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Review

Neuronal nicotinic receptors as novel targets for inflammation and neuroprotection: mechanistic considerations and clinical relevance

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A number of studies have confirmed the potential for neuronal nicotinic acetylcholine receptor (NNR)-mediated neuroprotection and, more recently, its anti-inflammatory effects. The mechanistic overlap between these pathways and the ubiquitous effects observed following diverse insults suggest that NNRs modulate fundamental pathways involved in cell survival. These results have wide-reaching implications for the design of experimental therapeutics that regulate inflammatory and anti-apoptotic responses through NNRs and represent an initial step toward understanding the benefits of novel therapeutic strategies for the management of central nervous system disorders that target neuronal survival and associated inflammatory processes.

Keywords: neuroprotection; inflammation; nicotinic receptors; second messengers
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Introduction

The role of neuroprotection may be central to the management of conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis, epilepsy, and ischemic optic neuropathy, as well as cerebrovascular disorders, traumatic brain injury, spinal cord injury, and retinal degeneration. Many of the underlying mechanisms responsible for damage to neural tissues are believed to be similar in a number of these conditions. Over 500 products have been investigated for neuroprotective effects, including those from the following categories: free radical scavengers, anti-excitotoxic agents, apoptosis inhibitors, anti-inflammatory agents, neurotrophic factors, and ion channel modulators. Extensive work assessing neuronal nicotinic acetylcholine receptor (NNR)-mediated neuroprotection supports a ubiquitous and broad role for NNRs in regulating the various pathways involved in cell survival, apoptosis, and death.

Inflammatory diseases affect more than 500 million patients in the major pharmaceutical markets, and the total prevalence is growing. Cytokine therapeutic agents play a major role in the treatment of these diseases. All existing or emerging therapies, including the T-cell modulators efalizumab and alefacept and the lymphocyte modulator abatacept, target specific cytokines, but none is directed toward several cytokines at once. Three recombinant anti-TNFα agents (etanercept, infliximab, and adalimumab) are currently used in the US for the treatment of inflammatory diseases such as rheumatoid arthritis (RA) and Crohn's disease (CD), and more than twenty other recombinant products that target 11 different cytokines are in clinical development for conditions such as RA, psoriasis, CD, asthma, multiple sclerosis, and osteoarthritis. In addition to inflammatory bowel disease, osteoarthritis, and sepsis, a number of clinical trials have also evaluated the role and contribution of anti-inflammatory agents in neurodegenerative diseases and other forms of dementia[1–4]. In addition, major public health conditions have been or are now being linked to insidious chronic inflammation. The "cholinergic anti-inflammatory pathway" and its role in immune responses and inflammatory cascades have attracted enormous interest due to the obvious relevance to a variety of debilitating human diseases, including atherosclerosis, diabetes, neurodegenerative diseases, osteoarthritis, sepsis, chronic obstructive pulmonary disease, and inflammatory bowel disease. Several clinical trials have also evaluated the role and contribution of anti-inflammatory agents in neurodegenerative diseases and other forms of dementia, potentially linking anti-inflammatory events with

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neuroprotective mechanisms[4, 5].

NNR and neuroprotection

NNRs are heterogeneous in biological systems, partly as a consequence of the genetic diversity of subunit-encoding genes, the variable stoichiometry of the pentameric structure, the intrinsic biophysical properties of the resulting ligand-gated ion channel, and the cell-dependent coupling to secondary and tertiary messenger systems. Nine of the sixteen human genes that encode the subunits comprising the pentameric structures are expressed exclusively in the human brain, with predominant, but not exclusive, presynaptic localization of the pentameric receptor, which is implicated in the heterologous modulation of various neurotransmitters. Presynaptic nicotinic acetylcholine receptors (nAChRs) heterotypically modulate the release of non-cholinergic chemical messengers such as GABA, glutamate, serotonin, norepinephrine, dopamine (DA) growth factors, and various cytokines[6].

The α4β2 and α7 NNR subtypes are the most abundant nicotinic receptor subtypes in the mammalian brain. Both appear to play a major role in cognitive processes such as learning and memory. Nicotinic receptors are found throughout the brain and have been shown to modulate multiple neuronal pathways involved in schizophrenia. Recent observations demonstrate that the gene products for the α4 and β2 subunits, which comprise the predominant mammalian brain NNR subtypes, can form heterogeneous targets[7, 8]. Human α4β2 NNRs expressed in transfected cell lines, as well as those expressed *in vivo*, are present as a mixture of two stoichiometries, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$. The former displays high sensitivity (HS), while the latter exhibits low sensitivity (LS) to agonist activation. The calcium permeability and affinity for nicotine of these two stoichiometries have also been shown to differ. The LS subtype has a lower affinity for nicotine and acetylcholine and displays high calcium permeability. Conversely, the HS subtype has a greater affinity for nicotine and acetylcholine and exhibits lower calcium permeability[9]. Although co-expression of the two isoforms in brain has been shown[10] their specific roles in biological processes remain unknown. To date, studies evaluating neuroprotection have yet to provide clear evidence identifying the isoform that is present during biological insult and following NNR-mediated recovery. Either the α4, β2, or α5 subunit may represent the fifth subunit partner in the α4β2* protein target. Further references to α4β2*below reflect this uncertainty.

