Interactive report

Nicotine prevents striatal dopamine loss produced by 6-hydroxydopamine lesion in the substantia nigra¹

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Abstract

While the work of several groups has shown the neuroprotective effects of nicotine in vitro, evidences for the same effects in vivo are controversial, mainly regarding neuroprotection in experimental models of Parkinson’s disease. In this context, we investigated the capability of various systemic administration schedules of nicotine to prevent the loss of striatal dopamine levels produced by partial or extensive 6-hydroxydopamine (6-OHDA) lesion of rat substantia nigra (SN). Eight days after 6- and 10-µg injections of 6-OHDA in the SN there was a significant decrease of dopamine concentrations in the corpus striatum (CS) and a concomitant increase in dopamine turnover. While 10 µg 6-OHDA produced an almost complete depletion of dopamine in the SN, 6 µg decreased dopamine levels by 50%. Subcutaneous nicotine (1 mg/kg) administered 4 h before and 20, 44 and 68 h after 6-µg 6-OHDA, prevented significantly the striatal dopamine loss. Administered only 18 or 4 h before or only 20, 44 and 68 h after, nicotine failed to counteract the loss of dopamine or the increase in dopamine turnover observed in the CS. Nicotine also failed to prevent significantly the decrease of striatal dopamine levels produced by the 10-µg 6-OHDA intranigral dose. Chlorisondamine, a long-lasting nicotinic acetylcholine receptor antagonist, reverted significantly the nicotinic protective effects on dopamine concentrations. These results are showing that putative neuroprotective effects of nicotine in vivo depend on an acute intermittent administration schedule and on the extent of the brain lesion. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Although initially introduced in Europe with tobacco plants for medicinal purposes, nicotine has become, through tobacco addiction, a harmful presence in our modern societies. The physiological effects of nicotine are varied, affecting the peripheral (cardiovascular, neuromuscular) and the central nervous system (CNS) [18], and are based on its ability to mimic the actions of acetylcholine at the nicotinic acetylcholine receptors (nAChR). The deleterious effects of tobacco use have precluded a comprehensive study of the effects of nicotine in the CNS. In recent decades, however, several studies demonstrated that nicotine could have a positive effect in several neuronal processes like attention, memory processing, pain, or neuroprotection [9]. The identification of more than 10 genes codifying the different sub-units of the nAChR gave a structural basis for the search of specific subunit combinations with beneficial effects in the CNS [9].

Several reports have shown that there is a negative correlation between smoking and the appearance of Parkinson’s disease. These epidemiological findings gave support to the hypothesis of a neuroprotective role of nicotine [12,36] and prompted the study of the effects of nicotine stimulation in several models of neurotoxicity. At present, numerous studies have confirmed the protection conferred by nicotine to neuronal cultures against toxic insults like excitotoxins [1,11,22,31,35,42] or β-amyloid toxicity [29]. Blockade of the neuroprotection effects by nAChR antago-
nists provided evidence for the specific mediation by different subtypes of nAChR [29,31,43].

In spite of this in vitro evidence, in vivo studies are still controversial when regarding the neuroprotective properties of nicotine treatments. While continuous nicotine infusion has been demonstrated to protect against neuronal loss produced by dopamine pathways hemitransection [25,26], the striatal depletion of dopamine after injection of 6-hydroxydopamine (6-OHDA) in the substantia nigra (SN) was unaltered by the same treatment [10]. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) systemic application in vivo resulted in a significant decrease of striatal dopamine content that was not prevented by nicotine in some studies [21], while another group showed that nicotine had protective effects against diethylthioucarbamate enhancement of MPTP lesions [34]. Moreover, in the MPTP model of experimental parkinsonism, nicotine has even shown an enhancement of the neurotoxicity [5,19].

Beyond these discrepancies, it is likely that the great variety of experimental conditions reported, differing in the schedule and method of nicotine administration, may in part explain the differences observed. Thus, reports using chronic treatments did not show effects in vivo [21,27], while the acute intermittent administration appeared to show protective effects in the models reported [27,34].

