The Role of Neuronal Nicotinic Acetylcholine Receptors in Acute and Chronic Neurodegeneration

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Abstract: In the last five years there has been a rapid explosion of publications reporting that neuronal nicotinic acetylcholine receptors (nAChRs) play a role in neurodegenerative disorders. Furthermore, there is a well-established loss of nAChRs in post-mortem brains from patients with Alzheimer's disease, Parkinson's disease and a range of other disorders. In the present review we discuss the evidence that nicotine and subtype selective nAChR ligands can provide neuroprotection in *in vitro* cell culture systems and in *in vivo* studies in animal models of such disorders.

Whilst *in vitro* data pertaining to a protective effect of nicotine against nigral neurotoxins like MPTP is less robust, most studies agree that nicotine is protective against glutamate and β-amyloid toxicity in various culture systems. This effect appears to be mediated by α7 subtype nAChRs since the protection is blocked by α-bungarotoxin and is mimicked by α7 selective agonists.

In vivo studies indicate that α7 receptors play a critical role in protection from cholinergic lesions and enhancing cognitive function. The exact subtype involved in the neuroprotectant effects seen in animal models of Parkinson's disease is not clear, but in general broad spectrum nAChR agonists appear to provide protection, while α4β2 receptors appear to mediate symptomatic improvements. Evidence favouring a protectant effect of nicotine against acute degenerative conditions is less strong, though some protection has been observed with nicotine pre-treatment in global ischaemia models. A variety of cellular mechanisms ranging from the production of growth factors through to inactivation of toxins and antioxidant actions of nicotine have been proposed to underlie the nAChR-mediated neuroprotection *in vitro* and *in vivo*.

In summary, although the lack of subtype selective ligands has hampered progress, it is clear that in the future neuronal nAChR agonists could provide functional improvements and slow or halt the progress of several crippling degenerative diseases.

Keywords: Nicotinic receptors, Parkinson's disease, Alzheimer's disease, neuroprotection, Aβ toxicity, cognition, stroke, nicotine

1. INTRODUCTION

As discussed by other authors in this journal issue nicotine and neuronal nicotinic acetylcholine receptor (nAChR) agonists have been shown to have effects in animal models of cognition, Parkinson's disease (PD), pain perception, vigilance, locomotor activity and schizophrenia as well as on many physiological parameters such as body temperature, respiration, cardiovascular, gastrointestinal tract function and electroencephalogram activity. Many of these effects may be due to modulation of other transmitter systems such as ACh, dopamine, noradrenaline, 5-HT, glutamate and GABA. In parallel with the neurochemical and behavioural work there is now a vast amount of literature on the role of neuronal nAChRs in neurodegeneration and neuroprotection in systems ranging from cell culture models *in vitro* through *in vivo* animal models of disease to evidence in human post-mortem brain. Taken as a whole, the accumulating evidence suggests that nAChRs play a greater role in chronic neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (AD) than in acute neuroprotection from stroke. However, there is some evidence that nicotine and nAChR agonists have effects in models of excitotoxicity *in vitro* (glutamate application to cortical cultures) and *in vivo* (rodent models of global and focal ischaemia). In this review we will focus on the *in vitro* and *in vivo* evidence for involvement of nAChRs in excitoxicity, Parkinson's disease and Alzheimer's disease.

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2. EVIDENCE FROM HUMAN POST MORTEM BRAIN

It is now well established that there is some loss of nAChRs in normal ageing brains, with the largest decreases being apparent in the cortex and hippocampus [1, and Graham *et al.* this issue]. This loss is much greater in neurodegenerative disorders and is documented in detail from patients that suffer from Alzheimer's disease or dementia with Lewy bodies (DLB) [1] and Parkinson's disease [2]. For example, there is a loss of high affinity agonist binding (nicotine, cytisine and epibatidine) in the cortex in both AD and PD and further loss in the striatum in PD (Graham *et al*. this issue). Normal cell loss in ageing brains is not limited to a particular receptor subtype, but in AD there is a selective loss of α4 containing subtypes. Less is known about the specific subtypes lost in PD but a recent study reported that there was no loss in immunoreactivity for α3, α4, α7 of β2 subunits in the caudate of PD patients despite a highly significant reduction in [^{3}H]-nicotine binding [3]. Epidemiological studies show that smokers who survive the detrimental effects of smoking have a reduced risk of developing AD and PD [4, 5]. Taken together, the post-mortem evidence has suggested that nAChRs may be good targets for slowing the progression of these chronic neurodegenerative diseases. Whether the experimental evidence from *in vitro* investigations and *in vivo* studies in animal models of these diseases back up these suggestions is considered in the remainder of this review.

3. EXCITOTOXICITY, STOKE AND TRAUMATIC BRAIN INJURY

3.1. *In Vitro* Models of Excitotoxicity

Glutamate-induced cell death (excitotoxicity) is one of the major contributors to neuronal cell death in both acute and chronic neurodegenerative diseases [6, 7]. Several lines of evidence suggest that nicotine can inhibit glutamate-induced neurotoxicity in rodent neurocortical [8, 9, 10] and hippocampal [11] or in striatal neuronal cultures [12]. Nicotine-induced neuroprotection has also been shown against N-methyl-D-aspartate (NMDA), but not against AMPA/kainate-induced cell death in primary neurocortical cultures [8, 13]. This agrees with the observation that in cultured cortical neurones, glutamate toxicity is mainly mediated through the activation of the NMDA subtype of glutamate receptors [14, 15]. In some *in vitro* systems neuroprotection has been shown to be mediated through nicotinic, rather than muscarinic AChRs. For example, atropine was ineffective, while mecamylamine and hexamethonium inhibited nicotine-evoked neuroprotection of cortical and striatal neurones [8, 12]. A substantial body of evidence shows that the effect of nicotine is α-bungarotoxin sensitive, suggesting the involvement of the α7 nicotinic receptor subtype [9, 10, 16, 17]. This is particularly important for designing cholinergic drugs for intervention in AD (expanded in section 5), because unlike the high affinity nicotinic receptors, α7 receptors do not disappear as AD progresses [18], therefore the molecular target remains accessible. Although the majority of the *in vitro* studies have been carried out in primary neuronal cultures of rodent origin, studies in human neuroblastoma cells (IMR32) have also demonstrated α7 receptor-mediated protected against glutamate toxicity by nicotine and ABT-418 ((S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole), another non-selective nicotinic agonist [9]. These data suggest that similar mechanisms are behind nicotine-induced neuroprotection in human and rodent neurones.

