 30 μM varenicline were significantly larger for human than for rat receptors, in striking contrast to the results obtained with cytisine.

3.4. Evaluations of the functional effects of the α5 SNP

3.4.1. Agonist-evoked responses

The α5 SNP D376N was studied along with the wild-type α5 in all of the agonist concentration–response studies (Fig. 7A–C). No functional effects of the α5 SNP were detected in those experiments (Tables 1–3). Although the curve fit values for nicotine net charge data suggest that there might be an effect, it should be noted that the curve for the wild-type α5 data is not well fit by the Hill equation since no clear plateau response was achieved at concentrations ≤3 mM nicotine.

3.4.2. Inhibition by an α7-selective agonist

Orthosteric ligands that are selective for the α7 nAChR have been proposed for a variety of indications from Alzheimer’s disease to asthma. In addition to producing selective activation of α7 receptors, most of these drugs also inhibit other nAChR subtypes such as α3β4* receptors (Horenstein et al., 2008). We evaluated the inhibitory activity of a prototypical agent in this class, GTS-21 (3-(2,4dimethoxybenzylidene)anabaseine) on α3β4 receptors incorporating either wild-type α5 or the α5 SNP as the accessory subunit (Fig. 7D). The IC50 for the GTS-21 inhibition of the concatamer co-expressed with wild-type α5 was 9.17 ± 1.08 μM, not significantly different than when co-expressed with α5D376N (8.63 ± 1.41 μM).

4. Discussion

It has been shown both in vivo and in heterologous expression systems that α3 will form receptors in various combinations with β2, β4, and α5 subunits. Unconstrained expression, when all of these subunits are present, results in a heterogeneous population of receptor subtypes both in neurons and in oocytes. We adopted the strategy of co-expressing a concatamer of β4 and α3 (β4–6–α3) with monomeric β2, β4, or α5 subunits and were able to confirm that we obtained pharmacologically distinct populations of receptors useful for drug characterization.

For the subtypes specifically associated with the α3–β4–α5 gene cluster, our approach to co-expression appeared to be largely successful at generating distinct, potentially homogeneous, populations of receptors. However, it may be noted that the acetylcholine response curve of the putative α3(2)β4(2)β2 receptors (Fig. 2) appears as though it may contain two populations of receptors. There appears to be a high sensitivity component, likely to contain β2 subunits, and an incomplete suppression of the α3β4 type receptors that occur when the concatamers were expressed alone.

We found that nicotine responses were affected by the identity of the α3β4* structural subunits, as previously reported for α4β2* receptors (Kuryatov et al., 2008). Receptors containing β2 structural...
ACh-evoked peak currents

Fig. 6. A) ACh concentration–response curves for rat and human α3β4 nAChR formed with the conventional method of co-expressing RNA for the subunit monomers at a 1:1 ratio. B) Cytisine and varenicline concentration–response curves for rat and human α3β4 nAChR formed with the conventional method of co-expressing RNA for the subunit monomers at a 1:1 ratio. All points represent the average data (±SEM) for at least four cells. Statistical analysis based on t-tests between the normalized responses of rat and human receptors indicated significance values of p < 0.01 (*), or p < 0.001 (**).

Table 4

Species dependent effects on α3β4 responses using monomeric constructs acetylcholine potency, from peak current data.

<table>
<thead>
<tr>
<th>Species</th>
<th>EC50 (µM)</th>
<th>Cytisine I+max</th>
<th>Varenicline I+max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human α3β4</td>
<td>131 ± 7</td>
<td>0.59 ± 0.03</td>
<td>5.6 ± 1.8</td>
</tr>
<tr>
<td>Rat α3β4</td>
<td>127 ± 6</td>
<td>1.10 ± 0.04</td>
<td>28 ± 3.2</td>
</tr>
</tbody>
</table>

subunits were most sensitive to low concentrations of nicotine. Our data also indicate that structural subunits will affect the channel-blocking activity of nicotine, as well as other non-competitive antagonists.

Our data indicate that the specific α3β4 receptor subtypes will have unique profiles of response to cytisine and related agents that are in development for smoking cessation. Importantly, our data highlighted a disparity between the activity of the agents on human α3β4 receptor subtypes and previous findings based on rodent receptors. It is particularly interesting that the species-specific differences for the two agents tested were in opposite directions and therefore likely to rely on different elements in the receptors. We typically use rodent models for preclinical testing of whole animal drug effects. In the context of our previous studies of mouse nAChR (Papke et al., 2010), we noted that cytisine and varenicline have similar activity profiles for rodent and human α4 and α7 type receptors. However, data for human α3β4 receptors were not available at that time. Our current results therefore highlight the potential importance of comprehensive cross-species validation of pharmacology before the translation of preclinical results to human therapeutics.

Constipation is a commonly reported side effect of varenicline-based smoking cessation programs. This side effect might easily be associated with depolarizing block of autonomic ganglia, consistent with the high sensitivity to varenicline we see for human α3β4 receptors. Cytisine (Tabex®) is commonly used as a smoking cessation agent in Europe, and reportedly has only mild side-effects, which may in part be due to the low sensitivity of human α3β4 receptors to cytisine. Interestingly, α3β4 receptors in the central nervous system (CNS) have also been linked to nicotine’s effects on appetite and weight loss (Mineur et al., 2011), and so differences in the α3β4 activity of specific smoking cessation agents may also affect the process of weight gain that often occurs following successful smoking cessation. Additionally, there have been numerous reports of adverse neuropsychiatric side effects for varenicline, while there have been fewer such reports for cytisine (Moore et al., 2011; Shytle et al., 2011). The potential importance of α3β4 receptors in the medial habenula and other parts of the brain associated with mood and behavior might suggest that the differences in the neuropsychiatric side effect profiles of varenicline and cytisine could also be related to their differing activity profiles for human α3β4 receptors.

