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Review

Mysterious α6-containing nAChRs: function, pharmacology, and pathophysiology

Ke-chun YANG[1,2], Guo-zhang JIN[2], Jie WU[1,*]

[1]Divisions of Neurology, Barrow Neurological Institute, St Joseph's Hospital and Medical Center, Phoenix, AZ 85013, USA; [2]Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

Neuronal nicotinic acetylcholine receptors (nAChRs) are the superfamily of ligand-gated ion channels and widely expressed throughout the central and peripheral nervous systems. nAChRs play crucial roles in modulating a wide range of higher cognitive functions by mediating presynaptic, postsynaptic, and extrasynaptic signaling. Thus far, nine alpha (α2-α10) and three beta (β2, β3, and β4) subunits have been identified in the CNS, and these subunits assemble to form a diversity of functional nAChRs. Although α4β2- and α7-nAChRs are the two major functional nAChR types in the CNS, α6*-nAChRs are abundantly expressed in the midbrain dopaminergic (DAergic) system, including mesocorticolimbic and nigrostriatal pathways, and particularly present in presynaptic nerve terminals. Recently, functional and pharmacological profiles of α6*-nAChRs have been assessed with the use of α6 subunit blockers such as α-conotoxin MII and PIA, and also by using α6 subunit knockout mice. By modulating DA release in the nucleus accumbens (NAc) and modulating GABA release onto DAergic neurons in the ventral tegmental area (VTA), α6*-nAChRs may play important roles in the mediation of nicotine reward and addiction. Furthermore, α6*-nAChRs in the nigrostriatal DAergic system may be promising targets for selective preventative treatment of Parkinson's disease (PD). Thus, α6*-nAChRs may hold promise for future clinical treatment of human disorders, such as nicotine addiction and PD. In this review, we mainly focus on the recent advances in the understanding of α6*-nAChR function, pharmacology and pathophysiology.

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) belong to the ligand-gated ion channel superfamily, which include GABA$_A$, glycine, and 5-HT$_3$ receptors[1]. nAChRs are widely distributed throughout the central nervous system (CNS) and activation of various nAChRs may play important roles in regulation of higher cognitive functions[2]. nAChRs are pentameric complexes made up of combinations of a number of different nAChR subunits, which can be classified as alpha subunits, containing two cysteine residues at positions analogous to Cys192 and Cys193, and non-alpha subunits ('structural' subunits), which can be defined as beta subunits when they are expressed in the vertebrate nervous system[3,4]. To date, nine alpha subunits (α2–α10) and three beta subunits (β2, β3, and β4) have been identified in the CNS[5]. A single subunit is about 600 amino acids long and has four separate transmembrane segments (TM1-TM4) with a large N- and a small C-termini facing the synaptic cleft[6,7]. Studies using affinity labeling and mutagenesis suggest that ligand-binding sites are located at the interfaces of the N-terminal hydrophilic domain of α subunit and its adjacent α/β subunit[8], and the wall of the ionic pore is formed by the second hydrophobic transmembrane segment (TM2) of each subunit[6,7]. The cation channel (mainly permeable to Na$^+$ and Ca^{2+}) can be opened only when the receptor is activated by endogenous acetylcholine (ACh) or exogenous ligand (eg nicotine) binding to the binding site (Figure 1)[7]. Furthermore, the β subunits also largely contribute to the physiological and pharmacological properties (such as desensitization, inward rectification, and functional rundown) of the receptors[9,10].

Physiological and pharmacological profiles of nAChRs range widely, depending on subunit co-assembly. nAChRs can be divided into two subfamilies, homomeric nAChRs (native α7 or heterologously expressed α7–9 subunits) and

* Correspondence to Jie WU, MD, PhD.
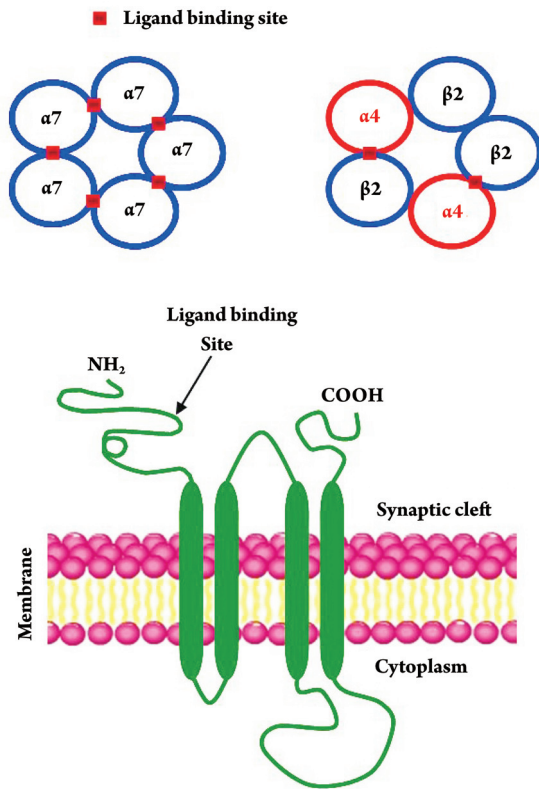E-mail Jie.Wu@chw.edu