Interestingly the neuroprotective concentration of nicotine varies with the culture system (two orders of magnitude in the case of cortical versus striatal cultures) [8, 12] and the timing of nicotine addition also appears to be a critical factor. In several cases, 2-3 or even 8 hours of pre-incubation with nicotine is required for neuroprotection [9, 10, 11], while others reported successful neuroprotection by nicotine employing a co-application [12, 19] or even a one hour post-incubation protocol [16]. These discrepancies suggest that different mechanisms may be responsible for the neuroprotective properties of nicotine in these model systems.

Although no unified mechanism of nicotine-evoked neuroprotection has emerged yet, several reports offer possible molecular mechanisms underlying this action (summerised in Fig **1**). Attenuation of excitotoxicity by nicotine is calcium-dependent [10, 16]. However, pre-incubation with nicotine does not affect the magnitude of the NMDA-induced calcium response, suggesting that the mechanism responsible for neuroprotection is downstream of NMDA receptor-mediated calcium entry [16]. Since calcium-activated neuronal nitric oxide synthase (nNOS) has been implicated in NMDA-induced excitotoxicity [20], nicotine may offer neuroprotection by interfering with NMDA-evoked nitric oxide (NO) production or the activation of downstream mediators (Fig. **1**). However, given that nicotine is able to protect against ionomycin-induced calcium overload, but not against NO-donor-induced cell death, it seems most likely that nicotine produces its effects upstream of NO generation [10]. Another possible mechanism to prevent excitotoxicity without attenuation of Ca^{2+} accumulation, is to increase the calcium buffering capacity of neurones. In hippocampal organotypic cultures, chronic pre-application of nicotine protected against excitotoxicity without altering NMDA-induced calcium responses [21]. However in these studies, nicotine did markedly increase the immunoreactivity of the Ca^{2+} binding protein calbindin-D28K [21]. Interestingly, the effect of nicotine does appear to depend on the developmental state of cells: α7 receptor activation, for example, is toxic to immature hippocampal neurones and progenitor cells, but neuroprotective to older mature neurones. This switch from toxic to neuroprotective effects of α7 receptor activation is in parallel with increases in the basal levels of calbindin-D28K, seen in mature neurons, supporting the potential involvement of calcium buffering in the neuroprotective actions of nicotine [22].

Another potential mechanism for nicotine-induced attenuation of excitotoxicity is via the production of growth factors. In cerebellar neuronal cultures, nicotine inhibited glutamate toxicity by preventing glutamate-induced proteolysis of microtubule-associated protein (MAP-2) in an α7-dependent manner [23]. Furthermore, activation of α7

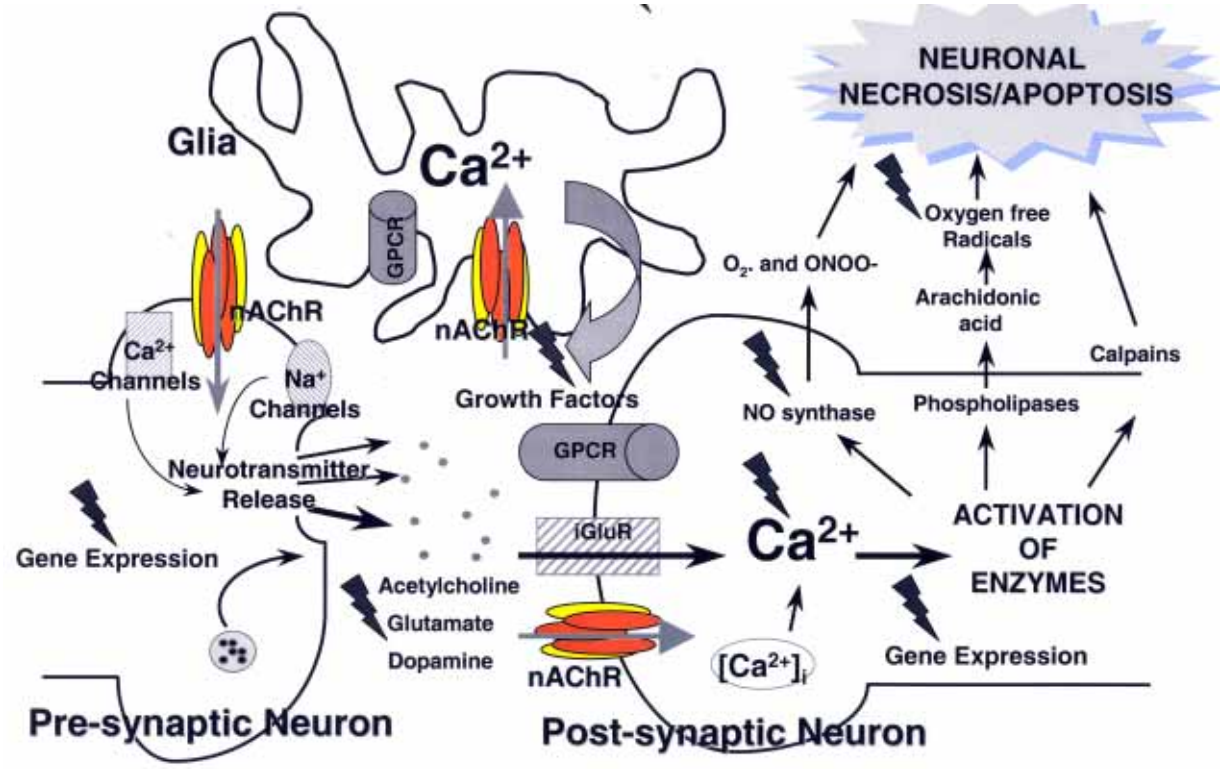

Fig. (1). Some potential sites ( ) of nAChR mediated protection.

and α4β2 receptors signal the production of neurotrophic factors, such as basic fibroblast growth factor (FGF-2) and brain-derived neurotrophic factor (BDNF) *in vivo* [24, 25]. Since in many cases more than 2 hours of pre-incubation with nicotine is required for the inhibition of neuronal cell death [9, 10, 11], such production of neurotrophic factors could well play a part in nicotine-induced attenuation of excitotoxicity even in *in vitro.* Finally, a direct effect of nicotine on apoptotic biochemical cascades cannot be ruled out since α7 receptor activation has also been demonstrated to prevent apoptosis induced by nerve growth factor withdrawal [26] or hypoxia [27] in PC12 cells and by arachidonic acid in cultured spinal cord neurones [28, 29].

