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Review

The subtypes of nicotinic acetylcholine receptors on dopaminergic terminals of mouse striatum☆

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ABSTRACT

This review summarizes studies that attempted to determine the subtypes of nicotinic acetylcholine receptors (nAChR) expressed in the dopaminergic nerve terminals in the mouse. A variety of experimental approaches has been necessary to reach current knowledge of these subtypes, including *in situ* hybridization, agonist and antagonist binding, function measured by neurotransmitter release from synaptosomal preparations, and immunoprecipitation by selective antibodies. Early developments that facilitated this effort include the radioactive labeling of selective binding agents, such as [^{125}I]-α-bungarotoxin and [^{3}H]-nicotine, advances in cloning the subunits, and expression and evaluation of function of combinations of subunits in *Xenopus* oocytes. The discovery of epibatidine and α-conotoxin MII (α-CtxMII), and the development of nAChR subunit null mutant mice have been invaluable in determining which nAChR subunits are important for expression and function in mice, as well as allowing validation of the specificity of subunit specific antibodies. These approaches have identified five nAChR subtypes of nAChR that are expressed on dopaminergic nerve terminals. Three of these contain the α6 subunit (α4α6β2β3, α6β2β3, α6β2) and bind α-CtxMII with high affinity. One of these three subtypes (α4α6β2β3) also has the highest sensitivity to nicotine of any native nAChR that has been studied, to date. The two subtypes that do not have high affinity for α-CtxMII (α4β2, α4α5β2) are somewhat more numerous than the α6* subtypes, but do bind nicotine with high affinity. Given that our first studies detected readily measured differences in sensitivity to agonists and antagonists among these five nAChR subtypes, it seems likely that subtype selective compounds could be developed that would allow therapeutic manipulation of diverse nAChRs that have been implicated in a number of human conditions.

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Alterations in nicotinic cholinergic receptor (nAChR) number or function have been implicated in psychopathologies such as anxiety, attention deficit hyperactivity disorder, depression, schizophrenia (reviewed in Refs. [1–3]), at least one form of familial epilepsy [4], and Parkinson's and Alzheimer's diseases [5]. It is not particularly surprising that nAChRs might play important roles in modulating several human diseases given that binding sites for nicotinic ligands such as [^{125}I]-α-bungarotoxin [6–8], [^{3}H]-nicotine [7,8], and [^{3}H]-epibatidine [9,10], are expressed throughout the brain and spinal cord. These binding sites are clearly functional since studies done in laboratory animals and model systems have demonstrated that nicotine treatment produces a broad array of behavioral and physiological effects that are blocked by pretreatment with nicotinic antagonists such as mecamylamine. For example, nicotine injection enhances several components of learning and memory in rats and mice [11–13], decreases anxiety [14] and pain perception [15,16], and increases or decreases locomotor activity, depending on dose, species and strain [17,18]. Nicotine treatment also protects animals and neuronal cells in culture from cell death produced by several neurotoxic chemicals [19–22].

The findings that nAChRs are broadly expressed in brain and that nicotine treatments evoke many physiological changes suggest that nicotinic compounds might prove to be useful for treating human disease. Nicotinic agonists, most notably nicotine and varenicline, have proven to be of some use in smoking cessation programs, but nicotinic agonists have not proven to be of much value when given to nonsmokers. Toxic actions (e.g. increases in heart rate, blood pressure and gastrointestinal tone [23–25], and seizures at high doses [26,27]) have served to limit the therapeutic usefulness of nicotinic agonists. These unwanted effects might be minimized, or eliminated, if agents with greater selectivity are developed.

Neuronal nAChRs are pentameric complexes that closely resemble the nAChR found at the neuromuscular junction [28,29]. Consequently, the presence of nine nAChR subunit genes (α2–α7, β2–β4) in mammalian brain (see [30] for a recent review) suggests that many, perhaps hundreds, of nAChR subtypes might be expressed in brain. Homomeric and heteromeric nAChRs expressed in *Xenopus laevis* oocytes or cell lines have demonstrated that subunit composition markedly affects biophysical properties (channel open time, desensitization rate, ion selectivity) as well as sensitivities to agonists and antagonists [31–34]. Studying functional properties of the naturally occurring (native) neuronal nAChRs has been important in identifying the subunit compositions and sites of expression of nAChRs. The observation that subunit composition has profound effects on the potencies and efficacies of routinely available nicotinic drugs suggests that new agents might be developed that are highly subtype selective and thereby could be used to treat disease with limited, or reduced, toxic side effects.

Electrophysiological evidence indicates that nAChRs are expressed on dendrites, cell bodies, axons as well as in perisynaptic and presynaptic sites [30]. Those receptors that are expressed on or near nerve terminals modulate calcium-dependent release of neurotransmitters [35–38] including dopamine [39–42], norepinephrine [43,44], glutamate [45,46], GABA [47], and acetylcholine [48,49]. Nicotinic agonist-stimulated release of dopamine has been studied extensively in the nucleus accumbens because these nAChRs may play a major role in regulating the reinforcing effects of nicotine [50–52]. In the striatum, modulation of dopamine release by nAChRs may affect symptoms as well as the pathological degeneration of the dopaminergic neurons in Parkinson's disease [53]. Interest in nicotinic regulation of the dopamine system has also been stimulated by the observations that some of nicotine's effects on learning and memory [54,55], anxiety [56,57] and locomotor activation [58,59] may result as a consequence of dopamine release. Additional interest in nAChR–dopamine interactions comes from the observations that individuals suffering from dopamine-related psychopathologies such as schizophrenia and attention deficit hyperactivity are frequently smokers (incidence $>$ 50–60%) [60,61] and that smoking may retard the onset of Parkinson's disease [62].

The development of subtype selective nAChR agonists and antagonists requires that the subunit compositions of native nAChR subtypes be identified. The experiments described here used molecular biological, biochemical and pharmacological approaches in a series of studies that were designed to identify the subunit compositions of the nAChR subtypes that are expressed in mouse brain dopaminergic nerve terminals. The results obtained indicate that a minimum of five different subtypes are expressed in dopaminergic nerve terminals. The pharmacological evaluations demonstrate that those nAChRs that include α6, α4, β2 and β3 subunits have the highest sensitivity for nicotine of any native receptors that have been described, to date [63].

