INTRODUCTION

Nicotine is an agonist of nicotinic acetylcholine receptors (nAChRs), which take their name precisely from their potent interaction with the alkaloid (1). nAChRs pertain to the super family of ligand-gated ion channels, classically related to the rapid flow of information in the central nervous system (CNS) (2). They are involved in several neuronal processes, like anxiety, attention, memory processing and pain (3). Lately other functions, such as neuroprotection, have started to be acknowledged. In view of the role of cholinergic transmission in CNS pathologies, the realisation that the nAChR can protect against a variety of insults like oxidative stress, excitotoxicity, apoptosis, etc. is of paramount importance. Since there is no general consensus over the interpretation of available evidence, this contribution is an attempt to answer some of the questions raised by putative nAChR neuroprotection in the 6-hydroxydopamine (6-OHDA) model of experimental parkinsonism in rats.

NEUROPROTECTION BY NICOTINE IN IN VITRO STUDIES

Numerous studies have confirmed the protection conferred by nicotine on neuronal cells in culture against diverse insults, such as excitotoxins, (4-6) or beta-amyloid toxicity (7). Blockade of the neuroprotective effects, by nAChR antag-
onists, provides evidence pointing to a specific mediation by different subtypes (8).

Literature has recently been published describing the intracellular mechanisms activated by the entrance of ions through the receptor channel. In this regard, calcium (Ca++) appears to play a critical role. It has been demonstrated that an independent Ca++ signal may be responsible for the neuroprotection nicotine confers on hippocampal neurones in culture against glutamate excitotoxic insult. The intracellular signals elicited are still debated, although mitogen-activated protein (MAP) kinase appears to be activated (9). On the other hand, a neuroprotective role for nicotine in cultures of spinal cord neurones undergoing apoptosis elicited by caspases due to arachidonic acid insult, has been described (10). The intracellular mechanisms activated appear to vary according to the neurone population involved.

In addition, it is known that nicotine induces an increase in some neuronal growth factors, like basic fibroblast growth factor (bFGF) and brain-derived neurotrophic factor (11,12), which could be mediating the neuroprotective effects.

NEUROPROTECTION BY NICOTINE IN IN VIVO STUDIES

In contrast with the uniform results obtained in in vitro studies, in vivo experiments investigating neuroprotection by nicotine have revealed discrepancies. Continuous nicotine infusion showed protective effects against neuronal loss provoked by hemitransection of dopamine (DA) pathways (13). On the other hand, following the same administration schedule, nicotine failed to revert the striatal depletion of DA provoked by the injection of 6-OHDA into the substantia nigra (SN) (14). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) systemic application in vivo resulted in a significant decrease in striatal DA content that was not reverted by nicotine (15). Furthermore, it has been shown that nicotine produces an enhancement of MPTP neurotoxicity (16,17). In another study nicotine had protective effects against diethyldithiocarbamate enhancement of MPTP lesion (18).

NEUROPROTECTION BY NICOTINE IN THE 6-OHDA MODEL OF EXPERIMENTAL PARKINSONISM

Even taking into account differences between models, factors like the route and schedule of nicotine administration appear to affect the results in in vivo studies. In an attempt to help further understanding of this controversy, we used the neurotoxin 6-OHDA injected into the SN as a model of experimental parkinsonism. We explored the neuroprotective capacity of nicotine by changing administration schedules, the extent of the nigral lesion and the time of assessment.

The influence of nicotine administration schedule and nAChR mediation

The different nicotine (1mg/kg, subcutaneous; sc) administration schedules used were:
a) in the eight-day assessment, i) 18 h before 6-OHDA, ii) 4 h before 6-OHDA, iii) 4h before, and 20, 44 and 68 h after 6-OHDA, iv) 20, 44, 68 h after 6-OHDA;
b) in the sixteen-day assessment, v) 4 h before and a daily dose for 12 days after 6-OHDA.

Eight days after the lesion and applying protocol iii for the administration of nicotine (4h before and 20, 44 and 68 h after 6-OHDA) a significant prevention in striatal DA loss was seen (Fig. 1). Concomitantly a significant decrement in the increased striatal dopamine turnover due to the lesion was also observed (data not shown). Neither of these two effects were seen in the sixteen-day assessment (Fig. 2).

Chlorisondamine (10 mg/kg), a long-lasting nAChR antagonist, administered sc 30 min before the first application of nicotine, reverted the neuroprotective effects of nicotine, demonstrating the
Neuroprotection by nAChR in experimental parkinsonism

**Fig. 1** - Striatal dopamine assessed 8 days after the injection of 6-OHDA (6 µg) expressed as percent (mean ± S.D.) of right vs left CS. Different protocols of subcutaneous nicotine administration were applied: 1) 18hrs before 6-OHDA; 2) 4hrs before 6-OHDA; 3) 4hrs before and 20, 44 and 68hrs after 6-OHDA; 4) 20, 44 and 68hrs after 6-OHDA. Each group is compared with its respective saline, *p<0.05. In each group no. = 8.