3.2 Models of Acute Brain Injury *in vivo*: Global and Focal Ischaemia and Traumatic Brain Injury

There is limited evidence for a role of nAChRs in models of excitotoxicity *in vivo*. The most common models that have "excitotoxicity" as their main hallmark include systemic or central (intercranial) injection of glutamate analogues (e.g. ibotenic acid, kainic acid) or models that mimic a disease state such as stroke (global and focal ischaemia) or traumatic brain injury (fluid percussion injury). In the mid 1990's it was reported that pre-treatment with nicotine could protect against kainic acid induced excitotoxic effects [30]. In that study nicotine (0.5mg/kg s.c.) 15 min before kainic acid (12mg/kg s.c., a dose widely used to induced temporal lobe convulsions), protected against "wet dog shakes" and also against a loss in acetylcholinesterase-positive neurons in the hippocampus. In support of these studies it has recently been reported that prolonged exposure to cigarette smoke reduced the number of kainic acid (10mg/kg i.p.) induced seizures, and degree of cell loss in the hippocampus [31]. This protection was mediated via nicotinic receptor activation as it was blocked by pre-treatment with mecamylamine (2 and 10mg/kg i.p.) in a dose-dependent manner [31].

Nanri and co-workers [32] reported that pre-treatment with nicotine, GTS-21 (DMXBA, 3-(2,4-dimethoxybenzyidene)-anabasine), an agonist at α7 receptors, or the acetylcholinesterase inhibitor THA (tacrine) attenuated impairments in passive avoidance and cell death in the gerbil model of global ischaemia (this mimics the situation of brain injury after cardiac arrest). In a more recent study it was reported that pre-treatment with nicotine (3-100μg/kg i.v.) attenuated ischaemia-induced decreases in regional blood flow and neuronal damage in the hippocampus [33]. The period of occlusion (3 min) used by Nanri and co-workers is less than the 5 min period which is routinely used and where several other pharmacological agents have proved effective. In addition both these studies used a pre-treatment protocol and never tried to see if these molecules could be administered after occlusion. While it is most likely that any new molecules used to treat stroke would need to work after the occlusion the above studies do at least suggest a positive prophylactic effect with nicotine.

The situation is more complex in models of focal ischaemia (mimics human stroke) as there is very limited data in animal models. However, epidemiological data suggest that smoking increases the risk of stroke and this appears to be supported by experimental data obtained to date. For example, nicotine treatment (4.5 mg/kg/day via mini-pumps for 14 days prior to ischaemia) decreased blood flow in the periphery of the ischaemic core during reperfusion, worsened neurological score and enhanced brain injury after transient (1hr) focal ischaemia in rats [34]. Additional studies reported that nicotine increased plasminogen activator inhibitor-1 in human CNS endothelial cells [35] again suggesting that smoking increases the risk of thrombosis.

As with stroke, the exact role of nAChRs in traumatic brain injury (TBI) is not clear. There is substantial evidence to suggest nAChRs can modulate growth factor expression and signal transduction pathways activated by growth factors that are know to provide robust protective effects in both focal ischaemia (middle cerebral artery occlusion in rats) and traumatic brain injury (fluid percussion in rats). Of more direct relevance may be the recent studies demonstrating enhanced recovery with FGF-2 in rodent models of stroke and TBI. If growth factors play a role, then nicotine or selective neuronal nAChR agonists (started after stroke) may indeed provide a useful intervention to enhance post-stroke and post-TBI recovery. In addition, these patients often have cognitive deficits and enhanced release of ACh and subsequent activation of nAChRs could attenuate these deficits and perhaps improve overall quality of life. The evidence in support of this comes from studies with acetylcholinesterase inhibitors. For example, ENA-713 (rivastigmine) has been reported to improve neurological scores after TBI in rats [36] and even more encouraging is data indicating that donepezil improves cognitive function (two patients) when administered several weeks after TBI [37].

3.3 Summary

In summary, activation of nAChRs, more specifically the α7 subtype, has a marked *in vitro* neuroprotective effect against excitotoxicity in a number of different cell culture systems. There is some evidence to suggest that nAChR agonists can be neuroprotective *in vivo*, but most studies to date have only used pre-treatment paradigms. Mechanistic studies suggest various signalling pathways and growth factors that may ultimately enhance recovery post-injury and may be involved in these neuroprotective actions (Fig. **1**). However, it is clear that much more pre-clinical and clinical work is required to confirm that nAChR activation post-injury could enhance recovery in stroke patients.

4. PARKINSON'S DISEASE

4.1. Clinical Perspectives

From a clinical perspective, tobacco smoking, or the use of nicotine patches or gum, has been shown to reduce the classical symptoms of PD sufferers, namely tremor, rigidity, bradykinesia and gait disturbances [38, 39]. This improvement in motor function most likely reflects the ability of nAChR activation to induce striatal dopamine release [40, 41]. However, the relief is not likely to be as effective as that achieved using the mainstay dopamine replacement therapies, L-DOPA or direct dopamine receptor agonists [42]. That said, nAChR agonists might also have additional beneficial effects on cognitive performance in patients, as recently demonstrated in parkinsonian primates [43]. Thus, nAChR agonists may be of valuable use in patients where PD is associated with dementia. More importantly, and of particular relevance to this review, nicotine is not only a CNS stimulant but may also be neuroprotective. This idea is proposed to explain the well-documented decreased incidence of PD among tobacco smokers [44, 45, 46, 47, 48]. The question of precisely which component of cigarette smoke is causing these effects is a topic of debate, with nicotine being the most likely candidate, although the effects of other components of cigarette smoke have not yet been ruled out.

