The Road to Discovery of Neuronal Nicotinic Cholinergic Receptor Subtypes

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Abstract The discovery that mammalian brain expresses the mRNAs for nine different nicotinic cholinergic receptor subunits (α2–α7, β2–β4) that form functional receptors when expressed in *Xenopus laevis* oocytes suggests that many different types of nicotinic cholinergic receptors (nAChRs) might be expressed in the mammalian brain., Using an historical approach, this chapter reviews some of the

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progress made in identifying the nAChR subtypes that seem to play a vital role in modulating dopaminergic function. nAChR subtypes that are expressed in dopamine neurons, as well as neurons that interact with dopamine neurons (glutamatergic, GABAergic), serve as the focus of this review. Subjects that are highlighted include the discovery of a low affinity α4β2∗ nAChR, the identity of recently characterized α6* nAChRs, and the finding that these α6∗ receptors have the highest affinity for receptor activation of any of the native receptors that have been characterized to date. Topics that have been ignored in other recent reviews of this area, such as the discovery and potential importance of alternative transcripts, are presented along with a discussion of their potential importance.

1 Introduction

Binding sites in brain and autonomic ganglia for the nicotinic cholinergic receptor (nAChR) antagonist, α-bungarotoxin (α-Bgt), were first identified over 40 years ago (reviewed in Oswald and Freeman 1981). However, these binding sites were an enigma because virtually every study that had attempted to detect α-Bgt-induced blockade of cholinergic activities in brain preparations and autonomic ganglia during this time period had yielded negative results (reviewed in Schmidt 1988). Molecular biological approaches turned the nAChR field on its head with the discovery of 12 nAChR subunit genes that formed functional receptors when expressed in *Xenopus laevis* oocytes (reviewed in Lindstrom 1998). Those genes that coded for subunits that included two vicinal cysteines in the extracellular domain were designated alpha (α) subunits, while those that coded for subunits without the vicinal cysteines were termed non-alpha or structural subunits (terms that were ultimately replaced by the beta [β] designation). Mammalian brain expresses nine of these nAChR subunit genes (α2–α7, β2–β4) (Patrick et al. 1989; Heinemann et al. 1991; Lindstrom 1998). The remaining three subunits, α8–α10, are not expressed in mammalian brain (Schoepfer et al. 1990; Sgard et al. 2002; Keyser et al. 1993; Elgoyhen et al. 1994). The α and β subunits expressed in "peripheral-type" receptors (skeletal muscle and electric organs) are designated α1, β1, γ, δ and ε; α2–α10 and β2–β4 were assigned their names based on order of discovery.

This chapter summarizes progress made in identifying the subunit compositions of native nAChRs expressed in mammalian brain. The discovery that nine different subunits are expressed in the brain suggests that many, perhaps hundreds, of different nAChR subtypes might be expressed in the brain, assuming that neuronal nAChRs are made up of five subunits like the peripheral-type receptors (Karlin 2002). However, the number of subtypes that are actually expressed is certainly less than hundreds due to factors such as rules of receptor assembly and limitations on sites of expression. Nonetheless, the finding that *X. laevis* oocytes form functional receptors with varying biophysical and pharmacological properties when injected with cDNAs for the various subunits (Leutje and Patrick 1991; Chavez-Noriega et al. 1997) transformed the nAChR field dramatically. We went from zero functional receptors to perhaps dozens of potentially different nAChR subtypes.

Identifying the subunit compositions, sites of expression, and pharmacological properties of native receptors is of continuing interest and much of this progress has been summarized in two excellent recent reviews (Gotti et al. 2006a, 2007). We will provide an overview of this progress in this chapter, while paying particular attention to those nAChR subtypes that regulate the function of dopaminergic neurons. However, we will also highlight issues that did not receive much attention in the reviews by Gotti et al. For example, we will emphasize topics such as heteromeric α7-type nAChRs and the recently discovered low affinity α4β2* nAChRs (Marks et al. 2007) that are not discussed in the reviews by Gotti et al. We have opted to take a historical approach when describing this progress because history can often serve as a blueprint for future successes, and also because many of the individuals who studied the actions of nicotine and nAChRs have played important roles in the development of modern neuroscience. One of the rewards associated with writing this review was reading papers written by scientific giants such as Claude Bernard, John Langley, C.C. Chang, Michael Raftery, and Jean-Pierre Changeux and learning that discoveries made between 25 and over 100 years ago are still directly relevant to research being done today.

2 Receptive Substance, the Beginnings of a Field of Study

Virtually every ongoing study of nicotine recognizes that the actions of nicotine arise as a consequence of binding to and either activating or inhibiting (desensitization or channel block) the protein complex normally activated by acetylcholine (ACh). These receptors are also activated/inhibited by nicotine; hence the name nicotinic cholinergic receptors. The notion that nicotine interacts with a specific receptor dates back to a 1905 paper that is arguably the most famous nAChR paper ever published (Langley 1905). In this early paper, Langley reported that nicotine produces short-term stimulation followed by long-term blockade of both intact and denervated skeletal (striated) muscle and rightly concluded that nicotine interacts with a "receptive substance" expressed on or in skeletal muscles. He noted that the receptive substance is found at a site very near a "synaptic substance." Langley's receptive substance theory evolved into receptor theory, which is one of the basic tenets of modern biology. It should be noted that Langley advanced the receptive substance hypothesis 16 years before Otto Loewi (1921) demonstrated that the decrease in heart rate that follows electrical stimulation of the vagus is produced by release of a chemical from the vagus nerve (vagusstoff), 21 years before Loewi and Navratil (1926) demonstrated that vagusstoff is ACh, approximately 30 years before ACh was identified as the neurotransmitter at all sympathetic ganglia (Kibjakow 1933) and at the neuromuscular junction (Dale et al. 1936), and nearly 50 years before it was determined that ACh is the neurotransmitter at parasympathetic ganglia (Perry and Talesnik 1953).

Langley's receptive substance paper described studies done at the neuromuscular junction. In earlier studies he had demonstrated that nicotine affected the ganglia of

both the sympathetic and parasympathetic branches of the autonomic nervous system (Langley 1890; Langley and Dickinson 1889, 1890a), and that nicotine elicits short-term stimulation followed by longer-term blockade (paralysis) when applied to the autonomic ganglia (Langley 1901). It is also clear that Langley recognized that nicotine stimulates the central nervous system. For example, in his study that compared the actions of pituri and nicotine (Langley and Dickinson 1890b) Langley reported that "nicotine first stimulates and then paralyzes the central nervous system, and that it has in general a similar effect upon peripheral ganglia." Thus, by 1905, Langley had identified the three major sites of nicotine's actions and had postulated that all of nicotine's actions occur subsequent to interaction between nicotine and a receptive substance.

3 Pharmacological Approaches Identify Receptor Subtypes

Evolutionary biologists have argued for many years that selection pressures have favored the development of plants that produce poisons, such as nicotine, because these poisons decrease the likelihood that insects or animals will eat the plant. The recent finding that insects will eat tobacco (*Nicotiana attenuata*) genetically modified not to produce nicotine, certainly supports this popular assumption (Steppuhn et al. 2004). It is absolutely the case that if it were not for chemicals (drugs) that might be described as gifts from Mother Nature, the identification and characterization of the nAChRs would have been incredibly slow. We owe the initial discovery of most of these poisons to unknown ancient people (early pharmacologists) who learned to use poisons derived from plants and animals to "capture" food or to alter the inner being. It is scientifically correct to use the term "pioneering" in describing Claude Bernard's (1856) studies with curare and John Daly's (Badio and Daly 1994) more recent studies with epibatidine; however these eminent scientists did not discover the drugs that they used in their work. Unfortunately, the identities of the individuals who discovered these very important tools cannot be designated by: (Genius et al. 4002 BC).

