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Pramipexole attenuates the dopaminergic cell loss induced by intraventricular 6-hydroxydopamine

T. Q. Vu[2], **Z. D. Ling**[1,2], **S. Y. Ma**[2], **H. C. Robie**[1], **C. W. Tong**[1], **E. Y. Chen**[2], **J. W. Lipton**[1,2,3], and **P. M. Carvey**[1,2]

Departments of [1] Pharmacology,
[2] Neurological Sciences, and
[3] Pediatrics Neuropharmacology Research Laboratories, Research Center for Brain Repair, Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL, U.S.A.

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Summary. The D_3 preferring dopamine agonist pramipexole has been shown to attenuate the cell loss induced by levodopa in vitro. Pramipexole was herein evaluated in the 6-hydroxydopamine lesion model to determine its in vivo effect. Rats were treated with pramipexole or saline before and after an intracerebroventricular 6-hydroxydopamine injection. In the preliminary study, 6-hydroxydopamine produced a 68% reduction in striatal dopamine and a 62% loss in tyrosine hydroxylase immunoreactive (THir) cell counts in the substantia nigra. Pramipexole treated animals exhibited a 29% and a 27% reduction in striatal dopamine and THir cell counts, respectively. THir cell counts and striatal dopamine were significantly correlated. In the stereological study, 6-hydroxydopamine reduced THir cell counts by 47% in saline treated animals and 26% in pramipexole treated animals. These data demonstrate that pramipexole attenuates the biochemical and THir cell changes normally produced by 6-hydroxydopamine consistent with its neuroprotective actions in vitro.

Keywords: Pramipexole, dopamine, 6-hydroxydopamine, Parkinson, rat, agonist, neuroprotection.

Introduction

Pramipexole (PPX, Mirapex™), the S(−) enantiomer of 2 amino-4,5,6,7-tetrahydro-6-propyl-amino-benzthiazole-dihydrochloride has recently been approved for monotherapy in patients with Parkinson's disease (PD). It is a full intrinsic-activity, direct-acting dopamine (DA) agonist at the D-2 receptor family (Mierau, 1992) possessing an approximately 7-fold preferential affinity for the D_3 receptor relative to its affinities for the D_2 and D_4 sites (Mierau, 1995; Camacho-Ochoa, 1995). Pramipexole (PPX) is also a potent antioxidant (Hall, 1996; Cassarino, 1998). As a DA agonist, PPX reduces DA release

(Piercey, 1996) and since it also acts as an antioxidant, it should theoretically reduce the oxidant stress on cells thought to accompany increased DA release (Adams, 1991; Olanow, 1992). Several in vitro and in vivo studies have examined this potential neuroprotective action.

We previously evaluated PPX's ability to inhibit the DA neuron loss produced by levodopa in mesencephalic tissue cultures. PPX was shown to attenuate levodopa-induced DA neuron loss in a stereoselective and dose-dependent fashion (Carvey, 1997a). Similarly, Zou et al. (1997) demonstrated PPX's ability to attenuate the levodopa-induced DA neuron loss in an immortalized mesencephalic cell line. We also showed that media conditioned by incubation of mesencephalic cultures with PPX enhanced the production of a soluble, heat-labile protein capable of increasing DA neuron growth and survival in recipient cultures (Carvey, 1997a). This "growth promoting activity" was not present in parietal cortex cultures or cultures pretreated with the DA neurotoxin 1-methyl-4-phenylpyridinium (MPP+) and was still inducible by PPX in cultures without glia (Carvey, 1997b). The neuroprotective effect PPX had in culture was enhanced and attenuated, respectively, by D_3 agonists (7-hydroxy-DPAT and PD 128,907) and the D_3 antagonist (U99194), while drugs acting at D_2 receptors were without effect (Carvey, 1997c; Ling, 1999). In addition, PPX dose-dependently attenuated the granule cell loss in cerebellar cultures normally produced by levodopa (Von Voigtlander, 1995) and Hall et al. (1996) showed the PPX pretreatment prevented the loss of DA neurons normally produced by high dose methamphetamine treatment. These results are consistent with our study in rats which demonstrated that 24 daily treatments with PPX, but not pergolide, dose-dependently increased the production of a mesencephalic-derived neurotrophic activity (Carvey, 1997b). We hypothesized that the D_3 agonist actions of PPX enhanced the production of a DA autotrophic factor that, in combination with its antioxidant properties (Hall, 1996; Cassarino, 1998), increased the production of a constitutively produced, 35 kDa, DA neuron-derived neurotrophic factor (Ling, 1998). To further test this hypothesis, we have now examined PPX's ability to attenuate the toxic effects of the DA neurotoxin 6-hydroxydopamine (6-OHDA) in the adult rat.

