Neuroprotective Effects of the MAO-B Inhibitor, PF9601N, in an *In Vivo* Model of Excitotoxicity

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SUMMARY

Background: PF9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] is an inhibitor of monoamine oxidase B (MAO-B), which has shown to possess neuroprotective properties in several *in vitro* and *in vivo* models of Parkinson's disease (PD). As there is evidence that excitotoxicity may be implicated in the pathophysiology of several neurodegenerative diseases, the aim of the present work was to investigate the effects of PF9601N in an acute *in vivo* model of excitotoxicity induced by the local administration of kainic acid during striatal microdialysis in adult rats. **Methods:** The basal and evoked release of neurotransmitters was monitored by HPLC analysis of microdialysate samples and tissue damage was evaluated histologically "*ex vivo*." **Results:** PF9601N (40 mg/kg, single i.p. administration) reduced the kainate-evoked release of glutamate and aspartate and increased taurine release, but it had no effect on the release of dopamine, DOPAC, and HVA. PF9601N pretreatment also resulted in a significant reduction in the kainate-induced astrocytosis, microgliosis, and apoptosis. **Conclusions:** The results suggest PF9601N to be a good candidate for the treatment of neurodegenerative diseases mediated by excitotoxicity.

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Introduction

Factors that may contribute to cell death in neurodegenerative disorders include genetic predisposition, environment, oxidative stress, inflammation, and energy failure. There is also some evidence that excitotoxicity, induced by glutamate, leading to calcium overload in affected neurons, may be an etiological factor in a variety of neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases, multiple sclerosis, and amyotrophic lateral sclerosis (see [1–6]).

Although most studies on the role of excitotoxicity in neurodegenerative disorders have concentrated on the NMDA receptors [7,8], non-NMDA receptors also appear to be involved [1,5,9,10]. It has also been suggested that kainate receptors might be a target for the development of new pharmacotherapeutic approaches in Parkinson's disease (PD) [11,12]. Kainate is a cyclic analogue of glutamate that is commonly used to study the mechanisms by which excitotoxicity induces cell death [13]. It provokes neuronal death by both apoptotic and necrotic processes [14,15]. It also increases astrogliosis and activates microglia [16–19].

Kainate-induced neurodegeneration in rodents has been considered a useful model to elucidate the pathogenesis of excitotoxicity in neurodegenerative diseases [20].

l-Deprenyl (selegiline) is a MAO-B inhibitory propargylamine derivative, which was introduced as an adjunct to levodopa therapy in PD and subsequently shown to possess neuroprotective properties in several PD models and to slow the rate of the disease progression (see [21,22]). Cruces et al. [23] described a series of propargylamine derivatives. Among these, the acetylenic tryptamine derivative PF9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] (Scheme 1) was shown to be a more potent and selective MAO-B inhibitor than *l*-deprenyl [24].

PF9601N (see Scheme 1) showed neuroprotective properties *in vivo*, against the toxicity induced by MPTP [25] and the intrastriatal injection of 6-hydroxydopamine [26]. *In vitro* studies showed PF9601N to prevent the cell death induced by mitochondrial complex I inhibition and to maintain the mitochondrial membrane potential [27]. It was also protective against endoplasmic reticulum stress [28,29] and able to prevent the dopamine-induced damage to SH-SY5Y cells [30] as well as to reduce reactive

Scheme 1 Chemical structure of PF9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine].

oxygen species [31]. The antiapoptotic effects of PF9601N appear to involve prevention of transcription factor p53 stabilization and its subsequent transcriptional activity [28].

In this study, we investigated the possible beneficial effect of PF9601N in an acute *in vivo* model of excitotoxicity induced by the intrastriatal perfusion of kainate during striatal microdialysis in adult rats. Changes in extracellular amino acids and monoamines were studied by HPLC analysis of the microdialysates. After microdialysis, cerebral tissue was evaluated immunohistochemically in terms of the ability of PF9601N to reduce the kainate-induced astrogliosis and microglial reactivity and to exert an antiapoptotic effect.

Materials and methods

Animal Housing

All experiments involving laboratory animals were performed according to the Italian Guidelines for Animal Care (D.L. 116/92), which were in accordance with the European Communities Council Directives (86/609/ECC), with all efforts to minimize animal suffering and the number of animals necessary to collect reliable scientific data. Formal approval to conduct the experiments was obtained from the animal subjects review board of the University of Florence. No alternatives to *in vivo* techniques are available for this type of experiment.

Male Wistar rats weighing 200–220 g (Harlan, Milan, Italy) were housed in transparent cages under controlled conditions of

temperature ($23 \pm 1^\circ\text{C}$) and humidity, with free access to food and water and with a 12-h light/dark cycle.

In vivo Experiments

Surgery and Microdialysis Procedure

As previously described [32], rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus. A

Figure 1 Time course of the output of aspartate (A), glutamate (B), and taurine (C) evoked by local administration of K⁺ and kainate (KA). The extracellular concentrations were expressed as percentage of their respective basal levels for illustrative purposes. Original concentration values, expressed as fmol/ μL of perfusate (nM), were used for statistical analysis. The time course reported on the x-axis shows the consecutively collected 20-min fractions, where -60 min, -40 min, and -20 min represent those collected under basal conditions, followed by those corresponding to the K⁺- and KA-stimulated period (from +20 min to +180 min), where +20 min represents the fraction collected immediately after the last basal fraction of -20 min, during which K⁺ was administered through the probe. The mean net stimulated output $\pm$ SEM, obtained from the area under the K⁺, from -20 min to +60 min, or kainate-evoked concentration time curve (AUC), from +80 min to +160 min, normalized to one time interval of 20 min, with basal output subtracted, are shown, together with their statistical analysis parameters, in Table 1. The concentration peaks of aspartate, glutamate, and taurine evoked by K⁺ and KA were statistically significant over basal levels. Concentration peaks were not statistically significant, either in the absence of stimulation or when the stimulation was applied to PF9601N pretreated animals.

concentric microdialysis guide cannula (CMA/Microdialysis AB, Stockholm, Sweden) was implanted vertically in the right neostriatum and fixed to the skull with self-curing acrylic (Kerr Italia, Salerno, Italy) and the skin was sutured. Stereotaxic coordinates for the neostriatum, relative to bregma, were AP 0.7, L 3.2, DV -5.5 mm [33].

