Venom based neural modulators (Review)

JIAO CHEN, XIAO-MING LIU and YUAN ZHANG

Department of Pediatric Internal Medicine, Xuzhou Children's Hospital, Xuzhou, Jiangsu 221002, P.R. China

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Abstract. Different types of neuronal nicotinic acetylcholine receptors (nAChRs) are expected to occur *in vivo*, most structure-activity relationship studies have been carried out for just a few neuronal subtypes. The present review enlightens current aspects of venom modulators of nAChRs. Important electronic databases such as PubMed or Google scholar were explored for the collection of latest studies in the field. Clinical and basic research has shown that cholinergic receptors play a role in several disorders of the nervous system such as chronic pain, Alzheimer's disease and addiction to nicotine, alcohol and drugs. Unfortunately, the lack of selective modulators for each subtype of nAChR makes their pharmacological characterization difficult, which has slowed the development of therapeutic nAChR modulators with high selectivity and absence of off-target side-effects. Animal venoms have proven to be an excellent natural source of bioactive molecules with activity against ion channels. The present review concludes that the presence of small-molecule nAChR modulators in spider venoms support the use of venoms as a potential source of novel modulators.

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Correspondence to: Dr Xiao-Ming Liu, Department of Pediatric Internal Medicine, Xuzhou Children's Hospital, 18 Sudibei Road, Xuzhou, Jiangsu 221002, P.R. China
E-mail: minglx@yeah.net

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1. Introduction

Recent studies indicate that imbalance of the cholinergic system in human brain via nicotinic acetylcholine receptors (nAChRs) are linked to various neurological disorders (1,2) while mutations in the nAChR can lead to frontal lobe epilepsy. These findings have prompted research into the development of drugs that target neuronal cholinergic receptors. Nevertheless, most of the chemical components under trial lack selectivity, or hardly improve the medical condition (3,4). The discovery of new therapeutic agents is currently a challenge because of the complex arrangement of subunits of neuronal cholinergic receptors expressed in human brain (5) and the fact that their roles under physiological and pathological conditions are not fully understood (6).

To facilitate studies of the pharmacology of nAChRs, a search for new nAChR modulators in spider and scorpion venoms was carried out using a high-throughput FLIPR assay to rapidly identify 'hit' venoms (7). The probability of finding native cholinergic modulators was expected to be high because arachnid venoms are estimated to contain approximately 1 million different components, and even though less than 0.01% of these molecules have been studied (8), many of them are peptidic toxins that act on diverse types of ionic channels (9).

2. Role of nAChRs in human disease

The role of ACh is as important as their molecular target, nAChRs (10). It is synthesised by the enzyme choline acetyltransferase that transfers an acetyl group from acetyl-CoA to choline. Its precursor, choline, is endogenously produced, although it is also absorbed in the small intestine to reach the levels required to accomplish several functions (11). For example, choline is a precursor for the biosynthesis of constituents of plasma membrane such as phosphatidylcholine and sphingomyelin (11). Furthermore, choline is a methyl group donor; and methylation is needed to regulate of expression of genes and mediate biosynthetic reactions. A deficiency in choline has been related to liver damage and impairment of cognitive functions in older people (12). Indeed, choline supplementation enhances cognitive functions in patients with schizophrenia (13). De-regulation of cholinergic function is linked to the function and expression of nAChRs. For instance, chronic administration of nicotine can induce an increase in the number of neurons containing α4β2 nAChRs in the glutamatergic subthalamic nucleus, a cerebral area related to

movement control (14), or downregulate the function of other subtype nAChRs. Below we discuss different pre-clinical and clinical evidence of the consequences of smoking, and links between de-regulation of nAChRs and major symptoms in neurodegenerative diseases.