In situ hybridization has been used to characterize mRNA expression patterns in brain tissue. Fig. 1 presents the results of *in situ* hybridization experiments that determined the mRNA expression patterns of the α2, α3, α4, α5, α6, α7, β2, β3, and β4 subunits in coronal sections of mouse (C57BL/6) brain [64]. The coronal sections shown in Fig. 1 include the ventral tegmental area (VTA) and the substantia nigra (SN), brain regions that have high concentrations of dopaminergic neurons. The VTA and SN express high concentrations of α4, α6, β2 and β3 mRNAs, intermediate levels of α5 mRNA, and low levels of the α3 and α7 mRNAs. No signal for α2 and β4 mRNA was detected. Le Novere et al. [65] obtained virtually identical results in a study that measured *in situ* hybridization of the α3, α4, α5, α6, β2, β3 and β4 mRNAs in catecholamine-rich rat brain regions. These findings suggest that more than one nAChR subtype is expressed in dopaminergic neurons, but this conclusion must be treated with caution because the *in situ* hybridization strategies that were used in the studies discussed above lack the anatomical resolution required to determine whether specific cell types express a given mRNA. This is a major problem given that these dopamine-rich brain regions also include high concentrations of GABAergic neurons [30].

The lack of anatomical resolution associated with standard *in situ* hybridization studies prompted two experiments that were designed to assess mRNA expression in dopaminergic cells. One of these used a double-label *in situ* hybridization approach where the mRNAs for tyrosine hydroxylase, the rate limiting enzyme involved in dopamine synthesis, and nAChR mRNAs were measured in rat brain SN and VTA [66]. These

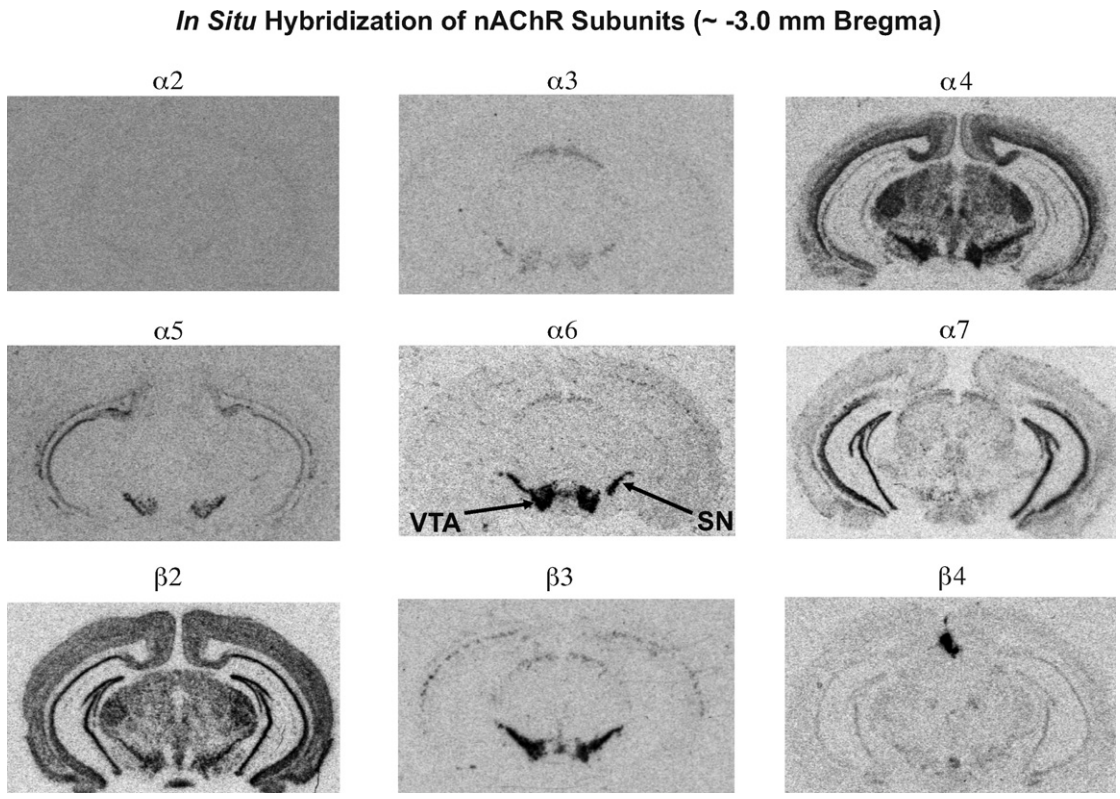

Fig. 1 – *In situ* at level of SN/VTA. Frozen sections (14 μm) from a C57Bl6 mouse at the level of SN/VTA (∼−3.0 mm bregma) were hybridized with full length riboprobes complementary to the α2, α3, α4, α5, α6, α7, β2, β3 and β4 nAChR subunits [64,100].

studies detected α4, α5, α6, β2 and β3 mRNAs in nearly every dopaminergic cell body in the VTA and SN. More than half of these neurons also expressed the α3 and α7 mRNAs and less than 10% expressed β4 mRNA. The second approach used single-cell reverse transcriptase polymerase chain reaction (RT-PCR) method to determine nAChR subunit gene mRNA expression in rat VTA and SN dopaminergic neurons [67]. This method detected α4 mRNA in virtually every SN and VTA dopaminergic cell body. Inconsistent results were obtained when β2 mRNA was measured; one oligonucleotide probe detected β2 mRNA in about 50% of the dopaminergic neurons whereas a second probe, deemed by the authors as more reliable, detected β2 mRNA in every dopaminergic cell that was studied. The α5, α6 and β3 mRNAs were detected in most (>70%) of the dopaminergic neurons, both α3 and α7 mRNAs were found in approximately 50% of dopaminergic neurons, β4 mRNA was encountered in approximately 12% of dopamine neurons and α2 mRNA was never detected. Thus, the mRNA measurement studies (*in situ* hybridization and RT-PCR) argue that nearly all dopaminergic neurons express α4, α5, α6, β2 and β3 subunit mRNAs and that α3 and α7 mRNAs are also expressed, but in fewer dopaminergic cells. Available evidence indicates that α2 and β4 mRNAs are rarely, if ever, expressed in dopaminergic neurons. These findings suggest that several, if not many, different nAChR subtypes may be expressed in dopaminergic neurons.

