1.3

Effects of incorporation of α5 subunits into α4β2 nicotinic acetylcholine receptors on pharmacological functions of nicotinic ligands

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To elucidate their role in diseases and disorders such as addiction, pain and attention deficit/hyperactivity disorder, the heteromeric α4β2 nicotinic acetylcholine receptors (NNRs) have been the focus of many studies. Recently, investigations have shown that other members of the NNR family, such as α5 expressed with α4β2 in the brain, may play important roles in these diseases. Receptor functions involving activation and desensitization are influenced by many factors including subunit composition which is likely modified during the course of diseases. In the present study, we co-transfected concatenated β2–α4 dimers with single α4, β2 or α5 monomers, to express low sensitivity (LS)-α4β2, high sensitivity (HS)-α4β2 or $(\alpha4\beta2)_2\alpha5$ (α4β2α5) NNRs respectively in HEK293F cells. Subsequently the effect of α5 incorporation on the pharmacological properties of α4β2 agonists varenicline, cytisine, and sazetidine-A was investigated by a membrane potential method. For varenicline and cytisine, the activation profiles were very similar on all three α4β2 NNRs. They partially activated LS-α4β2 with comparable potencies but had no detectable activities at HS-α4β2 NNRs. At activation of α4β2α5, similar potency and efficacy were observed as compared to their respective responses at LS-α4β2 NNRs. Sazetidine-A, in contrast, was a potent full agonist at HS-α4β2 with low activity at LS-α4β2. Its partial agonistic activity at α4β2α5 was similar to that at LS-α4β2. Interestingly, when cells were pretreated with these agonists and then challenged with acetylcholine (ACh), they all displayed similar desensitization efficacy, to a complete degree, at the three α4β2 NNRs, again with comparable potencies for varenicline and cytisine. Additionally, onset of the desensitization was within minutes for all agonists tested and fastest for sazetidine-A. We also characterized the influence of α5 on sensitivities of α4β2 NNRs to the antagonists dihydro-beta-erythroidine (DHβE) and TC-5280. When co-applied with ACh, DHβE and TC-5280 displayed IC_{50} values of 114 and 177 nM at HS-α4β2, 123 and 364 nM at LS-α4β2, and 85 and 439 nM at α4β2α5, respectively. Under pre-treatment condition, TC-5280 was more potent and selective to desensitize HS-α4β2 and α4β2α5 NNRs. In summary, for three agonists tested, incorporation of α5 shifted their activation responses to LS-α4β2-like and their desensitization responses to HS-α4β2-like. Understanding the changes in pharmacological functions influenced by receptor subunit composition will help us to understand the physiological role of such receptors and to better design drugs that target the relevant receptor subtypes involved in diseases.

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1.4

Comparison of binding and functional activity profiles for a set of nicotinic acetylcholine receptor ligands across multiple α6* expression systems

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It has been proposed that modulation of the nicotinic acetylcholine receptor α6* (where the asterisk indicates additional subunits) subtype may offer therapeutic opportunities in overcoming nicotine addiction and in alleviating motoric dysfunction associated with Parkinson's disease. Progress in the identification of selective α6* ligands has previously been hampered by the lack of robust, pharmacologically relevant high-throughput assays. Until recently, the only assay amenable to high throughput *in vitro* screening was the α6/4β4 chimera. Native α6* subtypes associated with dopaminergic activity are believed to contain an α6-β2 interface which is missing in the α6/4β4 chimera, calling into question the relevance of this data for predicting *in vivo* activity in animal models of addiction and Parkinson's disease. We have recently identified a number of compounds with moderate to high affinity for α6*-containing receptors through screening of previously published nicotinic ligands and Targacept proprietary database libraries in a variety of assay formats. These include novel α6β3β4α5 construct expressed in mammalian cells, rat native tissue from α6-rich brain areas, an α6/3β2β3 chimera expressed in mammalian cells, and autoradiography in rat striatum. The affinity and, consequently, selectivity versus other nicotinic receptor subtypes (α4β2, α3β4) varied greatly depending on the assay system. We present here the *in vitro* profiles of these diverse ligands across multiple α6* affinity measures and preliminary structure–activity relationships. The relevance of α6* binding affinity to α6* function, as measured by Calcium flux in the α6/3β2β3 chimera and the α-conotoxin MII-sensitive component of striatal dopamine release, will also be discussed.