Post-mortem studies in the brain of people who suffer from Alzheimer's and Parkinson's disease, epilepsy and schizophrenia showed that the density of α4β2 nAChRs is extremely low (15). The degeneration of cholinergic circuits was confirmed in human brains after the development of nicotine radioligands for imaging studies *in vivo*. For example, *in vivo* studies of brains of people suffering from Alzheimer's disease showed a decrease of α4β2 nAChRs and the density of nAChRs in arteries appeared to be affected as well (16). In patients suffering from Alzheimer's and Parkinson's disease, there is a notable loss of cholinergic projections into the cerebral cortex, and this has been considered as part of the reason for the impaired cognition observed in these patients, although the loss of the cortical cholinergic activity in people with Parkinson's disease is notable (17). In addition, depressive symptoms in patients with Parkinson's disease is strongly correlated to loss of α4β2 nAChRs in the neuronal frontal corticomesostriatal circuitry, while those patients with mild cognitive impairment were associated with low levels of α4β2 in the neuronal frontal corticobasal ganglia-limbic-cerebellar circuitry that includes some areas of the former circuitry. Furthermore, α4β2* nAChRs are widely distributed in the brain and are not restricted to cholinergic or dopaminergic neurons. Thus, downregulation of this heteromeric subtype may lead to dysfunction of other neuronal circuitries (18). Cholinergic deregulation in the hippocampus may be associated with cognitive problems because this cerebral region is related to functions of learning and memory (19) and this correlation between dysfunction of neuronal circuitry and cognitive functions is observed in various neuronal disorders.

nAChRs have also been identified in immune cells, and upregulation of its activity in these cells has been linked to cardiovascular diseases such as atherosclerosis in smokers (20). Recent evidence suggests that the mechanism underlying this process could be activation of α7 nAChRs that trigger transcription of cell-adhesion proteins on endothelial cells (21). These proteins, such as E-selectin, favour the attachment of monocytes, and it might lead to abnormal formation of fibrous plaques in vascular smooth muscle cells. Notably, the duration and amount of nicotine administered via smoking are dependent variables, and when these reach critical values it may facilitate the activation of numerous nAChR subtypes that are linked to signalling cascades and cause abnormal proliferation of neuronal and non-neuronal cells (22).

3. Modulators of nAChRs as drugs and insecticides

Several clinical trials are being carried out to evaluate potential drugs that target nAChRs. For instance, some nicotine analogues are been used to facilitate quitting smoking (23). Varenicline (trade name Chantix®) is currently being used for treatment of nicotine addiction and is a partial agonist of the α4β2 and α3* receptors. However, varenicline also activates the α7 nAChR (24), and it has been suggested that its mechanism of action involves is by modulation of several nAChRs (25) that contributes to decreasing the levels of dopamine that were previously increased by nicotine in the limbic system. This in turn prevents reward response induced by smoking (26). Imaging studies *in vivo* of smokers under treatment with varenicline showed that α4β2* nAChRs are saturated after administration of the drug at low or high doses, and withdrawal effects were not detected. Unfortunately, varenicline can also cause adverse effects in smokers under treatment; nausea is very common and is one of the primary reasons for withdrawal for treatment (27). The side-effect is likely to be mediated by activation of ganglionic nAChRs. This is likely to be a common problem when using therapeutic agents that target multiple subtypes of nAChRs distributed across the peripheral nervous system.

As previously mentioned, nAChRs are also important insecticide targets, and the so-called neonicotinoid insecticides that target insect nAChRs are one of the most successful classes of chemical insecticides. Nicotinoid insecticides are derived from nicotine, the major alkaloid derived from leaves and stems of the tobacco plant *Nicotiana tabacum*; nicotine is a secondary metabolite that is used by the plant to defend against herbivorous insects (28). The neonicotinoids are chemically similar to nicotine but they have low toxicity to mammals as they were engineered to bind selectively to insect nAChRs (29). However, like the majority of chemical insecticides, most neonicotinoids are broadly active against a wide range of insect pests, including beneficial insects such bees (30). Honeybees are efficient pollinators that mediate plant reproduction, and therefore it would be highly desired to engineer insecticides that preferentially target nAChRs in insect pests but not beneficial insects such as pollinators or predators of the targeted pest. Matsuda *et al* (31) compared the complex formed between imidacloprid, the most successful neonicotinoid, and α2β1 nAChRs from the honeybee *Apis mellifera* and the green peach aphid *Myzus persicae*. They found a remarkable structural difference between the binding sites for imidacloprid in the two nAChRs, with a hidden and broader binding groove in the aphid nAChR compared with the bee receptor. The authors suggested that linking imidacloprid to a molecular fragment that fits the groove of the aphid nAChR could improve the selectivity for insect pests over bees. To summarise, neonicotinoids are generally safe for humans but they could be toxic for small vertebrates such as birds and beneficial insects such as honeybees.

