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Review

Progress and challenges in the study of α6-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 α6 subunit. Extensive study by many researchers has indicated that a collection of α6-containing receptors representing a nicotinic subfamily 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 α6-containing receptors has lagged behind the state of knowledge of native α6 nAChR subunit composition. Several techniques, such as the expression of chimeric and concatameric α6 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 α6-containing receptors for smoking cessation, Parkinson's disease and other disorders.

Contents

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

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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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 α7 subunits and those containing α4 and β2 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 α6 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 (α1–10, β1–4, 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 $(\alpha7)_5$, and heteromeric combinations, such as $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$, have been described. For homomeric subtypes, there are five putative competitive ligand binding sites, one between each α7–α7 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 α6 subunit, which have generated much interest in the research and pharmaceutical communities. The α6 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 α6-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 subfamily of closely related nAChR subtypes containing the α6 subunit, which vary by the composition of other subunits in the pentameric complex. Subtle pharmacological differences between α6* 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 α6* receptor combination to fully explore differences in the pharmacology. Unlike α4β2 and α7 subtypes, however, it has been difficult to generate heterologous cell lines that express α6* receptors. Recent breakthroughs have been made in creating model systems for working with α6* subtypes, but there are still gaps in the tool set available. In addition, highly selective agonists and antagonists for the various α6* subtypes have yet to be identified and are critically needed for manipulating α6* receptor populations *in vivo*, *ex vivo* and *in vitro*. Radioligands selective for the various α6* subtypes are also needed to discriminate α6* subtypes containing closely related subunit combinations. These challenges have limited the study of receptor binding and functional properties of α6* nAChRs with *in vitro* expression systems and *in vivo* models.

2. Progress to date

Incremental progress has been made in the study of α6* nAChRs. Initial *in situ* hybridization studies identified the regional expression of α6 subunit mRNA. Early work to characterize α6* 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 α6* nAChRs and their physiological roles.

2.1. From cloning to transgenic animals

Cloning first identified the α6 subunit gene sequence [16], which has a high degree of homology to that of the α3 subunit. Once the sequence was determined, highly specific antisense oligonucleotide probes could be designed to measure mRNA expression in various brain regions. Le Novere et al. [17] determined that α6 subunit mRNA expression was restricted to a few areas of the rat brain. The greatest density of α6 mRNA was measured in catecholamingeric areas, such as dopaminergic nuclei (substantia nigra pars compacta, SNc; and ventral tegmental area, VTA) and noradrenergic nuclei (locus coeruleus; LC). In these areas, α6 mRNA density was higher than other nicotinic subunits. Further, α6 and β3 mRNA were typically co-expressed, suggesting that these two subunits may co-assemble in the same receptor complex. α6 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 α6-containing receptors and the authors speculated involvement of

α6-containing receptors in locomotor behavior and reward, based on the high density in dopaminergic cell bodies [17]. The mRNA expression of α6 and co-localization with β3 were confirmed in non-human primate brain, showing similar α6 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 α6-containing receptors. Many of the nuclei expressing α6 mRNA also expressed α4 and β2 mRNA [10]. Moreover, it was found that dopaminergic neurons in the rat SNc and VTA express a variety of α subunits and predominantly express β2 rather than β4 mRNA [20], suggesting a receptor complex containing α6, β2 and β3 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 α3 and β4 mRNA, often with α6 and other α subunits. Another LC cell type expressed α6, β3, β2 and, frequently, α4. Cells of the medial habenula and interpeduncular nucleus were shown to express β4 mRNA in higher density than β2, making it a more likely co-expression partner for α6 in this area [10,23]. Overall, these studies provided early evidence of the diversity of α6 receptors within different brain regions: those containing α6, β2, β3 and potentially other subunits in dopamine neurons and select LC neurons, and those containing α6, β4 and potentially other subunits in the remaining LC neurons and in the medial habenula/interpeduncular nucleus.

