Enhancement of nicotinic receptors alleviates cytotoxicity in neurological disease models

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Abstract: The common pathological mechanisms among the spectrum of neurodegenerative diseases are supposed to be shared. Multiple lines of evidence, from molecular and cellular to epidemiological, have implicated nicotinic transmission in the pathology of the two most common neurodegenerative disorders, namely Alzheimer's disease (AD) and Parkinson's disease (PD). In this review article we present evidence of nicotinic acetylcholine receptor (nAChR)-mediated protection against neurotoxicity induced by β amyloid (A β), glutamate, rotenone, and 6-hydroxydopamine (6-OHDA) and the signal transduction involved in this mechanism. Our studies have clarified that survival signal transduction, the α 7 nAChR/Src family/PI3K/AKT pathway and subsequent upregulation of Bcl-2 and Bcl-x, would lead to neuroprotection. In addition to the PI3K/AKT pathway, two other survival pathways, JAK2/STAT3 and MEK/ERK, are proposed by other groups. In rotenone- and 6-OHDA-induced PD models, nAChR-mediated neuroprotection was also observed, and the effect was blocked not only by α 7 but also by α 4 β 2 nAChR antagonists. We also document that nAChR stimulation blocks glutamate neurotoxicity in spinal cord motor neurons. These findings suggest that nAChR-mediated neuroprotection is achieved through subtypes of nAChRs and common signal cascades. An early diagnosis and protective therapy with nAChR stimulation could be effective in delaying the progression of neurodegenerative diseases such as AD, PD and amyotrophic lateral sclerosis.

Keywords: Alzheimer's disease, amyotrophic lateral sclerosis, β amyloid, glutamate, nAChR, nicotine, Parkinson's disease

Introduction

To date, available therapeutic agents against Alzheimer's disease (AD) are acetylcholinesterase inhibitors (AChEI), while the most reproducible epidemiologically relevant factor against Parkinson's disease (PD) is cigarette smoking habits [Quik, 2004; Dorn, 1959]. Both clearly show that placing the importance on modulation of acetylcholine systems must be the key therapeutic targets of these most common devastating neurodegenerative diseases, whose socio-economic impacts have been striking. Acetylcholine (ACh) is one of the major neurotransmitters in the central nervous system (CNS). ACh receptors are classified into two groups; nicotinic ACh receptors (nAChRs) and muscarinic ACh receptors (mAChRs). In the brain, nAChRs show additional complexity, as there are multiple receptor subtypes with differing properties and functions [Lindstrom *et al.* 1995;

Clarke *et al.* 1985]. At least nine α subunits (α 2– α 7, α 9, and α 10 in mammals; α 8 in chicks) and three β subunits (β 2– β 4) have been identified in the brain. Both α and β subunits are required to form functional heteropentameric receptors, with the exception of α 7– α 10 subunits, which apparently form functional homopentameric receptors. In the brain, α 7 homometric and α 4 β 2 heterometric nAChRs are the major two subtypes. Both α 4 β 2 and α 7 subtypes have been implicated in the mechanism of neuroprotection provided by nicotine (Figure 1) [Kihara *et al.* 2001, 1998]. Implication of heterometric nAChR-containing α 6 subunits, α 6 β 2*, is also emphasized in dopaminergic systems in the CNS (* indicates possible additional subunits) [Bordia *et al.* 2007; Bohr *et al.* 2005; Quik, 2004; Champiaux *et al.* 2002].

There is evidence that neuronal nAChRs are involved in synaptic plasticity as well as in

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neuronal survival and neuroprotection. Moreover, presynaptic nAChRs can modulate the release of many neurotransmitters, including dopamine, noradrenaline, serotonin, ACh, γ -aminobutyric acid (GABA), and glutamate. These neurotransmitter systems play an important role in cognitive and noncognitive functions such as learning, memory, attention, locomotion, motivation, reward, reinforcement, and anxiety. Thus, nAChRs are considered promising therapeutic targets for new treatments of neurodegenerative disorders. It is also known that $\alpha 4$ and $\beta 2$ nAChR genes, *CHRNA4* and *CHRNA2*, are causative genes of autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [De Fusco *et al.* 2000; Steinlein *et al.* 1995]. The benign nature of this form of epilepsy is explained by a compensatory mechanism of the nAChRs. Analyzing the polymorphism of the nAChRs genes in AD patients and controls, we concluded that genetic polymorphisms of the neuronal nAChR genes might be related to the pathogenesis of sporadic AD [Kawamata and Shimohama, 2002].

