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The Comparative Pharmacology and Up-Regulation of Rat Neuronal Nicotinic Receptor Subtype Binding Sites Stably Expressed in Transfected Mammalian Cells

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Running Title: **Pharmacology of nAChR Subtypes**

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Abbreviations: DHβE, dihydro-β-erythroidine; D MPP, 1,1-dimethyl-4-phenyl-piperazinium iodide; [^{3}H]EB, [^{3}H](±)epibatidine; I-A-85380, 5-iodo-A-85380; I-EB, iodo-(±)epibatidine; (±)MLA, methyllycaconitine; nAChR, nicotinic acetylcholine receptor

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Abstract

We stably transfected human embryonic kidney cells (HEK 293 cells) with genes encoding rat neuronal nicotinic receptor α2, α3 or α4 subunits in combination with the β2 or β4 subunit to generate six cell lines that express defined subunit combinations that represent potential subtypes of rat neuronal nicotinic acetylcholine receptors (nAChRs). These cell lines were designated KXα2β2, KXα2β4, KXα3β2, KXα3β4, KXα4β2 and KXα4β4. The K_d values of [^{3}H](±)epibatidine ([^{3}H]EB) binding to membranes from these six cell lines ranged from ~ 0.02 to 0.3 nM. The pharmacological profiles of the agonist binding sites of these putative nAChR subtypes were examined in competition studies in which unlabeled nicotinic ligands, including 10 agonists and 2 antagonists, competed against [^{3}H]EB. Most nicotinic ligands examined had higher affinity for the receptor subtypes containing the β2 subunit compared to those containing the β4 subunit. An excellent correlation ($r > 0.99$) of the binding affinities of the 10 agonists was observed between receptors from KXα4β2 cells and from rat forebrain tissue, in which [^{3}H]EB binding represents predominantly α4β2 nAChRs. More important, the affinities (K_i values) for the two tissues were nearly identical. The densities of the binding sites of all six cell lines were increased after a 5-day exposure to (-)-nicotine or the quaternary amine agonist carbachol. These data indicate that these cell lines expressing nAChR subunit combinations should be useful models for investigating pharmacological properties and regulation of the binding sites of potential nAChR subtypes, as well as for studying the properties of nicotinic compounds.

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Neuronal nicotinic acetylcholine receptors (nAChRs) are found throughout the CNS and peripheral nervous system. In the CNS they are located on neuronal cell bodies and dendrites, where they mediate fast excitatory postsynaptic responses to acetylcholine and other nicotinic agonists, as well as on axon terminals or preterminal sites. These receptors are often found associated with dopamine, norepinephrine, GABA, acetylcholine and glutamate neurons, where they can mediate the influence of acetylcholine and other nicotinic agonists on the firing of these neurons and the release of their transmitters. In the peripheral nervous system, nAChRs mediate excitatory neurotransmission at virtually all autonomic ganglia, the adrenal gland and at sensory ganglia; they are therefore critical for the normal function and adaptation of nearly all organ systems. In addition, these receptors are found in pineal gland (Marks et al., 1998; Hernandez et al., 1999; Perry et al., 2002), where they may influence melatonin secretion (Yamada et al., 1998), vascular endothelial and bronchial epithelial cells (Macklin et al., 1998; Maus et al., 1998) and skin keratinocytes (Grando et al., 1995), where their functions are not yet known.

nAChRs are composed of α and β subunits that form pentameric ligand-gated cation channels. Nine α subunits (α2 to α10) and three β subunits (β2 to β 4) have been identified in vertebrate neuronal tissues, and different combinations of these subunits define nAChR subtypes. The rules of subunit assembly are not yet well-established; therefore it's not known how many subtypes actually exist. Receptors composed of α4 and β2 subunits (the α4β2 subtype) and those containing α7 subunits (the α7∗ subtype) appear to represent the largest number of nAChRs in the CNS, but receptors containing α2, α3, α6, β3 and β4 subunits are also found in specific CNS nuclei and regions and very likely serve important physiological functions. In autonomic and sensory ganglia and in the adrenal gland, nAChRs containing α3 subunits in association with β2 or β4 subunits appear to predominate.

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All of the nAChRs receptors conduct Na^+, K^+ and Ca^{2+} when activated; however the different receptor subtypes have distinguishing pharmacological and/or functional properties, including binding affinities of ligands, efficacies and potencies of agonists and antagonists, channel conductances, and rates of desensitization and recovery (Lindstrom et al., 1996; Albuquerque et al., 1997; Colquhoun and Patrick, 1997).

Because most neuronal tissues express mRNA for multiple nAChR subunits and therefore have the potential to express more than one subtype of receptor, it is not possible to precisely compare the pharmacological properties across a broad range of receptor subtypes in native tissues. Expression of putative nAChR subtypes in Xenopus oocytes has allowed some systematic studies of the pharmacology of nAChR function (Chavez-Noriega et al., 1997) and binding sites (Parker et al., 1998) across subtypes. Similarly, the pharmacological properties of nAChR subtypes stably expressed in mouse fibroblasts (Whiting et al., 1991), human embryonic kidney (HEK293) cells (Gopalakrishnan et al., 1996; Stauderman et al., 1998; Wang et al., 1998; Xiao et al., 1998; Jensen et al., 2003) and human epithelial (SH-EP1) cells (Eaton et al., 2003) have been studied in some detail. Studies that compared the pharmacology of the subtypes have found that the potencies and affinities of any one drug can vary considerably across the subtypes. Here we extend that type of study by systematically comparing the binding affinities of twelve commonly used nicotinic drugs at six putative nAChR subtypes stably expressed in mammalian cells. We chose HEK 293 cells as the host for stably expressing rat nAChR genes because of their relatively high transfection efficiency, ease of culturing and good adhesion to plastic or glass surfaces. In addition, since there have been several human nAChR subtypes stably expressed in this cell line, it will eventually be possible to compare rat and human receptors

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expressed in the same cell line. The data presented here can serve as reference points for further systematic comparisons of drugs across nAChR subtypes.

Exposure to nicotine increases the number of nAChRs in rodent and human brain in vivo (Marks et al., 1983; Schwartz and Kellar, 1983; Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999), as well as in mammalian cell lines that express native (Peng et al., 1997; Avila et al., 2003) or heterologous (Peng et al., 1994; Wang et al., 1998; Meyer et al., 2001) nAChRs. To determine if this increase reflects a generalized property of nAChRs, we measured the effects of a 5-day exposure to nicotine on the number of nAChR binding sites for each of the six nAChR subtypes examined here. In addition, to determine if the increase in receptors is likely initiated by an action at the cell surface, the effects of exposure to carbachol, a quaternary amine nicotinic agonist that does not readily enter cells, was also measured.

