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Progress and Challenges in the Study of $\alpha 6$ -Containing Nicotinic Acetylcholine Receptors

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Abstract

Recent progress has been made in the understanding of the anatomical distribution, composition, and physiological role of nicotinic acetylcholine receptors containing the $\alpha 6$ subunit. Extensive study by many researchers has indicated that a collection of $\alpha 6$ -containing receptors representing a nicotinic sub-family is relevant in preclinical models of nicotine self-administration and locomotor activity. Due to a number of technical difficulties, the state of the art of *in vitro* model systems expressing $\alpha 6$ -containing receptors has lagged behind the state of knowledge of native $\alpha 6$ nAChR subunit composition. Several techniques, such as the expression of chimeric and concatameric $\alpha 6$ subunit constructs in oocytes and mammalian cell lines have been employed to overcome these obstacles. There remains a need for other critical tools, such as selective small molecules and radioligands, to advance the field of research and to allow the discovery and development of potential therapeutics targeting $\alpha 6$ -containing receptors for smoking cessation, Parkinson's disease and other disorders.

Keywords

alpha6; nicotinic receptor; Parkinson's disease; smoking cessation; addiction

1. Introduction

The neurotransmitter acetylcholine binds to two main classes of receptors, nicotinic and muscarinic, each named for the prototypical compound that interacts with the class. Nicotinic acetylcholine receptors (nAChRs) play critical physiological roles throughout the body and brain by mediating cholinergic excitatory neurotransmission, modulating the release of neurotransmitters, influencing second messenger systems and gene expression, and contributing to synaptic plasticity [1,2]. Although the action of nicotine at the neuromuscular junction was studied by John Langley in the early 1900s, the role of nAChRs in the central nervous system (CNS) remained in dispute for nearly 90 years. This occurred because expression of nicotinic receptors in the brain was considerably less dense than muscarinic receptors and because the field lacked appropriate tools to assess them adequately [3]. As the field progressed, multiple tools were developed, including molecular biology techniques, selective agonists and antagonists, radioligands, antibodies, heterologous expression systems and transgenic mice, which enabled research on specific

nicotinic subtypes in the CNS. Two neuronal nAChR subtypes have been examined extensively: those containing $\alpha 7$ subunits and those containing $\alpha 4$ and $\beta 2$ subunits. The respective roles of these subtypes have been sufficiently studied in various disease states to support the clinical development of therapeutics for smoking cessation, depression and cognitive disorders [4]. Research around other nAChR subtypes, including those containing the $\alpha 6$ subunit, is less advanced. With further work, it is anticipated that a better understanding of these subtypes will also lead to novel medications.

nAChRs encompass a family of ligand-gated ion channels consisting of a variety of subtypes. Each receptor subtype is formed from 5 separate protein subunits that co-assemble to form a pore permeable to cations such as Ca^{2+} , Na^{+} and K^{+} . There have been seventeen vertebrate (sixteen mammalian) nAChR subunits cloned ($\alpha 1$ – 10 , $\beta 1$ – 4 , γ , δ , ϵ), providing the potential for a large number of subunit combinations [5]. However, the assembly of nAChRs appears to be a highly regulated process, with certain subunit combinations favored based on subunit expression patterns, subunit interactions, post-translational modification and other cellular processes [2,6,7].

The pharmacology of each nAChR subtype is defined by the α and β subunits that make up the receptor. Each subunit protein is characterized by an N-terminal extracellular agonist-binding domain, four transmembrane spanning regions, which contribute to the channel and traverse the cell membrane, and a substantial intracellular domain composed of the loop between the third and fourth transmembrane sequences [2]. The subunit composition of each nicotinic receptor subtype determines the pharmacological characteristics of the ligand binding sites and the cation preference of the channel. Both homomeric combinations, such as $(\alpha 7)_5$, and heteromeric combinations, such as $(\alpha 4\beta 2)_2\alpha 4$ and $(\alpha 4\beta 2)_2\beta 2$, have been described. For homomeric subtypes, there are five putative competitive ligand binding sites, one between each $\alpha 7$ – $\alpha 7$ extracellular domain interface [8]. In the heteromeric combinations, there are two putative receptor binding interfaces that contribute to competitive ligand binding sites, each residing at the interface between the extracellular domains of neighboring α and β subunits. The fifth subunit is generally considered an accessory subunit and not a component of the orthosteric ligand binding sites. All five subunits in the pentameric complex contribute to channel kinetics, such as activation, inactivation, desensitization, channel open times, ion conductance and selectivity [2]. The receptor binding and functional properties of each subtype are unique, but overlap sufficiently to make distinguishing between them challenging with existing pharmacological agents. This is especially true for subtypes that have subunits in common or where differing subunits share a high degree of homology.

The diversity of nAChR subtypes is physiologically relevant, as it allows for a broad range of cellular roles. Changes in nAChR subunit expression in development [9], specificity of expression in different organs, across brain regions [10] and cellular compartments [11] and even changes in subunit composition in response to drug treatment [12,13] have been reported. This permits a wide variety of functional roles in normal and disease states and provides opportunities for pharmacological manipulation and drug design. Such is the case for receptors containing the $\alpha 6$ subunit, which have generated much interest in the research and pharmaceutical communities. The $\alpha 6$ subunit has a restricted expression pattern, being predominantly confined to dopaminergic neurons implicated in the reward pathway and motor behavior, but also found in noradrenergic neurons, the visual system and a few other regions. This makes $\alpha 6$ -containing nAChRs relevant for a number of CNS disorders such as drug addiction, Parkinson's disease (PD) [14] and potentially others [15].

Research over the last decade has revealed that there is a sub-family of closely-related nAChR subtypes containing the $\alpha 6$ subunit, which vary by the composition of other subunits

in the pentameric complex. Subtle pharmacological differences between $\alpha 6^*$ subtypes (the asterisk indicates the presence of other subunit types in the pentamer) have created specific challenges to research. Therefore, separate *in vitro* expression systems are needed for each likely $\alpha 6^*$ receptor combination to fully explore differences in the pharmacology. Unlike $\alpha 4\beta 2$ and $\alpha 7$ subtypes, however, it has been difficult to generate heterologous cell lines that express $\alpha 6^*$ receptors. Recent breakthroughs have been made in creating model systems for working with $\alpha 6^*$ subtypes, but there are still gaps in the tool set available. In addition, highly-selective agonists and antagonists for the various $\alpha 6^*$ subtypes have yet to be identified and are critically needed for manipulating $\alpha 6^*$ receptor populations *in vivo*, *ex vivo* and *in vitro*. Radioligands selective for the various $\alpha 6^*$ subtypes are also needed to discriminate $\alpha 6^*$ subtypes containing closely related subunit combinations. These challenges have limited the study of receptor binding and functional properties of $\alpha 6^*$ nAChRs with *in vitro* expression systems and *in vivo* models.