The microdialysis experiments were performed on freely moving rats 24 h later, starting at 9.00 AM. The artificial cerebrospinal fluid (aCSF) comprised the following: 140 mM NaCl, 3 mM KCl, 1.2 mM CaCl_2 , 1.0 mM MgCl_2 , 1.2 mM Na_2HPO_4 , 0.27 mM NaH_2PO_4 , and 7.2 mM glucose (pH 7.4). The dialysis probe (4 mm probe tip; CMA/Microdialysis AB) was inserted into the guide cannula and perfused with aCSF (rate 3 $\mu\text{L}/\text{min}$) via polyethylene tubing (i.d. 0.38 mm) connected to a 1-mL syringe mounted on a micro-infusion pump (CMA/100; CMA/Microdialysis AB). After a 90-min stabilization period, the dialysate samples were collected every 20 min. Three samples, shown in Figures 1 and 2 as -60 min, -40 min, and -20 min, were collected to measure the basal extracellular concentrations of neurotransmitters under resting conditions, before 50 mM KCl was locally applied during collection of the subsequent fraction indicated as $+20$ min, to represent the first 20-min fraction collected of the potassium-stimulated period. After collecting three more fractions, the rats were anesthetized (chloral hydrate 400 mg/kg i.p.), during collection of the 80-min fraction, just before an excitotoxic concentration (1 mM) of kainate was applied to the neostriatum for 20 min (during collection of the 100-min fraction) through the dialysis probe. The control PF9601N alone treated group also received chloral hydrate anesthesia during collection of the 80-min fraction. Further dialysate samples were then collected every 20 min, up to 180 min. PF9601N pretreatment consisted in a single i.p. administration of 40 mg/kg dissolved in the vehicle dimethylformamide, 3 h before the intrastratial kainate perfusion; the untreated group received an i.p. administration of the vehicle, 3 h before the application of kainate. The dialysate samples were either analyzed immediately or frozen before analysis. In some experiments, dialysates were split, using 10 μL for amino acid and 50 μL for amines and metabolites determinations.

Rats were killed by decapitation 48 h after kainate stimulation. Then, the striatum was isolated, fixed in 4% formalde-

hyde in phosphate-buffered saline (PBS) for 72 h, and embedded in paraffin. Finally, 5- μm -thick sections were cut using a microtome (Polaron, Watford, UK) and examined by light microscopy (Nikon Eclipse E800; Nikon Instrument SpA, Florence, Italy) to verify the correct placement of the probe. Only data obtained from rats with correctly implanted probes were included in the results.

For histological studies, sections were first deparaffinized, gradually hydrated, and finally washed twice in 0.1 M PBS (pH 7.4) containing 0.3% Triton X-100 (PBS-0.3Tx).

Figure 2 Time course of the output of dopamine (A), DOPAC (B), and HVA (C) evoked by local administration of K^+ and kainate (KA). The extracellular concentrations were expressed as percentage of their respective basal levels for illustrative purposes. Original concentration values, fmol/ μL of perfusate (nM), were used for statistical analysis. The mean net stimulated output $\pm$ SEM, obtained from the area under the K^+ , from -20 min to $+60$ min, or kainate-evoked concentration time curve (AUC), from $+80$ min to 160 min, normalized to one time interval of 20 min, with basal output subtracted, are shown in Table 2, together with their statistical analysis parameters. The concentration increases in dopamine evoked by K^+ and KA were statistically significant, and they were not affected by PF9601N pretreatment. The concentration of the metabolites of dopamine, DOPAC and HVA, was significantly reduced by K^+ and KA and was not affected by PF9601N pretreatment. PF9601N pretreatment, in the absence of K^+ and KA stimulation, did not affect the basal levels of dopamine and metabolites.

Table 1 Effects of PF9601N pretreatment on K⁺ (50 mM)- and kainate (1 mM)-evoked output of aspartate, glutamate, taurine, and GABA in rat striatum

Amino acid AUC (nM, 20 min)	K ⁺			KA		
	K ⁺ Vehicle (n = 13)	K ⁺ PF9601N (n = 5)	None PF9601N (n = 5)	KA Vehicle (n = 13)	KA PF9601N (n = 5)	None PF9601N (n = 5)
Aspartate						
Evoked (mean ± SEM)	120 ± 34	129 ± 39	−2 ± 17	138 ± 43	82 ± 42	7 ± 11
95% CI of the mean	(45–195)	(20–239)	(−50 to 45)	(45–231)	(−34 to 199)	(−23 to 37)
One-sample test	<i>P</i> = 0.0045	<i>P</i> = 0.0304	NS	<i>P</i> = 0.0070	NS	NS
Glutamate						
Evoked (mean ± SEM)	303 ± 96	38 ± 72	−23 ± 46	354 ± 81	33 ± 34	58 ± 53
95% CI of the mean	(94–512)	(−162 to 238)	(−151 to 105)	(176–531)	(−59 to 129)	(−89 to 205)
One-sample test	<i>P</i> = 0.0083	NS	NS	<i>P</i> = 0.0010	NS	NS
Taurine						
Evoked (mean ± SEM)	694 ± 111	784 ± 117	−34 ± 14	1295 ± 138	1852 ± 118	55 ± 24
95% CI of the mean	(452–935)	(459–1108)	(−74 to 6)	(994–1597)	(1525–2180)	(−12 to 122)
One-sample test	<i>P</i> < 0.0001	<i>P</i> < 0.0026	NS	<i>P</i> < 0.0001	<i>P</i> < 0.0001	NS
GABA						
Evoked (mean ± SEM)	68 ± 15	54 ± 15	0.8 ± 5	−2 ± 2	8 ± 3	13 ± 5
95% CI of the mean	(35–101)	(11–96)	(−12 to 14)	(−7 to 4)	(−0.1 to 14)	(−3 to 22)
One-sample test	<i>P</i> < 0.0010	<i>P</i> = 0.0243	NS	NS	NS	NS

K⁺, 50 mM KCl locally for 20 min; KA, 1 mM kainate locally for 20 min; PF9601N, 40 mg/kg i.p. [N-(2-propynyl)-2-(5-benzoyloxy-indolyl) methylamine] 3 h before KA; Vehicle, N,N-dimethylformamide i.p. 3 h before KA; Evoked, mean net output obtained from the area under the K⁺ or KA-evoked concentration time curve (AUC), normalized to one time interval of 20 min and subtracted of basal output; Concentration values were fmol/μL of perfusate (nM); 95% CL (Confidence Limits) not including zero and the probability level of the one-sample test were taken as an indication of the statistical significance of the mean evoked output of each treatment group; Means ± SEM were derived from number of observations/animals, n = 4–6; NS, not significant.