It is absolutely true that protein cannot be made without mRNA, but it is also true that the presence of mRNA does not guarantee that the protein product is formed or, if it is, how much is formed. Consequently, techniques that measure nAChR proteins must be employed to determine whether the mRNA is translated into a protein that is actually incorporated into a nAChR. Ligand binding assays have been used for over 40 years to detect and quantify the nAChR subunit proteins that are expressed in brain tissue. The first ligands that were developed, [^{125}I]-α-bungarotoxin [68] and [^{3}H]-nicotine [69], provided the first reliable evidence that mammalian brain might express nAChRs that resemble those that are found in the periphery. Early observations that the biochemical properties and anatomical distributions of [^{125}I]-α-bungarotoxin and [^{3}H]-nicotine binding differed from one another in both mouse [6] and rat [7] brain suggested that more than one class of nAChRs may be expressed in brain; i.e. nAChR subtypes exist. Almost all of the reports that described the cloning and sequencing of the eleven known nAChR subunit genes included studies that compared *in situ* hybridization and autoradiographic measurement of [^{125}I]-α-bungarotoxin and [^{3}H]-nicotine binding. These comparisons suggested that α7 subunits most likely play critical roles in forming the [^{125}I]-α-bungarotoxin site and that both α4 and β2 subunits make up the high affinity [^{3}H]-nicotine binding site. These conclusions were conclusively confirmed by the finding that deleting the α7 subunit resulted in elimination of the [^{125}I]-α-bungarotoxin binding site [70] and that null mutation of either the α4 [71] or β2 [72] subunit genes resulted in loss of the high affinity [^{3}H]-nicotine binding site.

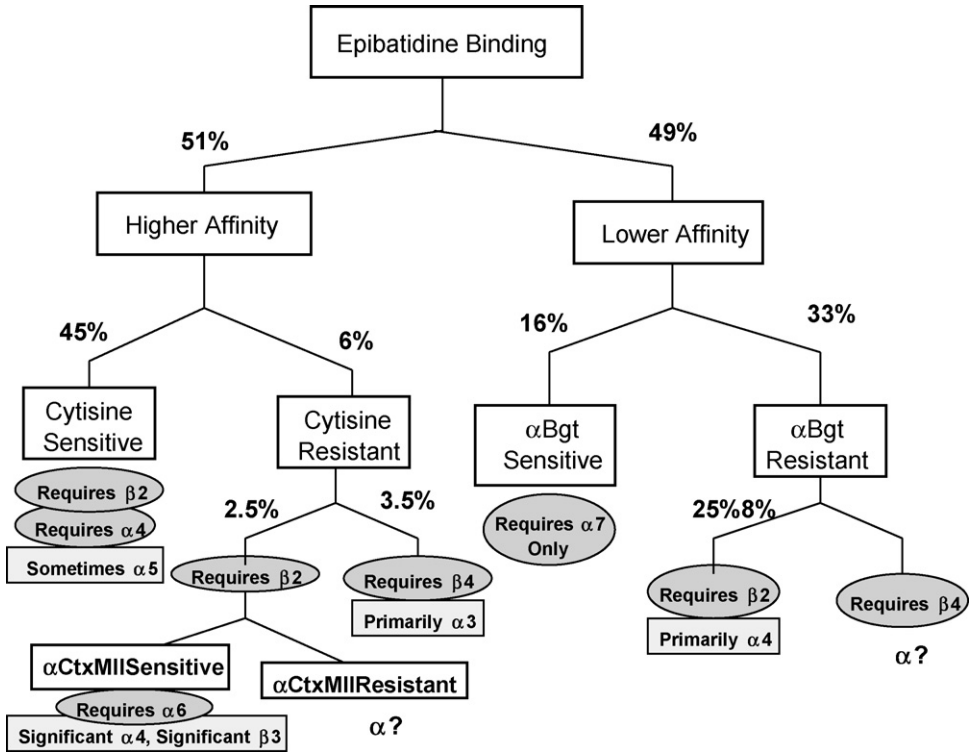

Fig. 2 – nAChR binding sites measurable with radiolabeled epibatidine. This diagram illustrates total mouse brain radiolabeled-epibatidine binding and the methods that can be used to subdivide this binding. Epibatidine binding in mouse brain is biphasic, with two sites differing significantly in affinity. Higher affinity refers to those receptor subtypes with K_D values of ~0.02 nM, while lower affinity receptors have K_D values of ~5 nM. Percentages throughout the diagram indicate the relative abundance of particular subsets in whole brain; however, note that relative abundance does vary among brain regions. "Required" subunits are determined by loss of binding in null mutant mice. Partial dependence of binding on a subunit is noted by "sometimes", "significant" or "primarily". Two of these subsets have been well characterized by other methods. Namely, higher-affinity cytisine-sensitive binding is the subset that is also measured directly by [^{3}H]-nicotine or [^{3}H]-cytisine binding, while the lower-affinity αBgt-sensitive subset is the nAChR measured directly by [^{125}I]-α-bungarotoxin binding. In addition, the α-CtxMII-sensitive subset can be measured directly by [^{125}I]-α-CtxMII binding (K_i < 1 nM). The α-CtxMII-resistant subset includes some receptors with moderate affinity for α-CtxMII (K_i > 10 nM).