In this context, and as a contribution to the characterization of the role played by nAChR in Parkinson’s disease, we studied the putative neuroprotective effects of nicotine in the 6-OHDA model of experimental parkinsonism, assessing whether the timing and schedule of nicotine administration as well as the extent of the lesion are factors that could effectively influence the neuroprotection profile.

2. Materials and methods

2.1. Animals

Experiments were carried out using male Sprague–Dawley rats (230–260 g). Animals had access to food and water ad libitum, and were housed in groups of six in a temperature controlled environment on a 12-h light/dark cycle.

2.2. Drugs and reagents

Chemicals for HPLC analysis, artificial cerebrospinal fluid (aCSF) and saline were purchased from Baker (Phillipsburg, PA, USA). Dopamine (hydrochloride), 3,4-dihydroxyphenylacetic acid (DOPAC), 6-OHDA, (−)-nicotine (tartrate) and L-ascorbic acid were obtained from Sigma (St. Louis, MO, USA). Chlorisondamine (CHL) was donated by Novartis Pharmaceuticals (NJ, USA). 6-OHDA solution for intranigral injection was prepared dissolving it in aCSF containing 0.2% ascorbic acid. Nicotine and CHL were dissolved in saline.

2.3. Intranigral injection of 6-OHDA

Animals were anaesthetized with halothane (Fluothane, Zeneca) and placed in a D. Kopf stereotaxic frame. Through a skull hole, the needle (0.022 mm o.d., 0.013 mm i.d.) of a Hamilton syringe (5 μl), attached to a micro-injection unit (D. Kopf), was lowered to the SN. Coordinates (H, −4.8; L, −2.2; V, −7.2) were determined from bregma, according to the atlas of Paxinos and Watson [37].

A total of 2.0 μl of a 6-OHDA solution (3 and 5 μg/μl for the 6- and 10-μg doses) was injected for 4 min and the needle was slowly withdrawn, allowing the drug to diffuse for another minute. Body temperature was maintained at 37°C using a temperature control system.

2.4. Experimental groups and nicotine administration schedule

2.4.1. Partial lesion (6 μg 6-OHDA in the SN)

Five groups of rats (n=8) injected with 6-OHDA (6 μg) in the right SN, received 1 mg/kg nicotine subcutaneously according to the following protocols: (1) nicotine 18 h before 6-OHDA; (2) nicotine 4 h before 6-OHDA; (3) nicotine 4 h before, and 20, 44 and 68 h after 6-OHDA; (4) nicotine 20, 44, 68 h after 6-OHDA; (5) similar administration schedule to (3) plus CHL 10 mg/kg s.c. 30 min before the first application of nicotine.

Each of the five experimental groups had its own control group of 6-OHDA plus saline instead of nicotine.

For control of 6-OHDA vehicle, six rats were injected with similar volumes of aCSF with 0.2% ascorbic acid.

2.4.2. Extensive lesion (10 μg 6-OHDA in the SN)

Animals injected with 10 μg 6-OHDA received nicotine or saline treatment according to the administration schedule (3).

2.5. Neurochemical analysis

For neurochemical analysis rats were decapitated 8 days after 6-OHDA injection, brains rapidly removed and the left and right SN and corpus striatum (CS) dissected out and kept at −70°C. Next day tissue samples were weighed, sonicated in perchloric acid 0.1 M (200 and 1000 μl for the SN and CS, respectively) and centrifuged (15 000×g) for 15 min at 4°C. Then, samples were injected into an HPLC system (PM-80 BAS, USA) equipped with a C-18 column (5-μm particles, 220 mm×4.6 mm; BAS, USA) and an electrochemical detector (LC-4C BAS) with oxidation potential at +0.75 V (glassy working carbon electrode versus an Ag/AgCl reference electrode). The mobile phase was composed of citric acid (0.15 M), sodium octyl
sulphate (0.6 mM), 4% acetonitrile and 1.6% tetrahydrofuran at pH 3.0; with a flow rate of 1.0 ml/min.

2.6. Statistical analysis

Dopamine is presented as percent of right versus left tissue levels, considering the left side (untreated) as 100%. In each experimental group comparison were made against controls as defined in Section 2.4. For turnover analysis, in each experimental group, the ratio DOPAC/DA of the ipsilateral (injected) side was compared with its control (contralateral side). Means comparison was performed using independent t-test (two-tailed). Statistical significance was chosen at $P<0.05$.

