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Pharmacology of the agonist binding sites of rat neuronal nicotinic receptor subtypes expressed in HEK 293 cells

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Abstract—The binding affinities of agonists at heteromeric nicotinic receptors composed of rat α2, α3 and α4 subunits in combination with β2 or β4 subunits were examined in stably transfected HEK 293 cells. In most cases, the affinities of agonists were higher at receptors composed of an α subunit in combination with the β2 subunit than the β4 subunit, and in some cases this difference was quite large (>250 times), suggesting the possibility of developing subtype-selective ligands and therapeutically useful drugs.

Neuronal Nicotinic receptors (nAChRs) are composed of different combinations of α and β subunits arranged as pentameric structures. Nine α and three β subunits are expressed in vertebrate tissues; therefore, under our current poor understanding of the rules of assembly, thousands of subunit combinations, representing potential nAChR subtypes are theoretically possible. Fortunately, this depressingly large number is only theoretical, and the actual number of nAChR subtypes that function in native tissues is likely to be much more manageable.

Nevertheless, developing the pharmacological profiles for even a more manageable number of receptors can present a daunting task. For one thing, native tissues from animals or from naturally occurring cell lines (e.g., PC12, IMR-32, SH-SY5Y cells) usually express more than one nAChR subtype, making it inherently difficult to know which specific receptor subtype one is measuring. A second major reason that the development of the nAChR subtype pharmacology has been somewhat delayed is that before 1994, the radioligands available (e.g., [^{3}H](−)nicotine and [^{3}H]cytisine) unambiguously labeled only one or two of the heteromeric subtypes (primarily the α4β2 subtype) because their affinities for other subtypes were simply too low to yield a useful signal (i.e., specific/nonspecific binding ratio). Thus, for example, nAChR subtypes in autonomic ganglia and the adrenal gland could not be measured with these radioligands. (The homomeric α7 nAChR could, of course, be labeled by [^{125}I] α-bungarotoxin, which has stimulated many advances in the pharmacology of this receptor.)

The discovery of epibatidine (EB) and its introduction into studies of nAChRs[1,2] led to the development of [^{3}H]EB, the first high affinity, broad-spectrum radioligand that labels most, if not all, nAChR subtypes.[3–8] In fact, the first systematic examination of the comparative pharmacology of the binding sites of multiple nAChR subtypes was carried out by Luetje et al.,[8] who measured competition for [^{3}H]EB binding sites in *Xenopus* oocytes expressing α2, α3, or α4 subunits in combination with β2 or β4 subunits.

As an initial approach to comparing the pharmacological profiles of nAChR subtypes, we developed a library of HEK 293 cells that stably express different combinations of rat α and β subunits. These cells allow detailed examination and comparison of the pharmacology of different potential nAChR subtypes, as defined by their specific subunit compositions. We have begun to examine the pharmacology of six of these subunit combinations, consisting of α2, α3 or α4 subunits in combination with β2 or β4 subunits (these are the same combinations examined in oocytes[8]). The binding sites were labeled

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Table 1. K_i values of nicotinic agonists at heterologously expressed nAChR subtypes and at receptors in rat forebrain. Values are the mean of 2 independent determinations

Ligand	Affinity ratio K_i (nM)						
	α2β2	α2β4	α3β2	α3β4	α4β2	α4β4	Rat forebrain
(±)-Epibatidine	0.026	0.087	0.025	0.48	0.063	0.16	0.064
(±)-I-Epibatidine	0.11	0.13	0.16	0.83	0.15	0.15	0.29
Acetylcholine	11	180	41	820	45	83	43
(−)-Nicotine	15	130	43	530	12	46	13
Cytisine	1.4	5.5	37	210	1.5	2.2	2.5
A-85380	0.074	17	0.24	72	0.14	7.1	0.22
I-A-85380	0.031	47	0.42	290	0.062	24	0.14
Carbachol	210	640	1100	4800	590	1300	450
DMPP	28	1300	42	800	90	1700	120
Choline	18,000	21,000	37,000	48,000	37,000	34,000	42,000