Ongoing research is attempting to identify and characterize new ligands that might be useful in measuring brain nAChRs that are not measured with [^{125}I]-α-bungarotoxin or [^{3}H]-agonists. Early attempts to develop new ligands demonstrated that agonists such as [^{3}H]-cytisine [73] and [^{3}H]-ACh [74] could be used to measure rat and mouse brain nAChRs. However, it quickly became apparent that [^{3}H]-cytisine and [^{3}H]-ACh bind to the same receptors (α4β2*) that bind [^{3}H]-nicotine with high affinity [73,75–79] demonstrating that what is routinely referred to as high affinity nicotine binding sites should be referred to as high affinity agonist binding sites. A little more than 10 years ago, two new high affinity ligands were introduced to the field, radiolabeled epibatidine [80,81] and α-conotoxin MII (α-CtxMII) [82,83]. [^{3}H]-epibatidine was originally described as being a very high affinity ligand that was useful, principally, for measuring α4β2* nAChRs [80,81] ('*' indicates possible additional subunits). However, [^{3}H]-epibatidine binding exceeded that of [^{3}H]-cytisine in several regions, and was present in some brain regions (optic nerve, optic chiasm, optic tract) that had no detectable [^{3}H]-agonist binding sites [9] suggesting that epibatidine labels sites in addition to α4β2-type nAChRs. Indeed, we have shown that [^{3}H]- or [^{125}I]-epibatidine can be used to measure at least seven different nAChR subtypes (see Fig. 2) [10,70,84,85]. These subtypes can be distinguished from one another on the basis of affinity for epibatidine (higher and lower affinity sites have been detected) and sensitivity to inhibition by other nicotinic compounds; e.g. cytisine is especially useful for distinguishing two subsets of higher affinity [^{3}H]-epibatidine binding sites. A85380 distinguishes between β2* and β4* nAChRs, and α-bungarotoxin is useful for distinguishing at least two classes of lower affinity binding sites.

The upper panel of Fig. 3 presents the results of experiments that evaluated the effects of α4, α5, α7, β2, β3 and β4 gene deletion on the cytisine-sensitive component of higher affinity [^{3}H]-epibatidine binding [70]. Striatal tissue was used for the studies described here because a primary goal of these studies is to identify the nAChR subtypes that are expressed in dopaminergic nerve terminals. As is evident from the results presented in Fig. 3, both α4 and β2 gene deletion resulted in total elimination of cytisine-sensitive [^{3}H]-epibatidine binding (α4β2*). Binding in heterozygous (±) mice was decreased by

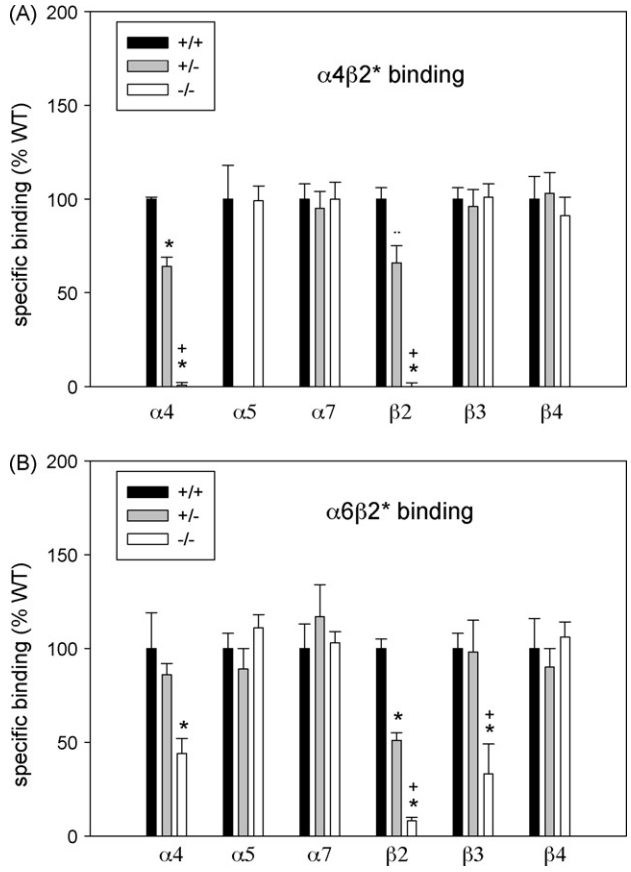

Fig. 3 – nAChR binding in striatum. Specific binding to nAChRs was measured in striatal membrane preparations from mice of the three genotypes (wildtype, heterozygous and null mutant) of each nAChR subunit null mutation indicated on the *x*-axis. (Panel A) Binding representing the α4β2* subtypes was measured by nicotine binding for the β3 null genotypes [100], and by higher-affinity cytisine-sensitive [^{3}H]epibatidine binding for the remaining genotypes (α5 data from [107]; β2 from Ref. [119]; α4, α7 and β4 unpublished data from M.J. Marks). (Panel B) Binding representing the α6β2* subtypes was measured by [^{125}I]-α-CtxMII binding [86]. For both panels, significant differences (one-way ANOVA) are indicated by the symbol '*' (different from wildtype $P < 0.05$) or '+' (different from heterozygote $P < 0.05$).

approximately 40%; i.e. a gene dose dependent decrease in binding was seen. The finding that α4 and β2 subunits are required to form the cytisine-sensitive high affinity [^{3}H]-epibatidine binding sites mimic exactly that obtained with [^{3}H]-nicotine [71,72] and clearly demonstrate that α4β2* nAChRs are expressed in the striatum. Deletion of the α5, α6, α7, β3 and β4 subunits did not result in a detectable change in cytisine-sensitive [^{3}H]-epibatidine binding.

We have also evaluated the effects of α4, α5, α7, β2, β3 and β4 gene deletion on [^{125}I]-α-CtxMII (α6β2*) binding in striatal membranes [86]. Deletion of the α4 and β3 subunits resulted in significant, but not total, elimination of [^{125}I]-α-CtxMII binding (lower panel of Fig. 3). These results suggest that at least some of the nAChRs that bind [^{125}I]-α-CtxMII with high affinity require these subunits for their formation. Binding was nearly absent in striatal tissues obtained from β2 null mutants. We suspect that β2 deletion results in total elimination of [^{125}I]-α-CtxMII binding-nAChRs, but we cannot be totally confident that this conclusion is accurate because this assay is plagued by high nonspecific binding [86]. Deletion of the α5, α7, and β4 subunits did not result in any detectable changes in binding.