4.2. *In Vitro* Studies of Relevance to Parkinson's Disease

The neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) or its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) can induce selective degeneration of the nigrostriatal tract *in vivo* to generate animal models of PD. These toxins are also applied to cells in culture to produce a model system in which to mimic (albeit crudely) the neurodegeneration typical of PD. However, unlike the vast amount of *in vitro* data available for the conditions covered elsewhere in this review, few studies of this nature have been performed in relation to PD. One such study of this kind [49] clearly demonstrated that 24 hour prior incubation of mesencephalic dopaminergic cells with 10μM nicotine afforded an approximate 20% protection against dopaminergic cell decline induced by subsequent exposure to 3μM MPP^+. Moreover, this protection was completely inhibited by pre-incubation with the nAChR agonist, d-tubocurarine indicating that activation of nAChRs mediated the protective response.

An alternative *in vitro* approach has been to consider the potential interaction between nicotine and the biochemical processes believed to contribute to the pathological degeneration of nigrostriatal dopaminergic neurones in PD, for example oxidative stress and enhanced hydroxyl free radical formation [50]. There are several known pathways culminating in the production of free radicals, but iron (Fe(II))-induced oxidative stress via the Fenton reaction is specifically implicated in PD [51, 52, 53, 54, 55, 56]. Using the *in vitro* salicylic acid trapping method, Ferger *et al.* [57] demonstrated that, in relatively high concentrations (5mM, but not 0.5mM) nicotine decreased hydroxyl radical formation attaining a significantly higher scavenging capability than the established radical scavenger α-phenyl-N-tert-butyl nitrone (PBN). However, the authors were cautious not to over interpret this finding since, in their hands, higher doses of nicotine administered *in vivo* (0.4 mg/kg but not 0.1 mg/kg) demonstrably enhanced the neurotoxicity of MPTP in mice [57; expanded in section 4.4.1] suggesting

that any radical scavenging activity might be overshadowed by such toxic mechanisms *in vivo*. Subsequent studies have used chromatographic means to confirm that nicotine can form complexes with Fe(II) ions, thereby reducing the likely production of free radicals via the Fenton reaction [58]. However, once again these authors were not able to confirm the functional significance of this effect since neither in rat neocortical cultures *in vitro*, nor in various regions of rat brain *in vivo* (including the striatum) were they able to demonstrate a nicotine-induced reduction in lipid peroxidation (measured by thiobarbituric acid reactive production formation) [58].

A more recent report has suggested that nicotine may exert its neuroprotective influences via effects on mitochondrial respiration. Using rat brain mitochondria, Cormier *et al.* [59] demonstrated that nicotine (100nM) reduced oxygen consumption via inhibitory effects predominantly on Complex I of the mitochondrial respiratory chain, more specifically through inhibition of NADH ubiquinone reductase activity. However, these effects of nicotine were deemed to be independent of nAChR activation since they were not inhibited by the nAChR antagonist hexamethonium and were exhibited by only some nAChR agonists (e.g. epibatidine but not cytisine or lobeline). Subsequent binding studies revealed that nicotine produced these effects via inhibition of NADH binding on Complex I. The resultant reduction in mitochondrial respiration and superoxide anion generation may provide another facet to the potential antioxidant effects of nicotine described above although the significance of these effects on mitochondrial respiration remain to be explored *in vivo*.

4.3. Symptomatic Relief in Animal Models of Parkinson's Disease

In view of the reported efficacy of tobacco smoking or nicotine patches/gum in reducing the motor deficits of PD patients [38, 39] and given that nAChR activation can increase dopamine release *in vitro* and *in vivo* [40, 41], it is not surprising that the ability of nicotine to provide symptomatic relief has been examined in animal models of PD. However, since this review focuses primarily on the neuroprotective ability of nicotine and nAChR agonists, details on these symptomatic studies will be kept to a minimum. In mice, chronic administration of nicotine (2mg/kg s.c. 4 x daily for 22 days) has been shown by Gao *et al.* [60] to significantly reduce MPTP-induced motor impairment. In our hands, in rats bearing a 6-OHDA-induced lesion of the nigrostriatal tract, acute nicotine administration stimulates ipsiversive rotational behaviour, indicative of a reversal of parkinsonian motor deficit (Murray & O'Neill, unpublished results). Our studies with a range of nAChR agonists further suggest that activation of β2 receptors, but not β4 or α7 receptors is responsible for mediating these functional effects (Table 1).

The MPTP-treated primate remains the most representative *in vivo* model of the human condition. In this model, low doses of nicotine or nAChR agonists failed to produce any movement when given alone but enhanced L-DOPA stimulated movement [42]. Consistent with our rodent studies, the β2-preferring agonist, SIB-1508Y, was more effective than nicotine at increasing L-DOPA stimulated movement and increasing striatal dopamine levels [42], supporting a major involvement of β2-containing nAChRs in mediating these responses. In contrast, Domino *et al.* [61] were able to demonstrate a small locomotor response in MPTP-treated monkeys given 6 daily injections of nicotine alone, although again this effect was greatly enhanced when nicotine was co-administered with L-DOPA. The authors postulated that the increased locomotor effects reflected increased striatal dopamine release, further facilitated by the administration of L-DOPA. As previously mentioned, Schneider *et al.* [43] have subsequently demonstrated that SIB-1508Y also improves cognitive functioning in these low dose MPTP-treated primates. These beneficial effects of SIB-1508Y persisted for up to 48h post-administration leading the authors to hypothesise that there may have been an increase in neurotrophic factor production or receptor levels in the CNS. Regardless of the underlying mechanism, these findings imply that nicotinic agonists may be useful in the treatment of both the motor and cognitive symptoms of PD. Whether α7-preferring nAChR agonists that have previously been shown to enhance measures of attentional ability, improve working memory and decrease distractibility in normal monkeys [62, 63, 64] perform similarly well in MPTP-treated primates remains to be seen.