Figure 1. Structure of nAChRs. nAChRs are formed by five subunits, which can be either homomeric (α) or heteromeric (α/β). (A) Organization of subunits in neuronal homomeric α7-nAChRs and heteromeric α4β2-nAChRs. (B) One subunit of the nAChR contains (1) a large N- and a small C-terminal extracellular domains, (2) four transmembrane domains (M1-M4), and (3) a long cytoplasmic loop between M3 and M4.

heteromeric nAChRs (α2–6 subunits combined with β subunits)[8, 11]. Although there are many possible combinations of neuronal α and β subunits, the majority of functional heteromeric nAChRs expressed throughout the brain are α4β2-containing nAChRs (α4β2*-nAChRs, *indicates the presence of possible additional subunits)[12]. Though α6*-nAChRs were characterized in the early 1990's[13, 14], it was not reported that α6 subunit could form functional heteromeric nAChRs until 1997[15]. Immunoprecipitation experiments demonstrated that not only α4β2-nAChRs, but also heteromeric α6*-nAChRs (*ie*, α6β2- and α4α6β2-nAChRs) are highly expressed in mesolimbic DAergic system[16]. More importantly, α6*-nAChRs expressed on DAergic neurons can be activated by endogenous ACh or exogenous nicotine and analogs, which suggest that the activation of α6*-nAChRs may play vital roles in central cholinergic circuits, including modulation of locomotor behaviour and drug addiction[17, 18]. In addition, α6*-nAChRs are particularly susceptible to

nigrostriatal damage, which may lead to Parkinson's disease (PD)[19, 20]. Accumulating evidence suggests that α6*-nAChRs might represent as potential therapeutic targets for treatment of PD and addictive behaviors[18–21].

Although α6*-nAChRs are abundant in the midbrain, they have been studied to a lesser extent than α4β2-nAChRs or α7-nAChRs[22]. In fact, we are only beginning to understand α6*-nAChR distribution, physiology and pharmacology, and the roles of these receptors in various diseases. In this review, we focus on recent advances in the understanding of α6*-nAChRs.

Anatomical distribution of α6*-nAChRs

Overwhelming experimental evidence demonstrates that neuronal nAChRs are present in a variety of regions of the brain, but the situation is much different for α6*-nAChRs, which are not abundantly expressed in the whole brain, but only detected in a restricted number of brain areas[23–25]. For example, in order to detect α6 subunit mRNA distribution in the CNS, Le Novere and colleagues explored the telencephalon, diencephalon, mesencephalon, and rhombencephalon of adult rat brain using *in situ* hybridization[23] and found that the amount of α6 subunit mRNA is particularly high in several catecholaminergic nuclei, including locus coeruleus, ventral tegmental area (VTA) and substantia nigra (SN). In reticular thalamic nucleus, supramammillary nucleus, interpeduncular nucleus, medial and lateral habenula, and mesencephalic V nucleus, α6 subunit mRNA can be detected, but at lower levels, while no detectable α6 subunit mRNA labeling is observed in the anterior pretectal area[23]. Based on these data, authors concluded that α6*-nAChRs are the primary α subunit expressed in DAergic cell groups within the midbrain[23]. After this initial report, subsequent studies confirmed that α6*-nAChRs are highly expressed in the SN and VTA, and particularly expressed on most midbrain DAergic neurons rather than on non-DAergic neurons, either by applying single-cell reverse transcription polymerase chain reaction (RT-PCR) and patch-clamp recording in slices from rats, wild-type mice and α6 subunit null mutant mice[25] or using double-labeling *in situ* hybridization in rats[24]. Additional in situ hybridization experiments using specific probes and stringent hybridization conditions demonstrated that α6 subunit mRNA is also abundantly expressed in neuroretina[26]. Other studies using [^{125}I]α-CTX MII binding indicate that high levels of α6*-nAChRs are expressed in the visual system, including retina, optic tract, and its terminal fields, including geniculate nucleus, zonal and superficial gray layer, and olivary pretectal nucleus[27]. Although nAChRs are widely distributed in the

peripheral nervous system (PNS)[28], no α6 subunit mRNA has been detected in the PNS (ciliary, superior cervical, sympathetic, dorsal root, nodose and petrous ganglia), except in trigeminal nucleus and trigeminal ganglion[26, 29]. Thus, we can draw the conclusion that the natural expression of α6*-nAChRs appears to be largely excluded from the PNS and mainly restricted to the CNS, and particularly enriched in midbrain catecholaminergic nuclei.