To date, mainly three possible mechanisms of nAChRs-mediated neuroprotection have been proposed, as follows [Buckingham *et al.* 2009]:

1. Phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homolog 1 (PI3K/AKT) pathway.
2. Janus kinase-2/signal transducer and activator of transcription-3 (JAK2/STAT3) pathway.
3. MEK (MAPK/ERK kinase; Mitogen-activated protein kinase/Extracellular signal regulated kinase kinase)/Extracellular signal regulated kinase (MEK/ERK) pathway.

In this review we focus mainly on the PI3K/AKT pathway initially proposed by our collaborators and present evidence for nAChR-mediated protection against β amyloid ($A\beta$)- and glutamate-induced neurotoxicity and in rotenone- and 6-hydroxydopamine (6-OHDA)-induced PD animal models, based mainly on our studies.

Alzheimer's disease

AD and nicotinic transmission

AD pathology is characterized by the presence of two hallmarks, senile plaques (SPs) and neurofibrillary tangles (NFTs), and by extensive neuronal loss [Giannakopoulos *et al.* 1996]. $A\beta$ is a major element of SP and one of the candidates for the cause of the neurodegeneration found in AD. It has been shown that the accumulation of $A\beta$ precedes other pathological changes and causes neurodegeneration or neuronal death *in vivo* [Yankner *et al.* 1990]. Several mutations of the $A\beta$ precursor protein (APP) are found in familial AD, and these mutations are involved in amyloidogenesis [Citron *et al.* 1992]. Also, familial AD mutations of presenilin 1 (PS-1) enhance the generation of $A\beta$ 1–42 [Tomita *et al.* 1997]. It is also known that $A\beta$ binds very strongly to $\alpha 7$ nAChR [Wang *et al.* 2000] and upregulation of $\alpha 7$ nAChR is observed in transgenic mice co-expressing mutant human presenilin 1 and APP [Dineley *et al.* 2002].

The cerebral cortex contains a dense plexus of cholinergic axon terminals that arise from the cells of the basal forebrain including the nucleus basalis of Meynert [Mesulam *et al.* 1983; Bigl *et al.* 1982]. Degeneration of this cholinergic projection is recognized as one of the most

Figure 1. Composition of two major nicotinic acetylcholine receptors, $\alpha 4\beta 2$ and $\alpha 7$, in the central nervous system.

prominent pathological changes in the AD brain [Rosser *et al.* 1982; Whitehouse *et al.* 1981]. In AD, the cholinergic system is affected, and a reduction in the number of nAChRs has been reported [Whitehouse and Kalara, 1995; Shimohama *et al.* 1986]. This, in conjunction with the memory-enhancing activity of nicotine and selective nAChR agonists such as the $\alpha 7$ nAChR agonist, 3-(2,4)-dimethoxybenzylidene anabaseine (DMXB) [Meyer *et al.* 1997], suggests a significant role for nAChRs in learning and memory. Therefore, it is generally recognized that downregulation of nAChRs is involved in the intellectual dysfunction in AD. Our studies showed that nAChR stimulation protected neurons from A β - and glutamate-induced neurotoxicity. This allowed us to hypothesize that nAChRs are involved in a neuroprotective cascade [Kihara *et al.* 2001, 1998, 1997; Kaneko *et al.* 1997; Shimohama *et al.* 1996; Akaike *et al.* 1994] as described in following sections.