Table 1. Functional Effects of nAChR Ligands in the 6-OHDA-Lesioned Rat Model of PD

Compound (Full name)	Compound (Short name)	nAChR Subtype(s)	Ipsiversive rotational behaviour
Nicotine	Nicotine	Broad spectrum	+
5-ethynyl nicotine or altiniciline	SIB-1508Y	β2 preferring	+
(E)-*N*-methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine	TC-2559	β2 selective	+
3-(2-azetidinylmethoxy)pyridine	A-85380	β2 preferring	+
((±-4-[[2-(1-methyl-2-pyrrolidinyl)ethyl]thio]phenol HCl	SIB-1553A	β4 preferring	-
(R)-(+)-5'-phenylspiro[1-azabicyclo[2.2.2]octane-3,3'(3'H)-furo[2,3-b] pyridine]	Astra ligand	α7 selective	-

(Data are a tabulated summary of in-house data generated at Eli Lilly & Co. Ltd).

4.4. Neuroprotective Effects in Animal Models of Parkinson's Disease

As yet, there are no reports of studies examining the neuroprotective ability of nicotine or nAChR agonists in MPTP-treated primate models of PD. However, many studies have been performed in the two rodent models of this condition.

4.4.1 Neuroprotective Effects in MPTP-Treated Mice

There is conflicting evidence on the role of nicotine in neuroprotection in the MPTP-treated mouse model of PD with some authors reporting marked neuroprotection yet others reporting enhanced toxicity with nicotine. These discrepancies have largely arisen as a result of a wide variation in protocols adopting differing doses, route of administration, treatment schedule and age of animals used. Thus, while it is clear that chronic infusion of nicotine via implanted mini-pumps (overall daily doses ranging from 14-72mg/kg) actually enhances MPTP-induced striatal dopamine depletion and neurotoxicity in mice [65, 66], chronic, intermittent nicotine administration has been shown to protect against MPTP, depending on the dose administered [57, 60, 66]. For example, Ferger *et al.* [57] found that chronic higher dose nicotine treatment (0.4mg/kg s.c. twice-daily for 14 days prior to MPTP and 7 injections of 0.4mg/kg on the day of MPTP) enhanced MPTP-induced loss of body weight and striatal dopamine depletion. In comparison, the same treatment schedule but with a lower dose of nicotine (0.1mg/kg s.c), which the authors likened to the possible concentration found in a smoker, tended to alleviate the MPTP toxicity. However, others have reported a partial (approximate 10-15%) protection against MPTP-induced striatal dopamine depletion with chronic intermittent administration of higher doses of nicotine (e.g. 4 daily injections of 2mg/kg s.c. for 22 days [60] or 5 daily injections of 2mg/kg for 1 week prior to and 3 weeks following MPTP [67]), so some discrepancies in dose-dependency clearly remain. In the study of Parain *et al.* [67], cotinine, a major metabolite of nicotine failed to protect against MPTP toxicity suggesting that nicotine *per se* is the protective factor. These data further suggest that the time of nicotine administration may be crucial to observing any neuroprotective effects if the metabolites are indeed inactive.

A number of mechanisms have been proposed for the observed enhancement of MPTP toxicity with nicotine; firstly, nicotine may, via the release of dopamine, lead to enhanced autoxidation and oxidative deamination of dopamine with the resultant generation of reactive oxygen species [57]; secondly nicotine may, by virtue of its similar chemical structure, compete with MPTP for its detoxification enzymes [62]. Similarly, more than one explanation has been suggested for the neuroprotective effects of nicotine. For example, these have been attributed to nicotine inducing functional desensitisation of the nAChRs, which would switch the low agonist affinity open channel state to a high-affinity binding state with a closed ion channel [60]. This in turn would reduce influx of cations into the cell, thus reducing the homeostatic energy demand on the cell, contributing to the increased survival of dopaminergic neurons. An alternative explanation is that the dopamine released by nicotine treatment could compete with MPP^+ for uptake into dopaminergic terminals, thus reducing MPTP toxicity [66]. A recent study adds strength to this argument. Quick & Di Monte [68] demonstrated that nicotine reduced striatal MPP^+ levels (up to 65%) in mice treated with MPTP. The authors suggested that increased dopamine release due to nicotine exposure, may result in increased expulsion of MPP^+ from terminals, producing the apparent decline in striatal MPP^+. Interestingly, these effects of nicotine were only apparent in 8-10 month old animals, and not 6-8 week old animals thus indicating that age may also be a variable in determining the likely neuroprotection afforded by nicotine.

4.4.2. Neuroprotective Effects in 6-OHDA-Lesioned Rats

Fewer studies have examined the neuroprotective effects of nicotine in rats bearing a 6-OHDA-induced nigrostriatal tract lesion. However, the data are largely supportive of a neuroprotective effect. For example, a recent study by Costa *et al.* [69] demonstrated that intermittent administration of nicotine (1mg/kg s.c. given 4 hour before and up to 68 hours after toxin administration) could protect against a partial 6-OHDA-induced lesion of the substantia nigra (50% decreased dopamine levels in the striatum). This protection was almost completely antagonised by the nAChR antagonist chlorisondamine, demonstrating that the protection is nAChR-mediated and suggesting that functional activation of the nAChR is necessary for neuroprotection. Interestingly, in this same study nicotine failed to protect against a full nigrostriatal tract lesion (~100% dopamine depletion in the striatum). We also failed to see protection with intermittent nicotine administration against a severe nigrostriatal tract lesion (induced by 12μg 6-OHDA in the medial forebrain bundle; Visanji *et al.* unpublished data), suggesting that any neuroprotective effects of nicotine may only be observed in the earlier stages of PD. Further studies have demonstrated that chronic infusion of low dose nicotine (0.75 or 1.5mg/kg/day for 7 days prior to and 7 days after 6-OHDA) was also protective against 6-OHDA induced nigrostriatal tract degeneration (assessed by ^{3}H-mazindol autoradiography) [70]. These authors also found that, as in the case of intermittent treatment, chronic infusion of higher doses of nicotine (3 and 30mg/kg/day) failed to offer protection, as noted previously by Blum *et al.* [71]. Ryan *et al.* [70] propose that this lack of efficacy of higher doses relates to their ability to desensitise the nAChR, providing further evidence that activation of nAChR underpins the observed neuroprotection of lower doses. However, it should be noted that other investigators suggest that nicotine affords neuroprotection by causing nAChR desensitisation resulting in decreased firing rate of the nigrostriatal neurones [72] and therefore reduced dopamine release [73] and reduced glucose utilization [74] so controversy still remains.