**Fig. 2** - Striatal dopamine assessed 8 and 16 days after the injection of 6-OHDA (6 µg) expressed as percent (mean ± S.D.) of right vs left CS. For 8 and 16 days (1) nicotine was applied 4hrs before and 20, 44 and 68hrs after 6-OHDA. For 16 days (2) nicotine was injected 4hrs before and a daily dose for 12 days after 6-OHDA. Each group is compared with its respective saline, *p<0.05. In each group no. = 8.
participation of this receptor (Fig. 3) (19). A limited, intermittent scheme of activation of nAChR appears to be critical to achieve protection.

The influence of the lesion extent, the time of assessment and the region of the dopaminergic pathway analysed.

In our experiments we used two different doses of 6-OHDA: 10 µg and 6 µg to produce 90% and 50% neuronal death, respectively. Nicotine prevented both the decrease in striatal DA concentrations and in DA turnover after a 6 µg dose. When the 10 µg dose was administered nicotine did not have these effects (Fig. 4).

The nicotine assays were performed eight and sixteen days after the lesion. At just eight days after 6-OHDA significant changes in striatal DA concentrations were seen (Fig. 2) in animals treated with nicotine versus saline treated animals.

Prevention of DA decrease after 6-OHDA was observed in the dopaminergic terminals in the corpus striatum (CS) and not in DA cell bodies in the SN (data not shown).

In summary, the effects of nAChR stimulation on the prevention of DA decrease after 6-OHDA only occurred in the dopaminergic terminal field with a lesion extent that affected approximately 50% of SN neurones.

PRESYNAPTIC INVOLVEMENT IN NICOTINE PREVENTION OF DOPAMINE DECREASE IN THE CORPUS STRIATUM

Through microdialysis assessment we explored the mechanisms of nicotine protection in

![Graph showing dopamine concentrations](image)
the CS. Our results showed that the basal extra-
cellular DA levels were kept similar to those of
non-lesioned animals. In addition, nicotine did
not modify significantly the basal extracellular
levels when compared to saline group (Fig. 5,
see over). When challenged with potassium chlo-
ride (KCl), 6-OHDA lesioned animals released a
significantly lower amount of DA compared to
control animals. Animals treated with 6-OHDA
and nicotine improved significantly the response
to the KCl challenge (Fig. 6, see over) (20).

These results could indicate either increased
DA availability at the terminal region or the
maintenance of more functional terminals. In the
first case, it would be likely that tyrosine hy-
droxylase (TH) is activated through nAChR
stimulation.

L-DOPA AND TOXICITY

To further understand the mechanisms in-
volved in increased presynaptic DA we decided
to study TH activity through the assessment of
L-dopa concentrations after inhibition of aromatic L-aminoacid decarboxylase (AADC) by
NSD-1015. When the inhibitor was adminis-
tered 30 min before tissue dissection, an in-
crease in the L-dopa levels was observed in 6-
OHDA treated animals. On the other hand, DA
levels were unaltered in both ipsilateral (treated)
and contralateral (untreated) CS. In contrast
with the expected results, when AADC inhibitor
was administered to animals injected with 6-
OHDA plus nicotine, the levels of L-dopa and
DA in the ipsilateral region showed a significant
decrease, whereas the contralateral side showed
no difference compared to the 6-OHDA group
(Figs 7, 8, see over).

LACK OF NEUROPROTECTION AT THE
NEURONAL CELL BODIES

Neuronal cell bodies in the SN were stained
for TH immunoreactivity, and also counted in
untreated control animals, after 6-OHDA and 6-
OHDA-nicotine treatment. A clear decrease in

Fig. 4 - Striatal dopamine assessed 8 days after the injection of 6-OHDA (10 µg) expressed as percent (mean + S.D.) of
right vs left CS. Subcutaneous administration of nicotine 4hrs before, and 20, 44 and 68hrs after 6-OHDA failed to coun-
teract significantly the dopamine decrease. In each group no. = 8.
Fig. 5 - Effect of different treatments on extracellular basal DA levels in the CS. Bars represent basal DA values of each experimental group (media + SEM, no. = 5-8 animals). No significant difference was found between nicotine and saline treated groups.

Fig. 6 - Effect of different treatments in the response of DA levels to KCl stimulus in the CS. Bars represent the media + SEM of 5-8 animals of the extracellular DA levels in response to KCl stimulus for each experimental group. *: Nicotine + 6-OHDA treatment statistically different from saline + 6-OHDA; o: saline + 6-OHDA statistically different from saline alone.
Neuroprotection by nAChR in experimental parkinsonism

Fig. 7 - Striatal dopamine tissue levels assessed 8 days after 6-OHDA (6 µg) and 30 min after NSD-1015 (100 mg/kg i.p.) expressed as percent (mean ± S.D.) of right versus left CS. In each group no. = 8. *: p<0.05. Nicotine treatment (4 hrs before and 20, 44, 68 hrs after 6-OHDA administration) statistically different from saline.