Convergence from many unrelated research areas has identified a primary role for one of the most abundant subtypes, the α7 nAChR, which is expressed in both the CNS and autonomic nervous system, in health and disease. Specifically, α7 NNRs are located in the hippocampus, thalamus, prefrontal cortex, subcortical basal ganglia, dopaminergic neurons in the ventral midbrain, and raphe serotonergic neurons. This nAChR subtype has been the subject of intense scrutiny in recent years, and it is becoming clear that it plays ubiquitous roles that range from cognitive processes to modulation of specific neurotransmitters and neuroprotection following various insults ranging from chemical toxicity to β-amyloid-induced cell death, normalization of sensory gating in schizophrenic patients and, more recently, as a central regulator of the inflammatory process. Similarly, a role for α4β2 in cognitive processes and neuroprotection has emerged, suggesting either redundant pathways within the same neurons or cell-specific expression of NNRs that regulate cell survival. Additional evidence, although limited, has suggested a potential role for α6β2* in protecting against nigrostriatal damage in mice. A number of excellent reviews have addressed various aspects of NNR-mediated neuroprotection [11–14] and have provided an exhaustive review of the literature, including references to some of the early pioneering work. In the present review, I attempt to restrict references to the most recent work, with particular emphasis on the body of work dedicated to understanding the molecular and cellular pathways involved in α7 and α4β2* NNR-mediated neuroprotection and anti-inflammatory potential.

Preclinical evidence for neuroprotection

The potential for neuroprotection is supported by numerous *in vitro* studies demonstrating NNR-mediated protection of cells from a variety of toxic challenges, including brain injury[15], oxygen-glucose deprivation[16–18], oxidative stress[16], β-amyloid toxicity (reviewed in[14]), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) kainite and glutamate excitotoxicity[19–21], ethanol exposure[22], and nerve growth factor (NGF) deprivation[23–26]. Similarly, a number of studies using *in vivo* models have demonstrated neuroprotection by the reduction of β-amyloid expression in the APPsw mouse (a transgenic model of AD)[27], surgically induced neuronal loss through nucleus basalis lesions[28], chemically induced neurotoxicity mediated by MPTP toxicity and systemic kainic acid-induced excitotoxic effects (reviewed in[19, 24]), glutamate toxicity in the spastic Han-Wistar rat[29], cerebral ischemia-reperfusion[30], chronic ischemia models, and paraquat toxicity[31]. Interestingly, transient forebrain ischemia, which is ameliorated through NNR

pathways, is associated with hyperphosphorylation of tau proteins in the hippocampus[32]. These broad neuroprotective effects following diverse insults can be recruited through several subtypes of the nicotinic receptor family of ligand-gated ion channels: the α4β2 nicotinic receptor[17, 33–35] and the α7 nicotinic receptor[13, 16, 18, 19, 36–40]. Cytisine, a partial agonist of α4β2 and a full agonist of the α7 nicotinic receptor, is protective against beta amyloid toxicity in rat cortical neurons[34].

The α7 NNR

In cultured cells, nicotinic agonists demonstrate neuroprotection against β-amyloid toxicity[37, 39, 41]. In addition, chronic administration of nicotine to APPsw mice for 5.5 months dramatically reduced β-amyloid plaque expression[27]. In follow-up studies, Nordberg's group reported that nicotine treatment reduced insoluble forms of β-amyloid by 80%[23], reduced GFAP-reactive astrocytes around plaques, and increased levels of the synaptic marker synaptophysin in as few as 10 days in the APPsw mice[42]. These data are relevant in that reduction of β-amyloid with anti-Aβ-antibodies leads to rapid recovery of associated neuritic dystrophy in living animals[43]. Furthermore, early cognitive deficits correlate with intracellular β-amyloid accumulation in 3×Tg-AD mice, and clearance of β-amyloid accumulation with immunotherapy reverses the early cognitive impairment[44]. Chronic administration of nicotine to 3×TG-AD mice (5 months via the drinking water) resulted in elevated levels of tau pathology[45]. No significant changes, however, have been observed in the tau pathology of humans chronically exposed to nicotine when compared to age-matched non-smokers[46–48]. In this respect, the 3×TG-AD mouse model may hold limited relevance to the effects of nicotine in humans. Shaw *et al*[39] were the first to report that tyrosine phosphorylation of JAK2, which subsequently forms a complex with α7, was the initial transducing step prior to activation of PI3 kinase and Akt phosphorylation. The α7-JAK2 pathway was later confirmed *in vivo*[49] and linked to the STAT3 pathway, suggesting an overlap between neuroprotective and anti-inflammatory pathways at the level of JAK2. β-amyloid binding to the α7 NNR subunit in hippocampal slices exposed to oxygen glucose deprivation has been shown to be necessary, as the neuroprotective effect of nicotine was lost in α7 knock-out mice[16]. Further studies in a mouse model of Alzheimer's disease have demonstrated clear inhibition of β-amyloid deposition and aggregation, and that these effects are mediated through a pro-survival cascade involving MAPK, Bcl-2, and NF-κB[13]. This cascade is activated by the α7 nicotinic receptor and is inhibited by β-amyloid[39]. The direct interaction of β-amyloid with the α7 nicotinic receptor is now well documented and has been demonstrated using electrophysiological and biochemical approaches. The interaction of nicotine with the α7 nAChR inhibits the interaction of Aβ(1–42) with the same receptor, and Aβ(1–42)-induced apoptosis is prevented by nicotine-induced activation of JAK2[37, 39]. These effects can be shown by measuring markers of cytotoxicity, including cleavage of nuclear protein poly(ADP-ribose) polymerase (PARP), induction of caspase 3, or cell viability. In addition, TC-1698 [2-(3-pyridyl)-1-azabicyclo[3.2.2]nonane], a novel α7-selective agonist, exerts neuroprotective effects via activation of the JAK2/PI-3K cascade[37]. Cross-talk between β-amyloid toxicity and tau hyperphosphorylation has also been demonstrated. Aβ1–42 can potentiate hyperphosphorylation of tau proteins in cell lines, as well as in transgenic mice. Aβ1–42-induced tau phosphorylation and increases in GSK-3β phosphorylation were attenuated by selective α7 nicotinic ligands, and these effects were blocked by the antagonists methyllycaconitine and α-bungarotoxin[50].