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1.5

Construction of cell line heterologously expressing the α6/3β2β3 nicotinic acetylcholine receptor (nAChR) subtype

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Natively-expressed α6* nAChRs are predominantly expressed in combination with β2 and β3 subunits (α6β2β3* nAChRs; asterisk denotes other possible subunits). Heterologous expression of functional α6* nAChRs in mammalian cell lines has historically proved difficult. In order to overcome this problem, we have applied a combination of approaches to increase expression of functional nAChRs, without using a gain-of-function mutation that could alter EC_{50} and IC_{50} values or change agonist efficacies. Native α6 subunits were replaced with α6/3 chimeric subunits, which have

been shown to express more readily in a variety of systems. The codon usage within the cDNA sequences of each subunit were optimized to increase expression in mammalian cells, areas of high GC content were altered to introduce greater AT sequence content, and predicted secondary structures were disrupted, while retaining the original protein sequence. Initially, α6/3β2 monoclones were selected, and several that expressed high levels of [^{3}H]epibatidine binding sites were identified. None of these clones produced function as measured by $^{86}Rb^+$ efflux, although single-cell patch-clamp electrophysiology identified low levels of function in one monoclone. Following introduction of the β3 subunit and further subcloning, [^{3}H]epibatidine binding site expression was increased. In an attempt to further increase nAChR expression, cells were incubated at 30 °C before testing. Multiple α6/3β2β3 nAChR monoclonal cell lines were identified as functional using $^{86}Rb^+$ efflux. The highest expresser was chosen for all further experiments. Preliminary testing was done with the agonists ACh, (−)-nicotine, carbachol, and cytisine. Each proved to be a potent agonist (EC_{50} values of 244 nM, 186 nM, 1.98 μM, and 238 nM, respectively). Cytisine was approx 50% efficacious, the others were fully efficacious. Potent antagonism was observed *vs.* 10 μM ACh activation for the α6-selective antagonists α-CtxMII and α-CtxPIA (nM IC_{50} values), while DHβE antagonism was of lower potency (7.8 μM IC_{50} value). These values closely resemble those measured at native α6β2β3* nAChRs. In addition, a set of novel compounds was tested for functional activity at α6/3β2β3, with a wide range of agonism, antagonism and potency observed. These data indicate that the new α6/3β2β3 cell line accurately reproduces native α6β2β3* agonist and antagonist pharmacology, and is well-suited for use in compound screening.

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1.6

Tethered pentamers—Low sensitivity α4β2-nicotinic acetylcholine receptors

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Nicotinic acetylcholine receptors (nAChR) containing α4 and β2 subunits appear to be expressed as two isoforms differing structurally in α4:β2 subunit ratios (3:2 and 2:3) and functionally in their sensitivity (low or high) for nicotinic agonists. When expressing α4β2-nAChR from loose subunits in *Xenopus* oocytes, variation in amounts of subunit cRNAs injected can bias expression toward a given isomer. However, no such control is possible in heterologous expression in mammalian cell lines from loose subunits. To overcome this shortcoming, we have designed and expressed cDNA constructs that encode concatenated subunits as covalently-linked or "tethered" pentamers. A construct designed to contain three α4 and two β2 subunits, when stably expressed in SH-EP1 human epithelial cells, encodes a product that conveys to cells low nicotinic agonist sensitivity for functional activation of whole-cell inward currents or $^{86}Rb^+$ efflux responses. These cells also display high affinity binding for radiolabeled nicotinic agonists. These studies suggest that the construct encodes tethered pentameric, functional and ligand binding, low sensitivity, $(\alpha4)_3(\beta2)_2$-nAChR. Further studies using this construct and cells expressing it will aid research on nAChR, help define roles played by low and high sensitivity α4β2-nAChR, and facilitate isoform-specific or -selective drug discovery with a view toward creation of novel therapeutics for treatment of psychiatric or neurological disorders.

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1.7

A methodological comparison of human α4β2 and α3β4 receptor properties using conventional and high-throughput patch-clamp electrophysiology techniques

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High-throughput screening for compounds with activity at neuronal nicotinic receptors using electrophysiology-based assays represents an important tool for biomedical research. The recent development and availability of high-throughput devices brings the need to validate these tools by demonstrating the ability to collect data that is consistent with results acquired through conventional electrophysiological methods. Population patch clamp (PPC) is a newly developed technique that allows for the simultaneous recordings from up to 64 cells per well. While PPC can greatly improve the success rate during automated electrophysiology experiments, it was not known whether the measured amplitude and kinetics from each well represented the sum of several uniform current responses from multiple individual cells, or an aggregate of varied responses resulting from different concentration transients across the cell population during application of the ligand. In this study, we compared the response properties of *h*α3β4 and *h*α4β2 nicotinic receptors to their endogenous ligand acetylcholine (ACh) using three separate electrophysiology platforms (Dynaflow, PatchXpress and IonWorks Barracuda). We found that in spite of the differences in methodological approaches among the Dynaflow (conventional electrophysiology), PatchXpress (medium-throughput electrophysiology) and IonWorks Barracuda (high-throughput electrophysiology) technologies, the values from the ACh dose-response curves (EC_{50}, Hill slope) were similar across all three platforms. In addition, we found that the decay kinetics due to desensitization of the receptors were also similar for all three applied techniques. This study provides the first data validating the consistency of results using low-, medium- and high-throughput electrophysiology platforms and supports their use for screening physiologically active compounds.

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1.8

Novel properties of neuronal nicotinic receptors revealed with brief pulses of acetylcholine

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Fast synaptic transmission within the central nervous system can occur on a sub-millisecond timescale. To effectively study these electrochemical events, ligand-gated receptors must briefly be exposed to concentrations of agonist that adequately re-create the endogenous physiological conditions. Synaptic properties such as