Table 2 Effect of PF9601N pretreatment on K⁺ (50 mM)- and kainate (1 mM)-evoked output of striatal dopamine, DOPAC and HVA in rat striatum

Amine AUC (nM, 20 min)	K ⁺			KA		
	K ⁺ /KA Vehicle	K ⁺ /KA PF9601N	None PF9601N	K ⁺ /KA Vehicle	K ⁺ /KA PF9601N	None PF9601N
Dopamine						
Evoked (mean ± SEM)	26.90 ± 6.77	23.03 ± 6.19	−0.007 ± 0.088	15.15 ± 4.38	14.41 ± 4.31	−0.001 ± 0.029
95% CI of the mean	8.09–45.71	7.11–38.94	−0.24 to 0.25	2.99–27.31	0.708–28.12	−0.083 to 0.080
One-sample test	<i>P</i> = 0.0165	<i>P</i> = 0.0137	NS	<i>P</i> = 0.0258	<i>P</i> = 0.0442	NS
DOPAC						
Evoked mean ± SEM	−98.46 ± 21.38	−144.20 ± 29.40	1.33 ± 23.67	−286.90 ± 37.72	−335.2 ± 43.15	2.47 ± 26.81
95% CI of the mean	−157.8 to −39.09	−219.8 to −68.60	−64.37 to 64.04	−391.6 to 68.59	−446.2 to −224.3	−71.98 to 76.92
One-sample test	<i>P</i> = 0.0100	<i>P</i> = 0.0045	NS	<i>P</i> = 0.0016	<i>P</i> = 0.0006	NS
HVA						
Evoked mean ± SEM	−50.62 ± 8.33	−72.70 ± 16.63	6.27 ± 8.96	−124.00 ± 35.70	−147.1 ± 27.11	−8.34 ± 13.03
95% CI of the mean	−77.13 to −24.10	−115.4 to −29.96	−18.61 to 31.16	−223.31 to −24.84	−223.3 to −71.81	−44.51 to 27.82
One-sample test	<i>P</i> = 0.0090	<i>P</i> = 0.0072	NS	<i>P</i> = 0.0255	<i>P</i> = 0.0056	NS

n, Number of animals; NS, not significant. K⁺, 50 mM KCl locally for 20 min; KA, 1 mM kainate locally for 20 min; PF9601N, 40 mg/kg i.p. [N-(2-propynyl)-2-(5-benzoyloxy-indolyl) methylamine] 3 h before KA; vehicle, N,N-dimethylformamide i.p. 3 h before KA; evoked, mean net output obtained from the area under the K⁺- or KA-evoked concentration time curve (AUC), normalized to one time interval of 20 min and subtracted of basal output; Concentration values were fmol/μL of perfusate (nM); 95% CL not including zero and the probability level of the one-sample test were taken as an indication of the statistical significance of the mean evoked output of each treatment group.

Amino Acid Determinations

The concentration of the excitatory amino acids, glutamate and aspartate, and the inhibitory amino acids, taurine and GABA, was measured by HPLC with fluorimetric detection, as previously described [34] with minor modifications. Briefly, one 10 μ L aliquot of each microdialysate sample was treated with mercaptoethanol and *o*-phthalaldehyde (OPA) to derivatize the amino acids. The OPA derivatives were then separated on a 5 μ m reverse-phase Nucleosil C18 column (EC 250 $\times$ 4.6 mm; Macherey-Nagel, Duren, Germany) at room temperature, using a mobile phase consisting of methanol and potassium acetate (0.1 M, pH adjusted to 5.48 with glacial acetic acid) at a flow rate of 1.0 mL/min in a three linear steps gradient (from 25% to 90% methanol). HPLC analysis was carried out using a Shimadzu (Shimadzu Italia S.r.l., Milan, Italy) HPLC system, comprising LC-10A_{VP} pumps, SIL-10AD_{VP} refrigerated autoinjector and RF-551 fluorescence detector (λ_{ex} = 340 nm and λ_{em} = 455 nm, for the amino acid OPA derivatives). The manufacturer's software (Class-VP™ 7.2.1 SPI Client/Server Chromatography Data System, Shimadzu Italia S.R.L., Milan, Italy) was used for controlling the system and for chromatographic peak recording and integration.

Determination of Dopamine and Metabolites

In some experiments, the concentrations of dopamine and its metabolites, HVA and DOPAC, in microdialysate samples were analyzed using HPLC with coulometric detection [35]. Aliquots (50 μ L) of the collected microdialysate fractions were injected into the HPLC apparatus, equipped with a Macherey-Nagel, 125/3 nucleosil 100-5 C18 AB column and a μ Bondapak 10 μ m 125A C18 Pre-column (Waters, Milan, Italy). The mobile phase, comprising 75 mM sodium dihydrogen phosphate monohydrate, 3 mM 1-octanesulfonic acid sodium salt, 1.2 mM EDTA, 8% acetonitrile, adjusted to pH 3.4 with phosphoric acid, was run isocratically, at a flow rate of 0.8 mL/min. The coulometric detector consisted of 3 ESA cells (Model 6210; Alfatech SpA, Genoa, Italy) (12 electrodes set at the following potentials: E1 -250 mV, E2 -200 mV, E3 -200 mV, E4 -80 mV, E5 -80 mV, E6 0 mV, E7 100 mV, E8 200 mV, E9 300 mV, E10 350 mV, E11 400 mV, E12 400 mV) and an ESA 5600A CoulArray Detector (Alfatech SpA). Chromatograms were processed using the CoulArrayWin MFC Application software. The detection limit was 0.20 nM for dopamine and 0.50 nM for its metabolites.

Immunohistochemistry

For astrocyte staining, after rehydration, tissue sections were treated with 0.5 M HCl for 30 min and incubated in a blocking solution, comprising 0.2 M glycine, 10% fetal bovine serum, 0.2% gelatin, and 0.3% Triton X-100 in PBS (PBS-0.3Tx) for 1 h. Subsequently, sections were incubated overnight at 4°C with anti-GFAP antibody (1:1000) in a solution containing 0.2 M glycine, 5% FBS, 0.2% gelatin, and PBS-0.3Tx. The sections were washed twice with PBS-0.3Tx containing 0.48 g/L levamisole, to block endogenous alkaline phosphatase activity, and then incubated for

1 h with the secondary anti-rabbit antibody coupled to biotin (1:400) in 0.1 M PBS also containing 0.48 g/L levamisole. After rinsing, the antigen-antibody complex was visualized using the Vectastain ABC-AP kit (1:200). Alkaline phosphatase was developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium chloride (NBTC) substrate solution until a specific blue color was developed.

For the histochemical staining of microglia, sections were incubated in 2% H₂O₂ and 70% methanol in 0.1 M PBS for 10 min to block endogenous peroxidase activity. After two further washes in PBS containing 0.1% Triton X-100, sections were incubated in 6 μ g/mL of biotinylated *Lycopersicon esculentum* lectin in the previous solution for 2 h at 37°C. Sections were washed again and incubated with HRP-streptavidin-peroxidase (1:200) in 0.1 M PBS for 1 h at room temperature. Finally, sections were washed and developed using 0.05% 3,3'-diaminobenzidine (DAB) and 0.01% H₂O₂ in 0.1M PBS. Sections were dehydrated in increasing concentrations of ethanol and cleared with xylene, and coverslips were mounted with DPX.