Neuronal nAChRs are located postsynaptically on the cell-body, where they mediate direct postsynaptic effects and/or regulate firing patterns of DAergic neurons[30], or presynaptically/preterminally on nerve terminals[16, 22, 31], where they modulate neurotransmitter release[5, 32–35]. Immunoprecipitation experiments have found that α6*-nAChRs account for 30% of ^{3}H-Epibatidine (Epi) binding sites in striatum but only 5% in SN/VTA[16]. Furthermore, quantitative immunoprecipitation experiments have shown that most of α6*-nAChRs (87%) disappeared in 6-hydroxydopamine lesioned (6-OH DA) striatum[36], further demonstrating the regulatory effects of presynaptic α6*-nAChRs on DA release[37, 38]. These results indicate that α6*-nAChRs appear to be preferentially addressed to DAergic nerve terminal compartments, since the majority of DAergic neurons in the SN project to the striatum[16, 36]. Our recent results using RT-PCR and patch clamp recordings in freshly dissociated VTA DAergic neurons[22] are in good agreement with these observations. Tissue RT-PCR data showed that nAChR α6 subunit mRNA levels are >20-fold higher in the VTA than that of other subunits[22], suggesting that α6*-nAChRs are mainly concentrated in midbrain catecholaminergic nuclei. However, 100 nmol/L α-conotoxin MII (α-CTX MII), an α6/α3-nAChR subtype-selective antagonist, had no significant effect on ACh (1 mmol/L) induced postsynaptic inward currents on all three subtypes of nAChRs on VTA DAergic neurons[22], suggesting that there are likely no functional α6*-nAChRs expressed on VTA DAergic neuronal somata under physiological conditions. Importantly, α-CTX MII (100 nmol/L) inhibited GABAergic spontaneous inhibitory postsynaptic currents in DAergic neurons containing GABAergic presynaptic boutons that were mechanically dissociated from the VTA[22], implicating that most of the functional α6*-nAChRs are located on presynaptic structures rather than on somata of DAergic neurons in the VTA, or that functional α6*-nAChRs are expressed on somatodendrites of VTA DAergic neurons under the natural conditions, but the expression level is too low to be detected using patch-clamp recording. One recent paper supports this hypothesis, which shows that after genetic enhancement of α6*-nAChR expression, the function of α6*-nAChRs can be clearly tested using whole-cell recording technique[39].

Therefore, nAChR α6 subunit mRNA is specifically expressed in DAergic neurons in the VTA and SN, and functional α6*-nAChRs are preferentially located on presynaptic nerve terminals.

Subunit composition of functional α6*-nAChRs

Studies using single-cell RT-PCR and patch-clamp recordings demonstrated that eight nAChR subunits (α3–7 and β2–4) are expressed on DAergic neurons in the VTA and SN[25], and the combination of some these subunits with α6 subunit can form several subtypes of nAChRs. One of them possesses a putative $\alpha4\alpha6\alpha5(\beta2)_2$ composition since the whole cell currents mediated by this kind of nAChR can be inhibited by both DHβE and α-CTX MII[25]. Immunoprecipitation results from the same group found that α4, α6, and β2 are the most abundant nAChR subunits in the striatum[16]. Thus, it is reasonable to believe that the composition of naturally expressed functional α6*-nAChRs is very complex.

As early as 1982, researchers began to study nAChRs heterologously expressed in *Xenopus* oocytes and demonstrated that functional nAChRs could be inserted in the oocyte membrane and that activity of functional nAChRs could be measured using voltage clamp recording[40, 41]. Since then, *Xenopus* oocytes have become one of the most practical and widely used systems to express and study the physiological and pharmacological properties of nAChRs[9, 15, 42–46]. It is quite difficult, however, to express functional α6*-nAChRs *in vitro*. Fifteen years later, Gerzanich and co-workers found that α6 may form detectable functional α6*-nAChRs when chicken α6 subunit is expressed together with human β4 subunit[15]. This was the first *in vitro* synthesized functional α6*-nAChRs, proving that the α6 is not the so called "orphan" subunit. Kuryatov *et al* have tested even more complex mixtures of α6 with several other nAChR subunits and found that the coexpression of α6, β4, and β3 subunit can produce the most efficient α6*-nAChRs with the largest and most consistent responses[44]. Meanwhile, a complex variety of functional α6*-nAChRs, including α6α3β2-nAChRs, α6α4β2-nAChRs, α6β2β4-nAChRs, α6β2α5-nAChRs, α6β4β3α5-nAChRs, α6β4-nAChRs, and chimeric α6/α3β2β3-nAChRs and α6/α4β2β3-nAChRs were synthesized[43–46]. But cotransfections of α6 and α3 without a β subunit can not yield functional receptors[26]. Interestingly, α6 subunit cotransfected with β2 and(or) β3 subunit either can form nAChR without gated ion channels thus can not further yield functional α6β2- or α6β2β3-nAChRs in *Xenopus* oocytes[15, 44], or can form α6β2-nAChRs with very poor function in transfected

cell line[26], even though these subunits can form different ligand binding sites with high affinity for Epi[44]. Thus far, *in vitro* synthesized functional α6*-nAChRs are very valuable preparations for development and testing specific α6*-nAChR antagonists[43, 45, 47]. However, *in vitro* synthesized functional receptors can only shed light on the possibilities of the *in vivo* receptors because complex subunit combinations of naturally expressed α6*-nAChRs that exist *in vivo* are not easily recreated in *in vitro* systems.