2. Progress to Date

Incremental progress has been made in the study of $\alpha 6^*$ nAChRs. Initial *in situ* hybridization studies identified the regional expression of $\alpha 6$ subunit mRNA. Early work to characterize $\alpha 6^*$ subtypes relied on a combination of relatively non-selective radioligand binding and/or functional assays that did not differentiate adequately from other nAChR subtypes. Eventually, more selective approaches were used such as subunit-null transgenic animals and peptide ligands, often in combination with immunoprecipitation assays using antibodies targeting nAChR subunits. These studies have provided valuable insights into the stoichiometry of various $\alpha 6^*$ nAChRs and their physiological roles.

2.1 From Cloning to Transgenic Animals

Cloning first identified the $\alpha 6$ subunit gene sequence [16], which has a high degree of homology to that of the $\alpha 3$ subunit. Once the sequence was determined, highly specific antisense oligonucleotide probes could be designed to measure mRNA expression in various brain regions. Le Novère et al. [17] determined that $\alpha 6$ subunit mRNA expression was restricted to a few areas of the rat brain. The greatest density of $\alpha 6$ mRNA was measured in catecholaminergic areas, such as dopaminergic nuclei (substantia nigra pars compacta; SNc, and ventral tegmental area; VTA) and noradrenergic nuclei (locus coeruleus; LC). In these areas, $\alpha 6$ mRNA density was higher than other nicotinic subunits. Further, $\alpha 6$ and $\beta 3$ mRNA were typically co-expressed, suggesting that these two subunits may co-assemble in the same receptor complex. $\alpha 6$ mRNA expression was also found in the reticular thalamic nucleus, the supramammillary nucleus, the mesencephalic V nucleus, medial habenula and interpeduncular nucleus. This study yielded the first clues to the potential role of $\alpha 6$ -containing receptors and the authors speculated involvement of $\alpha 6$ -containing receptors in locomotor behavior and reward, based on the high density in dopaminergic cell bodies [17]. The mRNA expression of $\alpha 6$ and co-localization with $\beta 3$ was confirmed in non-human primate brain, showing similar $\alpha 6$ mRNA distribution in the SNc, VTA, LC, medial habenula, and even in the cerebellum [18,19]

Additional *in situ* hybridization work identified expression of other nAChR subunits in these brain regions and hinted at the subtype complexity of $\alpha 6$ -containing receptors. Many of the nuclei expressing $\alpha 6$ mRNA also expressed $\alpha 4$ and $\beta 2$ mRNA [10]. Moreover, it was found that dopaminergic neurons in the rat SNc and VTA express a variety of α subunits and predominantly express $\beta 2$ rather than $\beta 4$ mRNA [20], suggesting a receptor complex containing $\alpha 6$, $\beta 2$ and $\beta 3$ in this area, potentially with other α subunits as well. Further work found that two types of neurons in the LC express nicotinic subunits, as determined by single-cell reverse transcription-PCR [21]. One type expressed $\alpha 3$ and $\beta 4$ mRNA, often with $\alpha 6$ and other α subunits. Another LC cell type expressed $\alpha 6$, $\beta 3$, $\beta 2$ and, frequently, $\alpha 4$.

Cells of the medial habenula and interpeduncular nucleus were shown to express $\beta 4$ mRNA in higher density than $\beta 2$, making it a more likely co-expression partner for $\alpha 6$ in this area [22,23]. Overall, these studies provided early evidence of the diversity of $\alpha 6$ receptors within different brain regions: those containing $\alpha 6$, $\beta 2$, $\beta 3$ and potentially other subunits in dopamine neurons and select LC neurons, and those containing $\alpha 6$, $\beta 4$ and potentially other subunits in the remaining LC neurons and in the medial habenula/interpeduncular nucleus.

Identification of the $\alpha 6$ subunit sequence enabled *in vivo* manipulation and yielded insights into the physiological role of $\alpha 6$ -containing nAChRs. Le Novere *et al.* [24] used continuous minipump administration of $\alpha 6$ antisense oligonucleotides into the cerebral ventricles of rats for several days to demonstrate a reduction in nicotine-induced locomotor activity. Manipulation of the subunit gene allowed the creation of transgenic mice with gene disruption (knock-out; KO) or addition of a mutated, hyper-sensitive $\alpha 6$ gene (gain-of-function; GoF). A combination of the two approaches was used to elucidate the $\alpha 6$ subunit combination relevant to locomotor activity [25,26]. The GoF manipulation used in this study involved mutation to serine of a leucine residue, 9 positions from the beginning of the M2 transmembrane domain of the $\alpha 6$ subunit, believed to form part of the ion-channel gate ($\alpha 6^{L9'S}$). Mutations of this type result in greater sensitivity to agonist activation and slower receptor desensitization [27]. Mice were engineered to express the $\alpha 6^{L9'S}$ subunit in addition to the usual native $\alpha 6^*$ nAChR population, under control of the native $\alpha 6$ promoter region to prevent ectopic expression. In contrast to wild-type and $\alpha 4$ KO mice, which exhibited normal home cage activity and minimal changes in activity in response to nicotine, $\alpha 6^{L9'S}$ mice exhibited exaggerated home cage locomotor activity in response to endogenous acetylcholine during their active phase (dark period) and in response to nicotine [25,26]. Further breeding was conducted to delete the $\alpha 4$ subunit gene ($\alpha 4$ KO) from this line of $\alpha 6^{L9'S}$ GoF mice [26]. This process likely removed multiple $\alpha 4^*$ subtypes: ($\alpha 4\beta 2$) $_2\alpha 4$, ($\alpha 4\beta 2$) $_2\beta 2$, ($\alpha 4\beta 2$) $_2\alpha 5$, $\alpha 6^{L9'S}\alpha 4\beta 2$ and $\alpha 6^{L9'S}\alpha 4\beta 2\beta 3$, leaving ($\alpha 6^{L9'S}\beta 2$) $_2\beta 3$ and $\alpha 6^{L9'S}\beta 2$ subtypes. In $\alpha 4$ KO/ $\alpha 6^{L9'S}$ mice, home cage activity was similar to wild-type, indicating that the $\alpha 4$ subunit is required for the hyperactivity observed in the $\alpha 6^{L9'S}$ mice. In addition, nicotine produced a reduced locomotor response and was less potent in $\alpha 4$ KO/ $\alpha 6^{L9'S}$ mice than in $\alpha 6^{L9'S}$ mice with $\alpha 4$ subunits. Thus, the most potent effects of nicotine on locomotor behavior in mice required the $\alpha 4$ subunit in combination with the $\alpha 6^{L9'S}$ subunit, suggesting the importance of nAChR with $\alpha 6\alpha 4\beta 2$ and $\alpha 6\alpha 4\beta 2\beta 3$ subunit combinations in motor activity [26]. These data highlight the importance of taking into account nAChR receptors containing both $\alpha 4$ and $\alpha 6$ subunits when interpreting the results of KO studies of either the $\alpha 4$ or $\alpha 6$ gene. It is also important to reiterate that these experiments were performed in mice expressing the $\alpha 6^{L9'S}$ subunit *in addition* to the native $\alpha 6$ population seen in wild-type littermates; backcrossing of the $\alpha 6^{L9'S}$ line onto an $\alpha 6$ -null background would provide a clearer picture of the effects of this GoF mutation.