with ~500 pM [^{3}H]EB and competition curves were generated with a variety of nicotinic agonists to determine their affinities. As shown in Table 1, most agonists have a wide range of affinities across these nAChR subunit combinations, but EB and iodo-epibatidine (the iodinated analogue of EB) have very high affinity ($\leq$1 nM) for all of them. In fact, these two compounds are the only ones that could reasonably be considered to be broad-spectrum ligands, useful for labeling all of these nAChR subtypes. For example, cytisine, which has quite high affinity for several of the subtypes, including α2β2 and α4β2 receptors, has much lower affinity for the receptors containing α3 subunits. Even at α3β2 receptors, cytisine's affinity of ~37 nM would make it difficult, if not impossible, to use as a radioligand because at the concentrations required to label a significant portion of the receptors, nonspecific binding would be too high. Similarly, A-85380 and its iodinated analogue, I-A-85380, have very high affinity for all three of the nAChR subtypes containing β2 subunits but much lower affinity for the subtypes containing β4 subunits. In fact, A-85380 and I-A-85380 are among the most selective drugs for β2-containing nAChRs we have examined.

As was found in previous studies carried out by Luetje and colleagues in *Xenopus* oocytes,[8] the binding affinity of nearly every agonist examined here was higher at subunit combinations consisting of any one of the α subunits paired with the β2 subunit than paired with the β4 subunit (Table 1). The binding affinity ratio for a ligand, calculated from its affinities at an α subunit paired with either the β2 subunit or the β4 subunit, represents a measure of the selectivity of that ligand with regard to the β subunits. These ratios are shown in Table 2. In some cases, the difference in a ligand's affinities for an α subunit paired with the β2 versus the β4 subunit was very small and the ratio was close to 1 (e.g., I-Epibatidine and cytisine at α4β4 versus α4β2 subtypes, and choline at all of the comparisons). However, in other cases the affinity differences were quite large, resulting in affinity ratios >200 (e.g., A-85380 and I-A-85380).

We also compared the affinities of these agonists for the heterologously expressed α3β4 subunit combination to their affinities for the rat forebrain receptor (Table 2). An α3β4 subtype is found in many sympathetic ganglia, while an α4β2 subtype is the predominant receptor in rat forebrain; therefore, the affinity ratios of drugs at these subtypes can help to predict the likelihood of possibly limiting autonomic nervous system side effects of drugs aimed at the predominant receptor in forebrain. As shown in Table 2, the affinity ratio of nearly every agonist examined is greater than 1, indicating that their affinity for at least one of the major ganglionic nAChRs is considerably lower than for the predominant nAChR in forebrain. Interestingly, this is also

Table 2. Binding affinity ratios for nAChR α subunits paired with β2 or β4 subunits. Also shown is the binding affinity ratio for the α3β4 subunit combination versus the rat forebrain (primarily α4β2). The ratios were calculated from the K_i values in Table 1

Ligand	Affinity ratio			
	α2β4/α2β2	α3β4/α3β2	α4β4/α4β2	α3β4/Forebrain
(±)-Epibatidine	3	19	3	8
(±)-I-Epibatidine	1	5	1	3
Acetylcholine	16	20	2	19
(−)-Nicotine	9	12	4	41
Cytisine	4	6	1	84
A-85380	230	300	51	327
I-A-85380	1516	690	387	2071
Carbachol	3	4	2	11
DMPP	46	19	19	7
Choline	1	1	1	1

true for the competitive antagonist dihydro-β-erythroidine, but not for methyllycaconitine (data not shown).