Expression of nAChRs in *X. laevis* has shown that α-CtxMII binds with high affinity to α3β2* nAChRs [82] and α6β2* nAChRs [33]. However, deletion of nAChR subunits clearly demonstrated that α6 is required to form those nAChRs that bind [^{125}I]-α-CtxMII with high affinity in dopaminergic neurons whereas α3 is not. Specifically, null mutation of the α6 subunit results in a total elimination of [^{125}I]-α-CtxMII binding in dopaminergic pathways [87] whereas null mutation of the α3 gene has no effect [88]. The observation that α3 gene deletion results in total elimination of [^{125}I]-α-CtxMII binding in a few brain regions (e.g. medial habenula, fasciculus retroflexus) and marked reductions in others (e.g. interpeduncular nucleus) clearly demonstrates that at least some native α3-containing nAChRs will bind [^{125}I]-α-CtxMII with moderate affinity. Thus, even though α3 mRNA is expressed in some SN and VTA dopaminergic cell bodies, α3-containing nAChRs that bind [^{125}I]-α-CtxMII with at least moderate affinity may not be formed in these brain regions or not axonally transported to dopaminergic terminals. Taken together, these results argue that α6, rather than α3, partners with β2 to form all of the nAChRs that bind [^{125}I]-α-CtxMII with high affinity, but moderate affinity binding may occur at α3β2* mouse nAChRs. The findings that α4 and β3 gene deletion result in partial decreases in [^{125}I]-α-CtxMII binding indicates that about half of the α6β2* nAChRs include the α4 subunit and approximately two-third include the β3 subunit. However, while it is clear that striatal tissue expresses nAChRs on dopaminergic nerve terminals [41,89,90], it is also readily apparent that other neurons, particularly GABAergic nerve terminals [47] also express nAChRs. Perhaps the best support for this assertion comes from the finding that approximately 50% of total epibatidine binding persists in mouse striatal tissue following treatment with MPTP doses that virtually eliminate [^{125}I]-α-CtxMII binding, dopamine transporters and nicotine-stimulated [^{3}H]-dopamine release, without affecting nicotine-stimulated [^{3}H]-GABA release from striatal synaptosomes [91]. It seems highly likely that α-CtxMII-binding nAChRs are expressed almost exclusively in dopaminergic neurons, given that treatment with the dopamine neuron neurotoxin, MPTP, results in decreases in mouse striatal [^{125}I]-α-CtxMII binding that closely parallel declines in the dopamine transporter and agonist-induced dopamine release without affecting GABA markers [91].

Failure to detect an effect of gene deletion on binding must be interpreted cautiously. For example, α5, α7, β3 and β4 gene deletion did not produce a measurable effect on cytisine-sensitive [^{3}H]-epibatidine and deletion of the α5, α7 and β4 subunits did not affect [^{125}I]-α-CtxMII binding, which may indicate that none of these subunits are absolutely required to form these receptors. It should be noted, however, that gene deletion may not elicit a detectable change in ligand binding if a neuron makes an alternative receptor (e.g. α4β2) where it

normally might make an α4α5β2 nAChR. Other methods may be necessary to resolve this issue.

Immunological approaches have also been used to investigate nAChR subtypes that are expressed in mouse striatum. These methods can help to verify which subunits combine to form a receptor subtype. Champtiaux et al. [41] used antibodies that were directed against the rat and human α4–α7, and β2–β4 subunits in immunoprecipitation experiments and identified three heteromeric receptors α4β2*, α6α4β2* and α6β2* in striatum. More recently, Gotti et al. [92] used the same battery of subunit specific antibodies to measure the nAChR subtypes that are expressed in wildtype and β3 null mutant striata. Three α6* nAChR subtypes (α4α6β2β3, α6β2β3, and a low concentration of α6β2) were identified. These α6* subtypes are, presumably, the nAChRs that bind [^{125}I]-α-CtxMII with high affinity. Comparable studies done with the rat identified α4α5β2, α4α6β2(β3) and α6β2(β3) nAChRs in striatum (the (β3) designation was used to indicate the possible presence of the β3 subunit) [93]. Thus, immunological approaches indicate that minimum of five nAChR subtypes (α4β2, α4α5β2, α4α6β2β3, α6β2β3 and α6β2) are expressed in rodent striatum. The likelihood that expressing multiple nicotinic receptors in dopaminergic nerve terminals is biologically important is enhanced by the findings that immunological methods have identified the same receptors in striatal tissue obtained from squirrel monkeys [94] and humans [95].

The subunit composition of nAChRs, expressed in *X. laevis* oocytes, has profound effects on biophysical (channel open time, ion conductance, desensitization rates) and pharmacological (sensitivity to agonists and antagonists) properties of nAChRs [31,32,96]. These findings have prompted several research groups, including ours, to develop biochemical methods that might be used to characterize the functional properties of native nAChRs. Nicotinic agonist-stimulated release of dopamine from brain has been studied intensively for over 40 years. Westfall [97] was the first to demonstrate nicotinic facilitation of dopamine release from rat brain striatal slices. Since that time, others have established that nicotine, and other nicotinic agonists, will elicit Ca^{2+}-dependent release of dopamine from striatal tissue slices (see, for examples [98,99]) and synaptosomes [39]. We have used a variant of the synaptosomal dopamine release assay that was originally developed in the Wonnacott laboratory [39] in a series of studies that characterized the pharmacological properties of dopamine release from striatum [40,89,90,100–102], as well as in other brain regions such as the nucleus accumbens, olfactory tubercles and frontal cortex [103]. Dose–response curves for agonist-stimulated dopamine release were obtained with many agonists, and, without exception, the results suggested that dopamine release is modulated by a single receptor subtype. However, the finding that α-CtxMII is a potent, but partial, inhibitor of nicotinic agonist-stimulated [^{3}H]-dopamine release from rat striatal synaptosomes [104] argued that, in spite of the agonist data, more than one nAChR subtype might be expressed on striatal dopaminergic nerve terminals. This finding prompted us to determine whether α-CtxMII also inhibits nicotinic agonist-stimulated [^{3}H]-dopamine release from mouse striatal synaptosomes [103]. As was the case with the rat, α-CtxMII proved to be a partial inhibitor of ACh-stimulated [^{3}H]-dopamine release from mouse striatal synaptosomes (Fig. 4A). Panel B of Fig. 4 shows concentration–effect curves for ACh-stimulated [^{3}H]-dopamine release from untreated synaptosomes (total release) and synaptosomes that had been incubated with 30 nM α-CtxMII before perfusion (α-CtxMII-resistant release). The α-CtxMII-sensitive component was calculated by subtracting α-CtxMII-resistant release from total release. The α-CtxMII-sensitive component of ACh-stimulated dopamine release accounts for approximately one-third of total release and the α-CtxMII-resistant component is approximately two-third of total release.