Identification of the α6 subunit sequence enabled *in vivo* manipulation and yielded insights into the physiological role of α6-containing nAChRs. Le Novere et al. [24] used continuous minipump administration of α6 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, hypersensitive α6 subunit (gain-of-function, GoF). A combination of the two approaches was used to elucidate the α6 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 α6 subunit, believed to form part of the ion-channel gate ($\alpha6^{L9'S}$). Mutations of this type result in greater sensitivity to agonist activation and slower receptor desensitization [27]. Mice were engineered to express the $\alpha6^{L9'S}$ subunit in addition to the usual native α6* nAChR population, under control of the native α6 promoter region to prevent ectopic expression. In contrast to wild-type and α4 KO mice, which exhibited normal home cage activity and minimal changes in activity in response to nicotine, $\alpha6^{L9'S}$ 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 α4 subunit gene (α4 KO) from this line of $\alpha6^{L9'S}$ GoF mice [26]. This process likely removed multiple α4* subtypes: $(\alpha4\beta2)_2\alpha4$, $(\alpha4\beta2)_2\beta2$, $(\alpha4\beta2)_2\alpha5$, $\alpha6^{L9'S}\alpha4\beta2$ and $\alpha6^{L9'S}\alpha4\beta2\beta3$, leaving $(\alpha6^{L9'S}\beta2)_2\beta3$ and $\alpha6^{L9'S}\beta2$ subtypes. In $\alpha4KO/\alpha6^{L9'S}$ mice, home cage activity was similar to wild-type, indicating that the α4 subunit is required for the hyperactivity observed in the $\alpha6^{L9'S}$ mice. In addition, nicotine produced a reduced locomotor response and was less potent in $\alpha4KO/\alpha6^{L9'S}$ mice than in $\alpha6^{L9'S}$ mice with α4 subunits. Thus, the most potent effects of nicotine on locomotor behavior in mice required the α4 subunit in combination with the $\alpha6^{L9'S}$ subunit, suggesting the importance of nAChR with α6α4β2 and α6α4β2β3 subunit combinations in motor activity [26]. These data highlight the importance of taking into account nAChR receptors containing both α4 and α6 subunits when interpreting the results of KO studies of either the α4 or α6 gene. It is also important to reiterate that these experiments were performed in mice expressing the $\alpha6^{L9'S}$ subunit *in addition* to the native α6 population seen in wild-type littermates; backcrossing of the $\alpha6^{L9'S}$ line onto an α6-null background would provide a clearer picture of the effects of this GoF mutation.

Gene deletion studies also revealed the potential physiological role of α6* in nicotine self-administration behavior. α6 KO mice appear to develop normally and do not have any overt behavioral deficits [28]. Unlike wild-type mice, however, α6 KO mice do not acquire nicotine self-administration [29]. When the α6 subunit was re-expressed by injecting a lentiviral vector into the VTA of α6-KO mice, the animals initiated nicotine self-administration. A similar pattern was seen with gene deletion and re-expression of α4 and β2 subunits, indicating the relevance of subtypes containing α6 and β2, as well as α4 and β2 subunits. Given that neither α6 nor α4 subunits were able to compensate for the loss of the other, the authors speculated that nAChRs containing α6, α4 and β2 subunits in the same pentamer are a key mediator of nicotine self-administration [29].

2.2. α-Conotoxin peptides

The previously discussed *in situ* hybridization studies described the regional localization of α6 mRNA, but did not provide information on distribution of the final protein product. In other words, questions remained as to whether the expression of α6 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 α6 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 α3β2* and α6β2* 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 [30]. Most α-conotoxins are nAChR competitive antagonists, meaning the α-conotoxin binding site overlaps with the agonist binding site [30]. α-CtxMII was originally isolated from the venom of the conesnail *Conus magus* and was initially characterized as a selective antagonist of α3β2 nAChRs, with 2–4 orders of magnitude higher affinity at this subtype vs. muscle, α2β2, α2β4, α3β4, α4β2, α4β4, and α7 subtype nAChRs [31]. 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) [32]. 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 α4β2*. 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 [33], extending the findings from *in situ* hybridization studies. Multiple investigators [57–62] determined the composition of this novel nAChR binding site using nAChR subunit-null mutant mice (see Section 2.3). Gene deletion of the α6

subunit, but not the α3 subunit, in transgenic mice substantially reduced [^{125}I]α-CtxMII binding in dopaminergic cell bodies and terminal fields [28,34]. The preceding findings demonstrated for the first time that, at least in dopamine-projection regions, α-CtxMII binding could be used to selectively identify α6β2* (but not α3β2* nAChRs), a finding confirmed in wild-type mice by the generation of α6-selective α-CtxMII derivatives [35]. Once this was established, α-CtxMII-sensitive, functional α6β2* nAChRs on mesolimbic dopamine projections were demonstrated to be on dopamine terminals [36], and to modulate the probability of dopamine release during both bursting and tonic activity [37–41]. In particular, α-CtxMII-sensitive α6β2* nAChRs were shown to have a predominant role in nicotinic modulation of dopamine release from nucleus accumbens dopamine terminals [2,42]. It is important to note that although rodent SN/VTA projection neurons express α6β2* in abundance with few α3β2* nAChRs, indicating regional selectivity for α6β2*, this is not the case for other brain regions, such as the medial habenula (discussed in Section 2.3), which express additional α-CtxMII-binding nAChR subtypes (including α3β2*) as well as α6β2*.