In the past decade, several research groups have tried to further define the possible compositions of α6*-nAChRs naturally expressed in neurons. Le Novere and co-workers reported the extensive colocalization of α6 and β3 subunits and were the first to propose the existence of heteromeric α6*-nAChRs containing both α6 and β3 subunits in catecholaminergic nuclei[23]. Further definition of the exact subunit compositions of naturally-expressed functional α6*-nAChRs remains challenging. Studies on rats and both wild type and several types of nAChR subunit-null mice (*eg* α4−/−, α6−/−, α4−/−α6−/−, and β2−/−), using combined single-cell RT-PCR, patch-clamp recording, *in vivo* microdialysis, and immunoprecipitation techniques, have demonstrated that a putative $\alpha4\alpha6\alpha5(\beta2)_2$ composition is present on the somata of DAergic neurons in the SNc and VTA[25]. Meanwhile, two types of α6*-nAChRs (α6β2*-nAChRs and α4α6β2*-nAChRs) are expressed in DAergic neuronal terminal fields located in the striatum[16]. Salminen and colleagues further demonstrated that the presence of presynaptic α-CTX MII-sensitive nAChRs (α6*-nAChRs) on DAergic nerve terminals in striatal synaptosomes plays important roles in mediating DA release. The more interesting finding is that in β2-null mutant mice(β2−/−), the α-CTX MII-sensitive DA release completely disappeared, while only 50% decrease of α-CTX MII-sensitive DA release resulted from β2+/− mutation, which indicates that the β2 subunit is an indispensable component for the α-CTX MII-sensitive nAChR-mediated DA release[37]. Additional studies found that deletion of β3 or α4 decreased the α-CTX MII-sensitive component of DA release by 76% or 55%, respectively. Neither β4 nor α7 gene deletion significantly altered α-CTX MII-sensitive DA release[37]. These results suggest that β3 and α4 rather than α7 and β4 subunits play important roles in forming naturally expressed functional α6*-nAChRs (α6β3β2 and α4α6β3β2) on DAergic presynaptic terminals[37]. The compositions of α6*-nAChRs in chick retina (from 1-day old chicks) are quite different from that in catecholaminergic nuclei. For example, one study showed that only a minor subpopulation of α6*-nAChRs (7.5%) contain the β2 subunit, but almost all of the α6 receptors contain the β4 subunit in chick retina[48]. The

presence of a mixture of different populations of α6*-nAChRs (surely α6β4; probably α6β4β3, α3α6β4, and/or α3α6β3β4) is found in chick retina[48]. However, Moretti *et al* reported that α6*-nAChRs expressed in rat retina (postnatal 21d) mainly contain the α6β3β2, α6α4β3β2, and α6α3α2β3β2 subtypes[49].

We have described the known diversity of naturally expressed functional α6*-nAChR subunit compositions, which vary in different brain regions or even within the same tissue from different species or different developmental periods within the same specie. This indicates the complex roles of α6*-nAChRs in physiological and perhaps in pathological states. As a result, the improving knowledge of subtype composition of α6*-nAChRs will be of considerable importance for development of selective and specific α6*-nAChR agonists and antagonists.

Analogs of α-conotoxins are subunit-selective antagonists of α6*-nAChRs

It is still a challenge to develop selective agonists and antagonists for α6*-nAChRs due to the complex subunit combinations of naturally expressed α6*-nAChRs and poor function in heterologous expression systems. Until now, the only reported selective agonist for α6*-nAChRs is TC 2429, which is a full agonist with 3-fold more selectivity at α6β2*-nAChRs compared to nicotine[39]. But α6*-nAChRs can be selectively inhibited by several analogs of α-conotoxins[25, 39, 43, 45]. Conotoxins can be divided into at least four superfamilies (A, M, O, and S) based on a conserved signal sequence and a characteristic disulfide framework that is distinct from the other superfamiles[50]. α-conotoxins, which are competitive nAChR antagonists, belong to the largest family of peptides in the A superfamily[50]. Several α-conotoxins are pharmacologically useful for distinguishing nAChR subtypes. For example, α7*-nAChRs can be inhibited by selective antagonist α-CTX ImI[51], α3β4*-nAChRs by antagonist α-CTX AuIB[52], and α6*-nAChRs by selective antagonist α-CTX MII and α-CTX PIA[39, 45].