All sections were visualized in a NIKON Eclipse TE 2000-E fluorescence microscope, and images were captured with a Hamamatsu C-4742-80-12AG digital camera. All slides were coded, so that quantitative analysis could be performed by an observer blinded to the treatment group. The number of positively stained cells, microglia or astroglia, was counted in six randomly chosen fields, using the Metamorph® software (Molecular Devices, Sunnyvale, CA, USA). Counts were expressed as the number of positive cells per mm².

TUNEL Assay

This was used to evaluate the degree of apoptotic damage, analyzing the extent of DNA fragmentation. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling immunohistochemistry, using the Klenow fragEL™ DNA Fragmentation Detection Kit (Inalco, Milano, Italia), was applied to identify apoptotic nuclei according to the manufacturer's instructions, as previously described [32]. Briefly, all steps were carried out at room temperature unless otherwise stated. Sections were first deparaffinized in xylene and rehydrated through a graded series of ethanol in water. Sections were then digested with proteinase K (1:100) for 20 min, washed in two changes in PBS, incubated for 5 min in 3% H₂O₂ in methanol (100%) to block endogenous peroxidase activity, and again washed twice with PBS. Section then underwent treatment with Klenow equilibration buffer (1:10) for 30 min, followed by incorporation of a mixture of biotin-labeled and unlabeled deoxynucleotides with the enzyme TdT (terminal deoxynucleotidyl transferase) for 90 min in a humidified chamber at 37°C. After two further washes with PBS, the sections were covered with 100 μ L of stop solution for 5 min to stop the reaction and then washed in three changes of PBS. Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase (HRP) conjugate (1X) (30 min); after two further washes with PBS the chromogen, 3,3'-diaminobenzidine (DAB: 1 mL H₂O, one tablet of DAB and one tablet H₂O₂/urea) was added and left from 5 to 15 min until the brain sections had turned pale brown. Counterstaining with methyl green (10 min) was per-

formed for the morphological evaluation and characterization of nonapoptotic normal cells.

Slides were dehydrated in increasing ethanol concentrations, cleared in xylene, and coverslipped with DPX mountant (VWR, Lutterworth, UK).

Slides containing tissue sections were coded, so that all quantitative analyses could be performed by an observer blinded to the treatment group. Apoptotic and nonapoptotic nuclei were identified by a brown and a light blue color, respectively, using a Nikon Eclipse E800 microscope (Nikon Instruments S.p.A, Firenze, Italy) equipped with a JVC SLR camera. Thus, two counts were made for each image, the number of TUNEL-positive and the number of methyl green-stained cells, in six fields randomly chosen. The number of TUNEL-positive nuclei was then expressed as a percentage of the total number counted (apoptotic + nonapoptotic). Quantitative image analysis was performed using the public domain software Scion Image (Scion Corporation, Frederick, MD, USA).

Statistical Analysis

Statistical analysis of amino acid and amine concentrations in microdialysate fractions was performed on the original concentration values (nM), whereas, for illustrative purposes only, concentrations were expressed as percentages of their respective basal values. The area under the concentration time curve (AUC), normalized to the time unit corresponding to one 20-min fraction, was used for statistical analysis. Mean basal values were obtained from the -20-min fraction, collected immediately before K^+ stimulation, or from the 80-min fraction, collected before kainate stimulation. Mean values for the stimulated K^+ - and kainate-induced output were obtained from the stimulated AUC values. Confidence intervals (95% CI) of means and the one-sample test were used for statistical significance of the evoked output. When appropriate, data were analyzed by ANOVA, followed by the Bonferroni's test for *post hoc* multiple comparisons, setting the probability level for statistical significance at $P < 0.05$ and using the program Prism 5.0 for Mac OS X (GraphPad Software Inc., La Jolla, CA, USA). N represents the number of animals used in each analysis.

Materials

The following commercial products were used: 17 amino acid stock solution (Pierce, Rockford, IL, USA). GABA, 2-aminoethanesulfonic acid (taurine), kainate, *o*-phthaldialdehyde, 2-mercaptoethanol, paraffin, NBTC, BCIP dipotassium salt, eosin, DAB, Hoechst 33258, N,N-dimethylformamide, hematoxylin, eosin, and all other chemicals not listed (Sigma-Aldrich, Milan, Italy); glacial acetic acid, chloral hydrate, and formaldehyde (Merck, Darmstadt, Germany); methanol (BDH, UK); xylene, ethanol, hydrogen chloride, hydrogen peroxide, gelatin from bovine skin (Panreac, Barcelona, Spain), Triton $\times 100$ (Probus, Cornwall, UK), glycine, Tris-HCl (USB), (-)-tetramisole hydrochloride (levamisole), magnesium chloride solution (Fluka, Milan, Italy), glutamine, fetal bovine serum (Gibco, Grand Islands, NY, USA), Vectastain ABC-AP (alkaline phosphatase) kit, Vectastain ABC-peroxidase kit, HRP-Streptavidin, anti-rabbit tyrosine hydroxylase (TH), antibody

anti-NeuN, goat anti-rabbit biotinylated IgG, horse anti-mouse biotinylated IgG (Vector Laboratories, Burlingame, CA, USA); anti-rabbit glial fibrillary acidic protein (GFAP) (Dako, Glostrup, Denmark), mouse anti-ssDNA (Apostain) (Bender Med Systems, Atlanta, GA, USA), Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). All other salts were from Merck, and [N-(2-propynyl)-2-(5-benzoyloxy-indolyl) methylamine] (PF9601N) was synthesised by the procedure of Cruces et al. [23].