Gene deletion studies also revealed the potential physiological role of $\alpha 6^*$ in nicotine self-administration behavior. $\alpha 6$ KO mice appear to develop normally and do not have any overt behavioral deficits [29]. Unlike wild-type mice, however, $\alpha 6$ KO mice do not acquire nicotine self-administration [30]. When the $\alpha 6$ subunit was re-expressed by injecting a lentiviral vector into the VTA of $\alpha 6$ -KO mice, the animals initiated nicotine self-administration. A similar pattern was seen with gene deletion and re-expression of $\alpha 4$ and $\beta 2$ subunits, indicating the relevance of subtypes containing $\alpha 6$ and $\beta 2$, as well as $\alpha 4$ and $\beta 2$ subunits. Given that neither $\alpha 6$ nor $\alpha 4$ subunits were able to compensate for the loss of the other, the authors speculated that nAChRs containing $\alpha 6$, $\alpha 4$ and $\beta 2$ subunits in the same pentamer are a key mediator of nicotine self-administration [30].

2.2 α -Conotoxin peptides

The previously-discussed *in situ* hybridization studies described the regional localization of $\alpha 6$ mRNA, but did not provide information on distribution of the final protein product. In other words, questions remained as to whether the expression of $\alpha 6$ subunit protein was restricted to cell bodies, or whether the protein was transported to distal synaptic terminals (in the case of DA neurons, from cell bodies in the SN/VTA to the projection fields of the striatum and nucleus accumbens). A breakthrough in the field of $\alpha 6$ receptor research, which helped answer these questions, occurred with the discovery of the α -conotoxin MII (α -CtxMII) peptide, the first tool that allowed positive identification of $\alpha 3\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs. α -Conotoxins are an extensive family of small peptides, derived from predatory marine snails, with exceptional selectivity for specific nAChR subtypes or defined groups of subtypes [31]. Most α -conotoxins are nAChR competitive antagonists, meaning the α -conotoxin binding site overlaps with the agonist binding site [31]. α -CtxMII was originally isolated from the venom of the conesail *Conus magus* and was initially characterized as a selective antagonist of $\alpha 3\beta 2$ nAChRs, with 2-4 orders of magnitude higher affinity at this subtype vs. muscle, $\alpha 2\beta 2$, $\alpha 2\beta 4$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, and $\alpha 7$ subtype nAChRs [32]. Shortly afterwards, α -CtxMII was shown to inhibit a portion of nicotinic agonist-induced [3 H]dopamine release from striatal synaptosomal preparations, although the specific nAChR subtype involved was not yet defined (see next paragraph) [33]. It is worth noting that these and subsequent similar experiments used a “subtraction” approach to identify α -CtxMII-sensitive nAChR populations, measuring a functional response in the presence and absence of α -CtxMII to distinguish activity of α -CtxMII-sensitive receptors from that of $\alpha 4\beta 2^*$. While this has been very useful, direct identification of α -CtxMII-sensitive nAChRs is preferred and would be facilitated by the development of selective agonists.

Development of a radiolabeled version of α -CtxMII ([125 I] α -CtxMII) permitted autoradiography studies that directly demonstrated an unique, highly restricted expression pattern of α -CtxMII-binding nAChRs, predominantly in projection areas of SN/VTA dopamine and retinal neurons, but also in the habenular-interpeduncular pathway [34], extending the findings from *in situ* hybridization studies. Multiple investigators [59-64] determined the composition of this novel nAChR binding site using nAChR subunit-null mutant mice (see section 2.3). Gene deletion of the $\alpha 6$ subunit, but not the $\alpha 3$ subunit, in transgenic mice substantially reduced [125 I] α -CtxMII binding in dopaminergic cell bodies and terminal fields [29,35]. The preceding findings demonstrated for the first time that, at least in dopamine-projection regions, α -CtxMII binding could be used to selectively identify $\alpha 6\beta 2^*$ (but not $\alpha 3\beta 2^*$ nAChRs), a finding confirmed in wild-type mice by the generation of $\alpha 6$ -selective α -CtxMII derivatives [36]. Once this was established, α -CtxMII-sensitive, functional $\alpha 6\beta 2^*$ nAChRs on mesolimbic dopamine projections were demonstrated to be on dopamine terminals [37], and to modulate the probability of dopamine release during both bursting and tonic activity [38-42]. In particular, α -CtxMII-sensitive $\alpha 6\beta 2^*$ nAChRs were shown to have a predominant role in nicotinic modulation of dopamine release from nucleus accumbens dopamine terminals [2,43]. It is important to note that although rodent SN/VTA projection neurons express $\alpha 6\beta 2^*$ in abundance with few $\alpha 3\beta 2^*$ nAChRs, indicating regional selectivity for $\alpha 6\beta 2^*$, this is not the case for other brain regions, such as the medial habenula (discussed in 2.3 section), which express additional α -CtxMII-binding nAChR subtypes (including $\alpha 3\beta 2^*$) as well as $\alpha 6\beta 2^*$.

Subsequently, α -CtxMII and its derivative, α -conotoxin MII[E11A] (E11A) have been used in *ex vivo* and *in vivo* studies to confirm the physiological importance of $\alpha 6\beta 2^*$ subtypes in the dopaminergic system and its relevance for PD and smoking cessation. E11A, like its parent compound, is a competitive nAChR antagonist. In contrast to α -CtxMII, E11A exhibits much improved selectivity for $\alpha 6\beta 2^*$ over $\alpha 3\beta 2$ subtypes, while maintain selectivity versus $\alpha 3\beta 4$, $\alpha 7$ and other nAChR subtypes [36]. Radiolabeled α -CtxMII was used to

demonstrate lower density of $\alpha 6\beta 2^*$ nAChRs in MPTP- induced Parkinsonism in animal models and in post-mortem tissue from PD subjects [44]. The loss of $\alpha 6\beta 2^*$ nAChRs, which are located on dopamine terminals in striatum, corresponded with the decline of SNc neurons [45]. E11A displaced [125 I]- α -CtxMII from control tissue in a biphasic manner, suggesting the presence of two $\alpha 6\beta 2^*$ sites recognized by [125 I]- α -CtxMII [44]. Further, the authors showed that only the higher-affinity phase of E11A displacement was abolished in $\alpha 4$ subunit-null mutant mice, suggesting that this corresponded to $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$, previously identified as having very high sensitivity to nicotine agonism [64], as opposed to the $(\alpha 6\beta 2)_2\beta 3$ subtype (see section 2.3). In a similar experiment in tissue from PD patients [44], the very high E11A-affinity $\alpha 6\beta 2^*$ nAChR site was selectively lost and thought to be $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ on the basis of the rodent studies. More severe nigrostriatal damage was necessary to observe complete deficits in non- $\alpha 6^*$ (predominantly $\alpha 4\beta 2^*$) nAChRs [46] and the dopamine transporter [44]. These findings indicate that the $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3^*$ nAChR site is a potential marker for selectively vulnerable dopamine neurons [44]. In a separate study of postmortem tissue from patients with Dementia with Lewy bodies, [125 I]- α -CtxMII binding was significantly decreased in the caudate and putamen, as well as in certain thalamic nuclei [15]. The [125 I]- α -CtxMII binding site decreases were apparent in patients with impaired consciousness, associated with fluctuations in cognition, but not in patients with visual hallucinations. The authors concluded that the loss of $\alpha 6^*$ sites may be related to the loss of dopamine neurons in Dementia with Lewy bodies and may contribute to the neuropsychiatric features of this disease [15].