Most studies designed to examine neuroprotection employ localized neurotoxin injection targeted to the medial forebrain bundle that are overwhelming to the system and not indicative of the type of cell loss seen in PD (Michel, 1990; Ling, 1996). As a result, potential neuroprotective effects become difficult to detect. We chose to use the intracerebroventricular (i.c.v.) 6-OHDA model in which neurotoxin diffuses to the target tissue. The i.c.v. model appears to produce a more gradual cell loss and the magnitude of cell loss is easy to control (Miller, 1981; Carvey, 1996). Since it is well known that partial DA cell loss can be compensated for biochemically (Kish, 1992; Calne, 1991) we examined not only striatal biochemical indices of DA, but also DA neuron counts in a preliminary study. In a stereological study we then evaluated THir neuron counts, volume of the substantia nigra (SN), and cell density in the mesencephalon using an unbiased stereological dissector counting technique.

Material and methods

Animals were allowed to acclimate to the animal facility for a period of at least 5 days before the onset of the experiments. All animals used were housed in a temperature and humidity controlled environment, allowed free access to food (Agway Rat Chow; Prolab, Syracuse, NY) and water and were kept on a 12 hour lights-on/12 hour lights-off cycle. The animals were weighed on Day 1 of the experiments and every other day thereafter. The protocols and procedures used in these studies were approved by the Institutional Animal Care and Utilization Committee (IACUC) for Rush University. Animals received an intra-peritoneal (i.p.) pentobarbital injection (Butler, Columbus Ohio) at 40 mg per kg body weight before surgery.

Preliminary study

In the first experiment, 18 Sprague-Dawley (Zivic-Miller, Allison Park, PA) male rats (225–250 g) were randomly assigned to one of three groups (n = 6/group): sham-lesioned/saline-treated, 6-OHDA lesioned/PPX treated, and 6-OHDA lesioned/saline treated. During days 1–14, the animals received daily injections of saline or PPX (3 mg/kg) in saline vehicle, administered intraperitoneally ([i.p.]; 1 ml/kg). One hour prior to surgery on day 15, each animal received an i.p. injection of desipramine HCl (25 mg/kg) to reduce the uptake of 6-OHDA into noradrenergic terminals (Evans, 1993). The animals were then anesthetized with pentobarbital (40 mg/kg; i.p.) and 5 µl of a 6-OHDA (150 µg 6-OHDA-HBr) [Research Biochemicals Inc.]) solution in ascorbate vehicle (0.02% ascorbate/saline) or 5 µl ascorbate was delivered into the right lateral ventricle stereotactically (A/P = 0.08 cm, M/L = 0.14 cm, D/V = 0.39 cm, relative to bregma) as described previously (Carvey, 1994).

Following surgery the animals continued to receive their same daily treatments on days 15–28. No drug treatments were given on days 29–31 (drug washout). After drug washout, all animals received pentobarbital (40 mg/kg) and were then perfused with saline as described previously (Carvey, 1997b) on day 32. After perfusion, the animals' brains were removed, immediately immersed in liquid methyl-butane until frozen, and stored at −80°C. The brains were subsequently mounted onto a cryostat chuck and three 750 µ sections through the striatum were rolled off. The striata ipsilateral and contralateral to the i.c.v. injection were dissected free from the frozen sections and immediately placed in 200 µl of ice cold antioxidant (0.4 N perchlorate, 0.05% EDTA, and 0.1% bis-metabisulfite), homogenized for 30 secs. on a Polytron GmbH homogenizer (setting 6; Kinematica; Luzern, Switzerland), and processed for HPLC and total protein as previously described (Carvey, 1996). Samples were run in duplicate. DA and homovanillic acid (HVA) were quantified by comparison to 6 point standard curves run daily and then expressed as ng/mg protein. The ratio of [HVA]/[DA] was also computed and served as an index of DA activity (Kish, 1992; Calne, 1991).

Post-fixation and Tyrosine Hydroxylase (TH) immunocytochemistry

The brain, still mounted on the cryostat chuck was sectioned until the accessory optic tract in the mesencephalon could be clearly identified. Sequential 40 µ sections of the mesencephalon were then collected and mounted on gelatin coated slides (Carvey, 1996). The mounted sections containing the substantia nigra (SN) at the level of the accessory optic tract (~6 slides) were allowed to dry overnight and were then post-fixed. The slides were placed in plastic mailers and dipped in Zamboni's fixative (7.5% saturated picric acid, 12 mM NaH_2PO_4, 88 mM Na_2HPO_4, and 4% paraformaldehyde) for 10 minutes, and then rinsed six times with dilution media (TBS and 0.25% Triton X). Then, the slides were bathed in 0.1 M sodium periodate for 20 min. After washing three more times with dilution media, the slides were exposed to blocker (dilution media and 3% normal horse serum) for a period of 60 minutes. Following blocker, the slides were incubated in primary

antibody (mouse anti-tyrosine hydroxylase, Incstar; 1:5,000) overnight. The following day, they were rinsed 9 times with dilution media. The tissue was placed in secondary antibody (biotinylated horse anti-mouse IgG; Vector Laboratories) for 60 minutes and then rinsed 6 times with dilution media. The tissue sections were placed in ABC (peroxidase conjugated avidin-biotin complex, Vector Laboratories) for 75 minutes. Following rinsing, the slides were immersed in 10 mM imidazole/50 mM acetate buffer (I/A; pH = 7.2) three times, each time for 10 minutes. Slides were then reacted with I/A solution containing nickel sulfate (2.5%, Sigma), 3,3′-diaminobenzidine (DAB, 0.05%, Sigma), and H_2O_2 (0.003%, Sigma) for a period of 5 minutes followed by 3 washes with I/A. Finally, the slides were dehydrated with a series of exposures to jars containing distilled water, 70% alcohol, 95% alcohol, 99% alcohol, and xylene. The slides were eventually cover-slipped with mounting media (Stephens Scientific) and allowed to dry overnight.