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Methods

Materials and chemicals. Tissue culture medium, fetal bovine serum, antibiotics, restriction endonucleases, modifying enzymes and molecular size standards were obtained from Invitrogen Corporation (Carlsbad, CA). [^{3}H](±)epibatidine ([^{3}H]EB) was purchased from PerkinElmer Life Sciences (Boston, MA). [α-^{32}P]CTP and [γ-^{32}P]ATP were obtained from Amersham Biosciences Corporation (Piscataway , NJ). 5-Iodo-A-85380 (I-A-85380) and (±)-exo-2-(2-iodo-5-pyridyl)-7-azabicyclo[2.2.1]heptane ((±)-I-epibatidine) were generously provided by Dr. J. Musachio of Johns Hopkins University. Electrophoresis reagents were purchased from Bio-Rad Laboratories (Melville, NY). All other chemicals and drugs were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Expression constructs. Five plasmids which carry the cDNA clones of rat neuronal nAChR subunits α2, α3, α4, β2 and β4 genes (pHYP16-9, pPCA48E-3, pHYA23-1E-1, pPCX49-1 and pZPC13, respectively) and which have been previously described (Boulter et al., 1987; Duvoisin et al., 1989), were generously provided by Dr. J. Boulter of University of California, Los Angeles. To constitutively express these rat neuronal nAChR subunits in mammalian cells, cDNA fragments containing entire coding sequences of the subunit genes were subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen Corporation, Carlsbad, CA). The resulting expression constructs with the α2, α3, α4, β2 and β4 subunit genes were designated pKXα2RC1, pKXα3RC1, pKXα4RC1, pKXβ2RC1 and pKXβ4RC1, respectively.

Cell culture and stable transfection. HEK 293 cells (ATCC CRL 1573) were maintained at 37^{o}C with 5% CO_2 in a humidified incubator. Growth medium for the HEK 293 cells was minimum essential medium supplemented with 10% fetal bovine serum, 100 unit/ml penicillin G, and 100 μg/ml streptomycin. Transfection of HEK 293 cells, selection and

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establishment of stable cell lines were carried out as described previously (Xiao et al., 1998). Stably transfected cell lines were grown in selection growth medium containing 0.7 mg/ml of Geneticin (G418). After selection, six stable cell lines engineered to express combinations of an α subunit gene and one of the β subunit genes, were designated KXα2β2, KXα2β4, KXα3β2, KXα3β4R2, KXα4β2 and KXα4β4. The expression pattern of each cell line was confirmed using multiple probe RNase protection assays as described previously (Xiao et al., 1998). In studies where cells were grown in the presence of nicotine hydrogen tartrate (nicotine) or carbamylcholine chloride (carbachol), these drugs were added to the selection growth medium on day 1 of a 5-day growth period.

Radioligand binding assay. Binding of [^{3}H]EB to nAChRs was measured as described previously (Xiao et al., 1998), with minor modifications. Briefly, cultured cells at >80% confluence were removed from their flasks (80 cm^2) with a disposable cell scraper and placed in 10 ml of 50 mM Tris·HCl buffer (pH 7.4, 4° C). The cell suspension was centrifuged at 1,000 x g for 5 min and the pellet was collected. For cells treated chronically with nicotinic agonists during culturing, the pellet was washed two more times by centrifugation in fresh buffer to remove residual drug. The cell pellet was then homogenized in 10 ml buffer with a Brinkmann polytron homogenizer (setting 6, 20 seconds) and centrifuged at 36,000 g for 10 min at 4° C. The membrane pellet was resuspended in fresh buffer, and aliquots of the membrane preparation equivalent to 30 to 200 μg protein were used for binding assays. Membrane preparations were incubated with [^{3}H]EB for 4 hr at 24^o C. The volumes for saturation and competition binding assays were 1 ml/tube and 0.5 ml/tube, respectively. Nonspecific binding was assessed in parallel incubations in the presence of 300 μM nicotine. Bound and free ligands were separated by vacuum filtration through Whatman GF/C filters treated with 0.5% polyethylenimine. The

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filter-retained radioactivity was measured by liquid scintillation counting. Specific binding was defined as the difference between total binding and nonspecific binding. Data from saturation and competition binding assays were analyzed using Prism 3 (GraphPad Software, San Diego, CA). Additional statistical analyses are indicated in the figure legends.

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Results

Binding of [^{3}H]EB to nAChR subtypes stably expressed in HEK-293 cells. Specific binding of [^{3}H]EB was found in membranes from cells expressing each of the α/β subunit combinations examined (see below); whereas, in contrast, binding was not detected in the parent HEK cells, HEK cells transfected with the pcDNA3 vector, or cells expressing any of the α or β subunits alone (Xiao et al., 1998); and data not shown). The binding of [^{3}H]EB over a concentration range of 1 to 3500 pM was examined in cells expressing each of the nAChR subtypes, as defined by their subunit combination. In each case, [^{3}H]EB specific binding was saturable and fit best to a model for a single high affinity site (Fig. 1 and Table 1). The affinities of [^{3}H]EB at the different subtypes ranged from ~ 0.02 nM at the α2β2 subtype to ~ 0.3 nM at the α3β4 subtype. The affinity of [^{3}H]EB was 2- to 8-fold higher at receptors comprised of any one of the α subunits paired with a β2 subunit compared to its pairing with a β4 subunit (Table 1). The K_d value of [^{3}H]EB binding at the heterologously expressed α4β2 receptor subtype of nAChR matches well with its K_d value in rat forebrain, which expresses predominantly the α4β2 subtype of receptor (Whiting and Lindstrom, 1987; Flores et al., 1992). Similarly, the K_d value at the heterologously expressed α3β4 subtype matches well with the value found in rat pineal gland, which appears to express this subtype nearly exclusively (Hernandez et al., 1999; Perry et al., 2002).

The density of the nAChR binding sites in these cells ranged from ~ 64 fmol/mg protein to > 9,000 fmol/mg protein, and for most subtypes examined here the density of sites was >300 fmol/mg protein (Table 1). The high density of sites makes these cells particularly useful for pharmacological studies of the binding properties of ligands and for biochemical or molecular studies of the receptors.