Since peptides are typically unstable and not bioavailable via systemic administration, *in vivo* work with the α -CtxMII peptide has mostly been limited to microinjections into the brain. Such studies have examined the effects of α -CtxMII on nicotine reward and withdrawal. Intracerebroventricular injection of α -CtxMII in mice, at concentrations that should achieve selectivity for $\alpha 6\beta 2^*$ nAChRs, dose-dependently attenuated the rewarding effects of nicotine in the conditioned place preference model without reducing locomotor behavior [47]. The same study showed that α -CtxMII attenuated the affective signs of nicotine withdrawal, but not the physical signs. In addition, α -CtxMII produced an anxiolytic effect in mice withdrawn from nicotine after chronic exposure. Mecamylamine-precipitated conditioned place aversion was also attenuated by α -CtxMII. In contrast, α -CtxMII did not reduce hyperalgesia or the somatic signs (e.g. tremors, jumping, ptosis) associated with nicotine withdrawal. Unlike mecamylamine, a non-selective nAChR antagonist, α -CtxMII itself did not precipitate nicotine withdrawal in chronically treated mice [47]. In other studies, microinjection of α -CtxMII directly into VTA [48] or the nucleus accumbens shell, a projection area of the VTA, attenuated *established* nicotine self-administration in rats [49] without non-specific changes in food responding [48]. These data extend previous findings that the $\alpha 6$ subunit is necessary and sufficient for the *acquisition* of nicotine self-administration behavior [30]. These data indicate that blockade of $\alpha 6\beta 2^*$ reduces the rewarding effects of nicotine in conditioned place preference, attenuates nicotine intake in a self-administration paradigm, and reduces anxiety associated with nicotine withdrawal, with implications for potential smoking cessation therapeutics. In addition, $\alpha 6\beta 2$ may be relevant for the rewarding effects of ethanol. When infused directly into the VTA, α -CtxMII decreased conditioned responding and voluntary ethanol intake in rodents [50,51]. Taken together, these studies underscore the role of $\alpha 6\beta 2$ in multiple aspects of drug abuse.

Despite its great utility, α -CtxMII does present some limitations. As noted, α -CtxMII interacts with $\alpha 3\beta 2^*$ and $\alpha 6\beta 2^*$ with similar potency, and thus distinguishes poorly between these subtypes in brain regions that express both subtypes. Fortunately, naturally-occurring α -conotoxins have provided a valuable source of further-selective pharmacological tools. Of note, α -CtxPIA is much more selective for $\alpha 6\beta 2^*$ vs. $\alpha 3\beta 2^*$ nAChR subtypes, and α -Ctx

BuIA may be used to distinguish $\alpha 6\beta 4^*$ vs. $\alpha 6\beta 2^*$ nAChRs (blocking norepinephrine release from hippocampal tissue, but not dopamine release from striatal tissue) [52-55]. Additional valuable conotoxin ligands are likely awaiting discovery [56]. Moreover, multiple studies demonstrate that it is possible to fine-tune conopeptide selectivity by modifying naturally-occurring, moderately-selective conotoxins [55,57,58]. These initial examples show that a combined discovery and modification approach of developing novel pharmacological agents to discriminate between individual members of the $\alpha 6^*$ nAChR subtype family is highly promising.

2.3 Identification of $\alpha 6$ -containing subtypes

A series of elegant studies by multiple investigators determined that there is a collection of diverse $\alpha 6^*$ subtypes in the brain. This work combined relatively unselective radioligand and/or functional binding assays in conjunction with more-selective techniques such as subunit-null mouse lines and/or immunochemical approaches. Initial work concentrated on the SN/VTA dopamine projection areas and demonstrated that, in these regions, $\alpha 6^*$ receptors are quite complex; being composed mainly of $(\alpha 6\beta 2)_2\beta 3$, which is assumed to have two $\alpha 6\beta 2$ binding interfaces and $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$, which is assumed to have one $\alpha 6\beta 2$ and one $\alpha 4\beta 2$ binding interface (also known in the literature as $\alpha 6\beta 2\beta 3$ and $\alpha 6\alpha 4\beta 2\beta 3$, respectively), perhaps with a small contribution from $\alpha 6\beta 2$ -only nAChRs [59-64]. In these regions, α -CtxMII-sensitive receptors ($\alpha 6\beta 2^*$) were shown to be more sensitive to ACh-stimulated dopamine release than α -CtxMII-resistant receptors ($\alpha 4\beta 2^*$, non- $\alpha 6$). Within the family of α -CtxMII-sensitive receptors, the traditional nAChR agonists nicotine and cytosine demonstrate high potency for dopamine release mediated by $(\alpha 6\beta 2)_2\beta 3$, and very high potency for dopamine release through $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ [64]. Further, there are distinct regional variations in subtype distribution, which hint at different physiological roles for these related subtypes: $(\alpha 6\beta 2)_2\beta 3$ predominate over $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ in the mesolimbic dopamine pathway, associated with reward, whereas $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ are much more prevalent in the nigrostriatal pathway, associated with locomotor behavior [48].

Outside of the SN/VTA dopamine projection regions, further evidence of α -CtxMII-sensitive nAChR diversity is developing. A study in optic tract-related regions demonstrates a complex mix of $\alpha 6^*$ and $\alpha 3^*$ nAChR subtypes [65]. Again, regional variations in the distribution of subtypes were observed, indicating potential physiological relevance of this diversity. In the medial habenula–interpeduncular nucleus tract, there is good evidence of $\alpha 6\beta 4^*$ nAChR expression [66], the significance of which is not yet known. In addition, there is evidence of $\alpha 6^*$ nAChR modulation of GABA release onto VTA dopamine neurons [11,67], and some indication of a similar phenomenon in the superficial layers of the superior colliculus [68]. These latter findings indicate that, even within the SN/VTA dopamine projections and optic tract, there is evidence for local diversity of $\alpha 6^*$ nAChRs, giving additional levels of control [48]. It is also possible that smaller, relatively dispersed populations of $\alpha 6^*$ nAChR-expressing neurons await discovery. Finally, the presence of $\alpha 6^*$ in non-neuronal tissues needs further investigation. There is preliminary evidence for $\alpha 6$ subunit expression in microglia from rhesus monkey retina, based on immunohistochemical studies [69]. The subunit composition of the $\alpha 6^*$ nAChRs involved is not well-resolved in any of these areas. Clearly, further conotoxin- and small molecule-based tools, such as radioligands, that are capable of distinguishing reliably between $\alpha 6$ -nAChR subtypes are desperately needed in order to understand the physiological roles of the extensive family of natively-expressed $\alpha 6^*$ (and $\alpha 3^*$) nAChR subtypes. This is true for the already identified, but under-studied, $\alpha 6\beta 4^*$ nAChRs, and also for the novel $\alpha 6^*$ subtypes that are still being discovered. As the distinct roles of different $\alpha 6^*$ nAChRs are identified, even within individual neuronal circuits, the opportunities for precise pharmacological intervention, and thus development of improved drug therapies, are likely to increase.