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 α6β2* 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 α6β2* over α3β2 subtypes, while maintaining selectivity vs. α3β4, α7 and other nAChR subtypes [35]. Radiolabeled α-CtxMII was used to demonstrate lower density of α6β2* nAChRs in MPTP-induced Parkinsonism in animal models and in post-mortem tissue from PD subjects [43]. The loss of α6β2* nAChRs, which are located on dopamine terminals in striatum, corresponded with the decline of SNc neurons [44]. E11A displaced [^{125}I]-α-CtxMII from control tissue in a biphasic manner, suggesting the presence of two α6β2* sites recognized by [^{125}I]-α-CtxMII [43]. Further, the authors showed that only the higher-affinity phase of E11A displacement was abolished in α4 subunit-null mutant mice, suggesting that this corresponded to (α6β2)(α4β2)β3, previously identified as having very high sensitivity to nicotine agonism [62], as opposed to the $(\alpha6\beta2)_2\beta3$ subtype (see Section 2.3). In a similar experiment in tissue from PD patients [43], the very high E11A-affinity α6β2* nAChR site was selectively lost and thought to be (α6β2)(α4β2)β3 on the basis of the rodent studies. More severe nigrostriatal damage was necessary to observe complete deficits in non-α6* (predominantly α4β2*) nAChRs [45] and the dopamine transporter [43]. These findings indicate that the (α6β2)(α4β2)β3* nAChR site is a potential marker for selectively vulnerable dopamine neurons [43]. In a separate study of post-mortem 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 α6* 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] (Fig. 1).

Since peptides are typically unstable and not CNS-available 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 α6β2* nAChRs, dose-dependently attenuated the rewarding effects of nicotine in the conditioned place preference model without

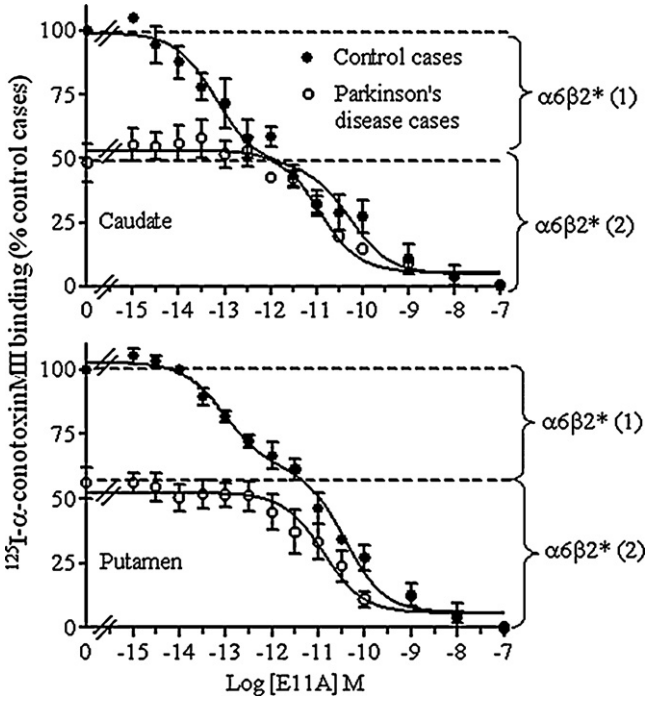

Fig. 1. Loss of the very-high-affinity ^{125}I-α-CtxMII binding component in striatum from Parkinson's disease cases. A decline in ^{125}I-α-CtxMII binding was observed in both caudate and putamen from Parkinson's disease cases. Competition of ^{125}I-α-CtxMII binding by E11A in control human striatum 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 ± 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. [43], reprinted with permission from the publisher.

reducing locomotor behavior [46]. 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, and ptosis) associated with nicotine withdrawal. Unlike mecamylamine, a non-selective nAChR antagonist, α-CtxMII itself did not precipitate nicotine withdrawal in chronically treated mice [46]. In other studies, microinjection of α-CtxMII directly into VTA [47] or the nucleus accumbens shell, a projection area of the VTA, attenuated *established* nicotine self-administration in rats [48] without non-specific changes in food responding [47]. These data extend previous findings that the α6 subunit is necessary and sufficient for the *acquisition* of nicotine self-administration behavior [29]. These data indicate that blockade of α6β2* 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, α6β2 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 [49,50]. Taken together, these studies underscore the role of α6β2* nAChRs in multiple aspects of drug abuse.