Fig. 8 - Striatal L-dopa tissue levels assessed 8 days after 6-OHDA (6 µg) and 30 min after NSD-1015 (100 mg/kg i.p.) expressed as percent (mean ± S.D.) of right versus left CS. In each group no. = 8. *: p<0.05. Nicotine treatment (4 h before and 20, 44, 68 h after 6-OHDA administration) statistically different from saline.
CONCLUDING REMARKS

The results of the experiments described above reveal a complex picture of the biochemical effects of nicotine – and nAChR – on the lesioned dopaminergic system. The effects are observed:
- only in the terminal striatal region
- after an early assessment
- after partial lesion
- after an intermittent administration schedule of nicotine (pre and post 6-OHDA).

These results could provide a basis on which to explain much of the present controversy surrounding neuroprotective nicotine – and nAChR stimulation – effects.

Intermittent and limited administration of nicotine appears to be essential in preventing the decrease in DA levels in the CS. It is important to stress that the effects were observed four days after the last dose of nicotine. This would indicate the occurrence of a plastic phenomenon probably triggered by a secondary signal elicited by the agonistic interaction with the receptor. An acute pharmacological effect can thus be discounted, a conclusion supported by the lack of prevention of DA decrease by intermittent daily doses of nicotine.

There is much evidence pointing to an α7 nAChR mediation of nicotine protective effects on cellular cultures, and this evidence could be taken as a starting point for further investigation (21-23). α7 nAChR shows higher permeability to Ca++ and could be the agent responsible for the first intracellular signal. Dajas-Bailador et al.
have demonstrated that the intracellular Ca++ signal elicited by nicotine can be identified even in the presence of a potent NMDA-elicited Ca++ entrance (9). It can be hypothesised that activation of a specific Ca++ domain would trigger a cascade of intracellular events leading to the activation of nerve growth factors. The particular administration schedule that prevents DA decrease in the CS is superimposed on the production of nerve growth factor (fibroblast growth factor - FGF) following nicotine administration (the expression of FGF peaks at 4hrs and returns to normal levels after 24 hrs) (24). Accordingly, a one-week continuous infusion of nicotine in an MPTP model of experimental parkinsonism failed to show neuroprotective actions due to a reduction in the production of FGF mRNA (25).

In our in vivo experiments, nicotine prevention of the decrease in DA concentrations is blocked by chlorisondamine, a non-specific nAChR antagonist (19). The weak specificity of nAChR antagonists makes it difficult to determine which of the subtypes of nAChR are involved in this prevention.

The fact that in the 6-OHDA model of parkinsonism the effects of nAChR are limited to terminal dopaminergic fields is important in the interpretation of the subjacent acting mechanisms. In the CS there is a very important local cholinergic innervation that regulates the release of neurotransmitters, including DA (26). This is a clear cut difference versus cell culture experi-
ments in which nicotine acts directly on the membrane of the cell to be protected. In the CS *in vivo*, nAChR provokes the release of DA and thus the neurotransmitter can itself elicit a secondary cascade of events. In any case the important interaction between cholinergic and dopaminergic systems in the CS could explain the marked activation of dopaminergic terminals observed in the model.

In spite of the fact that almost 50% of striatal dopaminergic terminals have been seen to be lost after 6-OHDA insult, microdialysis studies showed that extracellular DA concentrations are kept close to control levels in the CS. This would indicate the activation of the DA releasing mechanisms, very probably as a result of a marked increase in DA synthesis. The significant increase in the DA released by a KCL challenge in 6-OHDA-nicotine treated animals in the CS would confirm the further activation of the dopaminergic terminal. A hypothesis of increased survival of dopaminergic terminals cannot be discarded.

The fact that AADC inhibition causes a further loss of DA levels in the CS in 6-OHDA-nicotine treated animals compared with 6-OHDA alone would appear to demonstrate the fragility of the dopaminergic terminal sustaining the compensatory mechanisms. An acute and high increase of oxidative stress, caused by the sudden increment in intra-terminal L-dopa concentrations could be the cause of membrane lesioning and terminal loss. In any case, the biochemical changes observed at eight days are transient and no effects on the neurone population at sixteen days can be detected. A neuroprotective mechanism should keep more neurones alive after the toxic aggression. This does not apparently occur with nicotine and nAChR in our case. Nevertheless, is has to be borne in mind that the 6-OHDA insult is extremely aggressive. If under these conditions, after a precise administration schedule and with a partial lesion, nAChR can show a reversion of dopaminergic cell loss, it is conceivable that plastic cholinergic mechanisms are important in the early stages of Parkinson’s disease.

REFERENCES

9. Dajas-Bailador FA, Lima PA, Wonnacott S. Alpha nicotinic acetylcholine receptor subtype mediates nicotine protection against NMDA excitotoxicity in primary hippocampus cultures through a Ca++ dependent mechanism. Neuropharmacology 2000;39:2799-2807
10. Acid-induced caspase activation cytochrome C release in apoptosis of cultured cord neurons. J Neurochem 2001;76:1395-1403