In 2009, Dziewczapolski and colleagues reported interesting but controversial findings about the interaction between A β and $\alpha 7$ nAChR. They used a transgenic mouse model of AD, PDAPP, overexpressing a mutated form of the APP (both 'Swedish' and 'Indiana' mutations) and lacking the $\alpha 7$ nAChR gene. They reported that despite the presence of high amounts of APP and amyloid deposits, deleting the $\alpha 7$ -nicotinic subunit in the mouse model of AD rescues the mice from expressing the phenotype of the dysfunction in synaptic integrity and learning and memory behavior in aged stage (13–22 months) [Dziewczapolski *et al.* 2009]. One of the possible explanations is that pathological intracellular translocation of A β through $\alpha 7$ nAChR with high affinity binding is hampered by deleting

the $\alpha 7$ nAChR itself. Recently, Hernandez and colleagues reported that loss of $\alpha 7$ nAChR enhances A β oligomer accumulation, exacerbating early-stage cognitive decline in transgenic mouse model of AD, the Tg2576, overexpressing a mutated form of APP ('Swedish' mutation) [Hernandez *et al.* 2010]. Contribution of $\alpha 7$ nAChR to AD pathology may be different depending on the stage of neurodegeneration.

Protection against A β toxicity

A 48-hour exposure to 20 μ M neurotoxic A β 25–35 caused a significant reduction in the neuronal cells of rat fetal primary culture. Simultaneous incubation of the cultures with nicotine and A β significantly reduced the A β -induced cytotoxicity. The protective effect of nicotine was reduced by both dihydro- β -erythroidine (DH β E), an $\alpha 4\beta 2$ nAChR antagonist, and α -BTX, an $\alpha 7$ nAChR antagonist. The effect of a selective $\alpha 4\beta 2$ nAChR agonist, cytosine, and a selective $\alpha 7$ nAChR agonist, DMXB [Hunter *et al.* 1994], on A β cytotoxicity was examined. A β cytotoxicity was significantly reduced when 10 μ M cytosine or 1 μ M DMXB was co-administered. These findings suggest that both $\alpha 4\beta 2$ and $\alpha 7$ nAChR stimulation are protective against A β cytotoxicity [Kihara *et al.* 2001, 1998, 1997]. In addition, MK-801, an N-methyl-D-aspartate (NMDA) receptor antagonist, inhibited A β cytotoxicity when administered simultaneously with A β , suggesting that A β cytotoxicity is mediated via the NMDA receptor, or via glutamate in cultured cortical neurons (Figure 2).

Protection against glutamate cytotoxicity

Glutamate cytotoxicity is one of the most suspected causative pathways in neurodegenerative processes such as AD, PD, and amyotrophic

Figure 2. Proposed hypothesis for the mechanism of nicotinic acetylcholine receptor (nAChR)-mediated survival signal transduction against glutamate-induced necrosis. AChE, acetylcholinesterase; NMDA, N-methyl-D-aspartate; NO, nitric oxide; SNOC, NO-generating agent.

lateral sclerosis (ALS). It is also assumed that glutamate plays an important role in the neurodegeneration observed in hypoxic–ischemic brain injury [Meldrum and Garthwaite, 1990; Choi, 1988]. Several investigators have also suggested that cortical neurodegeneration in AD is attributable to glutamate [Mattson, 1988; Maragos *et al.* 1986]. Moreover, brief glutamate exposure induces delayed cell death in cultured neurons from certain brain regions such as the cerebral cortex and hippocampus. In these brain regions, the NMDA glutamate receptor subtype plays a crucial role in glutamate neurotoxicity. Several studies have indicated the existence of nitric oxide (NO) synthase in the CNS, including the cerebral cortex. NMDA receptor stimulation induces Ca^{2+} influx into cells through ligand-gated ion channels, thereby triggering NO formation. NO is also thought to diffuse to the adjacent cells, resulting in the appropriate physiological response and/or glutamate-related cell death [Dawson *et al.* 1991; Choi *et al.* 1989; Hartley and Choi, 1989]. Although there has been only limited information concerning the cholinergic interaction with glutamate neurotoxicity, Mattson and Olney and colleagues have demonstrated that stimulation of the muscarinic AChR potentiates neurodegeneration [Olney *et al.* 1991; Mattson, 1989].

