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In vivo modulation of dopaminergic nigrostriatal pathways by cytisine derivatives: Implications for Parkinson's Disease

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ABSTRACT

Nicotinic acetylcholine receptor agonists are considered potential pharmacological agents for Parkinson's Disease treatment, due to their ability to improve experimental Parkinson symptomatology, reduce 3,4-dihydroxy-L-phenylalanine-induced dyskinesias and stop the neurodegenerative process at an experimental level. In the present work, the ability of the nicotinic agonist cytisine and two halogenated derivatives (3-bromocytisine and 5-bromocytisine) to induce striatal dopamine release was characterized *in vivo* by microdialysis. Cytisine, 5-bromocytisine and nicotine were much more efficacious than 3-bromocytisine in eliciting dopamine release in response to their local application through the microdialysis probe. Moreover, the agonists were intermittently administered before and after an intranigral injection of 6-hydroxydopamine (6-OHDA), and striatal dopamine tissue levels were assessed 8 days after the lesion. Both cytisine and its 5-bromo derivative (but not the 3-bromo derivative) significantly prevented the decrease of striatal dopamine tissue levels induced by 6-OHDA. These results suggest that the efficacy of nicotinic agonists to stimulate dopamine release *in vivo* through presynaptic nicotinic receptors could be related to their potential to induce striatal protection.

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1. Introduction

The motor symptoms that are at the core of Parkinson's Disease – rigidity, bradykinesia and postural instability – are linked to the loss of nigrostriatal dopaminergic neurons (Singh et al., 2007). However, the administration of 3,4-dihydroxy-L-phenylalanine (L-DOPA) or dopamine receptor agonists, the key therapeutic strategies in use today to improve the dopaminergic functions in Parkinson's Disease, are unable to stop the neurodegenerative process (Singh et al., 2007).

Acetylcholine modulates the function of the nigrostriatal dopamine system through multiple subtypes of nicotinic acetylcholine receptors located postsynaptically on the neuronal cell bodies of the substantia nigra and presynaptically on terminals at the corpus striatum. Because nicotinic receptor activation increases the frequency of firing at the neuronal bodies (Lichtensteiger et al., 1982; Clarke et al., 1985) or causes neurotransmitter release at the dopaminergic terminals (Lichtensteiger et al., 1982; Clarke et al., 1985; Kaiser and Wonnacott, 2000; Wonnacott et al., 2000; Zhou et al., 2001; Zoli et al., 2002), nicotinic receptors are considered to be potential therapeutic targets for the treatment of Parkinson's Disease symptoms (Quik et al., 2007a, b). In addition, epidemiological studies have shown that smokers have a lower incidence of Parkinson's Disease and nicotine, a non-selective nicotinic receptor agonist, has been postulated to be responsible for this effect (Baron, 1986; 1996; Gorell et al., 1999).

Previously, we have shown that systemic administration of nicotine partially prevents the decrease of dopamine levels in the corpus striatum following administration of 6-hydroxydopamine (6-OHDA) (Costa et al., 2001; Abin-Carriquiry et al., 2002; Urbanavicius et al., 2007). As Quik et al. recently reviewed, other studies have confirmed these results in different experimental models of Parkinson's Disease (Visanji et al., 2006; Quik et al., 2006a, b, 2007c; Khwaja et al., 2007; Urbanavicius et al., 2007).

Depending on the experimental paradigm, both α7 and/or α4β2 subtypes of nicotinic receptors have been described as mediating protection by nicotine against various toxic insults in cell cultures (Kihara et al., 1997, 1998; Hejmadi et al., 2003; Stevens et al., 2003), consistent with a requirement for stimulation of more than one receptor subtype for protection (O'Neill et al., 2002; Dajas-Bailador and Wonnacott, 2004; Wonnacott et al., 2006). *In vivo*, although nicotine-induced protection against experimental parkinsonian lesions has been shown to be mediated by nicotinic receptor (Costa et al., 2001), identification of particular receptor subtypes has not

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been reported yet. Visanji et al. recently demonstrated that in contrast to the non-selective agonist nicotine, subtype-selective nicotinic agonists did not protect striatal dopamine terminals from a 6-OHDA insult in rats (Visanji et al., 2006), suggesting that interaction with multiple nicotinic receptor subtypes may be necessary to afford neuroprotection.