An alternative explanation for the neuroprotective effects of nicotine against nigrostriatal tract lesion in rats relates to nicotine's ability to induce the production of growth factors (as alluded to in section 3.1). In 1998, Maggio *et al.* [25], demonstrated that nicotine protects against striatal dopamine depletion induced by a different toxic paradigm, methamphetamine injection. In a parallel experiment, the

authors demonstrated that there was an increased induction of FGF-2 and BDNF mRNA in the striatum. FGF-2 is believed to be important for dopaminergic cell survival and differentiation and there is evidence for FGF receptor expression in both the striatum and substantia nigra of rats [75, 76]. Additionally FGF-2 has been shown to be severely depleted in the parkinsonian brain [77]. The increases in growth factor mRNA were antagonised by the nAChR antagonist mecamylamine, demonstrating the effects are nAChR mediated. These data have been supported and extended to the protein level in a subsequent study by Belluardo *et al*. [24]. These effects are not confined to nicotine but have also been demonstrated with epibatidine and ABT-594 ((R)-5-(2-azetidinylmethoxy)-2-chloropyridine, α4β2-preferring agonist) [78, 79, 80] and there is growing evidence for nicotinic modulation of nerve growth factor (NGF) production [80]. However, in other studies chronic infusion of nicotine has been demonstrated to reduce FGF-2 mRNA production in the striatum and substantia nigra of intact rats although these same authors also failed to demonstrate a neuroprotective effect of nicotine [71].

The question still remains as to which receptor subtypes are implicated in the neuroprotective effects of nicotine. The availability of subunit knockout mice has offered some insight into this area. It has recently been demonstrated [70] that in α4 subunit knockout mice the ability of acute nicotine treatment to protect against methamphetamine-induced neurodegeneration is lost, thus implicating this receptor subtype in the neuroprotective effects of nicotine. This suggestion is also consistent with the receptor subtypes implicated in the production of FGF-2 mentioned above [24]. However, nicotine still demonstrated a trend towards neuroprotection in the α4 knockout mice, although this failed to reach significance [70], suggesting that other receptor subtypes may also be involved in the neuroprotective response to nicotine albeit to a lesser extent. The α6 subunit is one such likely candidate. α6 mRNA is highly expressed in catecholaminergic neurones and it has been shown that α6 mRNA levels increase in the substantia nigra following MPTP treatment in monkeys [82]. Receptors containing this subunit may, therefore, make for excellent therapeutic targets. We have profiled the neuroprotective ability of nicotine and a range of nAChR agonists in a unilateral nigral 6-OHDA model of PD. The results (summarised in Table 2; Murray and O'Neill, unpublished results) indicate that chronic treatment with nicotine (1mg/kg daily for 14 days) or A-85380 (β2-preferring agonist) can provide some protection in this model. In contrast, various other subtype selective ligands (α7, β4-preferring) fail to show any protection. Interestingly, in our hands the broad spectrum agonist, nicotine, appears to provide the most robust protection again implying that more than one receptor subtype is involved (i.e perhaps α4, β2 and/or α6).

4.5 Summary

In conclusion, there is strong evidence that activation of nAChRs (including at least the α4β2 subtypes) can provide symptomatic improvement in PD. In addition, although inconsistent, there is a large amount of evidence suggesting that nAChR stimulation can provide some protection against various toxic insults commonly used as models of PD, both *in vitro* and *in vivo*. However, the data suggest that the protective effects of nicotine are modest, with no protection seen against severe nigrostriatal tract lesions. Moreover the effects appear to depend on the dosing schedules adopted and the absolute dose administered, with lower doses providing neuroprotection, but higher doses often displaying a neurotoxic profile. The mechanisms underlying the neuroprotective effects of nicotine remain to be clarified although various mechanisms ranging from antioxidant activities through to the induction of growth factors have so far been proposed.

5. ALZHEIMER'S DISEASE

Cholinergic deficit and β−amyloid deposition in the brain are among the hallmarks of AD. There is a substantial decrease in the amount of high-affinity nAChRs (mainly $\alpha_4\beta_2$), compared to α7 subtype nicotinic and muscarinic receptors as AD progresses [18, 83]. Consistent with the hypothesis of a neuroprotective and trophic role of nicotine, an increasing number of *in vitro* and *in vivo* studies together with epidemiological data have shown that drugs interacting with neuronal nAChRs have a potential to be useful in the treatment of AD. Since β-amyloid-induced neuronal loss can

Table 2. Effects of Various nAChR Ligands on Tyrosine Hydroxylase Immunoreactivity (Measuring Neuroprotection) in the 6-OHDA-Lesioned Rat Model of PD

Compound (Full name)	Compound (Short name)	nAChR Subtype(s)	Neuroprotective effect
Nicotine	Nicotine	Broad spectrum	+
5-ethynyl nicotine or altiniciline	SIB-1508Y	β2 preferring	-
3-(2-azetidinylmethoxy)pyridine	A-85380	β2 preferring	+
((±-4-[2-(1-methyl-2-pyrrolidinyl)ethyl]thio]phenol HCl	SIB-1553A	β4 preferring	-
(R)-(+)-5'-phenylspiro[1-azabicyclo[2.2.2]octane-3,3'(3'H)-furo[2,3-b] pyridine]	Astra ligand	α7 selective	-

(Data are a tabulated summary of in house data generated at Eli Lilly & Co. Ltd).

be one of the reasons why high-affinity nAChRs disappear during the course of the disease, neuroprotection by these compounds is a possible mechanism to explain how nAChR activation can ameliorate AD-like symptoms such as memory, learning and attention deficits.