3.1 Curare and Structure–Activity Analyses of Quaternary Ammonium Derivatives

Claude Bernard's early discovery (1856) that curare, the South American arrow poison (woorari), blocked muscle contraction elicited by stimulation of motor neurons, but not that elicited by direct stimulation of the muscle, provided the first demonstration that drugs could be used to study, what we now know are, nAChR-regulated functions. Bernard's early experiment served as the model for Langley's demonstration that the actions of nicotine on skeletal muscle could be blocked by pretreatment with curare (Langley 1880, 1907) and mimicked by pituri, the active

component of leaves from *Duboisia hopwood* that are chewed by Australian aborigines (Langley and Dickinson 1890b). These early studies established the concept of nicotinic agonists and antagonists and demonstrated that chemical structures might influence activity. Marshall (1913) established that the positively charged quaternary nitrogen found in most naturally-occurring nicotinic agents is vital for activity in his studies with tetraethyl ammonium (TEA). Nearly 40 years after Marshall's seminal findings, what might be viewed as follow-up structure–activity analyses of quaternary ammonium compounds resulted in the development of the bis-quaternary ammonium compounds $[(CH_3)_3N^+\text{-}(CH_2)_n - N^+(CH_3)_3]$ (Barlow and Ing 1948; Paton and Zaimis 1949). These first-ever structure–activity studies with nicotinic antagonists established that nAChR subtypes exist with the demonstration that ganglionic blockade is maximal when $n = 6$ (hexamethonium) and skeletal muscle blockade is maximal when $n = 10$ (decamethonium). Even today, the terms "C6" and "C10" are used to designate the ganglionic- and muscle-type nAChR subtypes.

3.2 α-Bungarotoxin (α-Bgt) and the Path to Identification of Neuronal Receptors

The need to develop better methods for treating snake bite, which had risen to epidemic proportions in Taiwan in the mid-twentieth century, led to the discovery that the "alpha" toxin derived from venom of the Taiwanese banded krait, *Bungarus multicinctus*, is a potent and irreversible inhibitor of electrical stimulation of the neuromuscular junction (Chang and Lee 1963). Subsequent studies showed that the toxin blocked carbamylcholine-induced depolarization of the electric organ of *Electrophorus electricus*; that these effects were blocked by pretreatment with *d*-tubocurarine, that the toxin blocked the binding of [^{3}H]-decamethonium to a protein extracted from the electric organ (Changeux et al. 1970); and that it had comparable effects at the neuromuscular junction (Miledi and Potter 1971). These results, coupled with the finding that α-Bgt did not block transmission in autonomic ganglia (reviewed in Schmidt 1988), added to the evolving data set that distinguished muscle-type receptors from ganglionic nAChRs.

α-Bgt provided an enormously powerful high-affinity tool that allowed purification and characterization of electroplaque (Heidmann and Changeux 1978) and skeletal muscle (Fambrough 1979) receptors. Purified receptors from these two sources were used to determine (among many things) that: (i) the nAChR is a pentameric assembly of four different subunits ($\alpha 1_2 \beta 1 \gamma \delta$ [neonatal] or $\alpha 1_2 \beta \varepsilon \delta$ [adult]); (ii) the α subunit contains a pair of disulfide-bonded cysteines that are separated by 13 amino acids (the Cys loop) in addition to a pair of vicinal cysteines that play a vital role in agonist binding; and (iii) each subunit has an N-terminal extracellular domain, four transmembrane domains, and two cyoplasmic loops between the first and second transmembrane domains (TM1–TM2), and a larger loop between TM3 and TM4 (reviewed in Karlin 2002). Purification of the four subunit proteins from *Torpedo californica* also allowed Raftery et al. (1980) to sequence the first 18 amino

acids of each of the four polypeptide chains obtained from *Torpedo* electric organ. Oligonucleotide probes based on the amino acid sequences were used to clone and then sequence the α1, β1 γ and δ subunit genes from the electric organs and skeletal muscle (Numa 1983). The peripheral-type nAChR gene sequences were then used to generate oligonucleotide probes that were used to clone and sequence the α2–α6 and β2–β4 subunits from rat brain (reviewed in Patrick et al. 1989; Heinemann et al. 1991) and α2–α4 and β2 from chick brain (Ballivet et al. 1988). Oligonucleotide probes generated from the amino acid sequence derived from an α-Bgt-binding protein isolated from chick brain (Conti-Tronconi et al. 1985) were used to clone the α7 and α8 cDNAs from chick brain (Schoepfer et al. 1990), α7 (Seguela et al. 1993) from rat brain, and both α9 (Elgoyhen et al. 1994) and α10 (Elgoyhen et al. 2001) from rat cochlear hair cells. Thus, α-Bgt played vital roles in identifying, cloning, and sequencing all of the known nAChR subunit genes.

4 Identification of α7* nAChRs

The α7-containing nAChRs were discovered earlier than the other neuronal nAChRs and are of enormous interest because of their unique properties (e.g., high permeability to Ca^{++}) and sites of expression.

4.1 [^{125}I]-α-*Bgt Binding*

Binding sites for [^{125}I]-α-Bgt had been described in autonomic ganglia (Patrick and Stallcup 1977) and in both mouse (Marks and Collins 1982) and rat (Clarke et al. 1985) brain, well before the nAChR subunit genes had been cloned and sequenced. The first report that [^{3}H]-nicotine binds with high affinity to rat brain also included the demonstration that α-Bgt did not block [^{3}H]-nicotine binding (Romano and Goldstein 1980). This, coupled with the findings that the regional distributions of [^{125}I]-α-Bgt and [^{3}H]-nicotine binding differ considerably in both mouse (Marks and Collins 1982) and rat (Clarke et al. 1985) brain, led to the conclusion that rodent brain expresses more than one nAChR subtype. Moreover, early pharmacological studies of [^{3}H]-nicotine binding (Romano and Goldstein 1980; Marks and Collins 1982) suggested that the brain nAChR(s) that bind nicotine with high affinity differ from the nAChRs found in autonomic ganglia, thereby raising the number of suspected nAChR subtypes to three.

4.2 α7 *mRNA Expression Patterns, in Situ Hybridization*

Early investigations of virtually all neuronal nAChR subunit genes, included in situ hybridization studies that determined mRNA expression patterns in rat [see, for

examples: α2 (Wada et al. 1988); α3 (Goldman et al. 1986); α4 (Boulter et al. 1986); α7 (Seguela et al. 1993); β2 (Deneris et al. 1988); β3 (Deneris et al. 1989)], chicken (Ballivet et al. 1988), and mouse (Marks et al. 1992) brain. Some of the mRNAs are expressed in both species in only very few brain regions (α2, β4), others (α3, α5) are readily identified in a significant number of brain regions, and others (α4, α7, β2) are expressed in many brain regions. The α6 (LeNovere et al. 1996; Azam et al. 2002) and β3 (Deneris et al. 1989; LeNovere et al. 1996; Azam et al. 2002) subunit mRNAs are expressed in very high concentrations in dopaminergic pathways as well as in visual pathways. Only a few brain regions (e.g., medial habenula and interpedunclear nucleus) seem to express virtually all the subunit mRNAs. These analyses suggest that some nAChR subtypes, particularly those that include α4, α7 and β2 subunits, might be broadly expressed in the brain, whereas others (e.g., α2β4) do not exist in appreciable numbers, if at all, in rodent brain.