Neuroprotective effects against amyloid-β toxicity by an α7-nAChR "antagonist"[51] also suggest involvement of mechanisms beyond the activation of channel opening. This indicates that the neuroprotective effects are associated with drug influences on messenger transduction and downstream signaling cascades. It is also possible that these effects are unrelated to channel function and changes in membrane potential.

The α4β2* NNR

A number of studies, however, have demonstrated that α4β2* neuronal nicotinic receptors function independently of α7 to induce neuroprotection and contribute to neuronal survival *in vivo*. Glutamate-induced neurotoxicity in primary cultures of rat cortical neurons[21] and hippocampal slices[52] was prevented in a time- and concentration-dependent manner by the α4β2* subtype. Evidence for this was shown using selective ligands and dihydro-beta-erythroidine, an α4β2* nAChR antagonist, whereas the α7 nAChR antagonist α-bungarotoxin had no effect. Furthermore, the neuroprotective effects required extracellular Ca^{2+}. Similar neuroprotective effects are observed during glutamate-induced excitotoxicity in adult pig retinal ganglion cells[20], suggesting potential opportunities for ophthalmic conditions. Transgenic mice have provided additional support for an independent role of α4β2* in mediating neuroprotection. Zanardi *et al*[53] found that mice lacking the β2 subunit demonstrate

increased susceptibility to hippocampal excitotoxic insult and diminished cognitive potential. Aged mice that lack the β2 neuronal nicotinic receptor subunit exhibit increased neuronal atrophy in the cortex and hippocampus[54]. In addition, compounds that are selective for α4β2* over α7 are neuroprotective *in vitro*. ABT-089, for example, is protective against glutamate toxicity in IMR-32 cells and rat cortical neurons[55]. Several Targacept compounds that are selective for α4β2* over α7, such as TC-1734, TC-2559, and TC-2403, are also protective against glutamate toxicity in cultured cells[17, 56], and these effects are blocked by the an α4β2* antagonist DHβE[34]. In cultured nigral dopaminergic neurons, nicotine partially protects against MPP^+ toxicity. This effect was blocked by the nicotinic antagonist d-tubocurarine, but not by the α7 antagonist α-bungarotoxin[57].

There is considerable evidence for nicotinic neuroprotection in several *in vivo* models of PD. Nicotine is neuroprotective against 6-OHDA lesions of the nigrostriatal tract[58–62] only at low doses[60, 63]. Nicotine also appears to be more effective against partial, but not complete dopaminergic lesions and when administered both before and after toxic insult[24, 59]. A-85380, SIB-1508Y (a β2-preferring agonists), TC-2403 (an α4β2 selective agonist), SIB-1553A (a β4-preferring agonist), and two selective α7 agonists were tested for neuroprotective effects in partial 6-OHDA lesion-induced rats[62]. Of the selective compounds, only A-85380 was effective alone, but it was not as effective as nicotine[24, 62]. At least one of the α7 compounds tested, however, was more potent at inducing desensitization than activation[64]. In addition, TC-2403 is known to undergo rapid first pass metabolism[65], and so it is likely that sufficient quantities at doses of 0.2 and 0.4 mg/kg failed to enter the brain and were therefore unable to provide any neuroprotective effect. In α4 knock out mice, nicotine neuroprotection of DA neurons is lost in a methamphetamine model of toxicity[60], supporting the relevance of the α4β2 subtype in neuroprotection.

In rodents with MPTP lesions of the nigrostriatal tract, the results of nicotine-induced neuroprotection are variable. This discrepancy in results can be attributed to the wide variety of protocols employed (reviewed in[24, 66]). For example chronic intermittent administration of nicotine is protective against MPTP toxicity, whereas chronic infusion of nicotine enhances MPTP toxicity[24, 66]. Nicotine, however, is always neuroprotective in primate models of MPTP toxicity. Chronic nicotine treatment administered via drinking water for several months before and during the toxic insult normalizes a number of parameters in the dopaminergic system. For example, nicotine administration attenuates the loss of tyrosine hydroxylase (TH; an enzyme involved in DA synthesis), DA, DAT (a marker of dopaminergic terminals), VMAT (another marker of dopaminergic terminals), and NNRs as a result of MPTP toxicity[67]. In addition, chronic nicotine administration in MPTP-treated primates normalizes nicotine-induced DA release, DA turnover, and synaptic plasticity[68]. These data imply that nicotine promotes an increase in DA neuronal processes and/or reduces nigrostriatal damage. Thus, it appears that nicotine administration reduces DA deficits resulting from nigrostriatal damage and supports the development of NNR ligands as promising therapeutics for PD.