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Pharmacology of the binding sites of six nAChR subtypes. The affinities of a number of nicotinic ligands at the receptor subtypes expressed in these cells was examined in binding competition studies against 500 pM [^{3}H]EB. Figure 2 shows representative binding curves for several of these ligands at each of the receptor subtypes in membrane homogenates. All of the ligands examined competed for >80% of the [^{3}H]EB binding sites over a concentration range spanning 2 log units, resulting in Hill coefficients close to 1 and indicating that they bound according to a simple model. Dihydro-β-erythroidine (DHβE), a competitive antagonist at nAChRs (Harvey et al., 1996) was a weaker competitor than most agonists at these receptors (Fig. 2).

Because the affinity of [^{3}H]EB at the different subtypes examined here varies by up to 14-fold (Table 1) and the binding competition assays were carried out using ~ 500 pM [^{3}H]EB for all of the nAChR subtypes, the IC_{50} values taken directly from the competition curves do not readily reflect the relative affinity of a ligand across the different receptor subtypes. Therefore, the K_i values, calculated from the IC_{50} values (Cheng and Prusoff, 1973), of these 12 nicotinic ligands at the six heterologously expressed receptor subtypes and in rat forebrain are provided in Table 2. Epibatidine and its iodinated analog had the highest affinities across all of the receptor subtypes and were also the least discriminating of the ligands at these subtypes. In general, the ligands examined had higher affinities for receptor subtypes containing the β2 subunit than for those containing the β4 subunit. It is also evident from Table 2 that, in general, the agonists had lower affinity for receptor subtypes containing the α3 subunit than for those containing α2 or α4 subunits. Thus, the affinity of every agonist ligand examined here except DMPP was lowest at the α3β4 subtype (Table 2).

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More specifically, as was seen with the binding of [^{3}H]EB, the affinity of nearly every ligand examined was higher at the receptor subtype formed by the combination of any of the α subunits with the β2 subunit than with the β4 subunit. The binding affinity ratio for a ligand, calculated from its affinities at an α subunit paired with the β2 subunit *vs* the β4 subunit, represents a measure of selectivity of that ligand with regard to the β subunits. Table 3 summarizes these ratios for the 12 ligands examined here. In some cases any difference in affinity was too small to measure, yielding ratios close to 1, for example iodo-epibatidine and cytisine at α4β2 and α4β4 receptors. But in other cases the differences were relatively large, for example the affinities of acetylcholine and nicotine were 9 to 15 times higher at α2β2 and α3β2 subtypes than at the corresponding β4 subtypes. The largest differences in affinities between the receptor subtypes containing β2 vs β4 subunits were seen with A-85380, which displayed affinity ratios of 58 to 370, and I-A-85380, which displayed ratios of 410 to 1300 and was the most selective ligand examined here. The one exception to this rule among the ligands examined was the antagonist MLA, which has higher affinity for α4β4 than α4β2 receptors (Table 3). MLA has high affinity for α7 nAChRs (~1 nM), where it is a relatively selective competitive antagonist (Ward et al., 1990; Davies et al., 1999). In contrast, its affinities at the six heteromeric receptors examined here are much lower, and even its highest affinities (1 – 2 μM), which are seen at the two receptor subtypes containing α3 subunits (Table 2), are 1,000-times lower than at the α7 subtype. The competitive antagonist DHβE has its highest affinity at the α4β2 subtype and is at least 6-fold selective for this subtype compared to the other subtypes examined (Table 2). Mecamylamine, a noncompetitive nAChR antagonist that blocks the receptor channel, did not compete effectively for the [^{3}H]EB binding site at any of the receptor subtypes at concentrations below 500 μM (Table 2).

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We also compared the affinity ratios of these ligands at α3β4 receptors, which mediate much of the cholinergic signaling in peripheral ganglia, and at the α4β2 receptors in rat forebrain (Table 3). The two A-85380 compounds had high affinity ratios, with I-A-85380 being the most selective between these two receptor subtypes, displaying a ratio of ~2500. Cytisine and DHβE also showed high selectivity (affinity ratios of ~120 and 850, respectively) for the receptors in forebrain compared to the α3β4 subtype (Table 3).

The utility of a cell line that expresses a nAChR subtype defined by its heterologous receptor subunits depends on how well that receptor reflects the pharmacology of native nAChR binding sites, thereby allowing them to serve as models of these receptors for at least some purposes. There are very few native tissues that express only a single subtype of nAChR of known subunit composition, which would allow the pharmacology of these cell lines to be compared. The rat forebrain comes close to fulfilling these criteria, since most (~90%) of the nAChRs measured with agonist radioligands in rat forebrain are the α4β2 subtype (Whiting and Lindstrom, 1987; Flores et al., 1992; Perry et al., 2002). Therefore, to help determine the fidelity of the agonist binding sites of the heterologously expressed α4β2 receptor subtype in these cells to their counterpart in native tissues, we compared the K_i values of ligands at these receptors. As shown in figure 3, the K_i values of the 10 agonists examined at the α4β2 receptors expressed in the cell line and those in rat forebrain were highly correlated ($r > 0.99$). Even more important, however, the best-fit line for the correlation was very nearly the line of identity, indicating that not only the rank order of the affinities but also the absolute K_i values of these agonists at the binding sites in the two tissues are virtually indistinguishable (see also Table 2). These data indicate that the pharmacology of the α4β2 receptor binding sites in these cells accurately reflects the pharmacology of agonists for these sites in forebrain. Interestingly, the affinities of the two competitive antagonists DHβE and

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MLA for the receptors in the forebrain and in these cells were not as close as the affinities of agonists. Nevertheless, the relative affinities of the two antagonists were similar for the α4β2 receptors in both rat forebrain and the cells, with the affinity of DHβE being ~ 50 times higher than that of MLA in both (Table 2).

A similar close correspondence between heterologously expressed α3β2 and α3β4 nAChRs and the native receptors in PC12 cells has been found. Thus, in PC12 cells grown in the presence of nicotine the pharmacology of the nAChR binding sites labeled by [^{3}H]EB is nearly identical to that in the KXα3β2 cells presented here; while in PC12 cells grown in the presence of nerve growth factor, the pharmacology of the nAChR binding sites is virtually identical to that of the receptors expressed in the KXα3β4 cells (Avila et al., 2003). Moreover, the potencies of nicotinic agonists to activate channel function measured by $^{86}Rb^{+}$ efflux in PC12 cells treated with nerve growth factor and in the KXα3β4 cells are nearly identical (Avila et al., 2003). Taken together, these data indicate that these heterologously expressed nAChRs accurately reflect the pharmacology of native nAChR binding sites.