The selectivity of some toxins for nAChRs has facilitated the development of fluorescent binding probes and radioligands for use in biological sciences and nuclear medicine (32). It has helped to determine the distribution of nAChRs in the brain of rodents and humans including in people suffering from Alzheimer's and Parkinson's disease. For example, α-bungarotoxin a 74-residue peptide isolated from venom of *Bungarus multicinctus*, was used to affinity purify the nAChR from the electric organ of *T. marmorata*. α-Bungarotoxin has also been useful for visualizing the distribution of nAChRs in the brain and in the electric organ from *Torpedo californica*, and for identifying clones expressing epitopes from nAChR in *Escherichia coli* (33). Recently, it was shown that Alexa Fluor 555-labelled α-bungarotoxin labels GABAA-Rs expressed in HEK-293 cells (32), and this binding is inhibited

by bicuculline, a competitive antagonist at GABAA-Rs, and *d*-tubocurarine, a non-selective nAChRs antagonist and a competitive antagonist at GABAA-Rs. This finding is not surprising because type-A GABAergic receptors are members of the pentameric Cys-loop ligand-gated ion channel family, like nAChRs (34). The selectivity of α-bungarotoxin has been used to discover new functional subunits of nAChRs, such as the α7 subunit of nAChRs. This is a receptor similar to the heteropentameric nAChR reported previously because α-bungarotoxin blocked its activation and was able to bind the subunit α7. Furthermore, iodinated α-bungarotoxin has been used in competition studies to evaluate the specificity of radioligands towards α7 nAChRs.

Nicotine radioligands are being used for imaging studies using positron emission tomography (PET) (35). Note that radioligands are typically used for imaging studies at sub-therapeutic doses and consequent safety rarely poses an issue. The first nicotine-based radioligand developed to label nAChR was [^{11}C]-nicotine. Other radioligands that are chemically-related to nicotine are still under development with some promising results, such as [^{18}F]-ZW-104 and [^{18}F]-AZAN-α. The first probe is a selective ligand for the β2 subunit of nAChR and has been tested pre-clinically. [^{18}F]-AZAN-α has been assayed in human studies with promising results (35). It reached the brain in <20 min after injection, and selectively bound the α4β2 subtype (36). In this study the specificity for α4β2 was evaluated through administration of varenicline, a selective partial agonist of α4β2 that is used to aid smoking cessation (37). The specificity of the radiotracer for the receptor was confirmed after the drug blocked totally the uptake of [^{18}F]-AZAN-α. A-85380 is another example of a radiotracer (38) that has high affinity for α4β2 nAChRs. It allowed mapping of the distribution of nAChRs in the brain of smokers and non-smokers. Furthermore, it has been used to confirm that expression of α4β2 in the brain of patients with Alzheimer's disease and dementia is significantly decreased, and is correlated with the degree of cognitive impairment (39).

The development of agents to be used for human brain imaging *in vivo* has been extended to design tracers selective for the α7 subtype. Some promising results has been obtained even where the homomeric α7 in the human brain is found in low concentrations compared to α4β2. For example, [^{18}F]-NS-10743 and [^{18}F]-ASEM showed high affinity toward α7 and the distribution of these radioligands were found in brain areas where α7 is densely expressed, such as frontal cortex and hippocampus. To prove the specificity of the agents, the specific ligand SSR180771, which is a selective and partial agonist at human and rodent subtype α7, was used in competition studies. The tracers showed their specificity for α7 when these were displaced by SSR180771. As discussed above, part of the problem with nAChR modulators as drug leads, insecticides and pharmacological tools is their lack of selectivity. This has made it difficult to dissect the role of nAChRs in different physiological processes as well as their role in pathophysiological processes such as cognitive dysfunction, depression, chronic anxiety, analgesia, inflammation and neurodegenerative diseases (40). Thus, there is still a great need to develop novel nAChR modulators that are both potent and selective. As discussed below, animal venoms have provided some of the most potent and selective nAChR modulators described to date and represent a potential source of new nAChR modulators.