Results

In vivo Experiments

Extracellular Levels of Amino Acids in the Striatum

In the absence of any stimulation or treatment, basal levels of aspartate, glutamate, taurine, and GABA were found to be stable over the entire period of sample collection (up to 180 min). Three groups of rats were used to study the effects of PF9601N on the basal and K^+ - and kainate-evoked release. Basal, K^+ -, and kainate-evoked outputs of the amino acids, aspartate, glutamate, taurine, and GABA, were monitored in one group in the absence and in one in the presence of PF9601N pretreatment, while in a third group of rats, PF9601N pretreatment was administered in the absence of K^+ and kainate stimulation to determine any possible effects on basal levels and their stability over time. The time courses of the extracellular concentrations of aspartate, glutamate, and taurine are shown in Figure 1A–C, respectively. The basal extracellular concentrations observed in the three groups, K^+ /kainate ($N = 13$), K^+ /kainate + PF9601N ($N = 5$), and PF9601N alone ($N = 5$), respectively, were (mean $\pm$ SEM, nM): 218 ± 36 , 292 ± 70 , and 243 ± 53 for aspartate, 858 ± 142 , 835 ± 88 , and 857 ± 66 for glutamate, 1459 ± 94 , 1052 ± 126 , and 1027 ± 180 for taurine, and 41 ± 5 , 48 ± 10 , and 45 ± 7 for GABA. None of these amino acids showed a statistically significant difference in the basal levels of the three treatment groups. Intrastriatal administration of K^+ (50 mM) or kainate (1 mM) induced an increase in the output of aspartate, glutamate, and taurine. The peak increase induced by K^+ was 179, 154, and 187% of basal values, for aspartate, glutamate, and taurine, respectively; that induced by kainate was 201%, 168%, and 207% of basal value, for aspartate, glutamate, and taurine, respectively. Mean evoked AUC values (nM, 20 min) $\pm$ SEM, together with their statistical parameters, are shown in Table 1. Pretreatment with PF9601N did not affect the K^+ -evoked release of aspartate or GABA but induced a significant reduction in the kainate-evoked release of aspartate and of both, the K^+ - and kainate-evoked release of glutamate. In contrast, PF9601N induced an increase in the kainate-evoked release of taurine, from 207 to 342% of basal values. This increase was shown to be statistically significant by ANOVA followed by the *post hoc* Bonferroni's multiple comparison test ($F_{2,20}=4.366$, $P < 0.0001$; kainate vs. kainate plus PF9601N, $P < 0.05$). Pretreatment with PF9601N alone with anesthesia did not affect the basal output levels of the 4 amino acids, which were stable for the whole collection period. However, as previously reported [32], no kainate-induced GABA

release could be observed when kainate stimulation was performed under anesthesia.

Extracellular Levels of Dopamine and Its Metabolites

The basal levels of dopamine and its metabolites, HVA and DOPAC, in the absence of any stimulation or treatment, were found to be stable over the entire experimental sample collection period (180 min). Time courses of the extracellular concentrations of dopamine, HVA and DOPAC are shown in Figure 2A–C, respectively. The basal concentrations observed in the three groups, K^+ /kainate, K^+ /kainate + PF9601N, and PF9601N alone, which were not significantly different, were (mean $\pm$ SEM, $n = 4$ –6) in nM: 1.76 ± 0.33 , 1.88 ± 0.10 , and 1.58 ± 0.30 for dopamine, 662 ± 36 , 800 ± 67 , and 807 ± 150 for DOPAC, 320 ± 25 , 453 ± 45 , and 399 ± 46 for HVA. As shown in Table 2, the intrastratial administration of 50 mM K^+ or 1 mM kainate induced a statistically significant increase in dopamine output together with a decrease in that of the metabolites, HVA and DOPAC. K^+ - and kainate-evoked changes were not affected by PF9601N pretreatment. PF9601N alone with anesthesia did not affect the basal output levels of

dopamine and metabolites, which were stable for the whole collection period.

Ex vivo Experiments

Striatal Immunohistochemistry of Glial Population

Representative microphotographs of striatal microglia (*Lycopersicon esculentum* lectin histochemistry) and astrocyte (GFAP immunostaining) response states are shown in Figure 3A. The numbers of activated microglia and astrocytes (mean $\pm$ SEM, number of cells/mm², $n = 4$) observed in the three groups, PF9601N alone, KA, and KA + PF9601N, are shown in Figure 3B,C. The increase in microglial and astrocytic reactivity observed in the striatum after local kainate application in rats pretreated with PF9601N or vehicle was evident, from the number of lectin- and GFAP-positive cells as well as their staining intensities and morphological features. The staining/morphology score was assessed using the scale of Colburn *et al.* [36]. According to that scale, rats treated with PF9601N alone were scored as “baseline,” resting conditions, being unperturbed and well spaced, consistent with the low counts/mm² of lectin⁺ (microglia)- and GFAP (astrocytes)-positive

Figure 3 Glial activation in response to kainate (KA) in the presence and absence of PF9601N.

(A) Representative microphotographs of microglial reactivity (upper panels; lectin histochemistry) and astrocytosis (lower panels; GFAP immunostaining) in the striatum of animals after local kainate (KA) administration. A significant increase in microglial activation and astrocytosis was apparent in the striatum of KA-treated rats (center panels) shown by both the number of positive cells and their staining intensity. PF9601N pretreatment was able to prevent the activation of microglial cells and astrocytosis (right panels). (B) Quantification of microglial activation (each treatment group: $n = 4$). (C) Quantification of astroglial activation (each treatment group: $n = 4$). One-way ANOVA followed by *post hoc* Bonferroni test for multiple comparisons. KA treated group: *significantly different from control ($P < 0.05$), ***highly significantly different from control ($P < 0.001$), ###highly significantly different from KA treated + PF9601N ($P < 0.001$).

Figure 4 Apoptosis in response to kainate (KA) in the presence and the absence of PF9601N. Representative microphotographs of TUNEL immunohistochemistry are shown in the upper panel with the quantitative analysis of apoptotic nuclei in the lower panel. The local administration of kainate (KA) in the striatum ($n = 6$) led to significant number of apoptotic nuclei, $70 \pm 13\%$ (mean $\pm$ SEM) of total counts (apoptotic + healthy nuclei). Pretreatment with PF9601N ($n = 6$) was able to prevent this, as the percentage of apoptotic nuclei found, $3.2 \pm 1.1\%$, was similar to the control group, $4.2 \pm 4.2\%$ ($n = 5$). One-way ANOVA followed by *post hoc* Bonferroni test for multiple comparisons. KA treated group: ***highly significantly different from control ($P < 0.001$), NS, not significantly different from control, ###Highly significantly different from KA treated + PF9601N ($P < 0.001$).

cells, 40.0 ± 0.9 and 30.7 ± 8.5 , respectively. The local application of KA induced significantly higher counts/mm² of both lectin⁺ (260.0 ± 21.8 , 650% of control) and GFAP-positive cells (156.7 ± 12.7 , 510% of control), together with an “intense” staining/morphological score (+++). However, the staining/morphological response induced by kainate in rats pretreated with PF9601N was evaluated as “mild” (+), and the lectin⁺ and GFAP-positive cell counts were much lower, at 115.5 ± 6.8 and 74.7 ± 3.3 cells/mm², 289% and 202% of control, respectively (Figure 3B,C).

Striatal Immunohistochemistry of Apoptotic Nuclei

Representative microphotographs of apoptotic nuclei in the striatum, determined by the TUNEL assay, are shown in Figure 4. Intrastriatal administration of kainate induced a significant increase in the presence of apoptotic nuclei, $70 \pm 13\%$ ($n = 6$), as compared to $5.2 \pm 3.4\%$ ($n = 5$) in the control group. Pretreatment with PF9601N reduced the number of apoptotic nuclei to control values (3.2 ± 1.1 ; $n = 6$) and those that received PF9601N alone (Figure 4 lower panel).