Cytisine, an alkaloid present in many plants of the Leguminosae family and a broad-specific nicotinic receptor agonist, has been shown to protect cells in culture against toxic agents (Kihara et al., 1998; Jonnala and Buccafusco, 2001). In this context, we recently studied a series of cytisine derivatives in striatal slices, showing that halogenation in positions 3 and 5 could increase or reduce respectively their potency to induce dopamine release without significant changes in their selectivity for nicotinic receptors subtypes (Abin-Carriquiry et al., 2006).

Given the potential of nicotinic receptor agonists as therapeutic agents in Parkinson's Disease it appeared meaningful to extend the characterization of cytisine and its bromo derivatives, studying their pharmacological profile *in vivo*. In the present study we explored the ability of cytisine, 3-bromocytisine (3-BrCy) and 5-bromocytisine (5-BrCy) to induce dopamine release *in vivo* by microdialysis, as well as their capacity to prevent the decline in dopamine tissue levels in experimental Parkinson's Disease.

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.

Experimental procedures were approved by the Committee on Ethical Care and Use of Laboratory Animals of the Instituto de Investigaciones Biológicas Clemente Estable in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

2.2. Materials

Chemicals for high performance liquid chromatography (HPLC) analysis, artificial cerebrospinal fluid and saline were purchased from Baker (Phillipsburg, PA, USA). Dopamine (hydrochloride), 3,4-dihydroxyphenylacetic acid, 6-OHDA, (−)-nicotine tartrate, and L-ascorbic acid were obtained from Sigma (St. Louis, MO, USA). Chlorisondamine was donated by Novartis Pharmaceuticals (NJ, USA).

2.3. Cytisinoids

Cytisine and its derivatives were obtained as previously described (Houlihan et al., 2001; Slater et al., 2003) and used as the hydrochloride salts. Briefly, cytisine was purified from the seeds of the Mexican plant *Sophora secundiflora* using standard methodology. Bromination of cytisine with a slight excess of molecular bromine in acetic acid led to the formation of a mixture of products containing a small amount of 3,5-dibromocytisine and mainly 3-BrCy and 5-BrCy. These were separated by column chromatography on silica gel, crystallized to homogeneity, and characterized by ^{1}H and ^{13}C NMR and HREIMS. Definitive structure assignments were based on ^{1}H–^{1}H COSY experiments.

2.4. Microdialysis

Animals were anaesthetized with ketamine (90 mg/kg)/xylazine (5 mg/kg) and placed in a D. Kopf stereotaxic frame. Through a skull hole, a guide cannula (BAS, MD-2250) was implanted into the dorsal striatum (caudate–putamen) and secured to the skull with steel screws and dental cement. The coordinates for cannula implantation were A/P=+0.6, L/M=+3.2, D/V=−3.2 from Bregma according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986). After surgery, the rats were housed individually into test cages and allowed to recover for 24–48 h. On the experimental day, a microdialysis probe (BAS MD-2204, membrane length=4.0 mm) was inserted into the cannula. The location of the tip of the microdialysis probe was −7.2. The probe was connected to a microperfusion pump and continuously perfused with artificial cerebrospinal fluid (147 mM NaCl, 3.4 mM $CaCl_2$, 4.0 mM KCl) at a flow rate of 2.0 μl/min. The sample collection (30 μl sample every 15 min) was started after a 2-h equilibration period. Concentrations of dopamine were determined immediately using an Epsilon Electrochemical Detector e5P with oxidation potential set at +0.650 V (glassy carbon working electrode versus a Ag/AgCl reference electrode). The column (Phenomenex Luna, 5 μm, C18, 4.6×100 mm) was kept at room temperature. The mobile phase consisted of citric acid (0.15 M), sodium octylsulphate (0.6 mM), 4% acetonitrile and 1.6% tetrahydrofuran at pH 3.0; with a flow rate of 1.0 ml/min. The average concentration of the first three dialysis samples was determined as baseline and defined as 100%. Nicotinic receptor agonist solutions were prepared in artificial cerebrospinal fluid adjusting the pH to 7.0 with HCl/NaOH and perfused through the dialysis probe during 15 min. Doses of nicotine utilized were according to the literature (Marshall et al., 1997; Quarta et al., 2007). At the end of each experiment, the probe was removed and the animal sacrificed. The brain was immediately removed, dissected and frozen for confirmation of probe localization (Abin-Carriquiry et al., 2002).