Neuroprotection conferred by nicotine in 6-OHDA and methamphetamine models of nigrostriatal damage is lost in α4 knockout mice[60]. Nigrostriatal protection through NNR has been consistent in studies using rat and non-human primates, but has demonstrated some discrepancies in mouse models of the disease[69]. The review by Quik *et al* reveals that the translational potential of cellular and *in vivo* models is of critical importance to understanding the clinical relevance of NNR-targeted therapies in various neurodegenerative conditions. For example, it is estimated that the α6* NNR represents approximately 40% of presynaptic nicotine-stimulated dopamine release in rodents, whereas, in non-human primates, the α6* component contributes as much as 70%[67]. Several NNR subtypes are emerging as likely candidates for modulating DA release and possibly nigrostriatal neuroprotection. These include α4β2*, α6-containing (α6*), and α7 NNRs, and studies with knockout mice indicate that a subpopulation of these receptors contains the α5 subunit (α4α5β2)[70]. The mRNA for the α7 subunit is found in DA cell bodies of the SN (reviewed in[71]); however, <3% of the nAChR binding sites in the striatum are attributable to α7[54]. These sites are not reduced after 6-OHDA lesions, suggesting that α7 NNRs are not located at dopaminergic presynaptic terminals[72]. Much less is known on the molecular pathways of α4β2*-mediated neuroprotection than for alpha7 possibly in part because there are no known *in vitro* cell system that naturally express the α4β2* to allow molecular dissection of putative signaling cascade.

Acetylcholinesterase inhibitors

Donepezil pretreatment has been shown to prevent acute glutamate- and ionomycin-induced neurotoxicity, but not *S*-nitrosocysteine-induced neurotoxicity, suggesting that donepezil protects neurons against acute glutamate neurotoxicity via nAChRs before nitric oxide synthase activation. Neuroprotection was inhibited by NNR antagonists or phosphatidylinositol 3-kinase (PI3K) signaling inhibitors, and

was associated with a decrease in the level of Akt phosphorylation. Neuroprotection was also inhibited by treatment with an inhibitor of mitogen-activated protein kinase (MAPK). These results suggest that donepezil protects neurons against moderate glutamate neurotoxicity via nAChR-PI3K-Akt and MAPK signaling pathways[73]. Kihara *et al* reported that nicotine and galantamine alone and in combination protected neurons against β-amyloid toxicity. Galantamine induced phosphorylation of Akt, an effector of PI3K, while PI3K inhibitors blocked the protective effect, as well as Akt phosphorylation. The FK1 antibody, which selectively blocks the allosterically potentiating ligand site on nAChRs, or suppression of α7 nAChR using an RNA interference technique significantly reduced galantamine-induced protection and Akt phosphorylation. These findings suggest that neuroprotection elicited by galantamine is mediated, at least in part, by the α7 nAChR-PI3K cascade. Galantamine has been shown to be neuroprotective in hippocampal slices subjected to oxygen glucose deprivation[74] and in a transient global cerebral ischemia model in gerbils[75]. Even when applied 3 h post-ischemia, galantamine significantly increased the number of living pyramidal neurons after ischemia-reperfusion injury by reducing TUNEL, active caspase-3, and SOD-2 immunoreactivity. This effect was blocked with the nicotinic antagonist mecamylamine.

In addition to putative effects through the α7-PI3P-Akt pathway, galantamine was found to prevent β-amyloid(1-40) and thapsigargin-induced cell death in the human neuroblastoma cell line SH-SY5Y and in bovine chromaffin cells by upregulating the expression of the α7 receptor and the anti-apoptotic protein Bcl-2[76]. The phosphatidylinositol 3-kinase-Akt pathway also mediates donepezil and galanthamine protection of cortical neurons against acute glutamate treatment[77]. Inhibition of acetylcholinesterase (AChE) results in an indiscriminate increase of ACh in cholinergic synapses. This is likely to result in indirect broad activation of muscarinic receptors as opposed to NNR ligands, which provide a more targeted approach. The pathways recruited through broad muscarinic activation may be additive or may potentially interfere with those recruited through selective NNR activation. A recent study found that activation of muscarinic receptors in astrocytoma cells modifies the expression of the p70S6K kinase involved in translational control[78]. Translational control is in part regulated by a cascade of phosphorylation-affecting proteins of the anti-apoptotic pathway controlled by mTOR (a mammalian target of rapamycin) and the pro-apoptotic pathway controlled by PKR. Muscarinic receptor activation with oxotremorine significantly increased the expression of phosphorylated p70S6K, eIF4E, and ERK without modifying mTOR activity in neuroblastoma cells or in the cerebral cortex and hippocampus of mice, suggesting stimulation of protein synthesis. Understanding the cross-talk between the mechanisms recruited through NNR and muscarinic activation may have clinical implications. Targeted selective activation of pathways such as α7 is preferable to broad non-selective recruitment of second and third messengers through indiscriminate cholinergic activation.