Despite its great utility, α-CtxMII does present some limitations. As noted, α-CtxMII interacts with α3β2* and α6β2* nAChRs 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 α6β2* vs. α3β2* nAChR subtypes, and α-Ctx BuIA may be used to distinguish α6β4* vs. α6β2* nAChRs (blocking norepinephrine release from hippocampal tissue, but not dopamine release from striatal tissue) [51–54]. Additional valuable conotoxin ligands are likely awaiting discovery [55]. Moreover, multiple studies demonstrate that it is possible to fine-tune conopeptide selectivity by modifying naturally occurring, moderately selective conotoxins [54,35,56]. These initial examples show that a combined discovery and modification approach for developing novel pharmacological agents to discriminate between individual members of the α6* nAChR subtype family is highly promising.

2.3. Identification of α6-containing subtypes

A series of elegant studies by multiple investigators determined that there is a collection of diverse α6* 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, α6* receptors are quite complex; being composed mainly of $(\alpha6\beta2)_2\beta3$, which is assumed to have two α6β2 binding interfaces, and (α6β2)(α4β2)β3, which is assumed to have one α6β2 and one α4β2 binding interface (also known in the literature as α6β2β3 and α6α4β2β3, respectively), perhaps with a small contribution from α6β2-only nAChRs [57–62]. In these regions, αCtxMII-sensitive receptors (α6β2*) were shown to be more sensitive to ACh-stimulated dopamine release than α-CtxMII-resistant receptors (α4β2*, non-α6). Within the family of α-CtxMII-sensitive receptors, the traditional nAChR agonists nicotine and cytisine demonstrate high potency for dopamine release mediated by $(\alpha6\beta2)_2\beta3$, and very high potency for dopamine release through (α6β2)(α4β2)β3 [62]. Further, there are distinct regional variations in subtype distribution, which hint at different physiological roles for these related subtypes: $(\alpha6\beta2)_2\beta3$ predominate over (α6β2)(α4β2)β3 in the mesolimbic dopamine pathway, associated with reward, whereas (α6β2)(α4β2)β3 are much more prevalent in the nigrostriatal pathway, associated with locomotor behavior [47].

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 α6* and α3* nAChR subtypes [63]. 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 α6β4* nAChR expression [64], the significance of which is not yet known. In addition, there is evidence of α6* nAChR modulation of GABA release onto VTA dopamine neurons [11,65], and some indication of a similar phenomenon in the superficial layers of the superior colliculus [66]. These latter findings indicate that, even within the SN/VTA dopamine projections and optic tract, there is evidence for local diversity of α6* nAChRs, giving additional levels of control [11,47]. It is also possible that smaller, relatively dispersed populations of α6* nAChR-expressing neurons await discovery. Finally, the presence of α6* in non-neuronal tissues needs further investigation. There is preliminary evidence for α6 subunit expression in microglia from rhesus monkey retina, based on immunohistochemical studies [67]. The subunit composition of the α6* 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 α6-nAChR subtypes are desperately needed in order to understand the physiological roles of the extensive family of natively expressed α6* (and α3*) nAChR subtypes. This is true for the already identified, but under-studied, α6β4* nAChRs, and also for the novel α6* subtypes that are still being discovered. As the distinct roles of different α6* 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 α6-containing 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 α6β2* nAChRs are problematic as these receptors are only expressed at low levels in a very restricted number of brain regions. Further, α6* expression is always accompanied by significant amounts of other nAChR subtypes [59,62,68]. 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 α6* nAChRs, however, the state of the art of *in vitro* model systems lags behind the state of knowledge of native α6 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 α6* nAChR sub-family have contributed to the problem. Assembly of the unique α6* nAChR 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 α6-containing receptors in oocytes