Measuring the binding of ligands at the different nAChR subunit combinations is an important step in the descriptive pharmacology of the nAChR subtypes and a reasonable start toward developing the pharmacology of their function. Furthermore, binding studies can provide essential structure–activity information that can lead to the development of new and subtype-selective drugs, which might have both research and therapeutic utility. Therefore, it is critical to know how well these heterologously expressed subunit combinations represent native nAChRs. There are only a few examples of tissues in which the predominant nAChR subtype is known. One of these is the rat forebrain where the majority of receptors that have high affinity for radiolabeled agonists are the α4β2 subtype.[9,10] As shown in Figure 1, comparison of the binding affinities (K_i values) of the agonists in Table 1 for the α4β2 nAChR subtype heterologously expressed in these cells and the native receptor in rat forebrain indicated a nearly perfect correlation ($r > 0.99$). More important, the best fit line for the correlation of the K_i values representing the 10 agonists examined was very nearly the line of identity, indicating that not only the rank order but the absolute values for the affinities of these agonists at the α4β2 nAChR binding sites in the cells and in rat forebrain are nearly identical.

Similarly, in PC12 cells treated with nerve growth factor the predominant nAChR has pharmacological characteristics of an α3β4 subtype, and here also the binding affinities of nicotinic drugs correspond very closely to their affinities at the heterologously expressed, defined α3β4 subtype.[11] Moreover, the potencies (EC_{50} values) of nicotinic agonists to stimulate function in these PC12 cells and the heterologously expressed α3β4 cell line are nearly identical.[11] A similar close correspondence in the pharmacology of the binding sites and function is found in comparisons between the predominant nAChR receptor in the rat pineal gland, which is also an α3β4 subtype,[12,13] and the heterologously expressed α3β4 receptor subtype (S. Hernandez and K. J. Kellar, in preparation). Interestingly, in PC12 cells treated with nicotine the predominant nAChR corresponds closely to the heterologously expressed α3β2 receptor.[11] Taken together, these comparisons indicate that the heterologously expressed nAChR subunit combinations reflect pharmacological properties of native nAChRs with high fidelity.

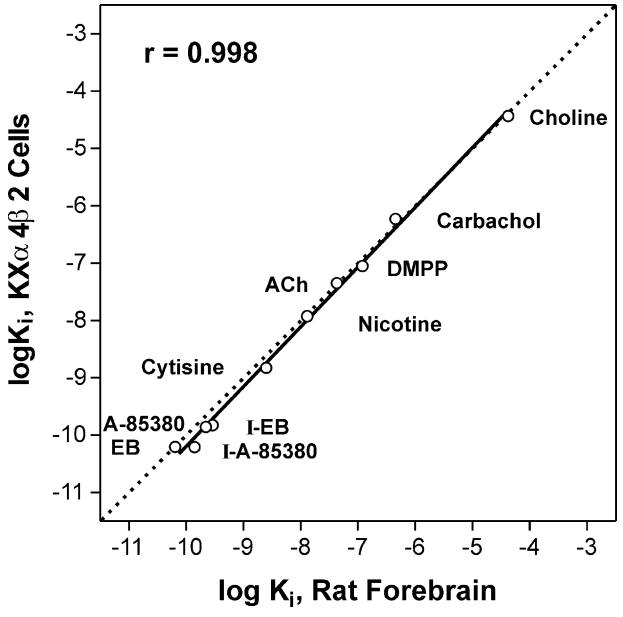

Figure 1. Correlation between the binding affinities of nicotinic agonists at heterologously expressed α4β2 nAChRs and the predominant nAChR subtype in rat forebrain. The K_i values are from Table 1. The dashed line is the line of identity.

Although the task of finding ligands and drugs that are highly selective for individual nAChR subtypes should not be underestimated, there are some promising lead compounds that might provide important information about subtype selectivity. For example, in addition to A-85380 and its derivatives, the competitive antagonist dihydro-β-erythroidine has much higher affinity for nAChRs containing β2 subunits than β4 subunits (refs 14, 15, Y. Xiao et al., submitted). Furthermore, as more is learned about the structure of nAChRs, the determinants of affinity and efficacy of a ligand at each receptor subtype will become clearer[14,16,17] and strategies for developing new subtype-selective compounds should become more rational.

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