4. Venom-derived modulators of nAChRs

Venoms from marine cone snails (genus *Conus*) have been extensively studied, although scarcely 0.1% of the predicted total number of venom compounds have been characterized so far (41). Conus venoms are replete with disulfide-bridged peptides, named conopeptides or conotoxins that target a wide range of voltage and ligand-gated ion channels, including nAChRs. Indeed, the venoms of marine cone snails are the largest natural source of potent and selective nAChR blockers (reviewed in ref. 42). There are at least seven families of α-conotoxins that bind specific muscle and neuronal nAChR subtypes with high selectivity (43). α-Conotoxins are the smallest conopeptides (generally 12-20 amino acid residues) that competitively binds to the ACh binding site of nAChRs. Their general framework is denoted CC-Xm-C-Xn-C, where C represents a cysteine residue and X corresponds to the two inter-cysteine loops that contain a variable region of amino acid residues. The number of amino acid residues is indicated as m/n and α-conotoxins with a 3/5 loop size combination are usually purified from the venom of *Conus* species that feed on fish and are typically active on mammalian neuromuscular nAChRs, while other conopeptides with 4/3, 4/4, 4/5, 4/6 and 4/7 loop combinations preferentially act on mammalian neuronal nAChRs (41,42).

Several studies of the interaction between α-conotoxins and AChBP have contributed to our understanding of how α-conotoxins can be selective for a particular nAChR (44). Basically, the different electrostatic interactions and hydrogen bonds between aromatic residues of α-conotoxins and amino acid residues outside of the ACh binding pocket are found in the conotoxins that block nAChRs while those conotoxins with fewer interactions are more likely to be agonists (45). These studies have also helped develop a structural model of the pharmacophore of α-conotoxins. It is delimitated by the two loops between the cysteine residues. One is conserved and determines the binding to the nAChR and the second loop consists of a variable sequence that determines their selectivity for a particular subunit of the nAChR (41,42).

5. nAChR modulators from other venomous animals

Snake venom is another large source of proteins and peptides that target nAChRs (46). The most abundant proteins described so far in elapid, colubrid and psammophid snakes are the three-finger toxins (3FTx) that contain between 60 to 74 amino acid residues with molecular masses ranging between 6000 and 8000 Da (47). Most 3FTxs target nAChRs. These toxins are divided into three types differentiated by the number of cysteines and the inter-cysteine spacing. Type I and type III α-neurotoxins with eight cysteine residues target neuromuscular nAChRs. Type II α-neurotoxins contain an extra pair of cysteine residues which form an additional disulfide bond that stabilises loop 2 (48). This additional disulfide bond facilitates interaction with neuronal α7 and α9-10 nAChRs, while maintaining affinity for the neuromuscular α1 nAChR. Another interesting group of 3FTxs are the so-called 'non-conventional

toxins' that have an additional disulfide bond that is not in the central loop II but instead in the N-terminal loop I (49). Examples of non-conventional toxins that block nAChRs are candoxin from *Bungarus candidus* and the weak toxin (WTX) from *Naja Kaouthia*.

6. Rationale for examining spider venoms as a source of novel nAChR modulators

Spiders are well known to produce venoms that contain diverse chemical structures such as peptides up to 10 kDa, proteins, amino acids and acylpolyamines (50). This complex and heterogeneous mixture of compounds allows spiders to prey on, and defend themselves against a wide range of prey and predators. The different repertoire of acylpolyamines appears to have evolved to allow predation of different species of invertebrates and small vertebrates.

Acylpolyamines could selectively block ionotropic glutamate receptors from insects and vertebrates including mammals (51). Because glutamate is the main neurotransmitter at the neuromuscular junctions in insects, block of glutamatergic receptors by acylpolyamines induces paralysis. However, acylpolyamine toxins are also reported to antagonise nAChRs. The basic backbone of acylpolyamines is an aromatic acyl group and the polyamine chain that form the essential part of the molecule. These two features are generally present in acylpolyamines derived from funnel-web, trap door and tarantula spiders (52). The length of the polyamine chain and the degree of hydrophobicity determines the affinity for nAChRs. Recently, a toxin denoted VdTX-1 was purified from the venom of the Brazilian theraphosid spider *Vitalius dubius* (53). This component has a molecular weight of 728 Da and it non-competitively blocks nAChRs. Surprisingly, this toxin was photosensitive, which might be why this toxin, and perhaps related toxins in other spider venoms, was not identified in previous studies.

7. Conclusions

There is clearly some evidence for the presence of small-molecule nAChR modulators in spider venoms, suggesting that these venoms could be explored more systematically as a potential source of novel modulators.

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