Regulation of nAChR subtype binding sites by nicotine. In vivo administration of nicotine increases the density of nAChR binding sites in rodent brain (Marks et al., 1983; Schwartz and Kellar, 1983), and a similar increase is found in brains from human smokers (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999). As a potential model for studying the mechanisms of this unusual effect of nicotinic agonists, several groups have demonstrated that in cells that stably express defined heterologous nAChR subtypes, incubation with nicotine for several hours to several days can increase the density nAChR binding sites in human α4β2 and α3β2 subtypes (Peng et al., 1994; Gopalakrishnan et al., 1996; Wang et al., 1998), as well as in the rat α3β4 subtype (Meyer et al., 2001). To compare the effect of nicotine treatment across

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the different subtypes and to determine whether carbachol, a quaternary amine agonist that does not readily cross cell membranes, could also affect nAChR binding sites, cells expressing each of the six subtypes were grown in the presence of nicotine (from 0.2 to 100 μM) or carbachol (200 μM) for 5 days before the number of nAChR binding sites in membrane homogenates was measured with [^{3}H]EB.

As shown in Figure 4, in cells grown in the presence of nicotine for 5 days [^{3}H]EB binding in membranes from each of the nAChR subtypes was increased in a concentration-dependent manner (ANOVA, $p<0.001$; post test for linear trend, $p<0.001$), and significant increases were seen in all subtypes at nicotine concentrations of 2 μM or less. But the extent of the increases varied markedly across the subtypes; and, although all of the increases were greater than what is usually found in brain tissues after in vivo treatment, the largest increases were seen in the three subtypes containing the β2 subunit, while the smallest increase was seen in the α3β4 subtype. At the highest concentration of nicotine tested, the increases in [^{3}H]EB binding varied from ~2.5-fold (α3β4 subtype) to ~ 60-fold (α3β2 subtype).

Nicotine might increase binding to nAChRs by acting at the agonist binding sites on the extracellular surface or, because it is lipophilic and readily crosses cell membranes, it might affect nAChRs from inside the cell. Therefore, to begin to examine the question of where agonists act to increase binding sites, [^{3}H]EB binding was measured in membranes from cells grown for 5 days in the presence of 200 μM carbachol, which does not readily cross cell membranes. Carbachol significantly increased [^{3}H]EB binding at all of the nAChR subtypes, and again the largest increases were seen in the β2-containing subtypes (Fig. 5).

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Discussion

Ligand binding measurements at different subunit combinations are an important step in the descriptive pharmacology of the nAChR subtypes and a reasonable start toward developing the pharmacology of their function. Moreover, 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. In this report, we have presented a comprehensive comparative study describing the pharmacological profiles of the agonist binding sites for six defined nAChR subtypes stably expressed in a mammalian cell line. The functional properties of three of these subtypes have been reported previously (Xiao et al., 1998; Zhang et al., 1999; Meyer et al., 2001; Fitch et al., 2003; Jensen et al., 2003).

The pharmacology of these binding sites varies substantially across the six subtypes examined here. Even epibatidine and its iodinated analog, which have high affinity for all of the subtypes and are among the least discriminating drugs overall, display 10- to 20-fold differences between the subtypes with the highest and lowest affinity. The rank order of the affinities of the drugs for the binding sites at these six nAChR subtypes agrees well with that found by Luetje and colleagues (Parker et al., 1998) in *Xenopus* oocytes expressing the same six rat receptors, and even the absolute values in the two expression systems agree reasonably well. Similarly, the affinity of [^{3}H]EB at the three subtypes containing rat β4 subunits is in good agreement with that found for these subtypes containing human β4 receptors expressed in HEK cells (Stauderman et al., 1998), and the affinities of 9 drugs measured in competition binding at the rat α4β2 receptor (Table 2) correlates very highly ($r>0.99$) with their affinities at the human α4β2 receptor (Eaton et al., 2003).

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How well any of these heterologous expression systems represent native receptors is not known with certainty because few native tissues express only one nAChR subtype, which confounds comparisons. However, the very close agreement between the affinities of the 10 agonists at the heterologously expressed defined α4β2 receptors in our cell line and at the receptors in rat forebrain, which expresses predominantly the α4β2 subtype (Whiting and Lindstrom, 1987; Flores et al., 1992; Perry et al., 2002), indicates that the heterologously expressed receptors accurately represent the binding site pharmacology of agonists at native α4β2 nAChRs. Similarly, previous studies have shown that the binding site pharmacology of the heterologously expressed α3β2 and α3β4 receptors in these cells is nearly identical to that of the predominant native receptors found in PC12 cells grown in the presence of nicotine and NGF, respectively (Avila et al., 2003). Taken together, these data indicate that these heterologously expressed nAChRs provide reliable information about the pharmacology of the agonist binding sites of native receptors.

The most discriminating of the drugs examined here were A-85380 and its iodinated analog, I-A-85380, both of which have much higher affinities at nAChR subtypes containing β2 subunits than β4 subunits. A particularly important comparison is between the affinities of drugs at nAChRs in rat forebrain, where the α4β2 subtype predominates, and at α3β4 receptors, which predominate in peripheral ganglia and which are also heavily represented in certain brain areas. All of the agonist drugs examined have higher affinity for α4β2 receptors than for α3β4 receptors, but A-85380 and I-A-85380 were by far the most selective in this regard, with affinity ratios of 310 and 2,500, respectively. Nicotine, cytisine and the competitive antagonist DHβE also were moderately to highly selective for α4β2 receptors over α3β4 receptors, displaying

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affinity ratios of ~37, 120 and 850, respectively. Interestingly, MLA did not follow this pattern and displayed a higher affinity for α3β4 receptors than for α4β2 receptors.

Drugs that discriminate between subtypes of nAChRs can be very useful in determining the distribution of subtypes in tissues that contain more than one subtype, such as the CNS. For example, relatively low concentrations of cytisine or A-85380 can nearly completely mask all α4β2 nAChRs, while leaving most of the receptors containing β4 subunits available for binding by radioligands (Marks et al., 1998; Perry et al., 2002). Moreover, because cytisine, unlike A-85380, has ~ 30-fold lower affinity for α3β2 receptors than for α4β2 receptors, under carefully chosen conditions it can be used to discriminate between these subtypes (Perry et al., 2002). It should be pointed out, however, that the pharmacology of the nAChRs containing α6 subunits is not yet known, and it could be similar to that of one of the other receptor subtypes described here.