Initial attempts to express functional α6-containing nAChRs in *Xenopus* oocytes were only modestly successful. The introduction of α6 and β4 subunits produced functional receptors [69], but function of α6β2 nAChRs in this system was extremely poor [70]. In an attempt to increase functional expression, chimeric subunits, containing the extracellular domain of the α6 subunit fused with the transmembrane and intracellular domains of the closely related α3 or α4 subunits, were employed [53,70,71]. Multiple laboratories have now demonstrated that functional nAChRs can be expressed in oocytes using α6/3 chimeric subunits in combination with β2 and/or β3 subunits [35,53,70–72]. This approach reproducibly increased expression compared to that seen with native α6 subunits, while closely retaining α6-like pharmacology. Furthermore, the pharmacology of the α6/3β2β3 combination has been differentiated from α3β2 pharmacology with conotoxin peptides such as α-CtxPIA [53], α-CtxMII[E11A] and related derivatives [35]. Another strategy used in oocytes was the combination of a mutant gain-of-function (GoF) β3 subunit (β3V273S) with wild-type α6β2 and α6β4 to improve functional responses over combinations containing wild-type β3 subunits [73]. Each of these approaches, pioneered in oocyte systems, has been applied in the engineering of functional α6* 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 α4β2 cell lines, which produce robust levels of receptor binding and function [74–77], previous attempts to express the native α6 subunit with β2 or β4 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 [13,69,70,78–81]. In addition, α6 and β2 subunits appeared to assemble into aggregates, rather than assembling into functional pentamers [13,69,70,82]. It has been reported that α6β2* 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 α6β2*-transfected cells expressed ACh-evoked currents, similar to the percentage of cells that exhibited cell surface expression of α6β2*, indicating poor cellular transport of the receptor complex to the cell surface. Sustained agonist exposure was also shown to upregulate α6β2* [13] and several labs have used this strategy to increase expression in their heterologous systems. Despite these extensive efforts, however, surface expression of α6β2* 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 α6* subtype combinations in mammalian cell lines. For example, a chimeric α6/4 subunit has been used to successfully express functional α6/4β4, but not α6/4β2 nAChRs [82]. With relevance to natively expressed $(\alpha6\beta2)_2\beta3$* nAChRs, a recent publication by Capelli et al. [83] demonstrated functional expression of the α6/3 chimeric nAChR subunit in combination with β2 and mutant β3 subunits. Inspired by previous findings in oocytes [73], the Capelli group used a β3 subunit with a GoF mutation (V273S which, similarly to the previously described $\alpha6^{L9'S}$ mutation, increases agonist activation potency and slows nAChR desensitization) in order to produce functional receptors. With the combined approach of using the chimeric α6/3 subunit and the β3 GoF subunit, they were able to generate robust responses to ACh and epibatidine, with blockade by the α6β2* 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 [84], and inevitably raises concerns over changed agonist and antagonist sensitivity and changes in efficacy [85]. Further, to cite the authors, "since only the extracellular N-terminal domain of the α6/3 chimera belongs to the α6 subunit, the $\alpha6/3\beta2\beta3^{V273S}$ cell line cannot be used to identify α6β2 channel blockers, or to study α6β2 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" [83]. Cross-comparison to native nAChR subtypes is clearly important, and the authors identified a set of compounds considered selective for $\alpha6/3\beta2\beta3^{V273S}$ nAChR functional activation for such testing. These were then assessed for their ability to inhibit [^{125}I]epibatidine binding to immunoimmobilized α6β2* nAChRs [83]. 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 vs. desensitized state in the binding assays).

One contributing factor for optimal expression of α6* subtypes may be that multiple nAChR subunits, such as α4, α5, β2, and β3, are needed in the receptor complex for efficient trafficking of the nAChRs to the cell surface, where they can be functional [7,13,78,79,86]. To circumvent these issues, a recent study employed modified α6 and β2 subunits in a mammalian cell line. The authors used a β2 subunit with two mutations to enhance export from the endoplasmic reticulum [87]. They also used an α6 subunit fused with enhanced green fluorescent protein and Sec24D-mCherry-labeled endoplasmic reticulum sites to visualize and select transfected cells. The α6-eGFPβ2 nAChRs were expressed in Neuro-2a cells and exhibited α6β2*-like pharmacology [87]. Additional experimentation on technical modifications should continue to yield improvements in the expression of α6-containing nAChRs in heterologous systems.