3. Technical challenges to the study of $\alpha 6^*$ nAChRs

A natural extension of understanding the subunit composition of most nAChR subtypes has been to investigate the pharmacological properties of the receptor from a native tissue source or in a heterologous expression system. Native sources of $\alpha 6\beta 2^*$ nAChRs are problematic as these receptors are only expressed at low levels in a very restricted number of brain regions. Further, $\alpha 6^*$ expression is always accompanied by significant amounts of other nAChR subtypes [61,64,70]. Heterologous expression systems, such as selective subtype expression in *Xenopus* oocytes or the creation of mammalian cell lines expressing those specific subtypes, typically allows detailed pharmacological characterization in an isolated system. Cell lines also enable high-throughput screening for receptor binding and function that, in turn, allows the identification of selective pharmacological tools and drug development candidates. In the case of $\alpha 6^*$ nAChRs, however, the state of the art of *in vitro* model systems lags behind the state of knowledge of native $\alpha 6$ nAChR subunit composition. There are several technical challenges that have contributed to this situation. Early attempts at heterologous systems produced simple subunit combinations, generally with low expression and little-to-no functional activity. The diversity and complex nature of the $\alpha 6^*$ sub-family has contributed to the problem. Assembly of the unique $\alpha 6^*$ subtypes, each containing up to four different subunits, in a reliable and reproducible way has been elusive but recent studies are making advances in this area.

3.1 Expression of $\alpha 6$ -containing receptors in oocytes

Initial attempts to express functional $\alpha 6$ -containing nAChRs in *Xenopus* oocytes were only modestly successful. The introduction of $\alpha 6$ and $\beta 4$ subunits produced functional receptors [71], but function of $\alpha 6\beta 2$ nAChRs in this system was extremely poor [72]. In an attempt to increase functional expression, chimeric subunits, containing the extracellular domain of the $\alpha 6$ subunit fused with the transmembrane and intracellular domains of the closely-related $\alpha 3$ or $\alpha 4$ subunits, were employed [54,72,73]. Multiple laboratories have now demonstrated that functional nAChRs can be expressed in oocytes using $\alpha 6/3$ chimeric subunits in combination with $\beta 2$ and/or $\beta 3$ subunits [36,54,72-74]. This approach reproducibly increased expression compared to that seen with native $\alpha 6$ subunits, while closely retaining $\alpha 6$ -like pharmacology. Furthermore, the pharmacology of the $\alpha 6/3\beta 2\beta 3$ combination has been differentiated from $\alpha 3\beta 2$ pharmacology with conotoxin peptides such as α -CtxPIA [54], α -CtxMII[E11A] and related derivatives [57]. Another strategy used in oocytes was the combination of a mutant gain-of-function (GoF) $\beta 3$ subunit ($\beta 3V273S$) with wild-type $\alpha 6\beta 2$ and $\alpha 6\beta 4$ to improve functional responses over combinations containing wild-type $\beta 3$ subunits [75]. Each of these approaches, pioneered in oocyte systems, has been applied in the engineering of functional $\alpha 6^*$ nAChR-expressing cell lines, as described in sections 3.2 and 3.3.

3.2 Expression in mammalian cell lines

Unlike expression of nAChRs in oocytes, stable transfection of nicotinic receptors in cell lines facilitates the generation of sufficient amounts of the receptor for ligand binding assays and high-throughput functional assays. In contrast to $\alpha 4\beta 2$ cell lines, which produce robust levels of receptor binding and function [76-79], previous attempts to express the native $\alpha 6$ subunit with $\beta 2$ or $\beta 4$ in mammalian cell lines (HEK, SH-EP1) yielded low cell surface expression, low receptor binding levels and poor functional responses under typical cell culture conditions [13,71,72,80-83]. In addition, $\alpha 6$ and $\beta 2$ subunits appeared to assemble into aggregates, rather than assembling into functional pentamers [13,71,72,84]. It has been reported that $\alpha 6\beta 2^*$ receptor binding levels and functional responses increase when the cellular incubation temperature is dropped from 37° to 30°C [13]. Even at 30°C, only a small percentage (15%) of $\alpha 6\beta 2^*$ -transfected cells expressed ACh-evoked currents, similar

to the percentage of cells that exhibited cell surface expression of $\alpha 6\beta 2^*$, indicating poor cellular transport of the receptor complex to the cell surface. Sustained agonist exposure was also shown to upregulate $\alpha 6\beta 2^*$ [13] and several labs have used this strategy to increase expression in their heterologous systems. Despite these extensive efforts, however, surface expression of $\alpha 6\beta 2^*$ nAChRs in cell line models has remained too weak to be easily used.

As with the oocyte-expression models, chimeric subunit approaches have also been used in the generation $\alpha 6^*$ subtype combinations in mammalian cell lines. For example, a chimeric $\alpha 6/4$ subunit has been used to successfully express functional $\alpha 6/4\beta 4$, but not $\alpha 6/4\beta 2$ nAChRs [84]. With relevance to natively-expressed $(\alpha 6\beta 2)_2\beta 3^*$ nAChRs, a recent publication by Capelli *et al.* [85] demonstrated functional expression of the $\alpha 6/3$ chimeric nAChR subunit in combination with $\beta 2$ and mutant $\beta 3$ subunits. Inspired by previous findings in oocytes [75], the Capelli group used a $\beta 3$ subunit with a GoF mutation (V273S which, similarly to the previously-described $\alpha 6^{L9'S}$ mutation, increases agonist activation potency and slows nAChR desensitization) in order to produce functional receptors. With the combined approach of using the chimeric $\alpha 6/3$ subunit and the $\beta 3$ GoF subunit, they were able to generate robust responses to ACh and epibatidine, with blockade by the $\alpha 6\beta 2^*$ antagonists, α -CtxMII and α -CtxPIA. However, important caveats apply to this approach. The GoF mutation used is similar to that employed to explore receptor stoichiometry [86], and inevitably raises concerns over changed agonist and antagonist sensitivity and changes in efficacy [87]. Further, to cite the authors, "since only the extracellular N-terminal domain of the $\alpha 6/3$ chimera belongs to the $\alpha 6$ subunit, the $\alpha 6/3\beta 2\beta 3^{V273S}$ cell line cannot be used to identify $\alpha 6\beta 2$ channel blockers, or to study $\alpha 6\beta 2$ receptors in terms of pore opening mechanism, desensitization and inactivation, properties that directly depend on residues in the transmembrane segments and the loops between them" [85]. Cross-comparison to native nAChR subtypes is clearly important, and the authors identified a set of compounds considered selective for $\alpha 6/3\beta 2\beta 3^{V273S}$ nAChR functional activation for such testing. These were then assessed for their ability to inhibit [125 I]epibatidine binding to immunoimmobilized $\alpha 6\beta 2^*$ nAChRs [85]. There was generally good correspondence between potencies in the two assay types, although they likely measured different states of the receptors (activated state in the functional assays versus desensitized state in the binding assays).