Electrophysiological experiments using *Xenopus* oocytes expressing mammalian neuronal nAChRs have demonstrated that α-CTX MII is a novel, potent, selective, and competitive antagonist for α3β2-nAChRs, which can reversibly block acetylcholine (ACh)-induced inward currents at very low concentration (IC$_{50}$ is 0.5 nmol/L)[53]. α-CTX MII is the first α-conotoxin known to target neuronal α3β2-nAChRs[53]. However, α-CTX MII is now widely used as a selective α6*-nAChR antagonist, especially in midbrain DAergic system because: (1) there is high structural similarity between the α3 and α6 subunits, 61%[17, 23] to 80%[47] residue identity in their

extracellular ligand-binding domains, and critical residues responsible for interaction with α-CTX MII[54] are conserved; (2) α6*- but not α3*-nAChRs are highly expressed in mid-brain DAergic system: studies demonstrated that α6 labeling is almost 20-fold more intense than α3 labeling in DAergic neurons[23], while α3 subunit only accounts for 2% of ³H-Epi binding sites at the DAergic terminal levels[16]. Moreover, electrophysiological recordings and *in vivo* microdialysis experiments showed that the inhibitory effect of α-CTX MII on ACh-induced inward currents and nicotine-induced DA release disappear in α6⁻/⁻ mice[16]; and (3) α-CTX MII is a selective antagonist for naturally expressed α6*-nAChRs: high-affinity [¹²⁵I]α-CTX MII binding sites are well pre-served in α3-nAChR subunit knockout mice[55], but can not be detected in α6-nAChR subunit knockout mice[16, 27]. Therefore, α-CTX MII, even at a high concentration, *eg* 100 nmol/L, can be used to study the function of α6*-nAChRs in midbrain DAergic neurons[39]. Recently, scientists dis-covered a novel α-conotoxin, α-CTX PIA, which has higher affinity and selectivity for α6* than α3* -nAChRs, exhibiting ~75-fold lower IC_{50} for α6/α3β2β3-nAChRs than for α3β2-nAChRs[45]. α-CTX PIA is able to specifically distinguish α6*-nAChRs from α3*-nAChRs due to its lower affinity for α3*-nAChRs. Another α-conotoxin, α-CTX BuIA, displays strong antagonistic effect on chimeric α6/α3β2β3 and α3β2-nAChRs[56, 57] with an IC_{50} of 0.26 nmol/L and 5.7 nmol/L for α6/α3β2β3-nAChRs and α3β2-nAChRs, respectively[57]. All of these α-conotoxins have the ability to selectively dis-criminate α6*-nAChRs from a variety of nAChR subtypes. In addition, the chemical structure of α-conotoxins (only 12–19 amino acids) is relatively simple (with highly conserved nature of the cysteine residues and conserved proteolytic process-ing sites), which has allowed them to be isolated and their structure sequences identified and synthesized[45]. Scientists are trying to modify the natural structure and synthesize new analogs of α-conotoxins. For example, substitution of Leu15 with Ala, Glu11 with Ala, and Ala for His9 shifts the selectiv-ity of α-CTX MII toward α6*-nAChRs, with approximately 37-, 54-, and 75-fold higher preference for α6*- than for α3*-nAChRs[38], respectively. In addition, Azam *et al* have suc-cessfully designed and synthesized α-CTX MII[S4A, E11A, L15A], which displays more selectivity for α6*-nAChRs with an IC_{50} of 1.2 nmol/L, and much lower affinity for α3β2-nAChRs with an IC_{50} of 1400 nmol/L[43].

In conclusion, several isolated natural and chemically-syn-thesized analogs of α-conotoxin have been used as powerful α6 subtype-selective antagonists to investigate physiological and pharmacological properties of the *in vitro* synthesized- and the *in vivo* naturally-expressed functional α6*-nAChRs. Such

pharmacological tools will, undoubtedly, be of considerable benefit to further understanding of α6*-nAChR function and pharmacology. A more detailed review by Azam *et al* of the applications of α-conotoxin analogs for nAChR studies can be found in this issue.

The potential role of presynaptic α6*-nAChRs in nicotine reward and dependence

The mesocorticolimbic system including VTA DAer-gic neurons and their projection areas is postulated to play a crucial role in regulation of cognitive functions, reward-based learning, and addiction[58–60]. Numerous nAChRs are expressed in the VTA and some of them are located extra-synaptically on somatodendritic regions and on pre-synaptic terminals. Somatodendritic nAChRs modulate neuronal excitation via membrane depolarization and can initiate short- and long-term changes by interfacing with Ca^{2+} signaling pathways or the firing pattern of DAergic neurons that determines the release of DA in the terminal regions[61, 62]. The modulation of neurotransmitter release by pre-synaptic nAChRs is one of the most well-investigated effects of nicotine in the CNS[33]. Activation of pre-synaptic nAChRs increases the release of many different neurotrans-mitters[33, 39, 61, 63, 64]. Exogenously-applied nicotinic agonists can enhance, while nicotinic antagonists often can diminish the release of ACh, DA, norepinephrine, serotonin, as well as glutamate and GABA[65]. The activation of pre-synaptic nAChRs initiates directly or indirectly intracellular Ca^{2+} signals that potentiate neurotransmitter release through the following mechanisms: (1) a small, direct Ca^{2+} influx via nAChR activity[66–68] that (2) may trigger Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores[69], and (3) the activa-tion of nAChRs further causes membrane depolarization that activates voltage-gated Ca^{2+} channels in pre-synaptic terminals[70]. The overall effect is that pre-synaptic nAChR activity elevates Ca^{2+} levels in presynaptic terminals, in turn leading to an increase in neurotransmitter release.