In general, the affinities of agonists and of DHβE were higher at binding sites formed with any one of the α subunits paired with a β2 subunit than with a β4 subunit. These results are in good agreement with studies of these receptor subtypes expressed in oocytes, which concluded that the β subunit exerts a large influence on the affinities of agonists at nAChR binding sites (Parker et al., 1998). However, the α subunit also appears to contribute to the pharmacology of the binding sites, although to less extent. In particular, holding the β subunit constant nicotine and cytisine have a somewhat lower affinity for receptors containing the α3 subunit than the α2 or α4 subunits. In contrast, MLA has a higher affinity for receptors containing α3 subunits.

Administration of nicotine to rats in vivo increases binding to some, though not all, nAChR subtypes (Flores et al., 1997; Davila-Garcia et al., 2003). In the cell lines examined here, exposure to nicotine or carbachol for 5 days increased the number of [^{3}H]EB binding sites

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of all of the nAChR subtypes, suggesting that an increase in response to agonists is an intrinsic property of the receptor. But the increases were usually much greater in the subtypes containing β2 subunits than in those containing β4 subunits (15- to 60-fold increases vs 2- to 10-fold increases).

It should be noted that the α4β2 nAChR, the major subtype increased in rat forebrain by nicotine administration in vivo (Flores et al., 1992) increased much more in these cells than in brain. Moreover, the α3β4 subtype, which represents one of the major nAChR subtypes in autonomic ganglia (Conroy and Berg, 1995; Mandelzys et al., 1995; Wong et al., 1995; Wang et al., 2002) and in the pineal gland (Hernandez et al., 1999; Perry et al., 2002), is significantly increased by exposure to even relatively low concentrations of nicotine in these cells; while in contrast, administration of nicotine to rats does not increase the nAChRs in the superior cervical ganglia, adrenal gland or pineal gland (Flores et al., 1997; Davila-Garcia et al., 2003). Previous studies suggested that different heterologous expression systems (e.g., oocytes vs mammalian cells) might influence the channel properties of nAChRs (Lewis et al., 1997), and our data here suggest that the cell type in which nAChRs are expressed might also influence the extent to which nicotine increases the density of the receptor binding sites.

The increased nAChR binding sites induced by nicotine are not accompanied by increased subunit mRNA in mice (Marks et al., 1992; Pauly et al., 1996) or in cultured cells (Peng et al., 1997; Avila et al., 2003), which suggests synthesis of new receptor subunits is not required. One way that nicotine could increase the density of nAChRs without altering their synthesis rates is by inhibiting their degradation (Peng et al., 1994). If this is the case, a very rapid turnover rate of the receptors would predispose them to a large nicotine-induced increase, as is seen in most of these heterologously expressed receptor subtypes. All heterologously

expressed nAChRs might display a relatively high intrinsic turnover rate, which could account for the observations of nicotine-induced increases in the different subtypes examined (Peng et al., 1994; Gopalakrishnan et al., 1996; Wang et al., 1998; Meyer et al., 2001), but the turnover rate of rat receptors expressed in human cells might be even higher, resulting in even greater increases when the cells are exposed to nicotine.

If the concept that the extent of nicotine's effect to increase nAChRs is related to the turnover rate of the receptors is applied to native nAChRs in mammalian tissues, it could help to explain the observations that in rat and mouse brain, administration of nicotine does not increase the presumed same α4β2 subtype equally in all regions (Kellar et al., 1989; Marks et al., 1992; Sanderson et al., 1993). For example, the same receptor subtype could have much different turnover rates depending on whether it's located on the neuron's cell body, dendrites or axon terminals. Furthermore, it could link this pharmacological effect of nicotine to intrinsic and extrinsic influences on neurons, such as neuronal activity, hormones or other drugs, that might affect the turnover rate of nAChRs. Thus, there could be a state-dependent aspect to nicotine's effects on receptors.

In conclusion, we developed a library of HEK 293 cells that stably express six different potential nAChR subtypes. Receptor binding studies indicate that some drugs may be useful as discriminators between certain subtypes. Exposure of these cells to nicotine or carbachol for several days increases the density of nAChR binding sites for all of the subtypes studied here. Moreover, the increase in each of the receptor subtypes after exposure to carbachol, which does not readily enter cells, suggests that the agonist-induced increase in nAChRs may be initiated by an action at the cell surface. Taken together, these studies indicate that these cells should be useful models with which to probe the receptor binding site pharmacology and possibly some

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aspects of the regulation of the receptor density. In addition, the high density of the receptors in these cells may provide enough material for structural and biochemical studies of the subunits and their associations.

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Footnotes

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Legends for Figures

Figure 1. Saturation binding of [^{3}H]EB in membrane homogenates from cells expressing six subtypes of nAChRs. Saturation binding assays were carried out as described in Methods over a [^{3}H]EB concentration range of ~1pM - 3000 pM . Binding Data were analyzed by nonlinear least-square regressions using Prism 3 software. The data were fit to both one-site and two-site saturation binding models. The data from these six cell lines fit best to the one-site model in all cases. Data shown are from a single representative experiment. See Table 1 for a summary of data from all experiments.

Figure 2. Ligand binding competition profiles in membrane homogenates from cells expressing these six nAChR subtypes. Competition binding assays were carried out as described in Methods, using a [^{3}H]EB concentration of ~ 500 pM. The data were analyzed by nonlinear least-square regression method using Prism 3 software. Data shown are representative of the ligands tested in one experiment. See Table 2 for a summary of K_i values from all competition experiments for 12 ligands tested.

Figure 3. Correlation between binding affinities of nicotinic agonists in rat forebrain and KXα4β2 cells. The logs of the K_i values from Table 2 for agonists competing for $\alpha4\beta2$ binding sites in rat forebrain and in the transfected cells were compared using Pearson two-tailed linear correlation analysis. The solid line represents the best fit through the values for agonists, and the dashed line represents the line of identity. The p-value for the correlation coefficient (r) shown is <0.0001. The values for the two nicotinic competitive antagonists DHβE and MLA are shown for comparison but were not included in the correlation analysis.