Discussion

The propargylamine derivatives *l*-deprenyl (selegiline) and rasagiline have been reported to delay the progression of the PD [22,37–39] and also to improve the motor complications in patients treated with *l*-DOPA [40,41]. They are currently licensed in Europe and North America for the symptomatic improvement of early PD, to reduce off-time in patients with more advanced PD and motor fluctuations related to levodopa therapy, but not to cover disease

modifications [see ref. 42]. As discussed by Schapira [42], because of the limitations of the currently available animal models of PD, further work will be necessary to show whether the experimental evidence that such compounds exert neuroprotective properties applies to the disease in humans [see 42]. The propargylamine derivative PF9601N has previously been shown to have neuroprotective properties in several *in vitro* and *in vivo* models of neurodegenerative diseases and also to prolong the effects of exogenously administered levodopa in different experimental models of PD [26,43,44].

The present studies indicate that the protective effects of PF9601N extend beyond PD. It also prevents the excitotoxic damage induced by kainate, in a process that involves decreasing the evoked release of the excitatory amino acids and increasing the output of the inhibitory and neuroprotective amino acid taurine. The significant decrease in the kainate-evoked release of the excitatory amino acids, aspartate and glutamate, resulting from PF9601N pretreatment suggests a protective effect against excitotoxicity. However, the effects of PF9601N may not be confined to the neuron, because both aspartate and glutamate are also present in some glial populations [45] and the present work has shown that the glial activation induced by kainate is prevented by PF9601N pretreatment. It has been previously reported that kainate can stimulate glial cells, and this may contribute to the neuronal damage induced by the toxin [17].

The kainate-evoked release of taurine may be a protective response that balances excessive stimulation and the corresponding osmotic disequilibria (see [46]). Much of this taurine release in the striatum appears to be from a non-neuronal source, possibly glial [47]. The physiological functions of taurine include osmoregulation and modulation of calcium transport. It has been claimed

to act as a neuromodulator, neurotransmitter, and neuroprotective molecule against glutamatergic toxicity [48]. This would be consistent with the increase in taurine release observed after PF9601N pretreatment having a protective function. The reduction in glutamate and aspartate release indicates that the effects of PF9601N are not at the level of kainate receptor activation, although future work should involve direct studies on its possible interactions with other glutamate-receptor subtypes.

Although GABA is released from striatal terminals under basal conditions [34] and by lower concentrations of kainate (100 μ M) [49], the failure to detect it in the present experiments can be attributed to anesthesia that was necessitated by the convulsive effects of the high concentration of kainate required for excitotoxicity [32]. Anesthesia with chloral hydrate is also known to reduce, but not to abolish, the release of aspartate evoked by 100 μ M kainate, with no significant effect on glutamate or taurine release [49]. In the case of dopamine, chloral hydrate anesthesia has been shown to be without effect on the recovery of dopamine [50] but to enhance the K^+ -stimulated release of dopamine in the striatum [51]. In contrast to the results of Hamilton et al. [52] who used the same anesthetic procedure with Sprague-Dawley rats (300–350 g) and reported chloral hydrate to reduce basal dopamine levels in the striatum, we could find no significant difference from controls.

The death of nigral dopaminergic neurons caused by kainate has been previously reported, but this appears to be a secondary phenomenon that does not arise for many days after the initial insult and represents a consequence of the primary toxic events, see [53–55]. The mechanisms underlying this indirect dopaminergic toxicity are not fully understood, and it has been suggested that it may result from the loss of striatal interneurons [55], although the high levels of released dopamine and the gliosis observed in the present work could also contribute. In the acute situation, studied in the present work, the protective effects of PF9601N do not appear to involve this system, because pretreatment did not, itself, have any significant effects on the basal levels of dopamine or its metabolites and neither did it significantly affect the kainate-evoked dopamine release. The large release of dopamine evoked by KA was of similar magnitude to that observed in microdialysis after the administration of other glutamate-receptor agonists [56,57], which was interpreted as being due to a facilitation of DA release. Such a mechanism has also been proposed by others [58–61], whereas Rodríguez et al. [62] suggested inhibition of DA uptake to be involved. The decreased levels of DOPAC observed in the present work, but not investigated in those previous microdialysis studies, would suggest inhibition

of dopamine uptake to be involved, because dopamine oxidation to DOPAC does not occur in the extracellular medium. Further work will be necessary to investigate the extent to which this dopamine release contributes, perhaps through the generation of reactive oxygen species, to the kainate toxicity toward other nerve cells.

PF9601N is a highly selective MAO-B inhibitor and the lack of significant effects on dopamine metabolites would be consistent with the dominant role of MAO-A in basal dopamine metabolism in the rat and mouse striatum [63,64]. The extent, if any, to which MAO-B inhibition may contribute to the protective effects would require further investigation, but the neuroprotective effects of propargylamine derivatives in several other systems have been shown to be independent of MAO inhibition (see [22]).

Pretreatment with PF9601N reduces the astroglial reactivity and microglial activation resulting from kainate excitotoxic damage. Microglial cells have been reported to be the first population to react against CNS lesions. Their reactions, which appear quite homogeneous and independent from the type of lesion, include changes in their morphology, proliferation and an increase in surface molecules [65]. It is difficult to distinguish p53 induction between microglia and macrophages, because they share intracellular and membrane markers, such as lectin domains and CR3 [66]. However, these results suggest a possible immunomodulatory effect of PF9601N, because it is able to prevent the microglial reactivity induced by kainate.

Thus, the protective effects of PF9601N, which have been previously observed in several models of PD, could also be of value in the therapy of neurodegenerative disorders that involve excitotoxicity. Further studies on the protective effects of chronic treatment with PF9601N would be merited.

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Conflict of Interest

The authors declare no conflict of interest.