The activation of nAChRs on VTA DAergic neurons by exogenously-applied nicotine results in increased DA release in the nucleus accumbens (NAc), which probably plays a key role in nicotine addiction[71, 72]. Studies indicate that dif-ferent subtypes of pre-synaptic nAChRs participate in the modulation of neurotransmitter release. It is supposed that by acting on presynaptic α7-nAChRs (desensitized less than non α7-nAChRs) located on glutamatergic terminals, nico-tine at concentrations experienced by smokers can produce long-term enhancement of glutamatergic transmission in the VTA[73], whereas activation of presynaptic non-α7 recep-

tors (possibly α4β2-nAChRs) only can transiently enhance GABAergic transmission. These non-α7 nAChRs become significantly and quickly desensitized during long-term exposure to low concentrations of nicotine[74]. As a result, GABAergic terminals, rather than glutamatergic terminals, become insensitive to tonically released ACh from cholinergic afferents from the pedunculopontine tegmental nucleus (PPTg) and laterodorsal tegmental nucleus (LDTg)[75, 76], which will in turn lead to long-term activation of glutamatergic input accompanied with depression of GABAergic input to VTA DAergic neurons that is experienced by tobacco smokers. Collectively, the differential desensitization properties of these two nicotinic receptor subtypes probably explain why low concentrations of nicotine tends to drive the activity of VTA DAergic neurons toward long-term excitation that underlie the course of nicotine addiction process[77]. Thus, *in vivo* experiments observed that a single exposure to nicotine increases DA release in NAc from VTA for more than one hour[78, 79].

Our current studies demonstrate that there are functional α-CTX MII and PIA sensitive nAChRs (α6*-nAChRs) located on GABAergic pre-synaptic boutons synapsing onto DAergic cell bodies in the VTA. Activation of these α6*-nAChRs by nicotinic agonists results in increased inhibitory postsynaptic currents (IPSCs) measured at the DAergic cell body using patch-clamp recordings. A 4-minute pretreatment with smoking-relevant concentrations of nicotine desensitizes rather than activates α6*-nAChRs and abolishes ACh-induced increases in spontaneuos IPSCs(sIPSCs) [80]. The results demonstrate that functional α6*-nAChRs are expressed on presynaptic GABAergic boutons in the VTA and likely play a critical role in mediating cholinergic modulation of GABA release. Their desensitization during chronic nicotine exposure may contribute to a disinhibition of VTA DAergic neuronal activity and enhanced DA release. Our findings suggest that α6*-nAChRs play important roles in nicotine-induced reinforcement through the modulation of GABAergic control on VTA DAergic neurons[80]. The observations are in good agreement with previous reports. An *in vivo* study of nicotine-induced increase in locomotor activity in a habituated environment found that 1 week administration of α6 antisense oligonucleotides (directed against the α6 subunit) by osmotic mini-pump suppresses 70% of the nicotine effect, which strongly suggests that enhanced locomotor activity elicited by nicotine is mediated at least in part via α6*-nAChRs[17]. Studies using striatal synaptosomes demonstrated a preponderant role of α4α6β2*-nAChRs in mediating the α-CTX MII-sensitive part of nicotine-elicited DA release[16], but the inhibitory effect of α-CTX MII on nicotine-induced DA release in α6 subunit knockout mice was

no longer observed[16]. α6*-nAChRs in the NAc also play a dominant role in DA release in an action potential frequency-dependent manner[81], which is the first direct evidence of the dominant role of α6*-nAChRs in dynamic filtering (frequency-sensitive regulation of DA neuronal activity and terminal DA release) of action potential-dependent DA release in the NAc. In addition, using patch clamp recordings in brain slice preparations from gain-of-function α6*-nAChR mice, Drenan et al demonstrated that in α6 transgenic mice, the α6*-nAChRs expressed on VTA DAergic neurons are ~10-fold more sensitive to nicotine than in locus coeruleus[39], which suggests that functional α6*-nAChRs can be detected on somatodendritic region of DAergic neurons after fuctional enhancement of α6*-nAChR expression. These results suggest that the up-regulation of functional α6*-nAChRs in the mesocorticolimbic system, such as the VTA and NAc, produces a hyperdopaminergic state that may play a critical role in nicotine dependence[82]. Meanwhile, nicotine self-administration investigated by Pons *et al*, using α6 and α4 knockout mice, highlighted the crucial roles of both α6*- and α4*-nAChRs in nicotine reinforcement[83]. It has been suggested that α6*- and α4*-nAChRs can modulate action potential evoked DA-release from either a low action potential threshold or a higher action potential threshold DAergic fiber, respectively[84]. Thus, nAChRs may exert their roles through 'filtering' action, which will lead to an increase in contrast in DA signals by switching the firing pattern of DAergic neurons from tonic activity to high frequency, reward-related burst activity, thus facilitating the reinforcement properties of nicotine[85, 86].