5.1. βAmyloid Toxicity *in Vitro*

In vitro, nicotine has been reported to be protective against β-amyloid fragment 25-35-induced cell death in rat neurocortical and hippocampal cultures [19, 84]. This protection was mediated by nicotinic, rather than muscarinic AChRs, since the protection was blocked by the selective AChR antagonists mecamylamine and hexamethonium, but not by the muscarinic antagonist atropine. More specifically, the α7 nicotinic receptor subtype is implicated in this response since the protection was also inhibited by α-bungarotoxin [19]. Interestingly, α4β2 nicotinic receptor activation by cytisine has also been found to be protective against β-amyloid-induced cell death in the same culture system [85]. Further support for a possible contribution of α4β2 receptors comes from findings that dyhidro-β-erythroidine also inhibited nicotine-induced protection against β-amyloid-evoked injury. However, the lack of selective pharmacological tools for α4β2 nicotinic receptors - e.g. cytisine is a partial agonist on α4β2 receptors, and also a full agonist on α7 receptors – makes elucidation of the contribution of this subtype difficult [86]. The contribution of α7 receptors seems far less equivocal. In addition to the study outlined above, activation of nAChR has also been shown to be neuroprotective against β-amyloid toxicity in other cell culture systems (rat PC12 and human SK-N-SH cell lines) and against other toxic APP fragments such as CT_{105} in an α7-mediated manner [87, 88, 89]. Moreover, Svensson *et al.* [88, 90] reported that compounds used successfully in the symptomatic treatment of cholinergic deficits in AD (e.g. acetylcholinesterase inhibitors, β−estradiol) also protect against β−amyloid toxicity in different cell lines in clinically relevant concentrations, and this effect is mediated by α7 nicotinic receptors [88, 90].

Despite the large number of studies that reported neuroprotective effects of nAChR activation against various insults, the underlying mechanisms of this neuroprotection remain largely unknown. In rodent primary neuronal cultures, β−amyloid 25-35 toxicity has been reported to be mediated by glutamate excitotoxicity, as the prototype NMDA antagonist MK-801 exerted a complete blockade of β−amyloid induced cell death [91, 92]. Although another toxic amyloid fragment, β−amyloid 1-42 has been shown not to be toxic to cortical neurones itself, co-applied with otherwise non-toxic doses of glutamate, it produced significant cell death which could also be prevented by NMDA-receptor antagonism [91]. Therefore, the underlying pathways of nicotine-mediated neuroprotection against glutamate-induced cell death discussed earlier in this review could also explain the effects of nicotine and nAChR agonists on β−amyloid 25-35 toxicity. Upregulation of anti-apoptotic proteins may provide an additional means of neuroprotection. For example, in the case of β-amyloid 1-42 toxicity, PI3 and Fyn kinases have been found to be associated with α7 nicotinic receptors, and α7 receptor activation induced the phosphorylation of the serine-threonine protein kinase akt and increased the levels of anti-apoptotic proteins, Bcl-2 and Bcl-x_L, providing possible means of neuroprotection [91, 92]. Although an effect of nAChR agonists on the beta-sheet structure of β-amyloid has also been considered as a potential mechanism to decrease the neurotoxic effect of amyloid fragments, no such modulation has been found in primary neurocortical cultures [93]. In addition to its direct protective effects, nicotine and α7 receptor agonists can also enhance the release of APPs, that are neurotrophic and neuroprotective, as shown in a study using the PC12 cell line [89].

Although neuronal loss is certainly an important factor in the cholinergic deficit and associated cognitive dysfunction in AD, studies on transgenic animals that overexpress human β−amyloid proteins containing mutations linked to early onset AD showed that learning deficits can occur well before neuronal cell death [94]. The lack of neuronal loss suggests that in the early phase of the disease, these impairments are more likely to be connected to disturbances in the normal signalling pathways involved in cognitive function, rather than to simply neuronal loss. Supporting this view, β−amyloid 1-42 has been reported to bind α7 nAChRs with very high affinity [95]. This interaction has been shown to be rather selective, since binding to α4β2 receptors showed a 5000 fold lower affinity [95]. β-amyloid has also been reported to be co-precipitating with nicotinic α7 receptors from human brain tissues [95]. This association can be inhibited by β-amyloid fragment 12-28, suggesting the position of a possible site of association. The exceptionally high affinity of β-amyloid to α7 nAChRs could serve as a trigger in the formation of amyloid plaques and contribute to selective cholinergic neurodegeneration. Moreover, binding of nanomolar concentrations of β−amyloid to α7 nAChRs reversibly blocked receptor function [96]. This block has been described as non-competitive, voltage-independent and mediated by the N-terminal extracellular domain of the receptor. Because of the role of α7 receptors in learning and memory, impaired receptor function could likely be an important factor in cognitive deficits in AD. Similar interactions between β−amyloid and α7 nAChRs were reported in hippocampal slice preparations [97]. It has also been demonstrated that β−amyloid 1-42 couples to the extracellular-signal regulated kinase-2 (ERK-2), a member of the MAP-kinase family, through binding to the α7 receptor [98]. This leads to the up-regulation of the α7 receptor itself, and a concomitant down-regulation of ERK-2. Diminished ERK-2 activity eventually causes decreased phosphorylation of a transcription factor CREB, known to be an important mediator of long term potentiation [99].

5.2. Effects on Cognition and in Cholinergic Lesion Models

The role of ACh in cognition has been well established with the development of acetylcholinesterase inhibitors which, have proven symptomatic actions on cognition in rodent models and in the clinic. For this reason several groups have investigated agents that increase ACh release in