The *in vitro* recovery of each nicotinic agonist was measured from a standard 1-mM solution by HPLC-UV. Eluate concentrations of nicotine, cytisine, 3-BrCy and 5-BrCy were determined immediately using a Gilson UV–Visible 118 Detector at 255 nm. The column (Phenomenex Luna, 5 μm, C18, 4.6×100 mm) was kept at room temperature. The mobile phase consisted of acetic acid (50 mM), sodium octylsulphate (0.6 mM) and 15% methanol at pH 3.7; with a flow rate of 1.4 ml/min. A similar recovery of 17% was found for the different nicotinic agonists.

2.5. Intranigral injection of 6-OHDA

6-OHDA lesions were provoked by the injection of 6-OHDA into the substantia nigra as previously described (Costa et al., 2001). Briefly, animals were anaesthetized with halothane (Halocarbon Laboratories, River Edge, NJ, USA) 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) was attached to a micro-injection unit (D. Kopf), and was lowered to the right substantia nigra (H, −4.8; L, −2.2; V, −7.2 from bregma, according to the atlas of Paxinos and Watson). A total of 2.0 μl of a 6-OHDA solution (3 mg/ml) prepared immediately before use, was injected over 1 min and the needle was slowly withdrawn, allowing the drug to diffuse for another 4 min. Body temperature was maintained at 37 °C using a temperature control system (Costa et al., 2001). Control rats for 6-OHDA lesions were injected with 2 μl vehicle (artificial cerebrospinal fluid with 0.2% ascorbic acid).

2.6. Agonists administration schedule

Rats (groups of 6–8) that had been injected with 6-OHDA (6 μg) in the right substantia nigra received nicotinic receptor agonist or saline subcutaneously, according to the following protocols: (1) 4 h before, and 20, 44 and 68 h after 6-OHDA; (2) the same administration schedule following treatment with the long-lasting nicotinic antagonist chlorisondamine (10 mg/kg s.c.) given 30 min before the first application of nicotinic receptor agonist or saline. After chlorisondamine administration, the motor activity of the rats decreased showing also bilateral palpebral ptosis. These effects lasted a few hours, with complete recovery afterwards. No other symptoms were observed.

The starting dose (1 mg/kg) for cytisine and 5-BrCy protection studies was similar to the one shown to be effective for nicotine *in vivo* (Costa et al., 2001). In the case of 3-BrCy, a dose of 1 mg/kg induced

tonic–clonic convulsions that lasted approximately 5 min. These effects limited the concentrations that could be evaluated *in vivo*.

Since even after receiving 0.3 mg/kg 3-BrCy, animals still showed a strong depressed motor activity and ptosis for approximately 1 h, the doses studied were lowered (0.01, 0.05 and 0.10 mg/kg).

2.7. Neurochemical analysis

To measure dopamine tissue levels, rats were decapitated 8 days after 6-OHDA injection, the brains rapidly removed and the left and right corpus striatum dissected and stored at −70 °C. On the next day tissue samples were weighed, sonicated in 1000 µl of perchloric acid (0.1 M) and centrifuged (15,000 *g*) for 15 min. Samples were then injected into an HPLC system (PM-80 BAS, West Lafayette, IN, USA) equipped with a C18 column (5 µm particles, 220 mm×34.6 mm; BAS, USA) and an electrochemical detector (LC-4C BAS) with oxidation potential set at +0.75 V (glassy carbon working electrode versus a Ag/AgCl reference electrode). The mobile phase was composed of citric acid (0.15 M), sodium octylsulphate (0.6 mM), 4% acetonitrile and 1.6% tetrahydrofuran at pH 3.0; with a flow rate of 1.0 ml/min (Costa et al., 2001).

2.8. Statistical analysis

2.8.1. Microdialysis

Dopamine levels were calculated from a standard solution and values were expressed as a percentage of basal levels (individual means of three pre-drug fractions). Statistical analysis was carried out using one- or two-way analysis of variance (ANOVA) for repeated measures of the dopamine values followed by post-hoc *t*-test when appropriate.

2.8.2. Tissue levels

Dopamine levels in the lesioned hemisphere of each animal were expressed as percent of the level in the unlesioned side. Comparison of the means was performed by ANOVA followed by Tukey–Kramer Multiple Comparison test.