The gene for the nAChR α5 subunit was first identified as part of the gene cluster with α3 and β4 in 1990 (Boulter et al., 1990). The predicted gene product was classified as an alpha subunit based on sequence similarity to other nAChR alpha subunits, most notably the presence of the two vicinal cystines in the structural subdomain of the ligand-binding site currently identified as the C-loop. The α5 gene was subsequently confirmed to be expressed in autonomic ganglia (Wang et al., 2002), hippocampus (Sudweeks and Yakel, 2000) and the cortex (Han et al., 2000), as well as the particularly nAChR-rich brain structures of the medial habenula and interpeduncular nucleus (Fowler et al., 2011; Grady et al., 2009). However, for many years the identification of the protein as a putative alpha subunit was something of a puzzle since it was not functional in heterologous expression systems either alone or in combinations with β2 or β4, subunits known to form functional receptors with neuronal alpha subunits. The α5 subunit was subsequently confirmed to be an obligatory structural subunit that could form functional receptors in combination with α4 and β2, or α3 and β4, and possibly other combinations of subunits which would not require α5 for function (Ramirez-Latorre et al., 1996) (Grinevich et al., 2005). Although α5 subunits were not required for function, the presence of α5 subunits has significant effects on
receptor pharmacology, in most cases increasing the agonist sensitivity of the receptors formed.

Based on immunoprecipitation and Western blot studies of wild-type and knockout mutant mice, it has been estimated that the nAChR of mouse autonomic ganglia contain approximately 55% $\alpha_3\beta_4$, 21% $\alpha_3\beta_4\beta_2$, and 24% $\alpha_3\beta_4\alpha_5$ nAChRs (David et al., 2010). Therefore, although $\alpha_5$-containing receptors play a role in autonomic transmission, the knockout of $\alpha_5$ does not produce a serious deficit in autonomic function, as seen with $\alpha_3$ or $\beta_4$ knockouts (Wang et al., 2002). Knockout of $\alpha_5$ however, does appear to impact nicotine-mediated behaviors relevant to development of nicotine dependence (Jackson et al., 2011) and adult brain circuitry required for attentional performance (Bailey et al., 2011). It may be the case that the role of $\alpha_5$, in combination with $\alpha_3$ and $\beta_4$ in the habenular/interpeduncular circuit, accounts for the knockout phenotype related to nicotine associated behavior, while the phenotype associated with attentional performance may relate to receptors containing $\alpha_4$ and $\beta_2$ in combination with $\alpha_5$ in the cortex, hippocampus, and other parts of the brain associated with cognitive function (Bailey et al., 2011).

The polymorphisms in the $\alpha_3$--$\beta_4$--$\alpha_5$ gene cluster associated with cancer risk and smoking behavior include several variations in untranslated sequence and a single salient mutation in the sequence coding for the $\alpha_5$ subunit protein. This SNP changes an aspartic acid to an asparagine at amino acid 376 (mature protein numbering, or 398 from start codon), a site within the putative amphipathic helix of the intracellular domain. In co-expression studies with $\alpha_4$--$\beta_2$ concatamers, Lindstrom and co-workers (Kuryatov et al., 2011) found this mutant in $\alpha_5$ lowered the calcium permeability of the receptors compared to wild-type and may have also affected the receptor’s kinetic properties. They studied the effects of the $\alpha_5$ SNP in co-expression with $\alpha_3$ and $\beta_4$ using the more conventional approach of expressing monomeric subunits at ratios hypothesized to give enriched populations of $\alpha_5$-containing receptors. Using that approach they found no clear effect of the $\alpha_5$ SNP on the calcium permeability of the putative $\alpha_3\beta_4\alpha_5$ receptors. Functional effects were also undetected when these subunits were co-expressed in HEK cells (Li et al., 2011). Consistent with these previous studies, no functional effects of the $\alpha_5$ SNP were detected in the current study on the agonist response profiles of the $\alpha_3\beta_4\alpha_5$ receptors. However, as with the introduction of any new experimental tool, our experiments touch upon only a small fraction of the questions that might be addressed.

5. Conclusion

Receptors containing $\alpha_3\beta_4$ nAChR subunits have long been considered strictly off target for nAChR targeting CNS therapeutics. However, as new roles are being discovered for these receptors in the CNS, in regard to nicotine use and appetite control (Mineur et al., 2011), it becomes important to characterize the $\alpha_3\beta_4$ receptor subtypes and understand how to target them selectively.
Therefore programs are being developed for high throughput screening of nACh-containing receptors, and the use of the P4–6–23 concatamer will permit detailed follow-up characterizations of potential new drug candidates that may be applied to smoking and other novel CNS indications. Our data additionally highlight the importance of cross characterization of potential useful therapeutic agents with in vitro tests of both the receptors relevant to animals models and the human receptor subtypes that will ultimately be the molecular targets of therapeutics.

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