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Figure 4. Nicotine-induced increase in [^{3}H]EB binding sites in membrane homogenates from cells expressing six subtypes of nAChRs. Cells were grown in the presence of 0.2 μM to100 μM nicotine hydrogen tartrate for 5 days before measurement of [^{3}H]EB binding sites. Concentrations of [^{3}H]EB used in measuring binding site density were ~ 3 nM. Results are expressed as a percent of control samples grown in parallel in the absence of nicotine. Values are mean ± SEM from 3 to 4 independent measurements. Exposure to nicotine increased [^{3}H]EB binding at all nAChR subtypes (ANOVA, $p<0.001$; post test for linear trend, $p<0.001$ for all subtypes; note that the y-axis differs for each subtype).

Figure 5. Carbachol-induced increase in [^{3}H]EB binding sites in membrane homogenates from cells expressing six subtypes of nAChRs. Cells were grown in the presence of 200 μM carbachol chloride for 5 days before measurement of [^{3}H]EB binding sites. Concentrations of [^{3}H]EB used in measuring binding site density were ~ 3 nM. Results are expressed as a percent of control samples grown in parallel in the absence of carbachol. Values are mean ± SEM from 4 independent measurements. Values for the carbachol treated groups are significantly different from control values ($P<0.05$, Student's t-test).

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Table 1. Comparison of saturation binding of [^{3}H]EB to membrane homogenates from six stably transfected cell lines and rat forebrain. Values shown are the mean ± SEM of at least three independent experiments. See Figure 1 for description of data analyses and curve fittings.

Subtype	K_d (nM)	B_{max} (fmol/mg protein)
α2β2	0.021 ± 0.003	340 ± 140
α2β4	0.084 ± 0.016	350 ± 70
α3β2	0.034 ± 0.006	64 ± 10
α3β4	0.29 ± 0.04	9700 ± 1400
α4β2	0.046 ± 0.012	430 ± 110
α4β4	0.094 ± 0.017	3500 ± 1300
Rat Forebrain	0.054 ± 0.006	86 ± 5

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Table 2. Comparison of K_i values of nicotinic receptor ligand binding to membrane homogenates from six stably transfected cell lines and rat forebrain. Values shown are the mean ± SEM of 3 - 8 independent experiments. See Figure 2 for description of data analyses and curve fittings.

Ligand	K_i (nM)						
	α2β2	α2β4	α3β2	α3β4	α4β2	α4β4	Rat forebrain
(±)-Epibatidine	0.025 ± 0.003	0.095 ± 0.017	0.035 ± 0.011	0.57 ± 0.12	0.061 ± 0.009	0.16 ± 0.01	0.059 ± 0.00
(±)-I-Epibatidine	0.11 ± 0.01	0.15 ± 0.03	0.18 ± 0.0.05	0.98 ± 0.27	0.15 ± 0.02	0.15 ± 0.01	0.33 ± 0.03
Acetylcholine	11 ± 1	110 ± 10	56 ± 15	850 ± 100	44 ± 9	99 ± 19	45 ± 5
(-)-Nicotine	12 ± 2	110 ± 20	47 ± 11	440 ± 60	10 ± 2	40 ± 6	12 ± 2
Cytisine	1.1 ± 0.3	5.4 ± 1.2	37 ± 9	220 ± 20	1.5 ± 0.3	2.1 ± 0.4	1.9 ± 0.5
A-85380	0.073 ± 0.011	18 ± 4	0.21 ± 0.04	78 ± 10	0.14 ± 0.03	8.1 ± 1.1	0.25 ± 0.05
I-A-85380	0.031 ± 0.008	41 ± 11	0.47 ± 0.11	280 ± 30	0.059 ± 0.009	24 ± 3	0.11 ± 0.03
Carbachol	260 ± 50	930 ± 170	1100 ± 400	4700± 700	590 ± 130	1100 ± 200	460 ± 60
DMPP	32 ± 4	1400 ± 200	35 ± 5	820 ± 120	82 ± 11	2700 ± 700	100 ± 10
Choline	14000 ± 3000	26000 ± 4000	49000 ± 12000	58000 ± 11000	35000 ± 5000	45000 ± 11000	43000 ± 900
DHβE	6200 ± 1200	190000 ± 40000	3800 ± 1300	110000 ± 10000	600 ± 70	17000 ± 4000	130 ± 50
MLA	52000 ± 19000	110000 ± 30000	1100 ± 300	2200 ± 500	28000 ± 8000	6400 ± 2200	6600 ± 1500
Mecamylamine	>500000	>500000	>500000	>500000	>500000	>500000	>500000

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Table 3. Relative selectivity of nicotinic ligands for nAChRs containing β2 vs β4 subunits. Selectivity was measured by their binding affinity ratios in membrane homogenates from the six stably transfected cell lines and rat forebrain**.** The affinity ratios were calculated from the K_i values in Table 2.

	Affinity Ratio			
Ligand	α2β4/α2β2	α3β4/α3β2	α4β4/α4β2	α3β4/Forebrain
(±)-Epibatidine	4	16	3	10
(±)-I-Epibatidine	1	5	1	3
Acetylcholine	10	15	2	19
(-)-Nicotine	9	9	4	37
Cytisine	5	6	1	120
A-85380	250	370	58	310
I-A-85380	1300	600	410	2500
Carbachol	4	4	2	10
DMPP	44	23	33	8
Choline	2	1	1	1
DHβE	31	29	28	850
MLA	2	2	0.2	0.3