The autoradiographic analyses of [^{125}I]α-Bgt binding done by Clarke et al. (1985) provided a very clear anatomical picture of brain regions that express the [^{125}I]-α-Bgt binding sites. These binding data were compared with the in situ hybridization patterns of the subunit mRNAs in virtually all "subunit discovery" papers. These comparisons are confounded if protein products are expressed in nerve terminals, given that mRNA is expressed principally in cell bodies. Nonetheless, Chen and Patrick (1997) were correct when they concluded that the α-Bgt-binding nAChRs are made up of α7 subunits when they noted that regional expression patterns for α7 mRNA and [^{125}I]-α-Bgt binding are very similar in rat brain. This result, coupled with the finding that both chick (Couturier et al. 1990) and rat (Seguela et al. 1993) α7 cDNA injected into *Xenopus* oocytes produced functional, homomeric receptors that were blocked by α-Bgt, led to the conclusion that the α-Bgt-binding nAChR is made up solely of α7 subunits. The assertion that α7 subunits are absolutely required to form the α-Bgt-binding receptor is proved by the finding that [^{125}I]-α-Bgt binding is absent in brain from α7 null mutant (gene knockout) mice (Orr-Urtreger et al. 1997). This conclusion is supported by findings that polyclonal antibodies directed against the α7 subunit detect only α7 subunits in rat brain (Chen and Patrick 1997) and affinity purification of PC12 cell-derived nAChRs yields only one type of subunit, although two-dimensional electrophoreses uncovered seven 35 kDa spots that differed in charge (pI value), which might reflect differences in posttranslational processing (Drisdel and Green 2000). The effects of these posttranslational modifications, if any, on receptor properties have not been determined.

4.3 Heteromeric α7 Receptors*

The first paper that described the cloning and sequencing of the α7 nAChR subunit gene from chicken brain identified two cDNA clones encoding two α-bungarotoxin–binding proteins, designated αBgtBP1 and αBgtBP2 (Schoepfer et al. 1990). Consistent with sequencing data, subunit-specific antibodies precipitated two receptor subtypes: approximately 85% included only αBgtBP1, the

remaining 15% contained both αBgtBP1 and αBgtBP2. Keyser et al. (1993) subsequently demonstrated that αBgtBP1 is the α7-encoded protein and αBgtBp2 the α8-encoded protein. Thus, approximately 15% of total α7* nAChRs formed in chick brain are α7α8 heteromers with unknown stoichiometry. When expressed in *Xenopus* oocytes, nAChRs that include the α8 subunit have lower affinity for α-Bgt (faster dissociation) and higher affinity for most agonists, including ACh and nicotine, than do homomeric α7 receptors (Anand et al. 1993).

Two groups (Yu and Role 1998a, b; Sudweeks and Yakel 2000) have speculated that heteromeric α7* nAChRs might exist, based on the findings that receptor function measured using native tissues differs dramatically from homomeric α7 receptors expressed in *Xenopus* oocytes. Yu and Role (1998a,b) suggested that chick autonomic ganglia may express α7α5 heteromeric nAChRs because the functional properties of chick ganglionic receptors that they measured differed from the functional properties reported for α7 homomeric receptors expressed in *Xenopus* oocytes and because these differences were lost following treatment with α5 antisense oligounucleotides. Given that immunoprecipitation techniques using subunit-specific antibodies do not detect α7α5 heteteromers (Pugh et al. 1995; Cuevas and Berg 1998), it does not seem likely that α7α5 receptors exist, at least in large numbers. It is likely (see Sect. 4.2) that heteromeric nAChRs made up of α7–1 and α7–2 alternative transcripts explain the data that prompted Yu and Role (1988a ,b) to suggest that α7α5 nAChRs might be formed in autonomic ganglia.

Yakel and colleagues (Sudweeks and Yakel 2000; Khiroug et al. 2002) also used functional data as the basis for their speculation that rat brain might produce α7β2* nAChRs. This argument was based on the findings that most rat brain regions that express the mRNA for α7 also express β2 mRNA (Sudweeks and Yakel 2000) and because α7 and β2 subunits coassemble to form receptors in oocytes with functional properties that resemble those of the α7* nAChRs expressed in hippocampal neurons (Khiroug et al. 2002). However, β2 gene deletion does not alter mouse brain [^{125}I]-α-Bgt binding (Zoli et al. 1998; Whiteaker et al. 2000) or a component of [^{3}H]-epibatidine binding that requires the α7 subunit (Marks et al. 2006). Further, no studies that have used antibodies directed against the β2 subunit have shown that α7 is precipitated along with the β2 subunit. Thus, the bulk of published data does not support the suggestion that heteromeric receptors made up of α7 and one or more of the other known α or β subunits are actually produced in autonomic ganglia or brain of mammals.

4.4 Alternative Transcripts and α7 Receptors*

The literature rarely, if ever, includes discussions that suggest that native heteromeric α7-type receptors might exist, even though early attempts at purification using α-Bgt (e.g., Conti-Tronconi et al. 1985) detected four α-Bgt-binding proteins with molecular weights ranging between 48,000 and 72,000. Reluctance to pursue the notion of heteromeric α7-type receptors may reflect the fact that several studies have presented compelling evidence that supported the conclusion that all α7-type

receptors are made up of α7 subunits only (Chen and Patrick 1997; Drisdel and Green 2000). We are persuaded that different types of α7* receptors might exist, at least in mouse brain, based on the results of a series of studies that have evaluated the effects of chronic nicotine or chronic glucocorticoid treatment on brain [^{125}I]-α-Bgt binding. We have reproducibly found that regulation of α7* receptor expression varies dramatically across brain regions following chronic nicotine treatment (see Marks et al. 1983, Pauly et al. 1991 for examples). Nicotine-induced increases (up-regulation) in mouse brain [^{125}I]-α-Bgt binding are dose-dependent, occur at higher doses than are required to produce an increase in [^{3}H]-nicotine binding, and vary dramatically across brain regions, with the hippocampus showing unique sensitivity. Similarly, mouse brain regions vary dramatically (hippocampus is the most sensitive) in chronic corticosterone-induced decreases in [^{125}I]-α-Bgt binding (Pauly et al. 1990a; Pauly and Collins 1993) and adrenalectomy-induced increases in α-Bgt binding (Pauly et al. 1990b). A convenient, but totally untested, explanation for these findings is that not all brain regions express identical α7* nAChRs.