Dual effects of α4β2 and α7 NNR

Agonists of α4β2*, such as TC-1734, are known to induce ACh release *in vivo*. Since ACh is an α7 agonist, such compounds have the potential to act as indirect agonists of α7 neuronal nicotinic receptors, recruiting this subtype for further neuroprotection. A distinguishing feature of the activation of α4β2* in enhancing ACh release in select brain regions versus the broad impact of AChE inhibitors may rest on the intrinsic biophysical properties of NNRs, which exhibit strong rectifying properties that allow activation of hypofunctional pathways and inhibition of hyperactive pathways. This would result in true normalization of synaptic tone. Taken together, these data may indicate the necessity of targeting multiple subtypes in order to achieve optimal neuroprotection[24, 62]. Amyotrophic Lateral Sclerosis (ALS, sometimes called Maladie de Charcot or Lou Gehrig's Disease in the US) is a progressive, usually fatal, neurodegenerative disease associated with inflammation. In preclinical models of ALS, nicotine-induced neuroprotection was inhibited by either dihydro-beta-erythroidin or α-bungarotoxin, suggesting that it is mediated through both α4β2* and α7 NNRs, both of which have been identified on rat spinal motor neurons. These findings are consistent with the emerging role of these NNR subtypes in neuroprotective mechanims. Furthermore, based on the plethora of data implicating them in neuroprotection and anti-inflammatory pathways, these data suggest that targeting NNRs may be a useful strategy in ALS treatment[79].

Mechanistic considerations

It has recently been shown that the correlation between AD pathology and cognitive decline represents a continuum from normal cognitive function to Mild Cognitive Impairment (MCI) in AD[71, 80]. In addition, progressive loss of cholinergic markers and severe depletion of cholinergic neurons (up to 85%) occur in AD patients[81]. Ideally, a therapy with neuroprotective capabilities should be administered at

prodromic stages, such as upon diagnosis of Age-Associated Memory Impairment (AAMI) or MCI, to prevent further neurodegeneration. Autopsy analysis of the cortical tissue of smokers reveals lower β-amyloid plaque densities compared to non-smokers[46, 47]. The underlying mechanisms responsible for the decrease in β-amyloid plaques remain unclear, and it should be noted that nicotine is a very poor ligand for α7 receptors. Furthermore, nicotine lack of selectivity is limiting. We now have evidence that α7 interaction with JAK2 and subsequent activation leads to several downstream signaling pathways, including Ras-mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt), GSK-3β modulation of tau phosphorylation, and transcription factor signal transducers and activators of transcription-5. In addition, the α7 NNR agonist and GSK-3β inhibitor have no additive effect[50]. These observations suggest that α7 modulation can influence Aβ1–42-induced tau phosphorylation, possibly involving GSK-3β. The overlap between mechanisms underlying Aβ1–42 toxicity and tau phosphorylation could, if confirmed *in vivo*, provide novel strategies for targeting the hallmarks of AD pathobiology. Several ongoing clinical trials are assessing α7 compounds with various selectivity profiles, but the clinical end-points are focused on acute cognitive amelioration rather than long-term neuroprotection.

The newly discovered linkage of α7 NNR to the JAK2/PI3P/Akt/STAT3/NF-κB pathway is highly reminiscent of the mechanisms reported for Erythropoietin (EPO). Interestingly, both α4β2 and α7 have been implicated in the regulation of myelo- and erythropoiesis in bone marrow. Erythropoietin is a 165-amino acid (~30 kDa) serum glycoprotein that is produced in fetal liver and adult kidney. It is responsible for the proliferation, survival, and differentiation of erythroid progenitor cells. The production and secretion of EPO is oxygen-dependent, and it stimulates the proliferation and maturation of erythroid cells. Upon binding of EPO to its receptor, it activates and causes dimerization of the receptor, as well as autophosphorylation of JAK2. EPO has been reported to have anti-inflammatory, anti-oxidative, anti-apoptotic, and neurotrophic properties that are relevant to cerebral ischemia. Its potential therapeutic role has been demonstrated in several animal models of cerebral ischemia, as well as in a clinical trial of ischemic stroke. The neuroprotective function of EPO is to target inflammation and improve cognitive and motor deficits manifested during traumatic brain injury[82].

The HMG-CoA reductase inhibitors ("statins") have been shown in preclinical models to reduce neuronal injury and infarct size during acute ischemic stroke. There is additional experimental and clinical evidence that statins have beneficial effects on endothelial function, cerebral blood flow, and inflammation. These observations have motivated the initiation of clinical trials such as the Neuroprotection with Statin Therapy for Acute Recovery Trial (NeuSTART) to evaluate its potential[83, 84].

Montaner *et al* [85, 86] conducted a double-blind, randomized, pilot clinical trial to study the safety and efficacy of simvastatin during the acute phase of ischemic stroke. They measured its efficacy on the evolution of several inflammatory markers (IL-6, IL-8, IL-10, monocyte chemoattractant protein-1, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, C-reactive protein, sApo/Fas, tumor necrosis factor-alpha, E- and L-selectin), however no differences were found among the biomarkers studied regarding treatment allocation. These results suggest that larger trials are needed to identify any effects of statins on inflammatory biomarkers.