We examined the effects of nicotine on glutamate-induced neurotoxicity using primary cultures of rat cortical neurons. Cell viability was decreased by treatment with 1 mM glutamate for 10 minutes followed by incubation in glutamate-free medium for 1 hour. Incubating the cultures with 10 μM nicotine for 24 hours prior to glutamate exposure significantly reduced glutamate cytotoxicity. To investigate whether nicotine-induced neuroprotection is due to a specific effect mediated by nAChRs, the effects of cholinergic antagonists were examined. An addition of DH β E or α -BTX to the medium containing nicotine reduced the protective effect of nicotine. We also examined the protection of nicotine against the effects of ionomycin, a calcium ionophore, and SNOC, a NO-generating agent. Incubating the cultures for 10 minutes in either 3 μM ionomycin- or 300 μM SNOC-containing medium markedly reduced cell viability. A 24-hour pretreatment with nicotine significantly attenuated the ionomycin cytotoxicity, but did not affect the SNOC cytotoxicity (Figure 2) [Shimohama *et al.* 1998, 1996; Kaneko *et al.* 1997; Akaike *et al.* 1994].

Protection against A β -enhanced glutamate toxicity

Whilst it is thought that PS-1 mutations enhance the generation of A β 1–42, it is controversial whether A β is directly toxic to neurons. We found that A β 25–35-induced neurotoxicity was inhibited by MK801. It can therefore be hypothesized that A β might modulate or enhance glutamate-induced cytotoxicity. Indeed, A β causes a reduction in glutamate uptake in cultured astrocytes [Harris *et al.* 1996], indicating that, to some extent, A β -induced cytotoxicity might be mediated via glutamate cytotoxicity.

In our previous study [Kihara *et al.* 2000], incubation of the cortical neurons with both A β 1–40 (1 nM) and A β 1–42 (100 pM) for 7 days did not induce cell death: these are the concentrations of A β in the cerebrospinal fluid (CSF) of AD patients [Jensen *et al.* 1999]. Although 20 μM glutamate alone did not significantly induce cell death, exposure to 20 μM glutamate for 24 hours caused a significant reduction in the neuronal cells in the A β -treated group, showing that A β itself is not toxic at low concentrations, but makes neurons vulnerable to glutamate. Conversely, co-incubation of the cultures with nicotine (50 μM for 7 days) and A β significantly reduced A β -enhanced glutamate cytotoxicity [Kaneko *et al.* 1997; Shimohama *et al.* 1996; Akaike *et al.* 1994].

Involvement of the phosphatidylinositol 3-kinase pathway in neuroprotection

To investigate the mechanism of the protective effect of nicotine, we focused on the phosphatidylinositol 3-kinase (PI3K) pathway because PI3K has been shown to protect cells from apoptosis [del Peso *et al.* 1997]. Long exposure to low concentrations of glutamate (50 μM for 24 hours) induced cytotoxicity. Incubating the cultures with nicotine (10 μM for 24 hours) prior to glutamate exposure significantly suppressed glutamate cytotoxicity. Simultaneous application of LY294002, a PI3K inhibitor, with nicotine cancelled the protective effect of nicotine. α -BTX, an α 7 nAChR antagonist, blocked the protection provided by nicotine and by DMXB, an α 7 selective nAChR agonist. Furthermore, this DMXB-induced protection was also reduced by LY294002. Although α 4 β 2 nAChR stimulation also had a protective effect on A β - and glutamate-induced cytotoxicity, this effect was not inhibited by LY294002, suggesting PI3K system is not directly involved in α 4 β 2

nAChR-mediated neuroprotection. PD98059, a mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor, did not reduce the protective effect of nicotine, also suggesting that the MEK/ERK pathway is not directly involved in the protective effect of nicotine (Figure 3). A nonreceptor tyrosine kinase inhibitor, PP2, did reduce the protective effect of nicotine, suggesting that Src is involved in the mechanism of the protective effect. Cycloheximide also inhibited the protection, implying that some protein synthesis is necessary for this effect.

AKT is a serine/threonine protein kinase and a putative effector of PI3K. To investigate the activation of AKT by nicotine through PI3K, we examined the level of phosphorylated AKT using an antiphosphospecific AKT antibody. The phosphorylated form of AKT appeared just after the application of nicotine. Nicotine-induced AKT phosphorylation was blocked by simultaneous application of LY294002, but not of PD98059, indicating that PI3K, but not MAPK is involved. The AKT phosphorylation is blocked by α -BTX, but not by DH β E, implying that nicotine-induced AKT phosphorylation is mediated by α 7 but not by α 4 β 2 nAChRs. PP2 also blocked AKT phosphorylation, which suggests the involvement of tyrosine kinase. The level of total AKT protein which was detected with anti-AKT antibody remained unchanged.