Alternative transcripts provide one potential explanation for the apparent heterogeneity in regulation of α7 expression across brain regions. Severance et al. (2004) have recently shown that alternative transcripts for α7 (designated α7–1 and α7–2) are expressed in rat autonomic ganglia and brain, and that receptors formed from these alternative transcripts have functional properties that resemble those seen with α7α8 heteromeric receptors. The α7–2 isoform includes an 87 base-pair cassette that is inserted in the exon that codes for the N-terminus of the α7–1 isoform (the "standard" isoform). When expressed in *Xenopus* oocytes, the α7–2 isoform produced receptors that desensitize slowly and exhibit a readily-reversible α-Bgt blockade. These properties closely resemble the properties of α7α8 nAChRs expressed in oocytes (Anand et al. 1993) and chick ganglionic α7* receptors (Yu and Role 1998a). The protein products for both α7–1 and α7–2 are expressed in all the brain regions that express α7 mRNA. Thus, mammalian brain may produce, using alternative transcripts, α7* receptors that serve the same purpose as α7α8 nAChRs do in chick.

Mouse brain also expresses at least two alternative transcripts for α7 (Saragoza et al. 2003). The nontraditional mouse α7 transcript, like α7–2 from rat, produces changes in the N-terminal domain. In this case, if produced, the variant protein would have a single amino acid substitution in the N-terminal domain. However, the unique transcript also contains an extra exon that arises from alternative splicing of intron 9. The protein product resulting from this alternatively processed RNA is truncated shortly after the third transmembrane domain. The alternatively spliced protein product acts as a dominant negative (i.e., inhibitor) of the α7 function when expressed along with the standard α7 in GH4C1 cells. Though highly speculative, it may be that heterogeneity across mouse brain regions in expression of receptors that include proteins derived from alternative transcripts, might explain why chronic drug-induced changes in [^{125}I]-α-Bgt binding differ so dramatically across mouse brain regions.

4.5 Sites of Expression and Function of α7 Receptors*

[^{125}I]-α-Bgt binding is found in many regions of both rat (Clarke et al. 1985) and mouse (Pauly et al. 1989) brain. Binding is particularly high in the hippocampus, where it is found on what are likely to be GABAergic interneurons in the stratum oriens and stratum radiatum, and on pyramidal neurons. Many of the α7* receptors are expressed somatodendritically on some, but not all, GABAergic interneurons (see, for examples Alkondon et al. 1999; Frazier et al. 1998; Zhang and Berg 2007). α7* nAChRs are also expressed on dendrites and cell bodies of some dopaminergic neurons in the ventral tegmental area (Wu et al. 2004). Functional and immunocytochemical data indicate that α7* nAChRs are expressed on the terminals of some, but not all, neurons that use glutamate as a neurotransmitter in hippocampus and VTA (Gray et al. 1996; Mansvelder and McGehee 2000; Fabian-Fine et al. 2001; Jones and Wonnacott 2004). These findings have sparked research that is geared towards understanding the role of α7* nAChRs in modulating learning and memory and addiction processes, and in the development of drugs that might be used to treat pathologies that are due to altered function of pathways that express these receptors.

5 Heteromeric Receptors Containing α4 and β2 Subunits: α4β2*

The earliest studies that attempted to determine whether α4 subunits formed functional receptors in *Xenopus* oocytes established that function was obtained only if the oocytes were also injected with either β2 or β4 cDNA, thereby establishing the concept of heteromeric neuronal nAChRs (Deneris et al. 1988, Connolly et al. 1992). The α4β2* nAChR has been studied extensively because: (i) it seems to be the most widely expressed nAChR subtype; (ii) it was considered, until recently, to be the highest affinity nAChR; and (iii) the number and function of these receptors are altered by chronic nicotine treatment.

5.1 Ligand Binding, In Situ Hybridization, and High Affinity α4β2 Receptors*

Early comparisons of α4 and β2 mRNA expression patterns and [^{3}H]-nicotine binding (Boulter et al. 1986; Deneris et al. 1988; Marks et al. 1992) suggested that nAChRs including these two subunits make up what was termed “high affinity” nicotine binding sites. This conclusion was accepted with suspicion however, because several brain regions have mismatches between binding and mRNA expression. Eventually much of this mismatch could be attributed to the fact that α4 and β2 mRNA are expressed in cell bodies, whereas many α4β2* nAChRs are expressed on nerve terminals where they modulate neurotransmitter (GABA and dopamine)

release. Other inexplicable mismatches between binding and mRNA expression still abound. The most notable of these exceptions is cerebellum, where massive levels of β2 mRNA are found with little or no detectable binding in mouse brain. This expression is not an artifact given that no cerebellar signal is detected in β2 null mutants (Picciotto et al. 1995). To this day, no one has provided a reasonable explanation for the massive expression of β2 mRNA in cerebellum, nor has a suitable α subunit that might be coexpressed with the β2 subunit been identified. As a result, the notion that α4 and β2 subunits make up the high affinity nicotine binding site was not generally accepted until it was shown that null mutation of both the α4 (Marubio et al. 1999) and β2 (Picciotto et al. 1995) subunit genes resulted in elimination of the [^{3}H]-nicotine binding site.

The [^{3}H]-nicotine binding sites, and α4β2* nAChRs, have been referred to for nearly 30 years as the high affinity nicotine receptor, a term used by Romano and Goldstein (1980) because the dissociation constant (K_d) for nicotine binding to rat brain membranes is in the low nanomolar range. Similar results are obtained in mouse brain; e.g., we consistently calculate K_d values of 2–5 nM for nicotine to mouse brain membranes (Bhat et al. 1994; Marks et al. 1986, 1991, 1992). The high affinity nicotine binding site is also referred to as the high affinity agonist binding site because other nicotinic agonists such as [^{3}H]-cytisine (Pabreza et al. 1991) and [^{3}H]-ACh (Schwartz et al. 1982) bind to the same sites as does nicotine in mouse (Marks et al. 1986) and rat (Pabreza et al. 1991) brain. Romano and Goldstein (1980) argued that nicotine binds to the high affinity, desensitized form of the receptor because the K_d values obtained in their studies were ten- to 100-fold less than EC_{50} values for receptor activation. Kinetic analyses of [^{3}H]-nicotine binding demonstrated that association rates are biphasic, which provided support for this postulate (Lippiello et al. 1987; Bhat et al. 1994). However, the best support for this postulate comes from the finding that the EC_{50} values for nicotine stimulation of current flow by α4β2 nAChRs expressed in *Xenopus* oocytes are approximately 100-fold higher than the K_d values determined for binding (Leutje and Patrick 1991; Connolly et al. 1992; Sabey et al. 1999; Rush et al. 2002) and the finding that subactivating concentrations of nicotine can fully desensitize α4β2 nAChRs with an EC_{50} value that is very similar to the K_d values reported for binding (Fenster et al. 1997). However, recent studies have shown that receptors that include only the α4 and β2 subunits are not the highest affinity nAChRs when receptor activation is measured. nAChRs that include α6 and β3 subunits, along with α4 and β2, have the lowest EC_{50} values of any native nAChRs that have been measured to date (Salminen et al. 2007).