The observation that α-CtxMII is a partial inhibitor of ACh-stimulated [^{3}H]-dopamine release was not surprising given that α-CtxMII binds with high affinity to α3β2* and α6β2* nAChRs, but not to α2*, α4β2* and α7* nAChRs, and that at least α6* and α4β2* are found in striatum. We chose to explore the postulate that the α-CtxMII-sensitive and -resistant components of [^{3}H]-dopamine release are modulated by different nAChRs by examining the pharmacological properties of the α-CtxMII-sensitive and -resistant components of the release process. Panel C of Fig. 4 illustrates the effects of application of varying concentrations of the four agonists on the α-CtxMII-resistant component of [^{3}H]-dopamine release. The agonists differed in potency and efficacy. Epibatidine (EPI) was the most potent agonist (EC_{50} = 12 nM) followed by cytisine (CYT) (EC_{50} = 470 nM), ACh (EC_{50} = 1.02 μM) and nicotine (NIC) (EC_{50} = 1.61 μM). The agonists also differed in maximal release (efficacy). Maximal release elicited by epibatidine was nearly four times that produced by cytisine and approximately 50% greater than the maximal release elicited by ACh and nicotine. Studies using expression systems have repeatedly demonstrated that cytisine has very low efficacy at α4β2* nAChRs [31,105,106]. Consequently, the finding that the maximal [^{3}H]-dopamine release elicited by cytisine is very low when compared with other agonists suggests that the α-CtxMII-resistant component of nicotinic agonist-stimulated dopamine release is modulated by an α4β2* nAChR. This conclusion is consistent with the observation (see Panel A, Fig. 3) that striatal membranes express high levels of cytisine-sensitive [^{3}H]-epibatidine binding which requires α4 and β2 subunits.

The effects of varying agonist concentrations on the α-CtxMII-sensitive component of [^{3}H]-dopamine release are provided in Panel D of Fig. 4. Once again, epibatidine was the most potent agonist (EC_{50} = 780 pM). The rank order of potencies (EC_{50} values) for the other three agonists is: cytisine (31 nM), ACh (150 nM) and nicotine (770 nM). This rank order differed from the results obtained with the α-CtxMII-resistant component of dopamine release. Differences between the agonists in efficacy were also observed, but these differences were not robust. Once again, epibatidine elicited the greatest maximal response, but robust differences in efficacy were not seen for the other three agonists. These findings argue that the α-CtxMII-sensitive component of agonist-elicited [^{3}H]-dopamine release is modulated by a different receptor(s) than is the α-CtxMII-resistant response. Moreover, these results demonstrate that those nAChRs that modulate the α-CtxMII-sensitive component of dopamine release are frequently more sensitive to the agonist properties of nicotinic ligands. This result is somewhat surprising since α4β2* nAChRs has the highest affinities in binding experiments.