Figure 3. Proposed three pathways for the mechanism of nicotinic acetylcholine receptor (nAChR)-mediated survival signal transduction. 1: PI3K/AKT pathway; 2: JAK2/STAT3 pathway; 3: RAS/RAF/MEK/ERK/MAPK pathway.

Bcl-2 and Bcl-x proteins are anti-apoptotic proteins that can prevent cell death induced by a variety of toxic attacks [Zhong *et al.* 1993]. It has been reported that AKT activation leads to the overexpression of Bcl-2 [Matsuzaki *et al.* 1999]. Because nicotine can activate AKT via PI3K, we examined the protein levels of Bcl-2 and Bcl-x. We found that treatment with nicotine for 24 hours increased the levels of Bcl-2 and Bcl-x, and this was inhibited by LY294002, which indicates the involvement of the PI3K pathway in nicotine-induced Bcl-2 and Bcl-x upregulation (Figure 3). These results suggest that nAChR stimulation protects neurons from glutamate-induced cytotoxicity by activating PI3K, which in turn activates AKT and upregulates Bcl-2 and Bcl-x.

Galantamine modulates nAChR and blocks β -enhanced glutamate toxicity

Galantamine is an AChEI that is currently used for the treatment of AD. In addition to the inhibition of AChE, galantamine binds to nAChRs and allosterically potentiates their synaptic transmission. Consequently, galantamine is called an allosteric potentiating ligand (APL) of nAChRs. This APL effect is present on both α 7 and α 4 β 2 nAChRs [Maelicke *et al.* 2001]. Thus, galantamine could stimulate cholinergic transmission in two ways: (1) by inhibiting AChE and increasing AChs; and (2) by potentiating cholinergic transmission through the APL effect. We demonstrated that galantamine protected cortical neurons against A β -enhanced glutamate toxicity by, at least partially, the α 7nAChR/PI3K/AKT pathway [Kihara *et al.* 2004].

Donepezil promotes internalization of NMDA receptors by stimulating α 7 nAChRs and protects against glutamate cytotoxicity

Donepezil is one of the most widely prescribed AChEIs for the treatment of AD and related dementias. Our group recently reported that in addition to upregulating the PI3K/AKT pathway, there is another mechanism underlying neuroprotection by donepezil: decreased glutamate toxicity through downregulation of NMDA receptors, following stimulation of α 7 nAChRs in primary rat neuron cultures [Shen *et al.* 2010].

Parkinson's disease

PD and nicotinic transmission

PD is the second most common progressive neurodegenerative disorder next to AD.

It is characterized by relatively selective degeneration of dopaminergic neurons in the substantia nigra and loss of dopamine in the striatum resulting in resting tremor, rigidity, bradykinesia, and postural instability [Obeso *et al.* 2010; Shimohama *et al.* 2003]. Although the pathogenesis of PD is still unclear, it is thought that the interaction of gene and the environment plays roles in causing the multifactorial disease. Rural residency, pesticides, and intrinsic toxic agents were reported as environmental risk factors for sporadic PD. Recent studies revealed several mutations in familial PD genes such as *α-synuclein*, *parkin*, *PINK1*, *LRRK2*, *DJ-1*, *UCHL1*, and *ATP13A2* [Hardy, 2010]. Epidemiological studies suggest that the use of pesticides increases the risk of PD, possibly via reduced activity of complex I in the mitochondrial respiratory chain in the substantia nigra [Mizuno *et al.* 1998; Mann *et al.* 1992; Parker *et al.* 1989]. The H₂O₂ pro-oxidant, 6-OHDA, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a mitochondrial complex I inhibitor, have been widely used to produce toxin models of sporadic PD. Chronic exposure to rotenone, a nature-derived pesticide, could be an appropriate animal PD model because rotenone-treated animals show slowly progressive dopamine neuronal loss, and Lewy body-like particles, which are primarily aggregations of *α-synuclein* [Inden *et al.* 2007; Mizuno *et al.* 1998].