One contributing factor for optimal expression of $\alpha 6^*$ subtypes may be that multiple nAChR subunits, such as $\alpha 4$, $\alpha 5$, $\beta 2$, $\beta 3$, are needed in the receptor complex for efficient trafficking of the nAChRs to the cell surface, where they can be functional [7,13,80,81,88]. To circumvent these issues, a recent study employed modified $\alpha 6$ and $\beta 2$ subunits in a mammalian cell line. The authors used a $\beta 2$ subunit with two mutations to enhance export from the endoplasmic reticulum [89]. They also used an $\alpha 6$ subunit fused with enhanced green fluorescence protein and Sec24D-mCherry-labeled endoplasmic reticulum sites to visualize and select transfected cells. The $\alpha 6$ -eGFP $\beta 2$ nAChRs were expressed in Neuro-2a cells and exhibited $\alpha 6\beta 2^*$ -like pharmacology [89] (this issue). Additional experimentation on technical modifications should continue to yield improvements in the expression of $\alpha 6$ -containing nAChRs in heterologous systems.

3.3 Expression of concatameric $\alpha 6^*$ constructs in oocytes and cell lines

Given that neurons are clearly able to assemble complex functional $(\alpha 6\beta 2)_2\beta 3$ and $(\alpha 4\beta 2)\beta 3$ nAChR subtypes, it seems likely that some, if not most, of the heterologous-expression difficulties are caused by incorrect assembly of the component subunits. Neurons express chaperone proteins that enforce correct associations between the subunits, ensuring proper assembly of functional nAChRs [90,91]. This may be the reason why some cell line backgrounds have been more amenable to $\alpha 6^*$ nAChR expression than others. In the absence of a full understanding of these neuron-specific factors, another way must be found

to impose the correct order of subunits in these complex nAChR subtypes. This is particularly important in the case of the $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ subtype, where uncontrolled assembly could result in the formation of multiple nAChR subtypes ($\alpha 4\beta 2$, $\alpha 6\beta 2$, $\alpha 4\alpha 6\beta 2$, all with or without the incorporation of $\beta 3$ subunits).

Recently, concatameric approaches have been used to enforce correct assembly of specific nAChR subunit arrangements. Here, subunits are tethered together into larger constructs using defined peptide linkers. As one might imagine, this can be a technically-challenging approach. The first published studies using concatameric linkers with nAChR subunits came from the laboratory of Jon Lindstrom [92]. Here, paired $\alpha 4$ and $\beta 2$ dimeric constructs were used in conjunction with either unpaired $\alpha 4$ or $\beta 2$ subunits, to investigate whether different $\alpha 4\beta 2$ stoichiometries ($(\alpha 4\beta 2)_2\alpha 4$ versus $(\alpha 4\beta 2)_2\beta 2$) resulted in receptors with different agonist affinity. Importantly, a comprehensive approach was taken to the preparation of the subunit dimers. Both $\alpha 4\beta 2$ and $\beta 2\alpha 4$ (reading N-terminal-to-C terminal) dimers were made. Further, the linker lengths between subunits were varied in an attempt to optimize expression. In total, four constructs were made: $\beta\text{-}\alpha$ (using only the C-terminal tail of the $\beta 2$ subunit for the linker), $\alpha\text{-(AGS)}_6\text{-}\beta$ (in which the C-terminal tail of $\alpha 4$ and six repeats of the sequence alanine-glycine-serine were used as a linker), $\beta\text{-(AGS)}_6\text{-}\alpha$, and $\alpha\text{-(AGS)}_{12}\text{-}\beta$. The $\beta\text{-}\alpha$ construct consistently produced no-or-lower expression than the others, indicating that the linker length was too short. Of the remaining constructs, $\beta\text{-(AGS)}_6\text{-}\alpha$ was the most successful. Functional expression using this construct was higher than for the others. Further, nAChRs arising from this construct retained the ability to be potentiated by 17- β -estradiol (seen for native $\alpha 4\beta 2$ nAChRs, and those made from unpaired subunits), and robust differences in agonist affinity were seen for $(\alpha 4\beta 2)_2\alpha 4$ versus $(\alpha 4\beta 2)_2\beta 2$ nAChRs made incorporating the dimer. The particular success of this construct was explained by the fact that the linker was of optimal length, and that, by reference to the acetylcholine binding protein structure [93], the β -to- α order enforced formation of the ligand binding pocket between the two subunits, formed using loops contributed from the (–), or “complementary,” side of the $\beta 2$ subunit and the (+), or “principal” side of the $\alpha 4$ subunit. Assembly of ligand-gated ion channel superfamily members from individual subunits has been demonstrated to occur in the endoplasmic reticulum via the formation of specific dimer and trimer intermediates [5,94-96], so this sensitivity to translation order might be expected.

Using a combined immunochemical, sucrose-density gradient, and electrophysiology plus reporter subunit approach [92] also demonstrated that using only subunit dimers results in the formation of multiple non-pentameric forms (dimers of pentamers, in which one tandem is shared between two pentamers, and also receptors with additional “dangling” subunits that are associated with, but not incorporated into, functional, pentameric, receptor structures). These concerns were confirmed and extended by other labs [97]. In some cases, expression of dimers in conjunction with single subunits can result in pentamers incorporating more than one monomer. These studies argued for the use of higher oligomers, such as dimer/trimer combinations, or fully pentameric concatamers. In fact, this same research group demonstrated the first successful expression of a pentameric construct ($\alpha 3\beta 4$, albeit with short linkers containing signal sequences, and low expression [98]. Applying the lessons learned from these earlier studies, well-optimized concatamers have been made that successfully reproduce the properties of different $\alpha 4\beta 2$ -stoichiometries and allow for detailed structure-function studies to be performed [99].

The Lindstrom laboratory has recently published the first demonstration of a combined $\alpha 6/3$ chimera and concatameric approach to recapitulate naturally-expressed $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ and $(\alpha 6\beta 2)_2(\beta 3)$ subtypes [100]. In Fig. 2, subunit orders (as per the linear peptide sequence) are shown, as are the linkers between subunits. For both $\alpha 6\beta 2\beta 3^*$ constructs shown, the authors concluded that subunit assembly must proceed in an anticlockwise manner from the initial

$\beta 3$ subunit. In this model, agonist binding pockets are formed at the interfaces between the principal faces of α subunits (+, which provide the conserved A, B, and C agonist binding loops), and the complementary faces of the following β subunits (–, which provide the conserved D, E, and F agonist binding loops; see [101,102,102,103] for detailed explanation of the importance of these receptor peptide loops). Note that this is the opposite subunit assembly order to that proposed in an earlier publication from the same laboratory [92]. In the earlier publication, optimal pentamer expression was obtained using $\beta 2$ - $\alpha 4$ dimers, in which a clockwise assembly of the $\beta 2(-)$ face to the following $\alpha 4(+)$ face must occur, plus loose $\alpha 4$ or $\beta 2$ subunits (Fig 13, *ibid*). In the later paper, longer linkers were used, likely allowing some flexibility with respect to which side of the initial subunit the following subunit may “choose” to associate [100]. On this basis, it is possible that the presence of an initial $\beta 3$ (accessory) subunit followed by an α subunit in the $\alpha 6\beta 2\beta 3^*$ concatamer forces the opposite assembly mode in these vs. $\alpha 4\beta 2^*$ concatameric nAChRs. The concatameric nAChR approach, although still under active development, clearly has great potential in enabling consistent heterologous expression of the diverse set of $\alpha 6^*$ nAChRs naturally expressed in the mammalian CNS.