As stated above, the JAK2/PI3K/Akt/STAT3/NF-κB pathway is recruited through a number of endogenous and external stimuli. The recruitment of these signaling molecules in order to achieve the optimum balance for minimizing inflammatory cascades and achieving neuroprotection, however, may require targeting specific transducing molecules positioned at critical points in the signaling pathways. Extracellular signal-regulated kinase 1/2 (ERK1/2), one of the most characterized members of the MAPK family, mediates a range of activities from inflammation to cell death and survival. It has been argued that ERK1/2 activity generated by endogenous inflammatory factors may have detrimental effects, whereas ERK1/2 activity produced by exogenous signals (*eg*, growth factors) favors neuroprotection[87].

Various additional strategies for neuroprotection have been used to attenuate brain inflammation via control of proliferation and production of proinflammatory cytokines, including derivatives of the insulin-like growth factor-1[88] and adenosine A2A analogues[89, 90]. Clinical proof of concept, however, remains to be established. Years of recreational human exposure to nicotine have provided some tantalizing observations of delayed onset in diseases such as Parkinson's disease where neurodegeneration and possibly inflammation are the primum movens of these conditions.

NNRs and inflammation

The "cholinergic anti-inflammatory pathway" and its role in immune responses and inflammatory cascades have attracted enormous interest due to their obvious relevance to a variety of debilitating human diseases. In this regard, evi-

dence has emerged showing that the central nervous system (CNS) modulates the immune system through the reticuloendothelial system. In particular, this CNS modulation is mediated through the vagus nerve, utilizing the major vagal neurotransmitter ACh, which acts on α7 nAChRs on macrophages. Nicotinic receptors, such as the α7 subtype, are at the apex of key cellular pathways, both central and peripheral, and are involved in anti-inflammatory processes, as well as cell survival. This opens the door for treating a broad array of intractable diseases and conditions with inflammatory components, such as rheumatoid and osteoarthritis, inflammatory bowel diseases, and sepsis. This cholinergic vagal pathway appears to play an important role in modulating inflammatory responses, as evidenced by the fact that vagotomy increases LPS (lipopolysaccharide)-induced TNF-α serum levels and hepatic TNF-α responses. Conversely, electrical stimulation of the vagus nerve or treatment of vagotomized animals with ACh prevents the increase in TNF-α release. The role of α7 nAChRs in cholinergic modulation of TNF-α in LPS-stimulated macrophages has been confirmed using antisense oligonucleotides to the α7 nAChRs. Indeed, when the expression of this receptor is blocked, ACh does not have an effect on LPS-induced TNF-α release. This observation has been extended to *in vivo* models, which demonstrate that vagus nerve stimulation does not inhibit TNF-α release in α7 knockout mice. The key role played by α7 nAChRs in inflammatory processes is further supported by the observations that nicotine and α7 nAChR agonists, such as CAP55 and GTS-21, are effective in models of inflammation and protective in models of sepsis. Furthermore, they have been shown to inhibit local leukocyte recruitment and decrease endothelial cell activation. The α7 NNR ligands inhibit LPS-induced TNF-α release in murine-derived microglial cells, an effect that is attenuated by α-bungarotoxin. Furthermore, this inhibition appears to be mediated by a decrease in phosphorylation of p44/42 and p38 MAPK[91].

The reduced incidence of ulcerative colitis among smokers suggests that nicotine may modulate the immune response that leads to this inflammatory bowel disease and that nicotinic receptors may be therapeutic targets for inflammatory diseases[92, 93]. The cellular and molecular mechanisms involved in the effects of nicotine, however, are not well understood, and attempts to treat ulcerative colitis with nicotine or other nicotinic ligands have provided conflicting results[92, 93]. Drug discovery efforts for these diseases have focused a great deal of energy on targeting TNF-α; however, other pro-inflammatory cytokines contribute to disease and may better reflect disease progression. One such late mediator is high mobility group box chromosomal protein 1 (HMGB1, historically known as an abundant non-histone architectural chromosomal protein), whose release from macrophages is stimulated by LPS treatment[94]. Nicotine attenuates HMGB1 release from stimulated macrophages *in vitro* and reduces serum HMGB1 levels in experimental sepsis. The reduced HMGB1 levels in sepsis correlate with increased survival. Splenectomy also inactivates the cholinergic anti-inflammatory pathway, demonstrating the involvement of the RES[95, 96]. The LPS-stimulated increase in systemic TNF-α production is eliminated in splenectomized animals and, interestingly, nicotine decreases the survival of splenectomized animals with sepsis and bacterial clearance in septic peritonitis[95, 96]. As our understanding of the tissues involved in the cholinergic anti-inflammatory pathway from the CNS to the RES has unfolded, advances have also been made in understanding the molecular mechanisms involved. For example, the neuroprotective effects of nicotine and the α7-selective ligand TC-1698 can be traced to α7 activation and transduction of signals to PI3K and AKT (protein kinase B) via JAK2[39], all of which participate in a key cell survival pathway. Immunoprecipitation experiments indicate that the α7 receptor and JAK2 interact directly (also see Figure1). Additional studies examined the effects of nicotine on LPS-treated and control peritoneal macrophages and found that nicotine treatment leads to phosphorylation of STAT3[49], another key component of the cellular anti-apoptotic cascade. This nicotine-mediated phosphorylation is inhibited by the α7-selective antagonists α-bungarotoxin and methyllycaconitine (MLA), as well as by AG490, a selective inhibitor of JAK2 phosphorylation. Immunoprecipitation studies support the previous findings showing that nicotine exposure recruits JAK2 and leads to an increased association between the kinase and α7 receptors. Studies examining LysM-Stat3$^{fl/-}$ mice, whose macrophages are deficient in STAT3, found that vagus nerve stimulation does not reduce peritoneal cytokine levels or intestinal inflammation, as it does in control animals. These data support the interaction of JAK2 and α7 and the critical role of STAT3 in the cholinergic anti-inflammatory pathway. LPS-stimulated release of TNF-α and neutrophil chemoattractant macrophage inflammatory proteins (MIP-1a and MIP-1b) are also inhibited by nicotine, and the mRNA levels of these inflammatory mediators are also modulated[97]. Therefore, cholinergic anti-inflammatory regulation occurs upstream of transcription. Nicotine also inhibits LPS-stimulated IκB phosphorylation, thereby preventing nuclear factor kappa B (NF-κB) activation, which is necessary for gene transcription of pro-inflammatory mediators[97, 98].

Secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related protein-1 (SLURP-1) is a 9 kDa secreted protein encoded by the ARS B gene that shows structural similarity to the snake venom toxin α-bungarotoxin[99]. Acetylcholine elicits current responses in control and SLURP-1-treated *Xenopus* oocytes expressing recombinant human α7 nAChRs. Furthermore, SLURP-1 significantly increases both ACh potency and efficacy (more than a 10-fold increase) via the α7 nAChR. It is localized to human skin, exocervix, gums, stomach, and esophagus and has been implicated in maintaining the physiological and structural integrity of the keratinocyte layers of the skin. Mutations in the SLURP-1 gene result in Mal de Meleda (MDM), a rare autosomal recessive genetic disease that is characterized by inflammatory palmoplantar keratoderma. SLURP-1 expression is regulated by retinoic acid, epidermal growth factor, and interferon-gamma[100–102]. The SLURP-1 protein has been identified in several biological fluids such as sweat, saliva, tears, and urine from normal volunteers[101]. In palmoplantar sections from MDM patients, as well as in their sweat, mutant SLURP-1, including the new variant R71H-SLURP-1, was either absent or barely detectable. Thus, SLURP-1 acts as a positive allosteric modulator of α7 NNRs[100] and most MDM mutations in SLURP-1 affect either the expression, integrity, or stability of the protein. This results in inflammatory manifestations, raising the intriguing possibility that, in addition to acetylcholine, endogenous peptides may actively modulate the α7 cholinergic cascade.

The role of α4β2 in inflammatory processes is also emerging. (E)-metanicotine (an α4β2-selective ligand[103]) inhibits IL-8 and TNF-α production in human macrophages and in cells of the inflamed muc osa[104]. Conversely, pro-inflammatory cytokines shift the neuronal nicotinic receptor assembly to the α4β2 configuration over α4β2 or α4β2β4, suggesting bi-directional regulation of cytokines and nicotinic receptor expression[105].

In addition, recent studies have implicated distinct participation of α4β2* and α7 in the regulation of B-lymphocyte development and activation and have suggested that the CD40 pathway contributes to these effects. These studies support a role for both α4β2 and α7 in the regulation of inflammatory/immune processes[106]. Lymphocytic cholinergic activity in the regulation of immune function is supported by studies in mutant mice and the emerging of cholinergic role in immune function may provide the basis for targeted immunotherapy[107].

Summary

A number of studies have confirmed the potential for nicotinic acetylcholine receptor-mediated neuroprotection and, more recently, its anti-inflammatory effects. The mechanistic overlap between these pathways and the ubiquitous effects observed following diverse insults have suggested that NNRs modulate fundamental pathways involved in cell survival (Figure 1). Neuroprotection mediated by α7 after a variety of cellular insults are initiated via activation of JAK2, triggering downstream cellular signaling events that include activation of phosphoinositide-3-kinase/Akt, GSK-3β, and the transcription factors STAT3 and NF-κB. This leads to inhibition of neuronal cell apoptosis or macrophage activation via the cholinergic anti-inflammatory pathway. Additional mechanisms through the MAPK/ERK pathways participate in the regulation of cell survival and apoptosis. These results have wide-reaching implications for the design of experimental therapeutics that regulate inflammatory and anti-apoptotic responses through the NNR. They also represent an initial step toward understanding the benefits of novel therapeutic strategies targeting neuronal survival and management of associated inflammatory processes for the management of CNS disorders. In addition, selective nicotinic ligands that affect the pro-inflammatory pathway from the transcriptional level upward provide a new therapeutic class possessing a potentially better mechanism of action for the treatment of inflammatory and autoimmune disorders. These ligands achieve this by modulating a broad array of cytokines and cellular pathways that are involved in cell homeostasis, an effect that would not be possible by targeting individual proteins. The mechanistic overlap between the signaling pathways involved in neuroprotective mechanisms and in controlling inflammation may provide the tools needed to control and break the vicious cycle of cell death and inflammation. The aptly named Janus kinase, the deity of gateways, beginnings, and endings in roman mythology, resides at the crossroad of these bimodal signaling cascades and may provide a convenient target via NNR modulation for novel therapies designed to manage the disruption of regulatory proteins that are central to cellular homeostasis. The present therapeutic armamentarium is lacking drugs directed toward some of the fundamental pathways involved in cell survival and chronic inflammation that have been increasingly implicated in some of the most devastating diseases, including atherosclerosis, diabetes, neurodegenerative diseases, chronic obstructive pulmonary diseases, inflammatory bowel diseases, and other untractable diseases. The potential to target neuronal survival and chronic inflammation could