3. Results

3.1. Striatal dopamine release evoked by cytisine and bromocytisines

The nicotinic agonists were evaluated for their ability to evoke dopamine release in the striatum by *in vivo* microdialysis following local application via the dialysis probe. Basal striatal extracellular dopamine levels of dialysate (1.91±0.34 nM) were similar to those obtained in our previous studies (Abin-Carriquiry et al., 2002). Local perfusion of nicotine, cytisine and 5-BrCy increased extracellular levels of dopamine in a concentration-dependent way (Fig. 1). 3-BrCy was the most potent and release was obtained already with 0.33 mM concentrations, showing little concentration-dependence over an extended concentration range.

The comparison of dopamine release induced by cytisine and its derivatives at 10 mM concentrations showed that 5-BrCy was significantly

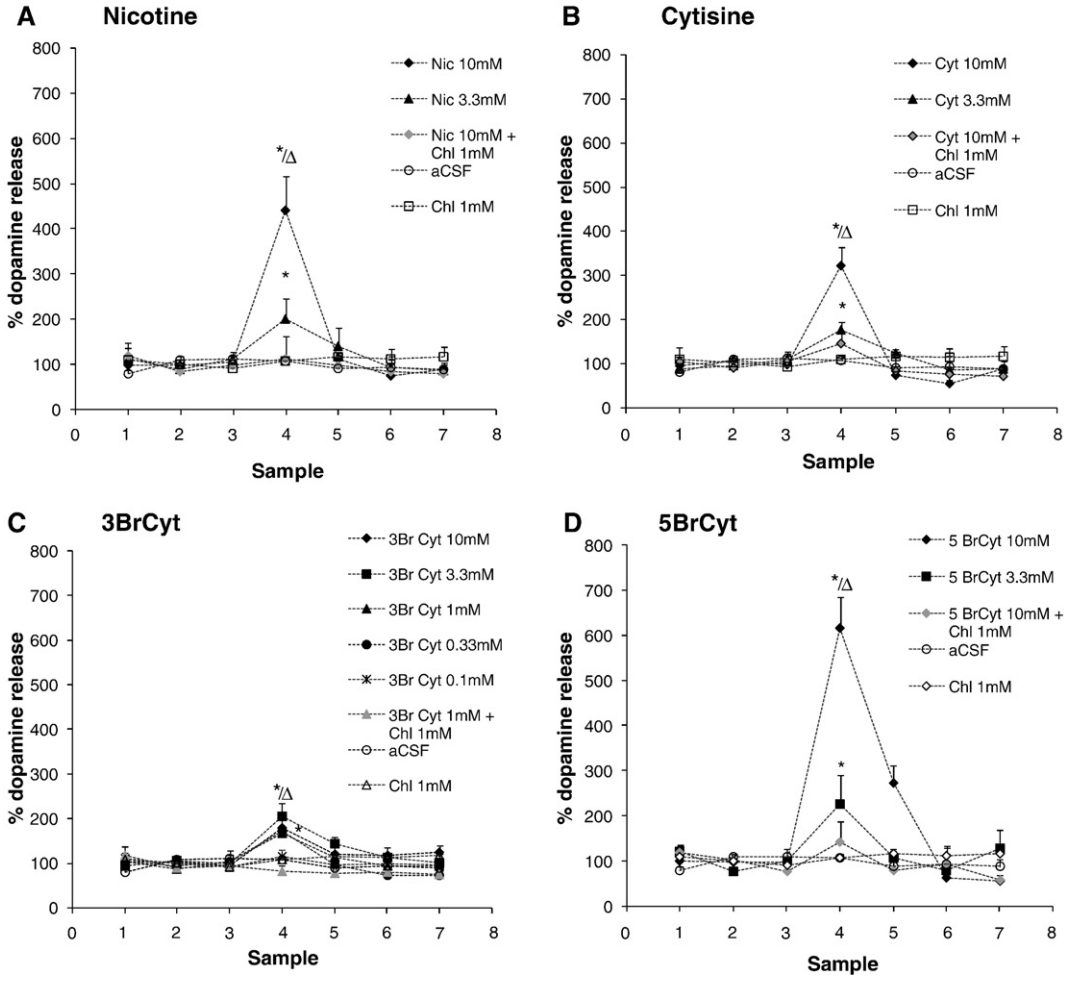

Fig. 1. Extracellular dopamine assessed by microdialysis in awake rats 24 h after the implantation of a cannula in the corpus striatum. Drugs were applied locally through the probe and dopamine release was monitored after the application of nicotine, cytisine, 5-BrCy and 3-BrCy at different doses after three stable basal samples. Data are expressed as percent over the basal levels (mean±S.E.M.). For each dose $n=4$–6. * denotes significant difference against controls (artificial cerebrospinal fluid, aCSF) (*$P<0.05$). Δ * denotes significant difference against agonist+chlorisondamine (Chl) 1 mM (Δ$P<0.05$).