5.2 [^{3}H]-Epibatidine Identifies Low Affinity α4β2 Receptors*

A little over 10 years ago radiolabeled epibatidine (Houghtling et al. 1995) was introduced as a new ligand with extraordinarily high affinity for α4β2* nAChRs. Early reports suggested that [^{3}H]-epibatidine binds to α4β2-type receptors only, but this assertion was quickly questioned when it was noted that epibatidine binding exceeds

that of [^{3}H]-agonist binding in several brain regions, and is present in some brain regions (optic nerve, optic chiasm, optic tract) that have no detectable [^{3}H]-agonist binding sites (Perry and Kellar 1995). This concern was enhanced by the observation that β2 gene deletion does not eliminate [^{3}H]-epibatidine binding in several brain regions that do not bind nicotine with high affinity (Whiteaker et al. 2000). As shown in Fig. 1a, saturation studies that use a broader range of ligand concentrations than were used in early [^{3}H]-epibatidine binding studies yield data indicating that more than one binding site exists. The biphasic nature of [^{3}H]-epibatidine binding can be readily seen when binding data are plotted using the Scatchard transformation (insert to Fig. 1a); the data yield the "hockey stick" shape that is characteristic of two sites that differ in affinity for the ligand. Epibatidine binding can be separated into higher (K_d = 10–20 pM) and lower (K_d = 10 nM) affinity classes; the ratio of these two major classes varies dramatically across brain regions (Marks et al. 2000).

Figure 1 also shows that the high and low affinity [^{3}H]-epibatidine binding sites can be further subdivided on the basis of sensitivity to inhibition by other nicotinic compounds. For example, Fig. 1b shows that cytisine is a potent inhibitor of [^{3}H]-epibatidine binding (results using 0.3 and 10 nM are shown). Note that more than 4 log units of cytisine concentration is required to attain total inhibition, a result that predicts that more than one binding site is being measured in these assays. Indeed, a two-site model provides the best fit to the inhibition data. This prompted us to use the terms cytisine-sensitive and cytisine-resistant to describe these two components of higher affinity [^{3}H]-epibatidine binding. Null mutation of both the α4 and β2 genes results in near-total elimination of the cytisine-sensitive component of higher affinity epibatidine binding, thereby demonstrating that these binding sites measure α4β2* nAChRs (Marks et al. 2006, 2007). Deletion of the α7, β4 (Marks et al. 2006), and α5 (Brown et al. 2007) subunits does not produce a detectable change in cytisine-sensitive higher affinity [^{3}H]-epibatidine binding. In contrast, a substantial fraction of the cytisine-resistant component is eliminated by β4 gene deletion (i.e., cytisine-resistant higher affinity [^{3}H]-epibatidine binding can be used to measure β4-containing nAChRs).

Figure 1c shows that the lower affinity site can be separated into components that are more or less sensitive to inhibition by *d*-tubocurarine (Marks et al. 1998; Whiteaker et al. 2000). Studies that evaluated the effects of gene deletion (i.e., null mutant analyses) on lower affinity binding yielded some results that were fully expected. For example, approximately 30% of the lower affinity binding sites are eliminated by α7 gene deletion and are blocked by α-Bgt (Marks et al. 2007). To our surprise, most (approximately 75%) of the remaining lower affinity [^{3}H]-epibatidine binding sites are eliminated throughout the brain by β2 (Marks et al. 2006) and α4 (Marks et al. 2007) gene deletion, indicating that these are α4β2* nAChRs that have low affinity for agonists. These low affinity [^{3}H]-epibatidine binding sites are found throughout the brain in numbers that are nearly equal to the high affinity [^{3}H]-nicotine binding sites that were first identified in the early 1980s (Romano and Goldstein 1980, Marks and Collins 1982; Clarke et al. 1985). The reports that α4 (Marubio et al. 1999) and β2 (Picciotto et al. 1995) eliminated the high affinity

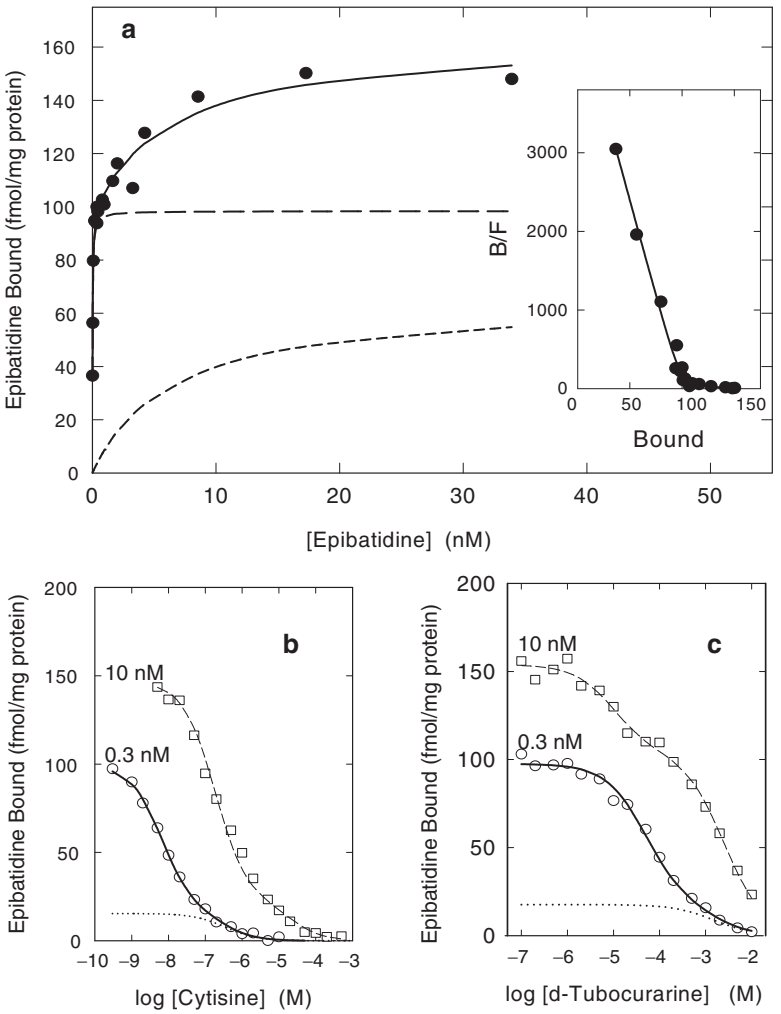

Fig. 1 Binding of [^{3}H]-epibatidine to membranes prepared from mouse brain. **a** depicts results of an experiment where varying concentrations of [^{3}H]-epibatidine were incubated with membranes prepared from whole mouse brain under equilibrium binding conditions (see Marks et al. 2006, 2007 for specifics of the assay). As concentration increased, saturation was achieved, but as is most readily seen by Scatchard analysis (*inset*); the data were best fit by a two-site model. **b** (higher affinity) and **c** (lower affinity) provide the results of competition binding experiments. The data presented in **b** show that the addition of varying concentrations of unlabeled cytisine to incubations that contained either 0.3 nM [^{3}H]-epibatidine (a concentration that fully saturates the high affinity epibatidine binding site) or 10 nM [^{3}H]-epibatidine (saturates the low affinity site) results in total inhibition of binding. However, more than 4 log units of cytisine were required to completely inhibit binding, leading to the conclusion that [^{3}H]-epibatidine binds to at least two nAChR subtypes that differ in affinity for cytisine (i.e., cytisine-sensitive and cytisine-resistant). **c** depicts the results of similar experiments that used *d*-tubocurarine to inhibit [^{3}H]-epibatidine binding

nicotine binding site had led to the apparently erroneous conclusion that all α4β2* nAChRs are always high affinity nAChRs. This clearly is not the case. At this point, all that is unequivocally known about these low affinity sites is they require both α4 and β2 and are found throughout the brain.