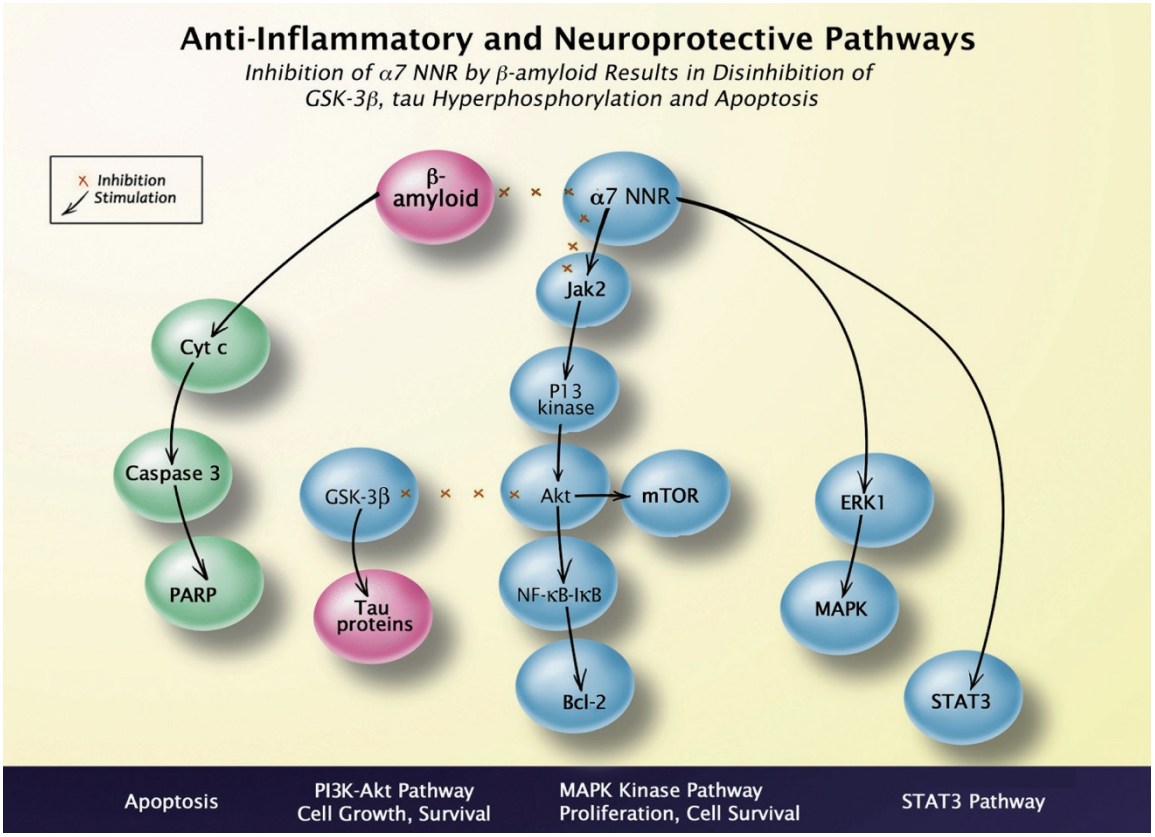

Figure 1. Relationship of α7 nAChRs to anti-apoptotic and anti-inflammatory pathways. Schematic showing α7 nAChR-mediated activation of JAK2 and cross-talk mechanisms between α7 nAChRs and β-amyloid-activated pathways. α7-mediated neuroprotection via the JAK2 pathway intersects with the anti-inflammatory pathway mediated through STAT3/NF-κB. Abbreviations: Akt (protein kinase B); Bcl-2 (B cell lymphoma 2 protein); IκB (Inhibitor kappa B); JAK2 (Janus kinase 2); NF-κB (nuclear factor kappa B and transcription factor complex); mTOR: mammalian target of rapamycin (kinase); STAT3 (Signal Transducer and Activator of Transcription3). PARP: Poly (ADP-ribose) polymerase.

be a turning point in our ability to manage some of the most costly public health issues of our time. Although there is mounting evidence for the potential of NNRs to target the hallmark of diseases that may constitute the biggest public health challenges, the regulatory path for such therapies remains to be established and only global pharmaceutical companies with a strategic interest in these areas have the means to undertake the task of extended clinical trials and to influence the regulatory bodies to pave the way for such new therapies.

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