3. Results

3.1. 6-OHDA

Intranigral injections of 6 and 10 μg 6-OHDA, produced a significant decrease of dopamine tissue levels in the right CS and SN (ipsilateral, treated side) when compared with the left, non-treated, one (contralateral) (Table 1). As shown in Table 1, the 10-μg dose produced an effect in the dopamine concentrations higher than the 6-μg dose. Control rats injected in the SN with 2 μl of aCSF (in 0.2% ascorbic acid), did not show any difference in dopamine levels between right and left CS (right, 17 583±1733; left, 17 651±2832 ng/mg of wet tissue weight). Dopamine turnover, expressed as the ratio DOPAC/DA, was significantly increased in the ipsilateral CS of 10 μg 6-OHDA treated rats (Fig. 4). In 6 μg 6-OHDA treated DOPAC/DA showed a marked tendency to increase with border line statistical significance ($P=0.06$).

3.2. 6-OHDA+Nicotine

3.2.1. Partial lesion (6 μg 6-OHDA in the SN)

In experimental group 3, nicotine treatment (4 h before, and 20, 44 and 68 h after 6-OHDA injection), prevented significantly the 6-OHDA effects on striatal dopamine tissue levels (Fig. 1), but not in the SN (Fig. 3). Nicotine also reverted the tendency of 6-OHDA treated to show an increased striatal dopamine turnover (Fig. 4). Nicotine by itself did not change dopamine levels and DOPAC/DA ratio in the contralateral CS and SN (data shown in the respective figure legend).

In experimental groups 1, 2 and 4, nicotine treatment only before (18 or 4 h); or only after (20, 44 and 68 h) 6-OHDA injection, failed to have any effect on striatal dopamine and DOPAC/DA ratio (Figs. 5 and 6). Nicotine by itself did not change dopamine levels and DOPAC/DA

| Table 1 |
| Dopa mine levels as ng/g of wet tissue weight±S.D. Ipsilateral dopamine level compared with contralateral one for each group |
| 6-OHDA, 6 μg | 6-OHDA, 10 μg |
| Cor trolateral | Ipsilateral | Con trolateral | Ipsilateral |
| Corpus striatum | 15 525±530 | 4775±3.790* | 16 291±2.511 | 458±465* |
| Substantia nigra | 1055±319 | 497±300* | 910±207 | 165±214* |

* $P<0.05$. For each group, n=8.
Fig. 3. Nigral dopamine assessed 8 days after the injection of 6-OHDA (6 or 10 µg) expressed as percent (mean±S.D.) of right versus left SN. Subcutaneous administration of nicotine 4 h before, and 20, 44 and 68 h after 6-OHDA failed to counteract significantly the dopamine decrease. For each group n=8. Control nigral concentration of dopamine (contralateral side): 6 µg 6-OHDA saline 1055±319 and nicotine 961±342; 10 µg 6-OHDA saline 910±207 and nicotine 789±285 (values are expressed as ng/mg of wet tissue weight).

Fig. 4. Bars represent the mean±S.D. of dopamine striatum turnover, expressed as DOPAC/DA ratio. Dopamine and DOPAC levels were assessed 8 days after injection of 6-OHDA in the right SN. Ipsilateral turnover compared with contralateral one for each group, *P<0.05. For each group, n=8.

Fig. 5. Striatal dopamine assessed 8 days after the injection of 6-OHDA (6 µg) expressed as percent (mean±S.D.) of right versus left CS. Different protocols of subcutaneous nicotine administration were applied. P1, 18 h before 6-OHDA; P2, 4 h before 6-OHDA; P3, 4 h before and 20, 44 and 68 h after 6-OHDA; P4, 20, 44 and 68 h after 6-OHDA. Each group compared with respective saline, *P<0.05. For each group, n=8. Control striatal concentration of dopamine (contralateral side): P1 saline 17 986±1450; nicotine 18 119±414; P2 saline 17 722±3321; nicotine 18 521±1788; P3 saline 15 525±530; nicotine 15 816±1996; P4 saline 14 404±1621; nicotine 12 649±1486 (values are expressed as ng/mg of wet tissue weight).