Grady and colleagues examined mouse brain using *in situ* hybridization to characterize the mRNA expression pattern. The ventral tegmental area (VTA) and substantia nigra express high concentrations of *α4* and *α6* and *β2*, and *β3* mRNAs, intermediate levels of *α5* mRNA, and low levels of the *α3* and *α7* mRNAs. No signal for *α2* and *β4* mRNA was detected [Grady *et al.* 2007; Le Novère *et al.* 1996]. They reviewed the subtypes of nAChRs on dopaminergic terminals of mouse striatum reporting five nAChR subtypes that expressed on dopaminergic nerve terminals, three of which are *α6*-containing subunits, namely *α4α6β2β3*, *α6β2β3*, and *α6β2*. The remaining two subtypes, *α4β2* and *α4α5β2*, are more numerous than the *α6*-containing subtypes. The *α6*-containing nAChRs, which do not contribute to dopamine release induced by nicotine, are mainly located on dopaminergic neuronal terminals and probably mediating the endogenous cholinergic modulation of dopamine release at the terminal level. In contrast, *α4β2* nAChR represent the majority

of functional heteromeric nAChRs on dopaminergic neuronal soma. *α7* nAChRs are present on dopaminergic neuronal soma, contributing to nicotine reinforcement [Champtiaux *et al.* 2003].

There are several studies analyzing the decline of specific nAChRs in PD patients. Court and colleagues reported the decline of *α3* subunits and no change of *α7* subunits [Court *et al.* 2000]. Gotti and colleagues as well as Court and colleagues reported a decreased level of *α4* subunits, in contrast to Guan and colleagues who did not report this [Guan *et al.* 2002; Court *et al.* 2000; Gotti *et al.* 1997]. Bordia and colleagues reported the decline of *α6* subunits in caudate and putamen. They further specified that the most vulnerable subtype in striatum of MPTP-treated mice and monkeys is *α6α4β2β3* rather than *α6β2β3* and further identified the specific loss of *α6α4β2β3* subtype in PD brains [Bordia *et al.* 2007].

These results seem to indicate that the decline of nigrostriatal specific *α6** subtypes is highly specific and relevant to the PD pathogenesis but not is *α7* subtype. Functional studies on *α6** nAChRs should be undertaken to confirm its pathological importance in PD.

Current drug therapy against PD is limited to supplementing dopamine or enhancing dopaminergic effect. Some may have neuroprotective effects, but their effects remain controversial [Iravani *et al.* 2006; Du *et al.* 2005; Quik, 2004]. It has also been reported that smokers have a lower risk for PD [De Reuck *et al.* 2005; Wirdefeldt *et al.* 2005], and nAChRs were decreased in the brains of PD patients [Fujita *et al.* 2006] and PD model animals [Quik *et al.* 2006]. Nicotine may upregulate dopamine release at the striatum from nigral dopaminergic neurons [Morens *et al.* 1995], followed by stimulation of *α4β2* nAChRs [Champtiaux *et al.* 2003]. Furthermore, nicotine could protect mitochondria and have a protective effect from oxidative stress [Xie *et al.* 2005; Cormier *et al.* 2003]. In studies made *in vivo*, stimulation of nAChRs resulted in neuroprotection in PD model animals [Parain *et al.* 2003]. Although several clinical trials to evaluate the possible therapeutic effect of nicotine in PD patients have been conducted, it is still controversial about whether it has therapeutic effects in PD. The relatively high-dose

administration of transdermal nicotine might have therapeutic effect in PD patients [Villafane *et al.* 2007].

Neuroprotection in rotenone-induced PD models

We investigated the neuroprotective effect of nicotine against nigral dopamine neuronal death induced by rotenone using a chronic rotenone-treated PD mouse model, and analyzed molecular mechanisms of the protection in dissociated cultures of the fetal rat ventral mesencephalon. Rotenone works as a mitochondrial complex I inhibitor. Acute lethal doses of rotenone eliminate the mitochondrial respiratory system of the cell, resulting in an anoxic status that immediately causes cell death. At sublethal doses it causes partial inhibition of mitochondrial complex I, and in this situation mitochondrial dysfunction leads to increased oxidative stress, decreased ATP production, increased aggregation of unfolded proteins, and then activated apoptotic pathway(s) that result in cell death [Betarbet *et al.* 2000], resembling dopamine neurodegeneration in PD.