4. Small Molecules Selective for $\alpha 6$ -containing Receptors

The anatomical distribution and physiological roles of $\alpha 6^*$ nAChRs in the studies described above suggests the relevance of these subtypes in drug abuse, PD and potentially other indications, making them important targets for research and pharmacotherapy development [14,41]. However, the most-selective ligands currently available are peptide antagonists [57,104], which are not readily bioavailable, making them unsuitable for most therapeutic applications. Availability of small molecule $\alpha 6^*$ -selective agonists and antagonists would allow better manipulation of $\alpha 6^*$ nAChRs *in vitro* and *in vivo*, which would yield new scientific insights and open up novel avenues for therapeutic and diagnostic development.

Several groups have used available tools and techniques to search for small molecules that interact with $\alpha 6^*$ nAChRs. Breining *et al.* [105] used displacement of [125 I] α -CtxMII from mouse tissue pooled from olfactory tubercles, striatum and superior colliculus (all dopamine projection regions enriched in $\alpha 6\beta 2^*$ nAChRs [106]) to determine receptor binding affinity of a diverse set of novel compounds. To determine the functional properties of these compounds, they assessed [3 H]DA release from striatal synaptosomes in the presence or absence of α -CtxMII to tease out the $\alpha 6\beta 2^*$ response from the $\alpha 4\beta 2^*$ response. These data were compared to receptor binding affinities at $\alpha 4\beta 2$ and $\alpha 7$ nAChRs, and functional responses at $\alpha 3\beta 4$ nAChRs to determine selectivity. They found that pyrimidine, but not pyridine substituents, on a number of scaffolds tend to enhance receptor binding affinity and/or function of native rodent $\alpha 6\beta 2^*$ receptors, while decreasing interaction with $\alpha 3\beta 4$ receptors. In addition, they identified two scaffolds with functional selectivity for $\alpha 6\beta 2^*$ compared to $\alpha 4\beta 2^*$ receptors. Zhang *et al.* [107] synthesized a series of tertiary amine analogs derived from lead azaaromatic quaternary ammonium salts and tested the compounds for antagonism of nicotine-evoked dopamine release. The lead compound from this series, bPiDI, antagonized dopamine release in a manner that was not additive with α -CtxMII, suggesting $\alpha 6\beta 2^*$ -like pharmacology. In addition, bPiDI attenuated nicotine self-administration in rats [108]. The selectivity of bPiDI relative to other nAChRs was not reported.

Lowe *et al.* [109] used the $\alpha 6/4\beta 4$ combination expressed in HEK cells [84] to test a series of [3.2.1]azabicyclic biaryl ethers. They found that an H-bond donor in the 5-position of the pyridine ring is needed to achieve potent binding and functional activity but may limit *in vivo* permeability, and thus, brain penetration. One compound, an extremely potent full agonist at both $\alpha 6/4\beta 4$ and $\alpha 3\beta 4$ subtypes, was effective in two models sensitive to

antipsychotic agents: the pre-pulse inhibition and mescaline-induced scratching assays. *In vivo* tolerability of these compounds with regard to activity at the $\alpha 3\beta 4$ subtype was not discussed. Most recently, the Capelli group [85] used their cell line expressing $\alpha 6/3\beta 2\beta 3^{V273S}$ to test a set of standard nicotinic ligands and a further library of candidate lead compounds. Based on the chemotypes that interacted with this combination, they proposed a pharmacophore model for $\alpha 6\beta 2^*$ antagonists consisting of a positive ionizable group, a hydrogen bond-acceptor and an aromatic ring. From this screening, they identified an antagonist as a potential lead for smoking cessation [85]. The results of these studies shed light on the structure-activity relationships needed for interaction with $\alpha 6^*$ and provide direction for additional drug discovery work.

Development of compounds that discriminate between individual natively-expressed $\alpha 6^*$ subtypes would allow fine-tuned manipulation of function. As previously noted, the two best-characterized $\alpha 6^*$ subtypes are thought to be $(\alpha 6\beta 2)_2\beta 3$ (high agonist affinity), which has two $\alpha 6\beta 2$ binding site interfaces and $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ (very high agonist affinity), which has both $\alpha 6\beta 2$ and $\alpha 4\beta 2$ interfaces. From a ligand binding standpoint, this raises several questions: Does the $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ receptor need both receptor sites to be occupied for the channel to open? Is it possible to design compounds that distinguish between $(\alpha 6\beta 2)_2\beta 3$ and $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$? For the $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ receptor, does the presence of the $\alpha 4$ subunit confer sufficient conformational change to the pentameric receptor complex to render the $\alpha 6\beta 2$ interface pharmacologically distinct from the $\alpha 6\beta 2$ interfaces in the $(\alpha 6\beta 2)_2\beta 3$ receptor? Along the same lines, does the presence of the $\alpha 6$ subunit in the $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ complex confer sufficient conformational change to the receptor complex to render the $\alpha 4\beta 2$ interface pharmacologically distinct from $(\alpha 4\beta 2)_2\alpha 4$ and $(\alpha 4\beta 2)_2\beta 2$ receptors? The pharmacological differences among $\alpha 6\beta 2^*$ subtypes are currently mere nuances and it will take the skills of very talented medicinal chemists and pharmacologists to develop small-molecule agents to differentiate between them.

5. Imaging Agents for $\alpha 6$ -containing Receptors

The primary imaging agents for evaluation of $\beta 2^*$ nAChRs in humans are halogenated derivatives of the agonist, A-85380: 5- ^{123}I -A-85380 for single photon emission computed tomography (SPECT) [85,110] and 2- ^{18}F -A-85380 for positron emission tomography (PET) [111]. These radiotracers have been used in research investigating the pathology of disease states and associated receptor changes. For example, 2- ^{18}F -A-85380 has been used to assess changes in $\beta 2^*$ nAChRs in PD patients, with significant decreases observed in dopaminergic regions (striatum, SN) and other areas [112,113]. In addition, 5- ^{123}I -A-85380 has been used to track changes in $\beta 2^*$ nAChRs at various time points during abstinence from smoking [114]. Imaging agents for drug targets can also be used in early clinical studies to assess receptor occupancy. This can guide appropriate dose selection for clinical studies, a critical factor in the success of CNS compounds with respect to efficacy and therapeutic index. 5- ^{123}I -A-85380 has been used in this way to optimize the dosing regimen of ABT-089 [115], once a candidate for Alzheimer's disease and attention deficit hyperactivity disorder [4,116].