It is also the case that the function of these binding sites is unknown, although it may be that low affinity epibatidine binding is measuring low affinity receptors that have been detected in mouse brain synaptosomal preparations using ion ($^{86}Rb^{+}$) flux assays (Marks et al. 1999). Figure 2 shows that agonist-induced $^{86}Rb^{+}$ flux can be separated into high and low affinity components (4 log concentrations are required to elicit maximal ion flux and the data are best fit by a two-site model) (Marks et al. 1999, 2000, 2007). It is clear (Fig. 2) that both components are modulated by α4β2* nAChRs given that both α4 and β2 gene deletion eliminate both the high and low affinity components (Marks et al. 1999, 2007). An analysis of both components of binding and ion flux that included 12 brain regions found a significant correlation between high affinity binding and high affinity agonist-induced ion flux and a

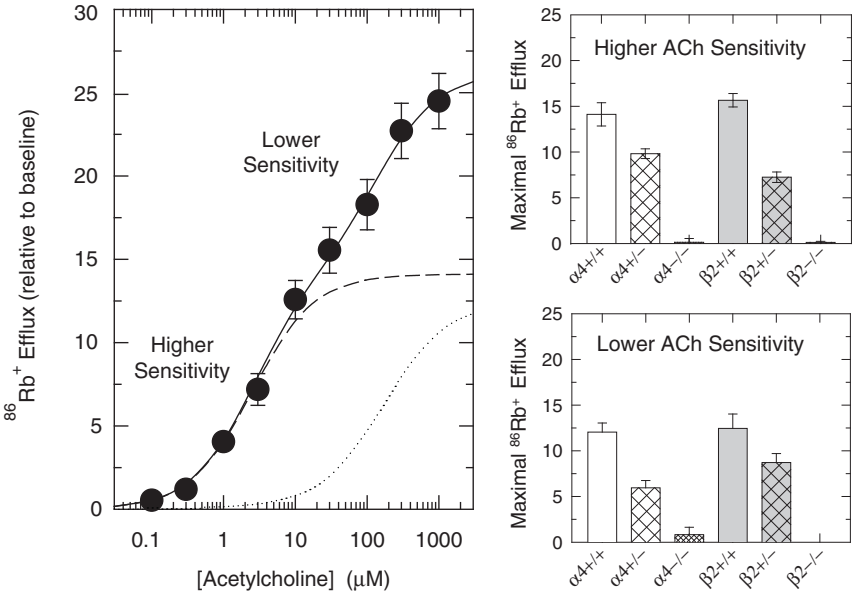

Fig. 2 Agonist-stimulated $^{86}Rb^{+}$ from mouse brain synaptosomes. Acetylcholine (ACh)-stimulated ion ($^{86}Rb^{+}$) efflux from synaptosomes prepared from mouse brain was done as described in Marks et al. (2007). The *left panel* of this figure demonstrates that 4 log units of agonist (ACh) were required to elicit maximal ion flux. These data are fit best by a two-site model indicating that higher and lower sensitivity components of the ion flux response exist. The *right hand panels* of the figure illustrate the effects of α4 and β gene deletion on the ion flux responses. Both α4 and β2 gene deletion resulted in total elimination of both the higher and lower sensitivity components of the ion flux response to ACh. ACh-stimulated release from synaptosomes prepared from mice that were heterozygous for the null mutations ($\alpha4^{+/-}$ and $\beta2^{+/-}$) showed intermediate levels of ion flux. These results demonstrate that α4β2* nAChRs are responsible for both components of the ion flux response

similar significant correlation between low affinity binding and low affinity ion flux (Marks et al. 2007). This finding suggests that the low affinity binding site may be measuring the same nAChR subtype(s) that modulates the low affinity component of ion flux.

Biphasic dose–response curves for agonist-induced increases in current flow and epibatidine binding have also been described for α4β2 nAChRs expressed in cell lines and *Xenopus* oocytes (Zwart and Vijverberg 1998; Buisson and Bertrand 2001; Nelson et al. 1992; Zhou et al. 2003). The expression system studies attempted to manipulate α4 and β2 subunit levels by altering mRNA ratios and by using receptors with α4 and β2 subunits linked together (concatamers). The results indicate that the high affinity components of binding and flux may be measuring nAChRs with two copies of α4 and three of β2 ($\alpha4_2\beta2_3$), and that lower affinity binding and flux may be measuring receptors made up of three α4 and two β2 subunit ($\alpha4_3\beta2_2$). Recently, we (Gotti et al. 2008) have reported results of experiments done with heterozygous α4 and β2 null mutant mice (i.e., $\alpha4^{+/-}$ and $\beta2^{+/-}$) that support the suggestion that $\alpha4_2\beta2_3$ and $\alpha4_3\beta2_2$ nAChRs are both found in mouse brain. Specifically, the ratios of the high and low affinity components of ACh-stimulated $^{86}Rb^{+}$ efflux as well as α4 and β2 protein expression are affected by the altered ratios of both α4 and β2 mRNAs that are seen in $\alpha4^{+/-}$ and $\beta2^{+/-}$ mice.

5.3 Heteromeric α4β2 Receptors that Include Other nAChR Subunits*

At least two heteromeric α4β2* nAChRs that include additional nAChR subunits have been identified to date. It is readily apparent from in situ hybridization studies that not all α4β2 nAChRs could possibly include the α5 subunit because α5 mRNA expression is limited when compared with α4 and β2 mRNA expression. However, RT-PCR studies have detected coexpression of the α4, α5, and β2 mRNAs in GABAergic neurons from rat cortex (Porter et al. 1999) and striatum (Klink et al. 2001). Recently, we (Brown et al. 2007) reported that subunit-specific antibodies will precipitate α4α5β2 nAChRs in approximately half of the 12 mouse brain regions that we studied. Ligand binding, antibody, and functional data have demonstrated that α4α5β2 nAChRs are expressed in dopamine neurons (see Sect. 6) and other (unpublished) work from our laboratory indicates that GABA release is modulated by α4α5β2 nAChRs in some, but not all, mouse brain regions. In addition, it has been established that some α4β2* nAChRs in dopaminergic neurons contain α6 and β3 subunits (see Sect. 6).

5.4 Alternative α4 Transcripts

One of the first studies that described the functional properties of α4β2 nAChRs indicated that rat brain forms two α4 transcripts, designated α4–1 and α4–2 (Connolly

et al. 1992). The C-terminal end of the α4 gene differs in these two alternate splice variants. The only report that describes the potential importance of these two transcripts suggests that receptors that include the C-terminal sequence coded for by the α4–2 transcript (same C-terminal as the human transcript) have increased sensitivity to the allosteric actions of some steroids (Paradiso et al. 2001).

5.5 Polymorphisms in the α4 and β2 Genes

Naturally occurring single nucleotide polymorphisms have been identified for virtually all of the human neuronal nAChR subunits, but most of these have not been studied in detail. Notable exceptions are the α4 and β2 polymorphisms that are linked with a rare form of epilepsy, autosomal dominant nocturnal frontal lobe epilepsy, ADNFLE (Steinlein et al. 1995; Phillips et al. 2001; De Fusco et al. 2000; Combi et al. 2004; Hogg and Bertrand 2004). Several linkage analyses have detected significant genetic associations between ADNFLE and α4 or β2 polymorphisms (Weiland et al. 2000; Steinlein 2007). These associations are provocative, especially since the mutant α4 or β2 genes have altered receptor function when expressed in vitro along with native β2 or α4 genes, respectively (Kuryatov et al. 1997; De Fusco et al. 2000; Rodriguez-Pinguet et al. 2005; Bertrand et al. 2005). More recently, mice with some of these polymorphisms have been generated and the results indicate that such mutations may be sufficient to cause phenotypic changes similar to ADNFLE (Klassen et al. 2006; Xu et al. 2006; Teper et al. 2007).