Fig. 6. Bars represent the mean±S.D. of dopamine striatum turnover, expressed as DOPAC/DA ratio. Dopamine and DOPAC levels were assessed 8 days after injection of 6-OHDA in the right SN. Different protocols of nicotine subcutaneous administration were applied. P1, 18 h before 6-OHDA; P2, 4 h before 6-OHDA; P3, 4 h before and 20, 44 and 68 h after 6-OHDA; P4, 20, 44 and 68 h after 6-OHDA. Ipsilateral turnover compared with contralateral one for each group, *P<0.05. For each group, n=8.

**3.2.2. Extensive lesion (10 µg 6-OHDA in the SN)**

When a dose of 10 µg 6-OHDA was used, nicotine treatment according to the administration schedule 3, neither restored significantly striatal dopamine levels (Fig. 2) nor dopamine turnover (Fig. 4). Nicotine by itself did not change dopamine levels and DOPAC/DA ratio in the contralateral CS and SN (data shown in the respective figure legend).

**3.3. 6-OHDA +nicotine +CHL**

When the nicotinic antagonist CHL was administered before nicotine and 6 µg 6-OHDA (experimental group 5), nicotine did not revert the striatal dopamine loss induced by 6-OHDA (Fig. 1). Dopamine and DOPAC/DA ratio both in ipsilateral and contralateral side did not change after CHL (data shown in the respective figure legend).

**4. Discussion**

The injection of 6-OHDA into the rat SN leads to a progressive and massive death of dopaminergic nerve cells and to a corresponding depletion of dopamine in the CS [3,13,28]. It is recognized that 6-OHDA insults involve at least two mechanisms of cell death: free radical production and/or reactivity of quinone products [13,23,30,32]. In agreement with these previous reports [3,13,39], the intranigral injection of 6-OHDA produced a significant decrease of dopamine levels in the ipsilateral CS, a reduction that can be directly correlated with a neuronal loss in the SN pars compacta [3,8]. The 6-OHDA treatments also produced an increase in the DOPAC/DA ratio, evidencing an accelerated dopamine turnover that likely
reflects an increase in the activity of the remaining dopaminergic terminals. This increase in dopamine turnover has been recognized as a functional response to the loss of dopaminergic terminals after neuronal death in the SN, to maintain the normal levels of extracellular dopamine [3,39].

The decrease of dopamine concentrations in the SN and CS produced by 6 μg 6-OHDA was utilized as the milder toxic stimulus for the assessment of nicotine protection. The 10-μg dose produced a more pronounced decrease of dopamine concentrations in the SN and the CS and was therefore used to test nicotine properties against a more toxic insult. Altogether, the utilization of two different doses of 6-OHDA, with marked differences in the extent of toxicity achieved, allowed us to assess the importance of the lesion extent in the nicotine protective effects.

Nicotine treatment prevented the striatal dopamine loss after the 6-μg 6-OHDA injection when administered 4 h before and 20, 44 and 68 h after the toxin. The nAChR antagonist CHL, administered 30 min before this administration schedule, almost completely reverted the nicotine protection after 6-OHDA. Previously, only Maggio et al. [34] had studied the blockade of nicotine protection by the antagonist mecamylamine, demonstrating its dependence on the activation of nAChR. CHL is a biquaternary amine, classically utilized as a nicotinic antagonist at autonomic ganglia, which produces a remarkably persistent blockade of central nicotinic responses [16,17,38]. Nicotine protection has been linked to different subtypes of nAChR, mainly the homopentameric α7 and the α4β2 [29,31,43]. It is not clear on which nAChR subtype CHL exerts its action, though evidence for a rather broad specificity, excluding the α7 subtypes, has been described [38]. Accordingly, the reversion of nicotine action in our results cannot be safely linked to any sub-population of nAChR subtypes.