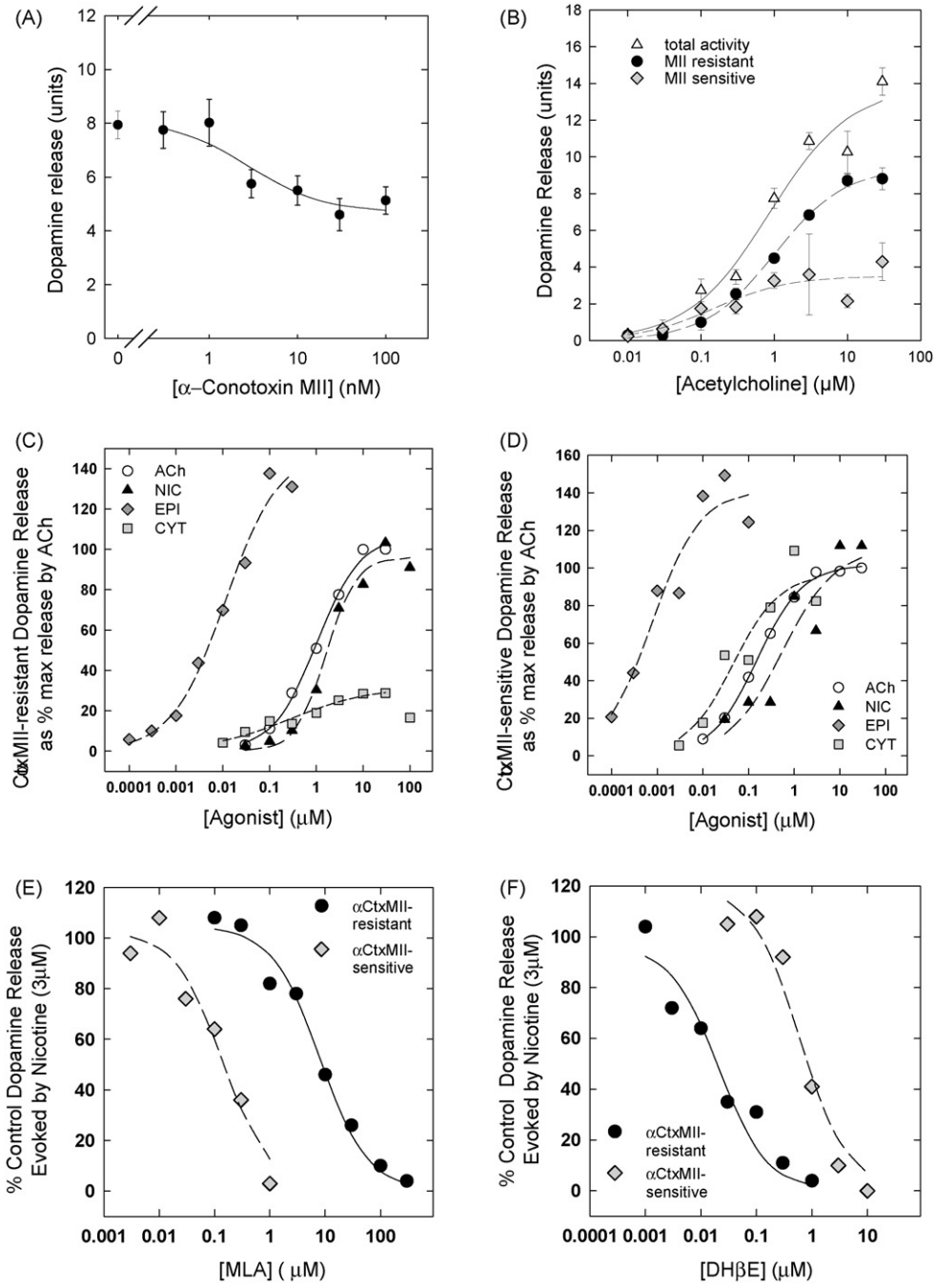

Fig. 4 – Agonist-stimulated dopamine release from striatal synaptosomes. (Panel A) α-CtxMII partially inhibits nicotine-stimulated dopamine release, evoked by a 10 s exposure to 10 μM ACh, with an IC_{50} value of 2.2 nM (data from Ref. [63]). Units indicate dopamine release as cpm normalized to cpm of baseline release. (Panel B) ACh-stimulated dopamine release measured from C57Bl striatal synaptosomes with (MII resistant activity) and without (total activity) prior exposure to α-CtxMII (50 nM for 5 min). The MII-sensitive activity is determined by difference [42]. Units are calculated as for Panel (A). (Panel C) α-CtxMII-resistant dopamine release stimulated by various agonists as % of maximal α-CtxMII-resistant release by ACh (replotted from Ref. [42]). (Panel D) α-CtxMII-sensitive dopamine release stimulated by various agonists as % of maximal α-CtxMII-sensitive release by ACh (replotted from Ref. [42]). (Panel E) Inhibition of both α-CtxMII-resistant and -sensitive dopamine release stimulated by nicotine (3 μM) by MLA [42]. (Panel F) inhibition of both α-CtxMII-resistant and -sensitive dopamine release stimulated by nicotine (3 μM) by DHβE [42].

Studies done with nicotinic antagonists provide further support for the conclusion that different nAChRs modulate the α-CtxMII-sensitive and -resistant components of dopamine release. Panel E of Fig. 4 shows that methyllycaconitine (MLA) pretreatment totally blocks both the α-CtxMII-sensitive and -resistant components of agonist-stimulated dopamine release. The IC_{50} value for inhibition of the α-CtxMII-sensitive component is approximately 0.5 μM whereas the IC_{50} for

inhibition of the α-CtxMII-resistant component is approximately 10 μM. Panel F of Fig. 4 depicts results obtained when the actions of dihydro-β-erythroidine (DHβE) were evaluated. DHβE pretreatment produced total blockade of agonist-stimulated dopamine release, but, in this case, the α-CtxMII-resistant component was more sensitive to inhibition than was α-CtxMII-sensitive component (IC_{50} values = .06 μM [α-CtxMII-resistant] and 0.9 μM [α-CtxMII-sensitive]).

Fig. 5 presents the results of experiments that evaluated the effects of deleting the α2, α4, α5, α7, β2, β3 and β4 genes on ACh-stimulated (10 μM) [^{3}H]-dopamine release from striatal synaptosomes. Panel A provides the results obtained when α-CtxMII-resistant dopamine release was measured. Deletion of the α2, α7 and β4 genes had no discernable effect. In contrast, α4 and β2 gene deletion resulted in total elimination of this component of dopamine release. These results indicate that the α-CtxMII-resistant component of dopamine release is modulated by an α4β2* nAChR. These results are also consistent with the finding that deleting these genes resulted in the total loss of cytisine-sensitive [^{3}H]-epibatidine binding. Unlike binding, however, α5 gene deletion produced a significant decrease in the α-CtxMII-resistant component of ACh-stimulated dopamine release. This finding argues that at least some of the nAChRs that modulate the α-CtxMII-resistant component of dopamine release are α4α5β2 nAChRs. A similar functional decrease, with no decrease in receptor number, is seen in the α5 null mutant mouse when function is measured by $^{86}Rb^+$ efflux [107]. Given that α5 gene deletion did not change cytisine-sensitive [^{3}H]-epibatidine binding (Fig. 3), we suspect that both α4β2 and α4α5β2 nAChRs are normally expressed in striatal dopaminergic nerve terminals and that α4β2 nAChRs replace the α4α5β2 nAChRs in the α5 null mutant mice. It has been repeatedly demonstrated that α4β2 nAChRs expressed in *Xenopus* oocytes or cultured cells can assemble in two stoichiometric forms with differing sensitivities to activation by agonists [108–110]. In neurons there is evidence for two forms of α4β2 receptors with functional differences ([111] and M.J. Marks, unpublished data); however, these two forms may have similar binding affinities (M.J. Marks, unpublished data). These properties of naturally occurring α4β2 receptors could explain the discrepancy between binding and function seen in the α5 subunit null mutant mouse where high affinity (α4β2*) binding appears unchanged but the high affinity function is decreased.

Panel B of Fig. 5 depicts the effects of gene deletion on the α-CtxMII-sensitive component of [^{3}H]-dopamine release. Once again, α2, α7 and β4 gene deletion produced no discernable effect. β2 gene deletion resulted in total elimination of this component of dopamine release, a finding that exactly mirrors the observation that α6 gene deletion eliminates α-CtxMII-sensitive dopamine release [41] indicating that all of the nAChRs that modulate the α-CtxMII-sensitive component of dopamine release are α6β2*. Both α4 and β3 gene deletion resulted in partial reductions in α-CtxMII-sensitive release, consistent with the immunological results that indicate that α4α6β2β3 and α6β2β3 nAChRs are expressed in mouse striatum.