6 Receptor Subtypes Expressed in Dopamine Neurons

Identifying and characterizing the nAChR subtypes expressed in dopaminergic neurons has been of primary interest, principally because dopaminergic systems presumably play a vital role in modulating the reinforcing effects of nicotine. Rapid progress has been made in this area in recent years and a very complex story has emerged: a minimum of five different nAChR subtypes are expressed in dopamine neurons.

6.1 [^{3}H]-Epibatidine Binding and mRNA Expression

Techniques that measure mRNA expression and ligand binding assays that measure receptor expression have been used extensively to identify those nAChR subtypes that are expressed in dopamine neurons. In situ hybridization studies using mouse (Marks et al. 1992; Grady et al. 1997) and rat (Le Novere et al. 1996) brain have detected the mRNAs for all of the known nAChR subunits, except α2 and β4, in

brain regions that are rich in dopamine cell bodies, such as the substantia nigra and ventral tegmental area. Techniques designed to measure mRNA in specific cell types have been used in other studies to identify those mRNAs that are expressed in dopamine neurons, in part because dopamine-rich brain regions also contain many GABAergic neurons that also express nAChRs. These methods, double-label in situ hybridization (Azam et al. 2002) and single-cell RT-PCR (Klink et al. 2001), have detected α4 and β2 mRNAs in virtually every dopaminergic cell body. A very high fraction (70–80%) also express α5, α6, and β3 mRNAs and approximately half of the dopamine neurons express α3 and α7 mRNAs. These findings suggest that dopamine neurons may express many different nAChR subtypes.

Ligand binding assays done with brain tissue obtained from nAChR subunit null mutant mice have provided critical data that have helped identify the subunit compositions of those nAChRs that are actually expressed in dopamine-rich brain regions and in dopaminergic neurons. Membrane-binding studies done with [^{3}H]-epibatidine as the ligand and brain tissue derived from α4, α5, α7, β2, and β4 null mutant mice have demonstrated that high levels of both high and low affinity α4β2* and intermediate levels of α7* nAChRs are expressed in dopamine-rich regions of mouse brain (Marks et al. 2006; 2007). These assays, while informative, measure all of the receptors that are expressed in these brain regions.

6.2 Binding and Functional Studies Using α-Conotoxin MII

Binding studies with radiolabeled α-conotoxin MII (α-CtxMII) have yielded the most informative results to date. These studies were built on the discovery that α–CtxMII binds with high affinity to, and blocks the activation of, α3β2* nAChRs (Cartier et al. 1996) and α6β2* nAChRs (Kuryatov et al. 2000) expressed in *X. laevis* oocytes. These observations, coupled with the demonstration that α-CtxMII blocks the release of [^{3}H]-dopamine from both rat (Kulak et al. 1997) and mouse (Grady et al. 2002) striatal synaptosomes suggest that α3β2* nAChRs (Cartier et al. 1996) and α6β2* nAChRs might be expressed in dopamine nerve terminals. 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 dopaminergic but not GABAergic markers, it seems highly likely that α-CtxMII-binding nAChRs are expressed almost exclusively in dopaminergic neurons (Quik et al. 2003).

Studies that evaluated the effects of nAChR gene deletion on [^{125}I]-α-CtxMII binding have helped identify the subunit compositions of α–CtxMII-binding receptors that are actually expressed in brain. Autoradiographic analyses showed that null mutation of the α6 (Champtiaux et al. 2002) subunit gene results in total elimination of [^{125}I]-α-CtxMII binding in dopaminergic pathways (Champtiaux et al. 2002), but α3 gene deletion has no effect (Whiteaker et al. 2002). The lack of effect of α3 null mutation is somewhat surprising given that the mRNA for this subunit is expressed in many dopaminergic neurons (Klink et al. 2001; Azam et al. 2002).

The finding that α3 gene deletion eliminates [^{125}I]-α-CtxMII binding in some brain regions (e.g., medial habenula, fasciculus retroflexus) and reduces binding in others (e.g., interpedunclear nucleus) (Whiteaker et al. 2002) demonstrates that [^{125}I]-α-CtxMII binds to native α3-containing nAChRs with high affinity. Thus, it may be that α3-containing nAChRs are not formed in dopaminergic neurons or it may be that α3* nAChRs are normally formed in dopaminergic neurons, but are replaced by α6*-AChRs in α3 null mutant mice.

The effects of α4, α5, α7, β2, β3, and β4 null mutation on [^{125}I]-α-CtxMII binding have been measured using membranes prepared from regionally dissected mouse brain (Salminen et al. 2005). Deletion of α5, α7, and β4 do not alter the binding of [^{125}I]-α-CtxMII to striatal membranes. In contrast, null mutation of β2 causes near-total loss of [^{125}I]-α-CtxMII binding in striatal membranes, indicating that most, if not all, of the nAChRs that bind [^{125}I]-α-CtxMII with high affinity require both the α6 and β2 subunits for their formation. Deletion of the α4 subunit results in a 50–75% decrease in [^{125}I]-α-CtxMII binding from striatal membranes, indicating that some α6β2* nAChRs include the α4 subunit (i.e., α4α6β2*). Deleting the β3 gene also results in a marked (approximately 65%) decrease in [^{125}I]-α-CtxMII binding, indicating that α4α6β2β3, α6β2β3, and α6β2 nAChRs are expressed in dopaminergic neurons in the mouse (see Fig. 3).

6.3 Immunological Approaches

Immunological approaches have been used to verify which subunits combine to form a receptor subtype. Champtiaux et al. (2003) used antibodies directed against rat and human α4–α7 and β2–β4 subunits in immunoprecipitation experiments to identify three heteromeric receptors α4β2*, α4α6β2*, and α6β2* in striatum. Gotti et al. (2005) identified α4α6β2β3, α6β2β3, and α6β2 subtypes in a study that evaluated the effects of β3 null mutation on [^{3}H]-epibatidine binding that was precipitated by these same antibodies. Similar immunological methods have identified all of these α6-containing receptors in striatal tissue obtained from rat (Zoli et al. 2002), squirrel monkeys (Quik et al. 2005), and humans (Gotti et al. 2006). A very recent report that used subunit-specific antibodies and α5 null mutant mice to demonstrate that many α4β2* nAChRs also contain the α5 subunit (Brown et al. 2007) adds to the immunological data to indicate that a minimum of five nAChR subtypes (α4β2, α4α5β2, α4α6β2β3, α6β2β3, and α6β2) are expressed in the striatum (Fig. 3).