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Figure 1

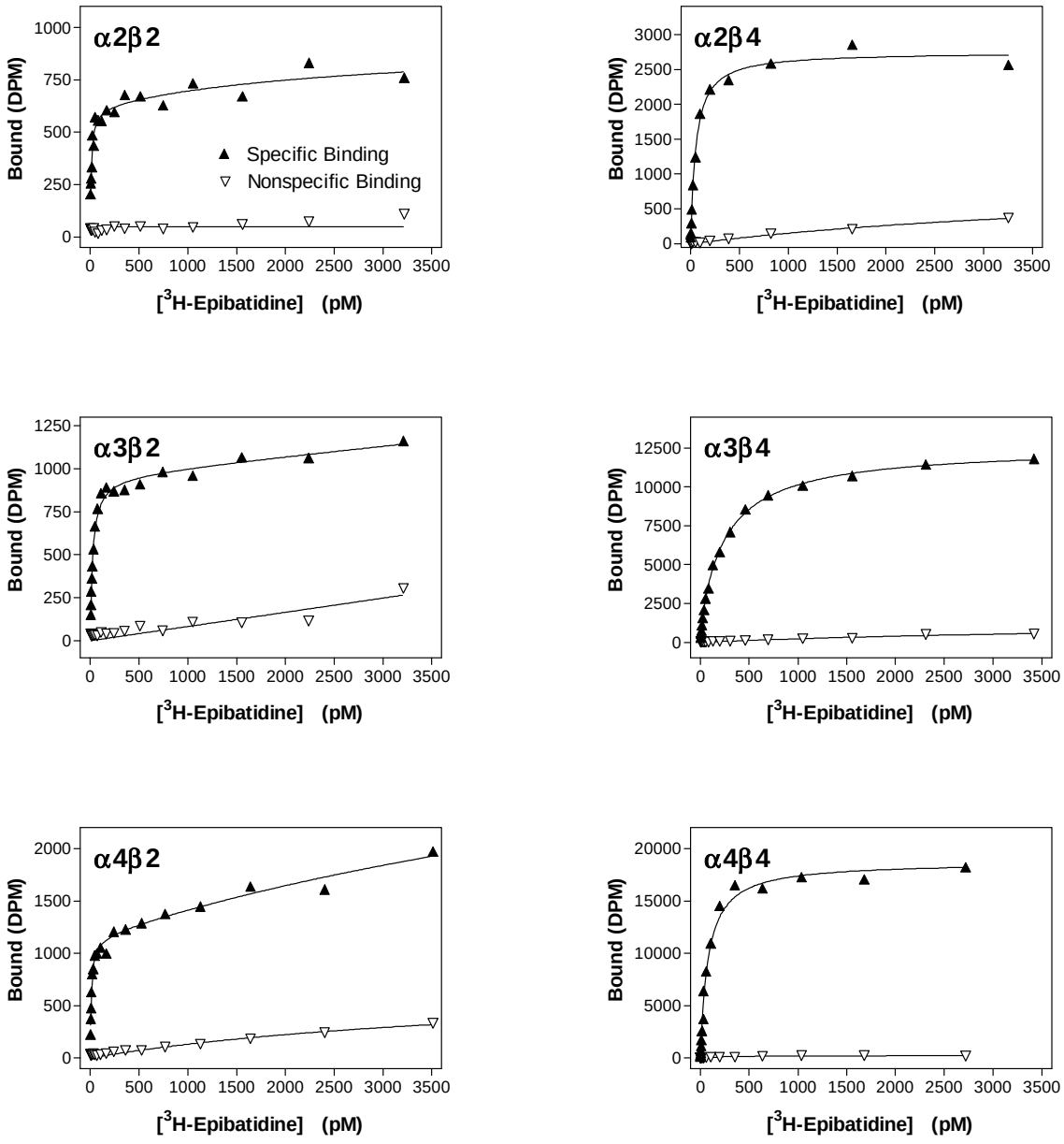

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Figure 2

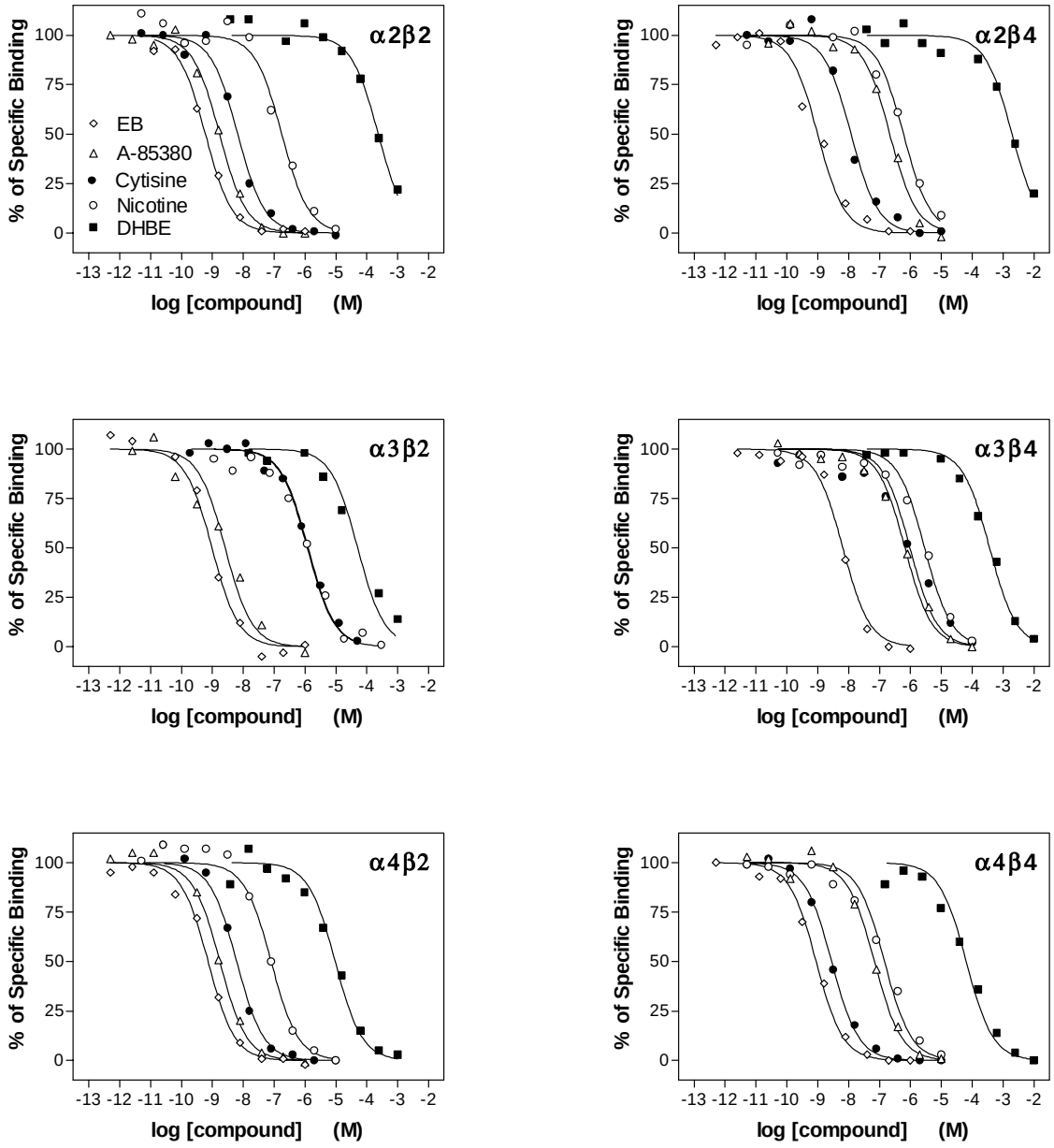