Although 5- ^{123}I -A-85380 recognizes both $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ subtypes, it is slightly more potent at the $\alpha 4\beta 2^*$ subtype [117]. It is important to independently delineate the roles of these two cholinergic receptor subtype classes ($\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$) because, based on different pharmacology and anatomical location in and around dopamine neurons in the striatum, they may play separate roles in PD and smoking cessation. To date, there has not been a selective small molecule radioligand developed for $\alpha 6\beta 2^*$ nAChRs. ^{125}I - α -CtxMII is currently used in basic research to assess $\alpha 6\beta 2^*$ binding. As previously discussed, it lacks the stability and bioavailability needed for systemic administration in human imaging

studies. Small molecule radiotracers with the appropriate characteristics of PET or SPECT radioligands are critically needed to advance studies relevant for $\alpha 6\beta 2$ and $\alpha 6\beta 4^*$ subtypes.

For smoking cessation, it would be valuable to better understand the distribution of $(\alpha 6\beta 2)_2\beta 3$ and $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ in brain regions relevant to reward, such as the nucleus accumbens shell and core. Knowledge of the anatomical distribution of the $\alpha 6^*$ subtypes in these areas would provide valuable information for the design of compounds as smoking cessation therapies. In addition, studies to examine the receptor occupancy of nicotine at $\alpha 6\beta 2^*$, as compared to $\alpha 4\beta 2^*$ [118] would yield a better understanding of the mechanisms of nicotine addiction.

By individually targeting $\alpha 6\beta 2^*$ subtypes with selective radiotracers, we could gain a better understanding of the pathophysiology of striatal degeneration occurring during the onset and progression of PD. As the field moves toward developing therapeutics aimed at disease modification of PD, it is becoming increasingly necessary to develop methods for early detection or confirmation of early stages of PD. The $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ subtype is selectively lost in tissue from PD patients (Fig. 1) and may be a more sensitive marker for PD-induced dopamine loss than the dopamine transporter [44], radiotracers for which are standard in the field of PD. These findings indicate that the $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ nAChR site is a potential marker for selectively vulnerable dopamine neurons [44] and may be an ideal target for a PET/SPECT ligand to be used in the identification of *early* dopaminergic degeneration in PD. If such a radiotracer targeting $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ can be developed, an important question to address will be whether the selective loss of the very high affinity receptor is associated with any of the pre-motor symptoms of PD, such as depression, cognitive deficits, abnormal REM sleep, and loss of olfaction. This knowledge would be critical to the development of novel and improved medicines for PD. Such a radioligand would also have utility in screening patients for inclusion in the clinical assessment of interventions designed to treat the symptoms of PD or to slow the progression of PD. Ultimately, a radiotracer targeting $\alpha 6\beta 2^*$ nAChRs may contribute to improved understanding of the pathophysiology of PD, for assessment of patients prior to presentation of PD symptoms and even to tracking of disease progression in longitudinal studies.

6. Remaining Gaps and Future Directions

Although recent advances have been made in our understanding of $\alpha 6^*$ nAChR subtypes, especially in the dopaminergic system, significant gaps remain. Consideration needs to be given to the relevance of $\alpha 6^*$ for other dopamine-related indications besides smoking cessation and PD, such as cognition [15], anxiety [47] and psychosis [109]. More work is needed to understand the role of $\alpha 6^*$ subunit composition in noradrenergic neurons, visual systems, the medial habenula-interpeduncular nucleus pathway and other regions, which may provide insights into the relevance of yet other disease states.

Development of better pharmacological tools will improve our understanding of the roles of individual $\alpha 6^*$ nAChR subtypes. Vital to all of these efforts will be development of improved *in vitro* model systems that accurately reproduce the native $\alpha 6$ nAChR subtypes that have been uncovered over the last decade or so. Along those lines, a better understanding of the cellular machinery that enables transport to and expression on the cell surface will enable the creation of better heterologous expression systems. Selective conotoxin- and small molecule-based antagonists, agonists and radioligands are needed as tools for investigating the various receptors in the $\alpha 6^*$ nAChR subfamily. Together, these tools should drive progress in understanding the physiological roles, the differing properties, and drug-development potential of individual $\alpha 6^*$ nAChR subtypes.

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Non-standard abbreviations

α-Ctx	alpha-conotoxin
CNS	central nervous system
GoF	gain-of-function
KO	knock-out
LC	locus coeruleus
MPTP	1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine
nAChRs	nicotinic acetylcholine receptors
PD	Parkinson's disease
PET	positron emission tomography
SNc	substantia nigra pars compacta
SPECT	single photo emission computed tomography
VTa	ventral tegmental area

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Figure 1.

Loss of the very-high-affinity ^{125}I - α -CtxMII binding component in striatum from Parkinson's disease cases. A decline in ^{125}I - α -CtxMII binding was observed in both caudate and putamen from Parkinson's disease cases. Competition of ^{125}I - α -CtxMII binding by E11A in control human striatum yielded biphasic curves (fit best to a two-site model). However, similar analyses of tissue from Parkinson's disease cases resulted in monophasic curves (fit best to a one-site model), suggesting the loss of the very-high-affinity E11A-sensitive component Parkinson's disease striatum. Symbols represent the mean $\pm$ S.E.M. of five control and four Parkinson's disease cases. Where the S.E.M. is not depicted, it fell within the symbol. Figure and legend from Bordia *et al.*, (2007) [44], reprinted with permission from the publisher.

Illustration of two fully-concatameric, functional, $\alpha 6\beta 2\beta 3^*$ subtypes described in Kuryatov and Lindstrom (2011) [100]. A) $(\alpha 6\beta 2)(\alpha 4\beta 2)\beta 3$ concatamer. B) $(\alpha 6\beta 2)_2\beta 3$ concatamer. The ACh binding pockets are formed between pairs of adjacent subunits. These contribute a set of six peptide loops that line the ACh binding pockets (loops A-C from the + side of the interface, and loops D-F from the - side of the interface; see [101-103]).

Figure 3.

A number of novel small molecules have been synthesized that interact with $\alpha 6$ -containing nAChRs. Breining *et al.* (2009) [105] found that pyrimidine (X = N), but not pyridine (X = CH) substituents, on a number of scaffolds tend to enhance receptor binding affinity and/or function of native rodent $\alpha 6\beta 2^*$ receptors, while decreasing interaction with $\alpha 3\beta 4$ receptors. They identified two scaffolds (a and b) with functional selectivity for $\alpha 6\beta 2^*$ compared to $\alpha 4\beta 2^*$ receptors. Wooters *et al.* (2011) [108] determined that bPiDI (c), an antagonist with α -CtxMII-like pharmacology, attenuated nicotine self-administration in rats. Lowe *et al.* (2010) [109] identified (d) as an extremely potent full agonist at both $\alpha 6/4\beta 4$ and $\alpha 3\beta 4$ subtypes, which was effective in two models sensitive to antipsychotic agents. Capelli *et al.* (2011) [85] identified several antagonists of the $\alpha 6/3\beta 2\beta 3^{V273S}$ construct, represented by the most potent and selective (e), as holding potential for starting templates for smoking cessation therapeutics.