6.4 Dopamine Release Assays

It is well established that nicotine, and other nicotinic agonists, will elicit Ca^{++}-dependent release of dopamine from striatal tissue slices (see, for examples

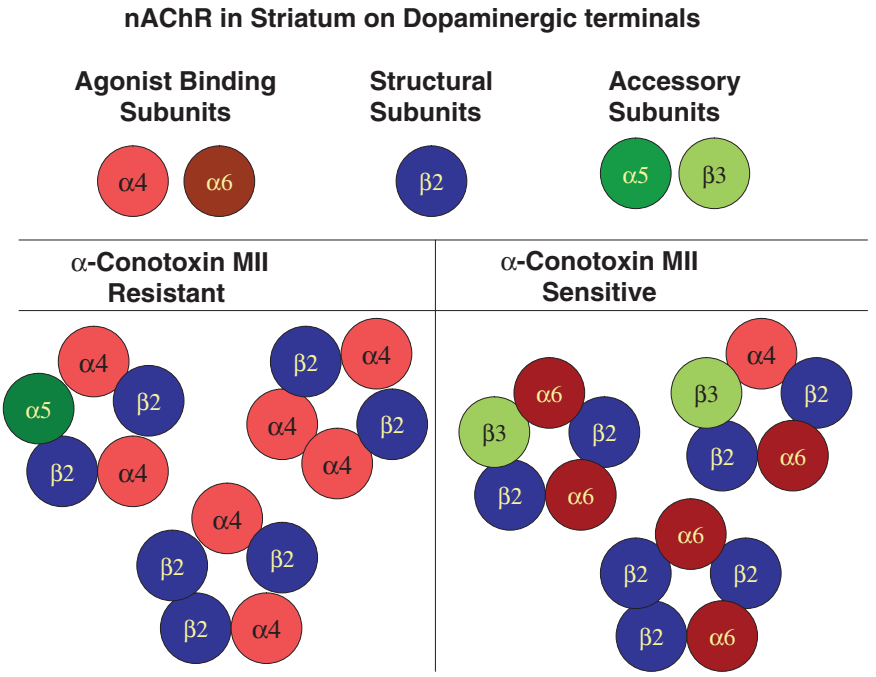

Fig. 3 Potential subunit compositions of nAChRs expressed in dopaminergic nerve terminals. A combination of ligand binding ([^{3}H]-epibatidine and [^{125}I]-α-conotoxin MII), immunoprecipitation, and dopamine release data have led to the conclusion that rodent brain expresses a minimum of five different nAChR subtypes. Three of these (the two forms of α4β2 and α4α5β2) do not bind α-conotoxin MII with high affinity (α-conotoxin MII-resistant). The three α6-containing subtypes bind α-conotoxin MII with high affinity (conotoxin MII-sensitive). In general, the conotoxin-sensitive nAChR subtypes are activated by lower concentrations of agonist than are required to activate the α-conotoxin MII-resistant subtypes (Salminen et al. 2007)

Giorguieff-Chesselet et al. 1979; Dwoskin et al. 1993) and synaptosomes (Rapier et al. 1988). We have used the synaptosomal dopamine release assay in a series of studies that characterized the pharmacological properties of dopamine release from striatum (Cui et al. 2003; Grady et al. 1992, 1994, 1997; Sharples et al. 2000; Whiteaker et al. 2000), and in one study that used the nucleus accumbens, olfactory tubercles, and frontal cortex (Grady et al. 2002). The finding that α-CtxMII is a potent, but partial, inhibitor of nicotinic agonist-stimulated [^{3}H]-dopamine release from mouse (Grady et al. 2002) and rat (Kulak et al. 1997) striatal synaptosomes suggested that more than one nAChR subtype might be expressed on striatal dopaminergic nerve terminals.

Recently, we (Salminen et al. 2004) evaluated the effects of deleting the α2, α4, α5, α7, β2, β3, and β4 genes on both the α-CtxMII-sensitive and resistant components of ACh-stimulated [^{3}H]-dopamine release from striatal synaptosomes. Deletion of the α4 and β2 subunit genes resulted in the total elimination and α5 gene deletion produced a significant decrease in the αCtxMII-resistant

component of ACh-stimulated dopamine release. Deletion of the α2, α7, and β4 did not alter αCtxMII-resistant dopamine release. These results indicate that α4β2 and α4α5β2 nAChRs modulate the αCtxMII-resistant component of dopamine release (Fig. 3).

The α-CtxMII-sensitive component of ACh-stimulated dopamine release is totally absent in striatal synaptosomes obtained from β2 null mutant mice. (Salminen et al. 2004). Identical effects are produced by α6 gene deletion (Champtiaux et al. 2003). Thus, all of the nAChRs that modulate the αCtxMII-sensitive component of dopamine release seem to be α6β2*. Deleting the α4 and β3 genes result in partial reductions in αCtxMII-sensitive release whereas deleting the α2, α7, and β4 genes has no effect (Salminen et al. 2004). These results suggest that dopaminergic nerve terminals express five nAChR subtypes, two that are resistant (α4β2, α4α5β2) and three that are sensitive (α4α6β2β3, α6β2β3, α6β2) to α-CtxMII (Fig. 3). This set of functional subtypes corresponds precisely to those identified with ligand binding and immunoprecipitation (Gotti et al. 2005). Recently, we (Salminen et al. 2007) reported the results of studies that used α4 and β3 null mutant and α4β3 double null mutant mice to evaluate the pharmacological properties of these receptor subtypes. The rank order of EC_{50} values for nicotine-induced dopamine release is: α4α6β2β3 < α6β2β3 ≅ α4(α5)β2 < α6β2.

7 Summary and Conclusions

The discovery that mammalian brain expresses the mRNAs for nine different nAChR subunits (α2–α7, β2–β4) that formed functional receptors when expressed in appropriate combinations in *Xenopus* oocytes suggested that brain tissue might express hundreds of receptor subtypes. This assumes that the brain nAChR(s) are pentameric assemblies that resemble the "peripheral-type" nAChRs that are expressed at the motor endplate or in the electric organs of marine species such as *Torpedo californica* or *Electrophorus electricus*. Fortunately, limited sites of expression and rules of receptor assembly have served to restrict this number enormously. Even so, ongoing research has identified more than ten different nAChR subtypes that differ in many ways. This chapter has summarized only some of the progress that has been made in identifying and characterizing native nAChRs. We have not covered any of the research that has focused on receptors that contain the α2, α3, or β4 subunits because they do not seem to be expressed in high quantities in dopaminergic neurons. We chose to emphasize those neuronal nAChR subtypes that are expressed in dopamine neurons or in neurons that directly interact with dopaminergic neurons in the ventral tegmental area and nucleus accumbens because dopamine seems to be very important in regulating the addiction process. Certainly, the recent discovery that nicotine activates certain α6* nAChRs at lower concentrations than are required for nicotine-induced activation of any of the other known nAChRs, including the nAChR that has been called the high affinity nicotine receptor for nearly 30 years (α4β2*) (Salminen et al. 2007), helps explain why the low doses of nicotine supplied by a single cigarette reinforce tobacco use.

John Langley's early work with nicotine led to the nicotinic receptor concept; he would probably be astonished at how complex the field that he originated has become. It is also likely, however, that he would be delighted to learn that his receptive substance is not a single entity and that nAChRs might play important roles in regulating vital behaviors such as learning and memory as well as psychopathologies such as anxiety, depression, and schizophrenia. Identifying the nAChR subtypes that modulate normal and abnormal behaviors and those that might influence the progression of neurodegenerative diseases could lead to newer and safer therapies.

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