the hippocampus in animal models of cognition and ageing. Thus, several studies have reported cognitive improvements with nicotine and broad spectrum nicotinic agonists (for review see [100]; Levin and Rezvani in this issue). Some of the most recent evidence has suggested that the improvement in cognition may be mediated by α7 nAChRs [101, 102]. It is worth noting that most of these studies were carried out using aged animals or models where there was either an ischaemic lesion or cholinergic deficit induced by nucleus basalis lesions or fimbria fornix lesions [103, 104]. Much of the older data was generated with GTS-21. GTS-21 is an agonist at α7 receptors and an antagonist at α4β2 and indeed the 4-hydroxy metabolite may be the active component [105]. As with many α7 ligands GTS-21 is also active at 5-HT3 receptors, but despite this has been widely profiled *in vivo* (for review see Baker *et al*. and Bencherif and Schmitt in this issue). As mentioned earlier in this section GTS-21 provided both functional and protective effects after global ischaemia [32, 106]. The compound has also provided cognitive improvements in some mice, rat and monkey models (see [107] for review) and this is sensitive to mecamylamine [101]. Further support for a role of α7 in cognitive performance has come from additional studies showing enhanced Morris water maze [108] and 17 arm radial maze [109] performance in aged rats with GTS-21. The most definitive link between all these studies and the potential for neuroprotection comes from data indicating that GTS-21 protects against neocortical neuronal cell loss after nucleus basalis lesions in rats [103]. Clearly, further studies like this are required to confirm the link with α7, but more recent studies have shown that the selective α7 agonist AR-R-17779 ((-)-spiro[1-azabicyclo[2.2.2]octane-3,5'-oxazolidin-2'-one]) also improves radial arm maze performance after fornix lesions in rats [104]. This data suggests that at least the functional improvements appear to be mediated by α7 receptors. Finally, it should be noted that there is an established literature on nicotinic receptor abnormalites in AD as reviewed recently [110, 111].

5.2. Summary

On reviewing the *in vitro* and *in vivo* data it is clear that nAChRs play an important role in memory formation and there is strong evidence that activation of these receptors may have neuroprotective actions. Taken together, activation of α7 nAChRs in particular has the potential to improve pathological conditions in AD, not only as symptomatic treatment by simply improving cholinergic function, but also potentially by i) providing neuroprotection against β–amyloid toxicity and ii) directly antagonising the detrimental effects of β–amyloid on signal transduction pathways involved in learning and memory. Further neuroprotective studies are required using *in vivo* models to confirm this effect. The availability of more potent and selective ligands and the use of transgenic models (e.g. APP transgenic mice) may help clarify the role of nAChRs in ageing and AD. As we prepare this review Nordberg and co-workers (2002) have reported that chronic nicotine treatment reduces β-amyloidosis in transgenic mice carrying the Swedish mutation of human amyloid precursor protein [112].

6. OVERALL SUMMARY AND CONCLUSIONS

This review has considered the *in vitro* and *in vivo* evidence relating to the potential neuroprotective effects of nicotine and nAChR activation in both acute and chronic forms of neurodegenerative disease. In conclusion, it is fair to say that although inconsistent, there is a large amount of evidence suggesting that nAChR stimulation can protect against various toxic insults used *in vitro* and *in vivo*. The compounds used to date have largely not been selective for the different nAChR subtypes (apart from some of the selective α7 agonists) and indeed some have both agonist actions on one receptor subtype coupled with antagonist actions on other subtypes (for example GTS-21 and metabolites). Thus it is not yet clear whether activation or desensitisation of nAChRs is important in mediating the protection or whether indeed the protection is mediated by production of growth factors, prevention of toxin access to neurones, enhancement of toxin metabolism or a whole host of other suggested mechanisms discussed above. Regardless of the cellular mechanisms underlying the response, these protective effects coupled with the functional effect on neurotransmitter release suggest that targeting neuronal nAChRs may be a novel approach to the treatment of both the symptoms and development of chronic neurodegenerative disorders. The potential of subunit specific drugs to have the same beneficial effects, while possibly having a more selective action and therefore a preferential side-effect profile provides an exciting new perspective for the future treatment of both the symptoms and progression of PD and AD.

APPENDIX 1: ABBREVIATIONS

Abbreviation	Full Name	IND / trade name
A-85380	3-(2(S)-azetidinylmethoxy)pyridine	
ABT-418	(S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole	
ABT-594	(R)-5-(2-azetidinylmethoxy)-2-chloropyridine	
ACh	acetycholine	
AChR's	acetylcholine receptors	
AD	Alzheimer's disease	

(Appendix). contd..

Abbreviation	Full Name	IND / trade name
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid	
APP	Amyloid precursor protein	
Astra II	(R)-(+)-5'-phenylspiro[1-azabicyclo[2.2.2]octane-3,3'(3'H)-furo[2,3-b] pyridine]	
BDNF	brain-derived neurotrophic factor	
AR-R-17779	(-)-spiro[1-azabicyclo[2.2.2]octane-3,5'-oxazolidin-2'-one]	
CNS	central nervous sytem	
CREB	cAMP regulatory element-binding protein	
ENA-713	(+)-(S)-N-ethyl-3-[(1-dimethylamino)ethyl]-N-methylphenylcarbamate	Rivastigmine
ERK-2	Extracellular-signal regulated kinase-2	
Fe	iron	
FGF-2	basic fibroblast growth factor (bFGF)	
GABA	γ-aminobutyric acid	
GTS-21	DMXBA or 3-(2,4-dimethoxybenzyidene)-anabasine	
5-HT	5-hydroxytryptamine, serotonin	
IMR32	human derived neuroblastoma cell line	
L-DOPA	L-3,4-dihydroxyphenylalanine	Levodopa
MAP-2	microtubule associated protein	
MK-801	(5*S*, 10*R*)-(+)-5-methyl-10,11-dyhydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine	dizocilpine
MPP^+	1-methyl-4-phenylpyridinium	
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine	
mRNA	messenger ribonucleic acid	
nAChR	nicotinic acetylcholine receptors	
NADH	nicotinamide adenine dinucleotide reduced form	
NGF	nerve growth factor	
NMDA	N-methyl-D-aspartate	
NO	nitric oxide	
nNOS	neuronal nitric oxide synthase	
6-OHDA	6-hydroxydopamine	
PBN	α-phenyl-N-tert-butyl nitrone	
PC12	rat adrenal pheochromocytoma cell line (designated PC12)	
PD	Parkinson's disease	
PI3	phosphatidyl inositol 3	
SIB-1508Y	5-ethynyl nicotine or altiniciline	altiniciline
SIB1553A	((±-4-[2-(1-methyl-2-pyrrolidinyl)ethyl]thio]phenol HCl	
TBI	Traumatic Brain Injury	
TC-2559	(E)-*N*-methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine	
THA	1,2,3,4-tetrahydro-5-aminoacridine	Tacrine

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