3.3. Expression of concatameric α6-containing constructs in oocytes and cell lines

Given that neurons are clearly able to assemble complex functional $(\alpha6\beta2)_2\beta3$ and $(\alpha6\beta2)(\alpha4\beta2)\beta3$ 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 [88,89]. This may be the reason why some cell line backgrounds have been more amenable to α6* 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 $(\alpha6\beta2)(\alpha4\beta2)\beta3$ subtype, where uncontrolled assembly could result in the formation of multiple nAChR subtypes (α4β2, α6β2, α4α6β2, all with or without the incorporation of β3 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 Lindstrom [90]. Here, paired α4 and β2 dimeric constructs were used in conjunction with either unpaired α4 or β2 subunits, to investigate whether different α4β2 stoichiometries ($(\alpha4\beta2)_2\alpha4$ vs. $(\alpha4\beta2)_2\beta2$) resulted in receptors with different agonist affinities. Importantly, a comprehensive approach was taken to the preparation of the subunit dimers. Both α4–β2 and β2–α4 (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: β–α (using only the C-terminal tail of the β2 subunit for the linker), α-$(AGS)_6$-β (in which the C-terminal tail of α4 and six repeats of the sequence alanine-glycine-serine were used as a linker), β-$(AGS)_6$-α, and α-$(AGS)_{12}$-β. The β–α construct consistently produced no-or-lower expression than the others, indicating that the linker length was too short. Of the remaining constructs, β-$(AGS)_6$-α 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 α4β2 nAChRs, and those made from unpaired subunits), and robust differences in agonist affinity were seen for $(\alpha4\beta2)_2\alpha4$ vs. $(\alpha4\beta2)_2\beta2$ 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 [91], 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 β2 subunit and the (+), or "principal" side of the α4 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 [5,92–94], so this sensitivity to translation order might be expected.

Using a combined immunochemical, sucrose-density gradient, and electrophysiology plus reporter subunit approach [90] also demonstrated that using only subunit dimers results in the

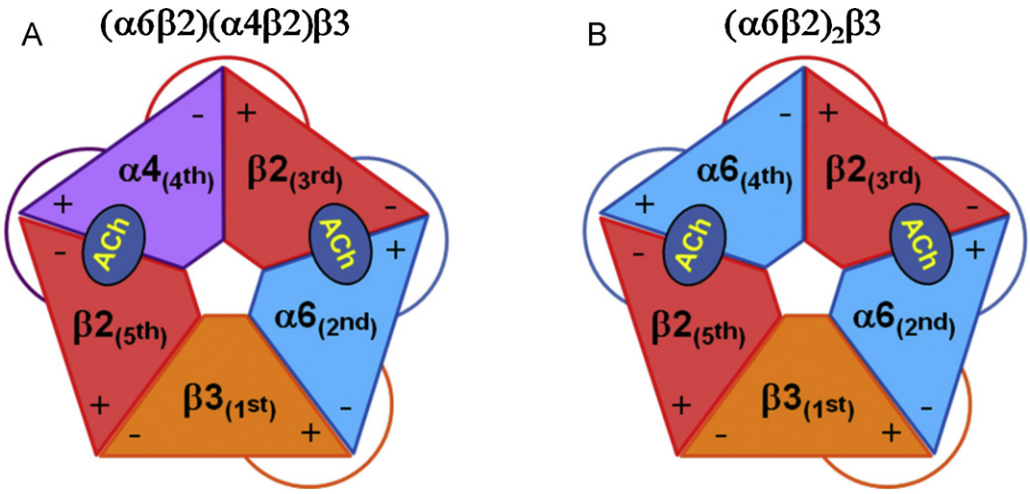

Fig. 2. Illustration of two fully concatameric, functional, α6β2β3* subtypes described in Kuryatov and Lindstrom [98]. (A) (α6β2)(α4β2)β3 concatamer. (B) $(\alpha6\beta2)_2\beta3$ concatamer. The ACh binding pockets are formed between pairs of adjacent subunits. These contribute a set of six peptide 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 [99–101].

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 [95]. 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 (α3β4), albeit with short linkers containing signal sequences, and low expression [96]. Applying the lessons learned from these earlier studies, well-optimized pentameric concatamers have been made that successfully reproduce the properties of different α4β2-stoichiometries and allow for detailed structure–function studies to be performed [97].