Our mouse model (oral rotenone 30 mg/kg for 28 days) showed motor deficits, dopaminergic cell death in the substantia nigra, and nerve terminal/axonal loss in the striatum. These findings are relevant to some previous reports about rotenone PD models [Ravenstijn *et al.* 2008; Schmidt and Alam, 2006]. Simultaneous subcutaneous administration of nicotine (0.21 mg/kg/day) rescued both motor deficits and dopamine neuronal cell loss in the substantia nigra of rotenone-treated mice. Using primary dopamine neuronal culture, we analyzed the molecular mechanisms of dopamine neuroprotective effect of nicotine against rotenone-induced toxicity. We found that dopamine neuroprotective effects of nicotine were inhibited by 1 μ M DH β E, 100 nM α -BTX, and/or PI3K/AKT/protein kinase B (PKB) inhibitors, demonstrating that rotenone toxicity on dopamine neurons are inhibited via activation of α 4 β 2 or α 7 nAChRs/PI3K/AKT/PKB pathways [Takeuchi *et al.* 2009]. Neuronal α 4 β 2 nAChR stimulation causes dopamine release [Champtiaux *et al.* 2003], and our data showed the neuroprotective effect also occurred via α 4 β 2 nAChRs, so the mechanism of neuroprotection could vary according to different receptor subtypes.

Synergistic effect of galantamine in the 6-OHDA-induced hemiparkinsonian rat model

Using the rat 6-OHDA-induced hemiparkinsonian model, the neuroprotective effects of galantamine and nicotine were evaluated. We injected 32 nmol 6-OHDA, with or without 4–120 nmol galantamine and/or 120 nmol nicotine, into the unilateral substantia nigra of rats. Although methamphetamine-stimulated rotational behavior and dopaminergic neuronal loss induced by 6-OHDA were not inhibited by galantamine alone, they were moderately inhibited by nicotine alone. In addition, 6-OHDA-induced neuronal loss and rotational behavior were synergistically inhibited by co-injection of galantamine and nicotine. These protective effects were abolished by mecamylamine, a nAChR antagonist. α 7 nAChR was expressed on both tyrosine hydroxylase (TH)-immunopositive and TH-immunonegative neurons in the rat SNpc. A combination of galantamine and nicotine greatly suppressed 6-OHDA-induced reduction of TH-immunopositive/ α 7 nAChR-immunopositive neurons. These results suggest that galantamine synergistically enhances the neuroprotective effect of nicotine against 6-OHDA-induced dopaminergic neuronal loss through an allosteric modulation of α 7nAChR activation [Yanagida *et al.* 2008].

Amyotrophic lateral sclerosis: stimulation of nAChRs protects spinal motor neurons

ALS is a fatal neurodegenerative disorder characterized by the rapidly progressive degeneration of motor neurons resulting in paralysis and, within a few years, death. To date, several causative genes of familial types have been reported including *superoxide dismutase 1 (SOD1)*, *Senataxin*, *TDP-43*, and *FUS*. Although the cause of sporadic ALS is still unknown, recent proteomic and pathological studies reveal that pathological phosphorylated TDP-43 is accumulated in cytoplasm of neurons, whereas physiological TDP-43 resides mainly in the nucleus [Arai *et al.* 2006; Neumann *et al.* 2006]. The only available therapeutic alternative is glutamate-release inhibitor, riluzole, and no effective treatments have been identified. There are numerous clinical and experimental studies suggesting the role of glutamate-induced excitotoxicity in ALS pathogenesis [Rothstein, 2009]. Our previous study demonstrated that rat spinal cord cultures exposed to long-term (24-h) low-dose (10 μ M) glutamate exhibit selective motor neuronal death and we proposed this paradigm as an *in vitro* model for ALS [Urushitani *et al.* 1998].

There is a study which demonstrates an early decrease in cholinergic input on motor neurons in the spinal cords of patients with ALS [Choi *et al.* 1989]. Therefore, we investigated the neuroprotective effect of nicotine and galantamine, an AChEI with APL properties, against spinal motor neuronal death induced by glutamate using dissociated cultures of fetal rat spinal cord.