Taken together, these studies suggest that nicotine at concentrations present in the plasma of tobacco smokers preferentially desensitizes both presynaptic α6* and α4β2-nAChRs on GABAergic neurons, whereas, nicotine has minimal desensitization effects on presynaptic α7-nAChRs located on glutamatergic terminals. Thus, endogenously released ACh can facilitate midbrain glutamate, but not GABA release, which leads to an increase in glutamate mediated excitatory inputs onto DAergic neurons in the VTA. As a result, nicotine induces both disinhibition and direct excitation of DAergic neurons, leading to an increased DA release in NAc (Figure 2).

The potential role of presynaptic α6*-nAChRs in Parkinson's disease

Parkinson's disease (PD) was first described as Shaking Palsy in 1817 by the British physician James Parkinson. PD is one of the most common progressive neurodegenerative disorders in the United States, affecting about one million people,
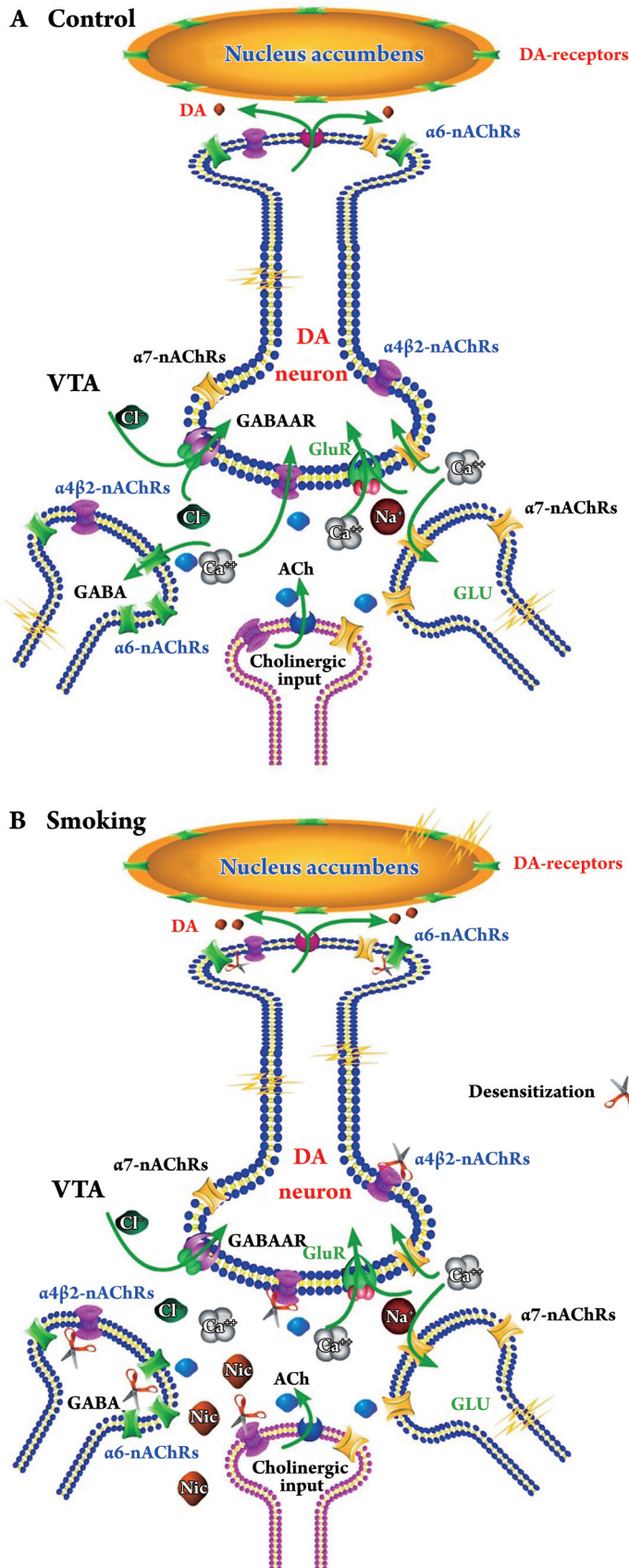
Figure 2. Simplified schematic diagram of the roles of nAChRs in the nicotine addiction process. In the VTA, α6*- and α4β2-nAChRs are located on GABAergic terminals and provide inhibitory inputs onto DAergic neuons, while α7-nAChRs are located on glutamatergic terminals and activation of these receptors enhances glutamate release and increases excitability of DAergic neurons. Endogenous ACh released from cholinergic terminals projected from PPTg and LDTg can modulate the excitability of both GABAergic and glutamatergic terminals. A: Under control conditions, endogenous ACh can activate α6*- and α4β2-nAChRs on GABAergic terminals and α7-nAChRs on glutamatergic terminals. Thus postsynaptic DAergic neurons will receive balanced inhibitory and excitatory inputs. B: In smoking conditions, α6*- and α4β2-nAChRs, rather than α7-nAChRs, are desensitized rapidly after chronic exposure to low concentrations of nicotine, thus inhibiting GABAergic inhibitory inputs (disinhibition). But endogenous ACh can still significantly enhance glutamatergic inputs onto the DAergic neurons. As a result, the increased excitation of DAergic neurons will result in a net increase in DAergic neuron firing and more DA release in NAc.