Gene deletion did not always result in a decrease in agonist-stimulated [^{3}H]-dopamine release. Deleting the β3 gene resulted in an unexpected increase in the α-CtxMII-resistant component of ACh-stimulated dopamine release. This increase was not accompanied by a discernable change in cytisine-sensitive [^{3}H]-epibatidine binding (Fig. 3) or in a detectable change in brain mRNA concentrations for any of the other subunits (α2-α7, β2, β4) that are normally expressed in mouse brain [100]. Thus, β3 gene deletion elicited an increase in a response that appears to be modulated by α4(α5)β2 nAChRs, but it did not change the number of receptors that are measured by a binding assay that requires expression of α4 and β2 subunits. Similar findings were obtained when the effects of α5 gene deletion were evaluated. Deleting the α5 gene resulted in an increase in α-CtxMII-sensitive dopamine release, but α5 gene deletion did not produce a detectable change in [^{125}I]-α-CtxMII binding (Fig. 3). In both cases, as expected, gene deletion resulted in loss of function for those components of dopamine

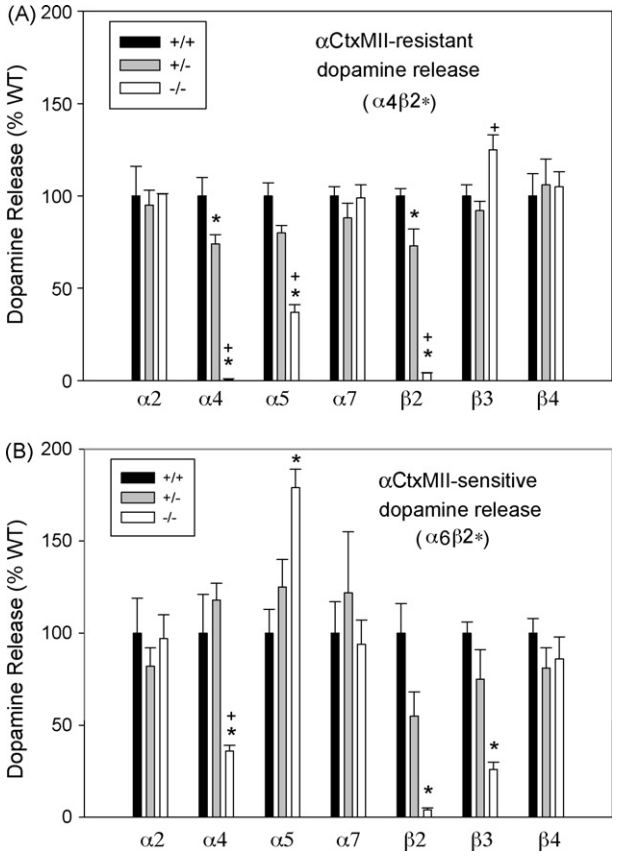

Fig. 5 – Effect of nAChR subunit null mutations on agonist-stimulated dopamine release from striatal synaptosomes. (Panel A) Effects on α-CtxMII-resistant dopamine release by ACh (10 μM) with prior exposure to α-CtxMII (50 nM for 5 min) as % of wildtype response (replotted from Ref. [42] and unpublished data from S.R. Grady). (Panel B) Effects on α-CtxMII-sensitive dopamine release stimulated by ACh (1 μM) by difference between samples of synaptosomes with and without prior exposure to α-CtxMII (50 nM for 5 min) as % of wildtype response (replotted from Ref. [42] and unpublished data from S.R. Grady). For both panels significant differences (one-way ANOVA) are indicated by '*' (different from wildtype $P < 0.05$) or '+' (different from heterozygote $P < 0.05$).

release that are modulated by receptors that normally include the deleted subunit gene (see Fig. 5). Unexpectedly, in both cases, a compensatory biochemical increase in release is seen in that component modulated by receptors that do not contain the deleted gene product (see Fig. 5). These biochemical compensations occur without detectable alterations in the number of receptors.

As indicated previously, nAChRs are expressed on cell bodies, dendrites, and axons, as well as close to and at the nerve terminal [30]. A series of studies have attemptal to evaluate which of these expression sites is most important in regulating dopamine release and conflicting results have been obtained. Several studies [112–114] yielded results that questioned whether presynaptic nAChRs expressed in the nerve terminals play a role in regulating dopamine release. All of these studies evaluated the effects of systemic nicotine injection on dopamine release in the rat nucleus accumbens using *in vivo* microdialysis and all of these studies showed that inhibiting nAChRs expressed on cell bodies, or blocking action potentials with tetrodotoxin, resulted in virtual elimination of nicotine-induced dopamine release in the accumbens. Other studies that evaluated the effects of nicotine infused into dopaminergic nerve terminals (accumbens, striatum, frontal cortex) resulted in readily detected increases in dopamine release [115,116]. These contradictory findings might be explained by the more recent observation that used cyclic voltametry to obtain a better resolution of time-dependent effects of nicotine application on dopamine release. The data obtained using striatal [117] and nucleus accumbens [118] slices are identical: nicotine produces a decrease in dopamine release following a single electrical stimulation and an increase in release following a burst of five stimulations. These observations argue that both somatodendritic and nerve terminal nAChRs play important roles in modulating the release of dopamine from dopaminergic neurons.

In summary, the studies outlined here demonstrate that dopaminergic nerve terminals in the striatum express as many as five different nAChR subtypes ($\alpha4\beta2$, $\alpha4\alpha5\beta2$, $\alpha4\alpha6\beta2\beta3$, $\alpha6\beta2\beta3$ and $\alpha6\beta2$). It is not clear what advantages accrue from having so many subtypes expressed in dopaminergic nerve terminals. Perhaps multiple subtypes allow a very fine control of dopaminergic activity or perhaps different dopaminergic nerve terminals express different subtypes. For example, multiple subtypes could allow release differentially associated with voltage-sensitive calcium channels [38]. These questions have not been addressed, but our data indicating that the $\alpha4(\alpha5)\beta2$ and $\alpha6\beta2^*$ subtypes clearly have different pharmacological properties suggest that subtype selective compounds might be developed. Such compounds might result in more effective smoking-cessation therapies as well as treatments for psychopathologies and neurodegenerative diseases that involve the nigro-striatal dopamine system.

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