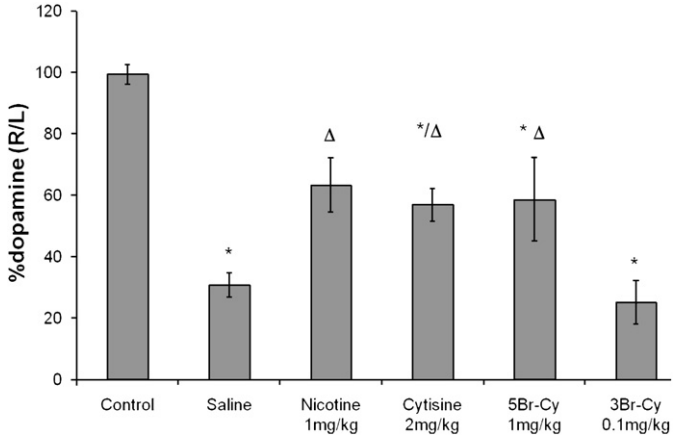

Fig. 2. Striatal dopamine assessed 8 days after the injection of 6-OHDA (6 μg) in the substantia nigra. Agonists (nicotine, cytisine, 5-BrCy and 3-BrCy) were subcutaneously administered 4 h before, and 20, 44 and 68 h after 6-OHDA injection. Data are expressed as percent (mean ± S.E.M.) of lesioned versus intact corpus striatum. For each treatment $n=6–8$. * denotes significant difference against the control group ($^*P<0.05$). Δ denotes significant difference against the saline group ($\Delta P<0.05$).

more efficacious than cytisine and 3-BrCy, being 3-BrCy the less efficacious (Fig. 1).

Perfusion of chlorisondamine (1 mM), a non-specific nicotinic receptor antagonist, prevented the increase in dopamine release induced by every agonist (Fig. 1). Chlorisondamine alone did not affect significantly the basal levels of dopamine (1.61 ± 0.60 nM).

3.2. Prevention of striatal dopamine decrease after 6-OHDA injection

Eight days after intranigral injection of 6 μg of 6-OHDA, there was a significant decrease in dopamine tissue levels in the ipsilateral striatum (lesioned side) when compared with the contralateral (non-lesioned side), (Fig. 2). Control rats injected in the substantia nigra with 2 μl vehicle did not show any difference in dopamine levels between ipsilateral and contralateral striatum (17.5 ± 1.7 and 17.6 ± 2.8 ng/mg of wet tissue weight, respectively).

As reported previously (Costa et al., 2001), intermittent nicotine administration (1 mg/kg, nicotine tartrate salt); significantly attenuated the 6-OHDA-induced decrease in striatal dopamine tissue levels (Fig. 2). Cytisine (2 mg/kg) and 5-BrCy (1 mg/kg), administered according to the same schedule, prevented similarly the decrease of striatal dopamine levels. In contrast, 3-BrCy (0.10 mg/kg) was unable to prevent the decrease of striatal dopamine tissue levels induced by 6-OHDA (Fig. 2). Lower doses of 3-BrCy (0.01, 0.05 mg/kg) did not prevent the dopamine decrease either (data not shown).

The protection afforded by cytisine and 5-BrCy was prevented by the prior administration of the long-acting non-specific nicotinic receptor antagonist chlorisondamine (10 mg/kg). Dopamine levels (expressed as percent of lesioned versus intact corpus striatum) were 19.0 ± 3.9%, 28.3 ± 8.7% and 20.5 ± 8.0% for saline, cytisine and 5-BrCy treatments, respectively.

4. Discussion

Our data showed that local application of cytisine and its bromo derivatives induced dopamine release in the corpus striatum, being cytisine and 5-BrCy, as well as nicotine significantly more efficacious than 3-BrCy.