with more than 50 000 new diagnosed cases each year[87]. The pathogenesis of PD typically is slow-paced but relentlessly progressive loss of DAergic neurons in the nigrostriatal DAergic system in the ventral midbrain[88]. Our understanding of the etiology of PD is still limited. Therefore, interventions to slow, halt or reverse the progression of the disease are crucial. Importantly, epidemiological studies indicate that cigarette smoking offers some protection against developing PD, as smokers with the longest duration of smoking and the highest daily consumption of cigarettes have the lowest PD risk[89], which indicates that the protective effects of nicotine are dose- and time-dependent, and the protective effects wane after smoking quit. In addition, results of both prospective and retrospective studies demonstrate that the decreased incidence of PD in smokers does not appear to be due to the increased smoking-related mortality[90–93].

Accumulating lines of evidence indicate that smokers may have a lower incidence of PD through the following possible mechanisms: (1) nicotine directly activates nAChRs expressed on nigrostriatal DAergic neurons, stimulating DAergic neurons to release more DA[94, 95], which could partly overcome the nigrostriatal DAergic dysfunction in the disorder; (2) *in vitro* and *in vivo* studies suggest that nicotine exposure is neuroprotective against glutamate excitotoxicity, ischemic damage, and DAergic neurotoxic compounds such as 1-methyl-4-phenyl-1-,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine, paraquat, and methamphetamine[90], thus producing a neuroprotective effects[96, 97]; (3) nicotine may exert its neuroprotective effects on DAergic neurons through anti-inflammatory actions via decreased microglial activation[98], which appears to play a possible role in initiating or amplifying DAergic neuronal injury[99-101]; (4) nicotine administration can significantly ameliorate PD symptoms such as tremor, rigidity, bradykinesia, and gait disturbance including frozen gait[102–104] and attenuate levodopa-induced dyskinesias[90, 105]; and (5) nicotine may also act through non-receptor-mediated actions by decreasing ROS generation and oxidative stress and promoting mitochondrial function[106–108]. Collectively, evidence suggests that nicotine mainly produces its beneficial effects on PD through nAChRs expressed in nigrostriatal DAergic system. In order to gain further insight into the nAChR subtypes involved in modulating DAergic function and characterization of changes in their expression with nigrostriatal damage, the development of PD therapies that slow or prevent the disorder by using nAChR ligands could be a useful strategy.

Experiments carried out on rodents, primates, and humans have shown that α6*-nAChRs and α4β2*-nAChRs are the major nAChR subtypes in the nigrostriatal system[16, 37, 109–114], while α7-nAChRs are expressed to a lesser extent[110, 111, 115].

Nicotine may act through α6*-nAChRs mainly expressed on DAergic terminals and stimulate DA release from striatal synaptosomes[38]. More importantly, α6β2*-nAChRs, but not α4β2*-nAChRs, are significantly decreased and this decrease closely accompanies nigrostriatal DAergic deficits caused by paraquat treatment[116], suggesting that chronic nicotine administration may produce its neuroprotective effects on nigrostriatal DAergic neurons through the actication of a select population of α6*-nAChRs in mice[116]. The same phenomena is seen in both monkeys and humans based on Bordia and coworkers' finding that α6α4β2β3-nAChR is preferentially vulnerable to nigrostriatal damage in monkey treated with MPTP and patients with PD[111]. These observations indicate that α6*-nAChRs in the nigrostriatal DAergic system are promising targets for selective preventive treatment of PD. The development of selective α6*-nAChR ligands is attracting attention and will hold promise for PD therapies.

Conclusion

Neuronal nAChRs are richly expressed in the central nervous system, however, the α6*-nAChRs are found at the highest concentrations in both mesocorticolimbic and nigrostriatal pathways, and are particularly present in presynaptic nerve terminals. Functional α6*-nAChRs in mesocorticolimbic and nigrostriatal DAergic systems may play crucial roles in nicotine addiction and be of potential therapeutic value in PD. However, we are only beginning to understand the distribution of nAChR α6 subunit in neuronal networks and know very little about its physiological functions and pharmacological properties. At present, the daunting challenge is the development of α6*-nAChRs selective ligands for both basic research and future clinical treatment of human disorders in which α6*-nAChRs have been implicated, such as nicotine reinforcement and PD.

Acknowledgements

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