References

- Lau A, Tymianski M. Glutamate receptors, neurotoxicity and neurodegeneration. *Pflügers Arch* 2010;**460**:525–542.
- Mattson MP. Excitotoxic and excitoprotective mechanisms: abundant targets for the prevention and treatment of neurodegenerative disorders. *Neuromolecular Med* 2003;**3**:65–94.
- Nicotera P, Leist M, Ferrando-May E. Apoptosis and necrosis: different execution of the same death. *Biochem Soc Symp* 1999;**66**:69–73.
- Caudle WM, Zhang J. Glutamate, excitotoxicity and programmed cell death in Parkinson's disease. *Exp Neurol* 2003;**220**:230–233.
- Johnson KA, Conn PF, Niswender CM. Glutamate receptors as therapeutic targets for Parkinson's disease. *CNS Neurol Disord Drug Targets* 2009;**8**:475–491.
- Blandini F. An update on the potential role of excitotoxicity in the pathogenesis of Parkinson's disease. *Funct Neurol* 2010;**25**:65–71.
- Turski L, Bressler K, Rettig KJ, Löschnmann PA, Wachtel H. Protection of substantia nigra from MPP+ neurotoxicity by N-methyl-D-aspartate antagonists. *Nature* 1991;**349**:414–418.
- Koutsilieri E, Riederer P. Excitotoxicity and new antglutamatergic strategies in Parkinson's disease and Alzheimer's disease. *Parkinsonism Relat Disord* 2007;**13** (Suppl 3):S329–S331.
- Michaels RL, Rothman SM. Glutamate neurotoxicity in vitro: antagonist pharmacology and intracellular calcium concentrations. *J Neurosci* 1990;**10**:283–292.
- Leist M, Nicotera P. Apoptosis versus necrosis: the shape of neuronal cell death. *Results Probl Cell Differ* 1998;**24**:105–135.
- Jin XT, Smith Y. Activation of presynaptic kainate receptors suppresses GABAergic synaptic transmission in the rat globus pallidus. *Neuroscience* 2007;**149**:338–349.
- Matute C. Therapeutic potential of kainate receptors. *CNS Neurosci Ther* 2011;**17**:661–669.
- Portera-Cailliau C, Price DL, Martin LJ. Non-NMDA and NMDA receptor-mediated excitotoxic neuronal deaths in