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Figure 3

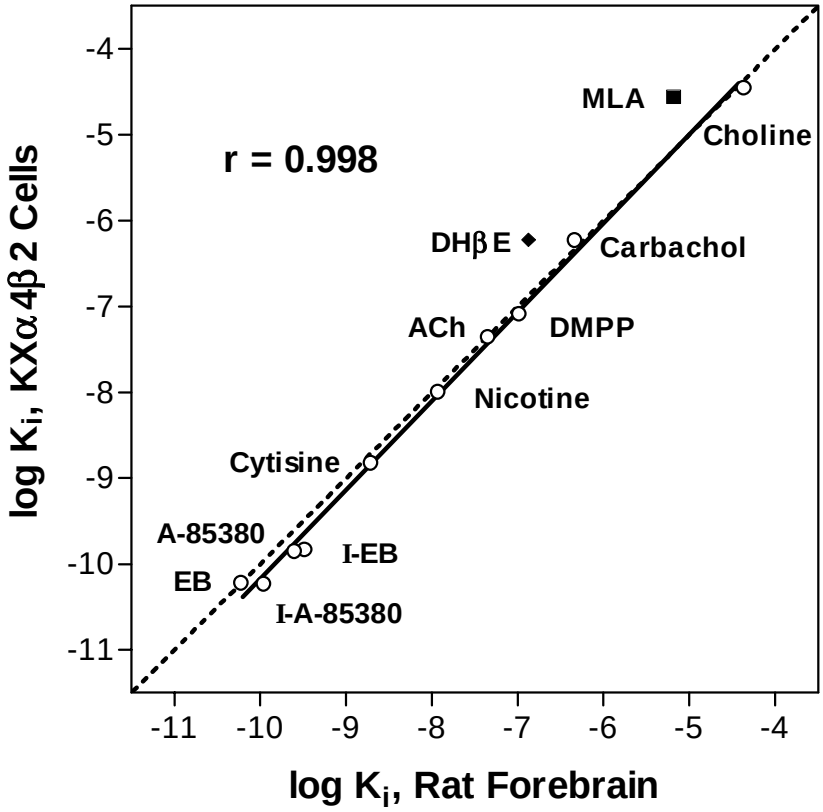

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Figure 4

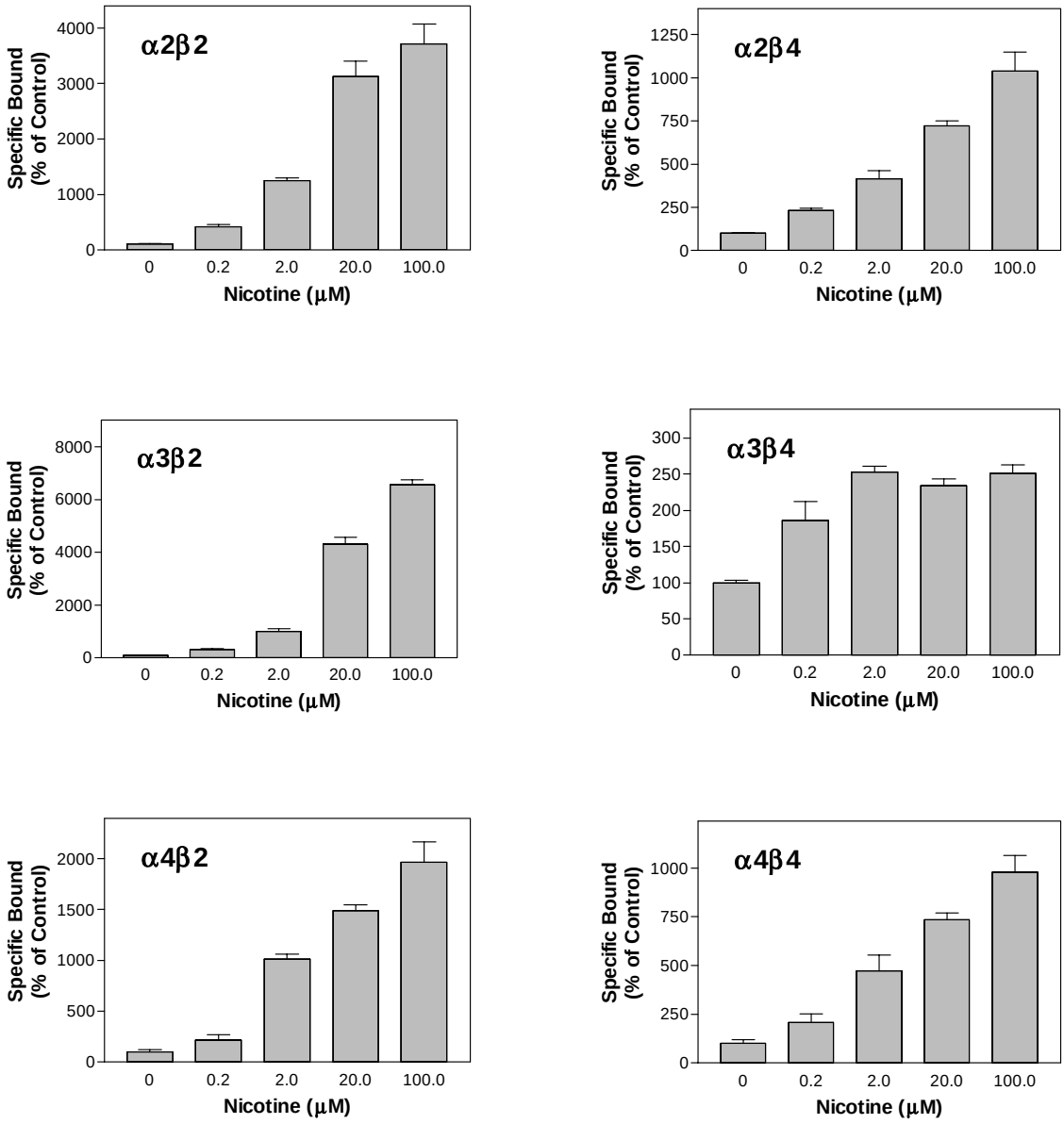

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Figure 5