When nicotine was injected only 18 or 4 h before or only 20, 44 and 68 h after, it did not prevent the striatal dopamine loss. The dissimilar effects observed after diverse nicotine treatments suggests that the schedule and means of nicotine administration are crucial in the achievement of nicotine protection. In a previous study [10], nicotine infusion in a chronic and continuous manner, failed to protect dopamine depletion in the CS after 6-OHDA lesion in the SN. In addition, several studies assessing nicotine protection in MPTP-induced experimental parkinsonism reported that chronic continuous nicotine did not prevent the dopamine loss, and even worsened it [5,10,21,27]. In contrast, acute intermittent application [27,34] appeared to protect SN neurons. Concurring with these latter reports, our results are showing that an acute intermittent administration schedule is meaningful for restoring dopamine concentrations and turnover in an experimental model of Parkinson’s disease. In addition, and besides being intermittent, our data suggest that the nicotine stimulus should be applied before and also after the nigral lesion. Although not yet proved, the desensitization of nAChR has been postulated as the basis for the lack of effects of continuous and/or chronic nicotine treatment [25]. As it is known, nicotine administration to rats produces an increase in nAChR population in the CNS, which appears to be a result of nAChR receptor desensitization [20,42]. Desensitization has also been utilized as an explanation for the protective effects of nicotine at the level of postsynaptic receptors in the SN, in view that it would decrease the activation tone of neurons, probably contributing to their survival after 6-OHDA lesion [25]. However, desensitization cannot explain the protection given by nicotine, when considering that nAChR blockade with CHL, not only failed to be protective per se, but also significantly prevented the protection observed after nicotine treatment. Accordingly, our results suggest that the functional activation of nAChR is deemed necessary for the achievement of nicotine protection.

The intracellular mechanisms by which nicotine exerts its effects are not yet fully understood. In vitro, some studies have shown that actions of nicotine are dependent upon the entry of calcium to the cell [14,15], but not much progress has been observed regarding the intracellular cascades activated by increased cytoplasmic calcium. In vivo, nicotine or nicotine agonist treatments, have been shown to produce regional increases in trophic factors, like basic fibroblast growth factor (FGF-2) [6,7]. In this regard, it has been shown that the glial cell line-derived growth factor (GNDF), can protect SN neurons against 6-OHDA neurotoxicity [40,41,44]. Other trophic factors have been shown to be protective in models of ischemia, excitotoxicity and hypoxia [2,4,24]. In agreement with the lack of protection after continuous nicotine treatment mentioned above, the growth factor increase obtained with acute nicotine treatment is not seen after chronic continuous infusion [10,27]. Moreover, the induction of FGF-2 expression peaked 4 h after an injection of nicotine and returned to normal levels within 24 or 48 h [6,7]. Consequently, our protective administration schedule of nicotine would coincide with the peak production of growth factors, suggesting that they could ultimately be the active protective substances.

While nicotine prevented the effect of the 6-μg injection of 6-OHDA, it failed to achieve the same protection when the higher dose of 6-OHDA (10 μg) was injected. Moreover, the most significant recovery on dopamine levels after partial lesions, is only seen in the CS, and not in the SN. These results are indicating that during mild toxic insults a high percentage of the neuronal population would still be responsive to the protective actions of nicotine stimulation, a situation that is probably not occurring when higher doses of 6-OHDA are tried. During the progression of degenerative diseases like Parkinson’s, neuronal loss has a progressive development, most probably resembling the effects of a milder dose of 6-OHDA.

Previous reports have shown that 6-OHDA oxidative
insult peaks soon after the 6-OHDA injection [13], starting a self-maintained process of neuronal damage. The increased dopamine turnover of surviving neurons would partially preserve functional activity, while contributing to further neuronal damage through increased oxidative stress. The protection given by nicotine is not only reflected in the dopamine tissue levels, but also in the tendency to stabilize dopamine metabolism. The improvement of this latter index of neuronal function would indicate that nicotine is not only preventing neuronal death, but also rectifying the functional abnormalities that occur during 6-OHDA neurotoxicity.

In summary, our results show that the acute and intermittent activation of central nAChR can support neuronal survival after 6-OHDA-evoked neurotoxicity. Together with previous observations of nicotine neuroprotection they lend support for the concept that nAChR may exert a trophic role in neuronal development and survival, and encourage the view that nAChR are convincing targets for neuroprotective therapies [33].

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