The Lindstrom laboratory has recently published the first demonstration of concatameric approach to recapitulate naturally expressed (α6β2)(α4β2)β3 and $(\alpha6\beta2)_2(\beta3)$ subtypes [98]. In Fig. 2, subunit orders (as per the linear peptide sequence) are shown, as are the linkers between subunits. For both α6β2β3* constructs shown, the authors concluded that subunit assembly must proceed in an anticlockwise manner from the initial β3 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 [99–101] 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 [90]. In the earlier publication, optimal pentamer expression was obtained using β2–α4 dimers, in which a clockwise assembly of the β2(−) face to the following α4(+) face must occur, plus loose α4 or β2 subunits (Fig. 13 in [90]). 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 [98]. On this basis, it is possible that the presence of an initial β3 (accessory) subunit followed by an α subunit in the α6β2β3* concatamer forces the opposite assembly mode in these vs. α4β2* 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 α6* nAChRs naturally expressed in the mammalian CNS.

4. Small molecules selective for α6-containing receptors

The anatomical distribution and physiological roles of α6* 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 [14,40]. However, the most-selective ligands currently available are peptide antagonists [35,33], which are not readily bioavailable, making them unsuitable for most therapeutic applications. Availability of small molecule α6*-selective agonists and antagonists would allow better manipulation of α6* 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 α6* nAChRs. Breining et al. [102] used displacement of [^{125}I]α-CtxMII from mouse tissue pooled from olfactory tubercles, striatum and superior colliculus (all dopamine projection regions enriched in α6β2* nAChRs [103]) 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 α6β2* response from the α4β2* response. These data were compared to receptor binding affinities at α4β2 and α7 nAChRs, and functional responses at α3β4 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 at native rodent α6β2* receptors, while decreasing interaction with α3β4 receptors. In addition, they identified two scaffolds with functional selectivity for α6β2* compared to α4β2* receptors (Fig. 3a and b). Zhang et al. [104] 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 (Fig. 3c) antagonized dopamine release in a manner that was not additive with α-CtxMII, suggesting α6β2*-like pharmacology. In addition, bPiDI attenuated nicotine self-administration in rats [105]. The selectivity of bPiDI relative to other nAChRs was not reported.

Lowe et al. [106] used the α6/4β4 combination expressed in HEK cells [82] to test a series of [3.2.1]azabicyclic biaryl ethers. They

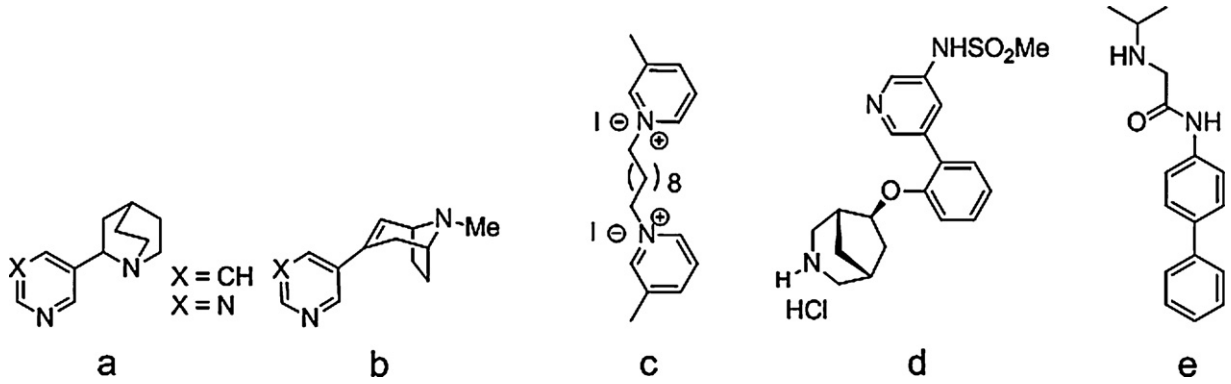

Fig. 3. A number of novel small molecules have been synthesized that interact with α6-containing nAChRs. Breining et al. [102] found that pyrimidine (X = N), but not pyridine (X = CH) substituents, on a number of scaffolds tend to enhance receptor binding affinity and/or function at native rodent α6β2* receptors, while decreasing interaction with α3β4 receptors. They identified two scaffolds (a and b) with functional selectivity for α6β2* compared to α4β2* receptors. Wooters et al. [105] determined that bPiDI (c), an antagonist with α-CtxMII-like pharmacology, attenuated nicotine self-administration in rats. Lowe et al. [106] identified (d) as an extremely potent full agonist at both α6/4β4 and α3β4 subtypes, which was effective in two models sensitive to antipsychotic agents. Capelli et al. [83] identified several antagonists of the α6/3β2β3^{V273S} construct, represented by the most potent and selective (e), as holding potential for starting templates for smoking cessation therapeutics.