- adult brain are morphologically distinct: further evidence for an apoptosis-necrosis continuum. *J Comp Neurol* 1997;**378**:88–104.
14. Liu W, Liu R, Chun JT, et al. Kainate excitotoxicity in organotypic hippocampal slice cultures: evidence for multiple apoptotic pathways. *Brain Res* 2001;**916**: 239–248.
 15. Bonfoco E, Kraine D, Ankarcrona M, Nicotera P, Lipton SA. Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. *Proc Natl Acad Sci* 1995;**92**:7162–7166.
 16. Nakai M, Qin ZH, Chen JF, Wang Y, Chase TN. Kainic acid-induced apoptosis in rat striatum is associated with nuclear factor-kappaB activation. *J Neurochem* 2000;**74**:647–658.
 17. Matija E. Morphologic evidence of a primary response of glia to kainic acid administration into the rat neostriatum; studied in vivo and in vitro. *Exp Neurol* 1986;**92**:609–623.
 18. McBean GJ, Doorty KB, Tipton KF, Kollegger H. Alteration in the glial cell metabolism of glutamate by kainate and N-methyl-D-aspartate. *Toxicol* 1995;**33**:569–576.
 19. Zhang XM, Zhu J. Kainic acid-induced neurotoxicity: targeting glial responses and glia-derived cytokines. *Curr Neuroparmacol* 2011;**9**:388–398.
 20. Zheng XY, Zhang HL, Luo Q, Zhu J. Kainic acid-induced neurodegenerative model: potentials and limitations. *J Biomed Biotechnol* 2011;**2011**:10.
 21. Ebadi M, Sharma S, Shavali S, El Refaey H. Neuroprotective actions of selegiline. *J Neurosci Res* 2002;**67**:285–289.
 22. Youdim MBH, Edmondson D, Tipton KF. The therapeutic potential of monoamine oxidase inhibitors. *Nat Rev Neurosci* 2006;**7**:295–309.
 23. Cruces MA, Elorriaga C, Fernandez-Alvarez E. Acetylenic and allenic derivatives of 2-(5-hydroxyindolyl) methylamines: synthesis and in vitro evaluation as monoamine oxidase inhibitors. *Eur Med Chem* 1991;**26**:33–41.
 24. Perez V, Marco JL, Fernandez-Alvarez E, Unzeta M. Relevance of benzyloxy group in 2-indolyl methylamines in the selective MAO-B inhibition. *Br J Pharmacol* 1999;**127**:869–876.
 25. Perez V, Unzeta M. PF 9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine], a new MAO-B inhibitor, attenuates MPTP-induced depletion of striatal dopamine levels in C57/BL6 mice. *Neurochem Int* 2003;**42**:221–229.
 26. Cutillas B, Ambrosio S, Unzeta M. Neuroprotective effect of the monoamine oxidase inhibitor PF 9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] on rat nigral neurons after 6-hydroxydopamine-striatal lesion. *Neurosci Lett* 2002;**329**:165–168.
 27. Battaglia V, Sanz E, Salvi M, Unzeta M, Toninello A. Protective effect of N-(2-propynyl)-2-(5-benzyloxy-indolyl)methylamine (PF9601N) on mitochondrial permeability transition. *Cell Mol Life Sci* 2006;**63**:1440–1448.
 28. Sanz E, Quintana A, Battaglia V, et al. Anti-apoptotic effect of Mao-B inhibitor PF9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] is mediated by p53 pathway inhibition in MPP(+)-treated SH-SY5Y human dopaminergic cells. *J Neurochem* 2008;**105**:2404–2417.
 29. Sanz E, Quintana A, Hidalgo J, Marco JL, Unzeta M. PF9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] confers MAO-B independent neuroprotection in ER stress-induced cell death. *Mol Cell Neurosci* 2009;**41**:19–31.
 30. Sanz E, Romera M, Bellik L, Marco JL, Unzeta M. Indolalkylamines derivatives as antioxidant and neuroprotective agents in an experimental model of Parkinson's disease. *Med Sci Monit* 2004;**10**:BR477–BR484.
 31. Bellik L, Dragoni S, Pessina F, Sanz E, Unzeta M, Valoti M. Antioxidant properties of PF9601N, a novel MAO-B inhibitor: assessment of its ability to interact with reactive nitrogen species. *Acta Biochim Pol* 2010;**57**:235–239.
 32. Freinbichler W, Colivicchi MA, Fattori M, et al. Validation of a robust and sensitive method for detecting hydroxyl radical formation together with evoked neurotransmitter release in brain microdialysis. *J Neurochem* 2008;**105**:738–749.
 33. Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. Compact third edition. San Diego: Academic Press, 1997.
 34. Bianchi L, Della Corte L, Tipton KF. Simultaneous determination of basal and evoked output levels of aspartate, glutamate, taurine and 4-aminobutyric acid during microdialysis and from superfused brain slices. *J Chromatogr B Biomed Sci Appl* 1999;**723**:47–59.
 35. Ward RJ, Colivicchi MA, Allen R, et al. Neuro-inflammation induced in the hippocampus of "binge drinking" rats may be mediated by elevated extracellular glutamate content. *J Neurochem* 2009;**111**:1119–1128.
 36. 3Colburn RW, DeLeo JA, Rickman AJ, Yeager MP, Kwon P, Hickey WF. Dissociation of microglial activation and neuropathic pain behaviors following peripheral nerve injury in the rat. *J Neuroimmunol* 1997;**79**:163–175.
 37. Parkinson Study Group. A controlled trial of rasagiline in early Parkinson's disease: the TEMPO study. *Arch Neurol* 2002;**59**:1937–1943.
 38. Parkinson Study Group. A controlled, randomised, delayed-start study of rasagiline in early Parkinson's disease. *Arch Neurol* 2004;**61**:561–566.
 39. Macleod AD, Counsell CE, Ives N, Stowe R. Monoamine oxidase B inhibitors for early Parkinson's disease. *Cochrane Database Syst Rev* 2005;CD004898.
 40. Rascol O, Brooks DJ, Melamed E, et al. Rasagiline as an adjunct to levodopa in patients with Parkinson's disease and motor fluctuations (LARGO, lasting effect in Adjunct therapy with Rasagiline given once daily, study): a randomised, double-blind, parallel-group trial. *Lancet Neurol* 2005;**356**:947–954.
 41. Marconi R, Bonnet AM, Vidallhet M, Agid Y. The IMAO-B MDL 72.974th in Parkinson's disease. *J Neurol Neurosurg Psychiatry* 1992;**55**:1096–1097.
 42. Schapira AH. Monoamine oxidase B inhibitors for the treatment of Parkinson's disease: a review of symptomatic and potential disease-modifying effects. *CNS Drugs* 2011;**25**:1061–1071.
 43. Perez V, Romera M, Lizcano JM, Marco JL, Unzeta M. Protective effect of N-(2-propynyl)-2-(5-benzyloxyindolyl) methylamine (PF 9601N), a novel MAO-B inhibitor, on dopamine-lesioned PC12 cultured cells. *J Pharm Pharmacol* 2003;**55**:713–716.
 44. Prat G, Perez V, Rubi A, Casas M, Unzeta M. The novel type B MAO inhibitor PF9601N enhances the duration of L-DOPA-induced contralateral turning in 6-hydroxydopamine lesioned rats. *J Neural Transm* 2000;**107**:409–417.
 45. Tanaka K. Roles of glutamate transporters in astrocytes. *Brain Nerve* 2007;**59**:677–688.
 46. Della Corte L, Crichton RR, Duburs G, et al. The use of taurine analogues to investigate taurine functions and their potential therapeutic applications. *Amino Acids* 2002;**23**:367–379.
 47. Bianchi L, Colivicchi MA, Bolam JP, Della Corte L. The release of amino acids from rat neostriatum and substantia nigra in vivo: a dual microdialysis probe analysis. *Neuroscience* 1998;**87**:171–180.
 48. Foos TM, Wu JY. The model of taurine in the CNS and the modulation of intracellular calcium homeostasis. *Neurochem Res* 2002;**27**:21–26.
 49. Colivicchi MA, Stefanini C, Freinbichler W, et al. Monitoring extracellular amino acid neurotransmitters and hROS by in vivo microdialysis in rats: a practical approach. In: Di Giovanni G, Di Matteo V, editors. *Microdialysis techniques in neuroscience*. Neuromethods, vol. **75**. New York: Humana Press, 2013;225–248.
 50. Pan WH, Lai YJ. Anesthetics decreased the microdialysis extraction fraction of norepinephrine but not dopamine in the medial prefrontal cortex. *Synapse* 1995;**2**:85–92.
 51. Chen HT, Kandasamy SB. Effect of chloral hydrate on in vivo KCl-induced striatal dopamine release in the rat. *Neurochem Res* 1996;**21**:695–700.
 52. Hamilton ME, Mele A, Pert A. Striatal extracellular dopamine in conscious vs. anesthetized rats: effects of chloral hydrate anesthetic on responses to drugs of different classes. *Brain Res* 1996;**597**:1–7.
 53. Foster JA, Bezin L, Groc L, Christopherson PL, Levine RA. Kainic acid lesion-induced nigral neuronal death. *J Chem Neuroanat* 2003;**26**:65–73.
 54. Schwarcz R, Coyle JT. Striatal lesions with kainic acid: neurochemical characteristics. *Brain Res* 1977;**127**:235–249.
 55. Friedle NM, Kelly PH, Moore KE. Regional brain atrophy and reductions in glutamate release and uptake after intrastriatal kainic acid. *Br J Pharmacol* 1978;**63**: 151–158.
 56. Bruton RK, Ge J, Barnes NM. Group I mGlu receptor modulation of dopamine release in the rat striatum in vivo. *Eur J Pharmacol* 1999;**369**:175–181.
 57. Verma A, Moghaddam B. Regulation of striatal dopamine release by metabotropic glutamate receptors. *Synapse* 1998;**28**:220–226.
 58. Borland LM, Michael AC. Voltammetric study of the control of striatal dopamine release by glutamate. *J Neurochem* 2004;**91**:220–229.
 59. Kulagina NV, Zigmund MJ, Michael AC. Glutamate regulates the spontaneous and evoked release of dopamine in the rat striatum. *Neuroscience* 2001;**102**:121–128.
 60. Wagner AK, Sokoloski JE, Ren D, et al. Controlled cortical impact injury affects dopaminergic transmission in the rat striatum. *J Neurochem* 2005;**95**:457–465.
 61. Wülner U, Testa CM, Catania MV, Young AB, Penney JN Jr. Dopamine terminal loss and onset of motor symptoms in MPTP-treated monkeys: a positron emission tomography study with 11C-CFT. *Exp Neurol* 1994;**126**:305–309.
 62. Rodriguez M, Morales I, Gomez I, Gonzalez S, Gonzalez-Hernandez T, Gonzalez-Mora JL. Heterogeneous dopamine neurochemistry in the striatum: the fountain-drain matrix. *J Pharmacol Exp Ther* 2006;**319**: 31–43.
 63. Lamensdorf I, Youdim MB, Finberg JP. Effect of long-term treatment with selective monoamine oxidase A and B inhibitors on dopamine release from rat striatum in vivo. *J Neurochem* 1996;**67**:1532–1539.
 64. Fornai F, Chen K, Giorgi FS, Gesi M, Alessandri MG, Shih JC. Striatal dopamine metabolism in monoamine oxidase B-deficient mice: a brain dialysis study. *J Neurochem* 1999;**73**:2434–2440.
 65. Stoll G, Jander S. The role of microglia and macrophages in the pathophysiology of the CNS. *Prog Neurobiol* 1999;**58**:233–247.
 66. Flaris NA, Densmore TL, Molleston MC, Hickey WF. Characterization of microglia and macrophages in the central nervous system of rats: definition of the differential expression of molecules using standard and novel monoclonal antibodies in normal CNS and in four models of parenchymal reaction. *Glia* 1993;**7**: 34–40.