Besides, cytisine and 5-BrCy prevented the decrease of striatal dopamine tissue levels 8 days after the intranigral injection of 6-OHDA in rats. To the extent of our knowledge, this is the first experimental evidence of *in vivo* protection by nicotinic receptor agonists other than nicotine. This protection was mediated by nicotinic receptor, since it was blocked by chlorisondamine administration. However, pharmacological equivalent doses of 3-BrCy (taking into account that its potency to induce dopamine release is more than one order of magnitude higher than cytisine and 5-BrCy) did not have protective effect against the 6-OHDA lesion.

In a previous work we compared the effects of cytisine halogenation at C3 and C5 on α4β2 and α7 nicotinic receptor binding, showing that both 3-BrCy and 5-BrCy presented higher affinity for heteromeric α4β2 than for homomeric α7 nicotinic receptors. Additionally we evaluated their ability to evoke [^{3}H]dopamine and [^{3}H]noradrenaline release in striatal and hippocampal slices, respectively, showing that both agonists presented greater potency as releasers of [^{3}H]dopamine than of [^{3}H]noradrenaline. Nevertheless, both derivatives do not differ significantly in subtype specificity between themselves or with cytisine or nicotine (Cassels et al., 2005; Abin-Carriquiry et al., 2006).

In recent years an important line of experimental evidence has shown the protective capacity of nicotine in *in vivo* experimental models of Parkinson's Disease, an effect mediated by nicotinic receptor agonism (Costa et al., 2001; Visanji et al., 2006; Quik et al., 2006b, 2007a,c). Our results suggest that the prevention of striatal dopamine decrease is not an exclusive property of nicotine but also of other non-subtype specific nicotinic receptor agonists. Moreover we showed that protection afforded by nicotinic agonists is blocked by chlorisondamine, a non-specific nicotinic receptor blocker (Costa et al., 2001). In this sense Visanji et al. showed that neither the modulation of α4β2 nor α7 subtypes alone appears to provide protection *in vivo* (Visanji et al., 2006). Taken together these results would suggest that broad nicotinic receptor subtype specificity would be necessary for prevention of dopaminergic terminal degeneration.

Nicotine, cytisine and 5-BrCy, which attenuated dopamine loss after 6-OHDA were also the most efficacious in evoking dopamine release. This latter effect was clearly dose-dependent and was blocked by chlorisondamine. 3-BrCy, which failed to induce protective effects in a wide concentration range, was also the least efficacious inducer of dopamine release. In this sense, the agonist dopamine releasing efficacy (reflecting efficacy at nicotinic receptors) appears to be more relevant than potency itself to induce the plastic changes on the dopaminergic pathway.

As an explanatory hypothesis, the experimental evidence suggests that activation of nicotinic receptor leads to Ca^{2+} entry into the terminal (Rapier et al., 1990; Grady et al., 1992; Puttfarcken et al., 2000). The greater efficacy shown by cytisine and 5-BrCy inducing dopamine release, could be associated to a major Ca^{2+} entry to the cell, leading to activation of several steps in the synaptic vesicle cycle (Smith et al., 1998; Stevens and Wesseling, 1998; Turner, 2004) and triggering specific intracellular signalling cascades related to plastic changes in dopamine metabolism and other key protective pathways (Dajas-Bailador and Wonnacott, 2004).

However an indirect pathway, involving the activation of dopaminergic autoreceptors (Bozzi and Borrelli, 2006; Scheller et al., 2007) could also explain the relationship between efficacy and protection.

Evidence based largely on experimental *in vitro* and *in vivo* rodent studies is showing that dopamine agonists may have neuroprotective properties in addition to their symptomatic effects (Le and Jankovic, 2001; Schapira, 2002). In this sense, ropinirole has been shown to protect mouse striatal neurons against 6-OHDA toxicity, by stimulating the increase of glutathione, catalase and superoxide dismutase antioxidant activities in the striatum, and this effect was mediated through dopamine D_2 receptor (Iida et al., 1999). Nevertheless, further experiments are required in order to discriminate between both mechanisms.

The results obtained in the present work show for the first time *in vivo*, that efficacious non-selective nicotinic receptor agonists, other than nicotine, are able to reduce striatal dopamine depletion induced by 6-OHDA injection in the substantia nigra and efficaciously evoke dopamine release *in vivo*.

The protective effect of cytisine and 5-BrCy is a challenging result that provides a new lead for understanding the central nervous system plasticity mediated by nicotinic receptors, suggesting their potential for Parkinson's Disease treatment.

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