The study demonstrated that administration of nicotine prevented glutamate-induced motor neuronal death in primary cultures of the rat spinal cord. The 10 μ M nicotine-induced neuroprotection was inhibited by either 30 nM DH β E or 1 nM α -BTX, suggesting that it is mediated through both α 4 β 2 and α 7 nAChRs. Both α 4 β 2 and α 7 nAChRs were identified on rat spinal motor neurons by immunohistochemical methods. We also demonstrated that galantamine prevented glutamate-induced motor neuronal death [Nakamizo *et al.* 2005]. Aberration in the PI3K/AKT signaling system has been reported in both ALS patients [Wagey *et al.* 1998] and ALS transgenic mice [Warita *et al.* 2001]. Thus, it is possible that the PI3K/AKT pathway is also involved in nAChR-mediated neuroprotection against glutamate-induced spinal motor neuronal death.

Enhancement of nAChRs against neurodegenerative diseases through three pathways

PI3K/AKT pathway

Our studies showed that nAChR stimulation protected neurons from A β -, glutamate-, rotenone-, and 6-OHDA-induced neurotoxicity. From the experimental data, our hypothesis for the mechanism of nAChR-mediated survival signal transduction is as follows: activation of α 7 nAChRs stimulates the Src family, which in turn activates PI3K. PI3K phosphorylates AKT, which causes upregulation of Bcl-2 and Bcl-x. α 4 β 2 nAChR stimulation also causes neuroprotection cascade without direct involvement of PI3K system.

JAK2/STAT3 pathway

Other properties of nicotine proposed by other groups are anti-inflammatory potentials and modulating innate immune pathways mainly via α 7 nAChR. Nicotine exerts its anti-inflammatory effect in activated immune cells, macrophages and microglia, by interacting with α 7 nAChR. Activated α 7 nAChR binds directly to JAK2 and triggers the JAK2/STAT3 pathway to interfere with the activation of TLR-induced NF- κ B,

which is responsible for pro-inflammatory cytokine transcription [Cui and Li, 2010].

MEK/ERK pathway

The importance of the MEK/ERK pathway in nicotinic neuroprotection has been also emphasized by several groups [Buckingham *et al.* 2009; Wang *et al.* 2003]. Dajas-Bailador and colleagues reported nicotine stimulation leads to PKA activation through α 7 nAChR and a further Raf-1/MEK/ERK signaling pathway [Dajas-Bailador *et al.* 2002]. Another group showed that stepwise activation of Ras/Raf-1/MEK/ERK cascade provides for an increased cytoplasmic concentration of STAT3 due to an upregulated expression [Arredondo *et al.* 2006] activating the JAK2/STAT3 pathway in human oral keratinocytes.

Stimulation of nAChRs initiates these survival signal cascades in addition to their role as neurotransmitter receptors. Cholinotherapy is currently being applied with clinically symptomatic benefit in terms of AChE inhibition in AD and related disorders. It has been suggested that some of the inhibitors used in this therapy, including galantamine, which has additional nAChR modulating properties [Maelicke *et al.* 1995], may have disease-slowing effects. Selective α 7 nAChR agonists, including DMXB [Meyer *et al.* 1997], S 24795 [Wang *et al.* 2009], R3487/MEM3454 [Rezvani *et al.* 2009], and ABT-107 [Bitner *et al.* 2010], have proven to be effective in AD animal disease models and may be regarded as therapeutic alternatives next to AChEIs. As shown in this review, recent studies suggest that nAChR stimulation could protect neurons in rotenone or 6-OHDA induced PD models as well as from A β and glutamate toxicity.

Conclusion

We have been studying the PI3K/AKT pathway in AD, PD, and ALS models in *in vitro* and *in vivo* systems to elucidate the mechanism of nAChR-mediated survival signal transduction as follows: activation of α 7 nAChRs stimulates the Src family, which in turn activates PI3K. PI3K phosphorylates AKT, which causes upregulation of Bcl-2 and Bcl-x. Considering the facts, together with other two pathways proposed by other groups, it is promising that protective therapy by nAChR stimulation could attenuate the progress of neurodegenerative diseases such as AD, PD, and ALS.

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Conflict of interest statement

The authors have no conflicts of interest.

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