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 α6/4β4 and α3β4 subtypes (Fig. 3d) 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 α3β4 subtype was not discussed. Most recently, the Capelli group [83] used their cell line expressing α6/3β2β3^{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 α6β2* antagonists consisting of a positive ionizable group, a hydrogen bond-acceptor and an aromatic ring. From this screening, they identified an antagonist (Fig. 3e) as a potential lead for smoking cessation [83]. The results of these studies shed light on the structure–activity relationships needed for interaction with α6* and provide direction for additional drug discovery work.

Development of compounds that discriminate between individual natively expressed α6* subtypes would allow fine-tuned manipulation of function. As previously noted, the two best-characterized α6* subtypes are $(\alpha6\beta2)_2\beta3$ (high agonist affinity), which has two α6β2 binding site interfaces and (α6β2)(α4β2)β3 (very high agonist affinity), which has both α6β2 and α4β2 interfaces. From a ligand binding standpoint, this raises several questions: Does the (α6β2)(α4β2)β3 receptor need both receptor sites to be occupied for the channel to open? Is it possible to design compounds that distinguish between $(\alpha6\beta2)_2\beta3$ and (α6β2)(α4β2)β3? For the (α6β2)(α4β2)β3 receptor, does the presence of the α4 subunit confer sufficient conformational change to the pentameric receptor complex to render the α6β2 interface pharmacologically distinct from the α6β2 interfaces in the $(\alpha6\beta2)_2\beta3$ receptor? Along the same lines, does the presence of the α6 subunit in the (α6β2)(α4β2)β3 complex confer sufficient conformational change to the receptor complex to render the α4β2 interface pharmacologically distinct from $(\alpha4\beta2)_2\alpha4$ and $(\alpha4\beta2)_2\beta2$ receptors? The pharmacological differences among α6β2* 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 α6-containing receptors

The primary imaging agents for evaluation of β2* nAChRs in humans are halogenated derivatives of the agonist, A-85380: 5-[^{123}I]-A-85380 for single photon emission computed tomography (SPECT) [83,107] and 2-^{18}F-A-85380 for positron emission tomography (PET) [108]. 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 β2* nAChRs in PD patients, with significant decreases observed in dopaminergic regions (striatum, SN) and other areas [109,110]. In addition, 5-[^{123}I]-A-85380 has been used to track changes in β2* nAChRs at various time points during abstinence from smoking [111]. 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 [112], once a candidate for Alzheimer's disease and attention deficit hyperactivity disorder [4,113].

Although 5-[^{123}I]-A-85380 recognizes both α4β2* and α6β2* subtypes, it is slightly more potent at the α4β2* subtype [114]. It is important to independently delineate the roles of these two cholinergic receptor subtype classes (α4β2* and α6β2*) because, based on different pharmacology and anatomical locations 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 α6β2* nAChRs. [^{125}I]-α-CtxMII is currently used in basic research to assess α6β2* binding. As previously discussed, it lacks the stability and CNSavailability 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 α6β2 and α6β4* subtypes.

For smoking cessation, it would be valuable to better understand the distribution of $(\alpha6\beta2)_2\beta3$ and (α6β2)(α4β2)β3 in brain regions relevant to reward, such as the nucleus accumbens shell and core. Knowledge of the anatomical distribution of the α6* 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 α6β2*, as compared to α4β2* [115] would yield a better understanding of the mechanisms of nicotine addiction.

By individually targeting α6β2* 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 (α6β2)(α4β2)β3 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 [43], radiotracers for which are standard in the field of PD. These findings indicate that the (α6β2)(α4β2)β3 nAChR site is a potential marker for selectively vulnerable dopamine neurons [43] 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 (α6β2)(α4β2)β3 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 α6β2* 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 α6* nAChR subtypes, especially in the dopaminergic system, significant gaps remain. Consideration needs to be given to the relevance of α6* for other dopamine-related indications besides smoking cessation and PD, such as cognition [15], anxiety [46] and psychosis [106]. More work is needed to understand the role of α6* 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 α6* nAChR subtypes. Vital to all of these efforts will be development of improved *in vitro* model systems that accurately reproduce the native α6 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 α6* nAChR subfamily. Together, these tools should drive progress in understanding the physiological roles, the differing properties, and drug-development potential of individual α6* nAChR subtypes.

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