Slides were then matched within each group to find sections where the accessory optic tract dissected the mesencephalon. All tyrosine hydroxylase immunoreactive (THir) cells were counted in the area lateral to the accessory optic tract which was operationally defined as the substantia nigra compacta. For each animal, three sections were counted by an individual blinded to treatment history. Only THir cells with morphologies typical of DA neurons were counted. In order to generate an estimate of the total number of cells in the SN, the formula of Konigsmark was used ($Nt = Ns \times [St/Ss]$; Nt = total number of cells; Ns = number of cells counted; St = total number of sections through the counted; Ss = number of sections in which cells were counted) (Konigsmark, 1970).

Stereological study

When results were collected from preliminary study, we recognized that the changes seen at the level of the accessory optic tract might not be indicative of the entire SN so we performed a second study using a pre-fixation technique and counted sections sterologically. Twenty-eight animals were assigned to one of four treatment groups: Sham lesioned/PPX treated (N = 8); 6-OHDA lesioned/PPX treated (N = 8); sham lesioned/saline treated (N = 6); 6-OHDA lesioned/saline treated (N = 6). Observing a PPX effect in the preliminary study prompted the inclusion of a fourth group of sham lesioned/PPX treated animals in this study to determine if PPX by itself could alter THir cell counts. The treatment and surgical schedule was identical to that described above. However, after the four day drug washout interval, the animals were perfused first with saline followed by Zamboni's fixative. The brains were subsequently removed and allowed to soak in Zamboni's fixative for a period of approximately 25 hours. The brains were subsequently refrigerated in 10, 20, and 30% sucrose/TBS solution for three successive nights as described similarly by Kordower et al. (1991, 1994).

Tissue section preparation for optical dissector counts

The entire mesencephalon was sliced into consecutive 40 μ sections and placed into a 24 well falcon tray containing cryoprotection solution (0.1 M PBS, pH 7.2 plus 30% sucrose and 30% ethylene glycol). Every sixth section was stained for THir using a technique described previously (Kordower, 1991, 1994). Briefly, endogenous peroxidase activity was eliminated with a 20-minute incubation in 0.1 M sodium periodate (Sigma Chem. Co.). Background staining was then inhibited by a 1-hour incubation in 3% normal horse serum after which the tissue was incubated for 48 hours in the primary antibody (Incstar; 1:5,000). The immunohistochemical procedure was continued by using a biotinylated anti-mouse IgG (0.5%; Vector Laboratories) for 1 hour and peroxidase conjugated avidin-biotin complex (Vector Laboratories) for 75 minutes. The chromogen solution used to complete the reaction consisted of 0.05% 3.3′-diaminobenzidine (DAB) and 0.003% H_2O_2 in I/A solution. The sections were washed and mounted on gelatin-coated slides, dehydrated through graded alcohols (70%, 95%, 99%), cleared in xylenes, and cover-slipped with Permount.

Optical dissector procedure

The number of THir cell bodies was counted by an observer blinded to treatment history using a stereological procedure described previously (Gundersen, 1998; Ma, 1995a,b, 1996, 1997, 1999a,b). The system used for stereological counting was an Olympus BX-60 microscope that was hard-coupled with a Prior H128 computer-controlled x-y-z motorized stage, a high sensitivity Hitachi 3CCD video camera system (Hitachi, Japan), and a Macintosh 8500 computer. THir cell counts were carried out using Neuro-Zoom stereological software and four sections were randomly selected from each brain. The instruments were calibrated prior to each series of measurements. The SNc was outlined under a low magnification (4X) lense and approximately 10% of the outlined region was measured with a systematic random design using dissector counting frames (9,890 μm^2). The average thickness of the sections evaluated was 30.1 μm. Total THir neurons were estimated using the optical dissector method employing a 100× planapo oil immersion objective with a 1.4 numerical aperture. Under the dissector principle, about 200 THir neurons were sampled by optical scanning using a uniform, systematic and random designed procedure for all measurements. Once the top of the section was in focus, the z-plane was lowered 1–2 μm. Care was taken to ensure that the top forbidden plane was never included in the analysis. The total number of THir cells (N) within the SN was calculated using the following formula: $N = Nv \cdot Vsn$ where the Nv is the density and Vsn is the volume of the SN determined by the Cavalieri principle (Cavalieri, 1966; Gundersen, 1988; Pakkenber, 1991; Emborg, 1998). The variability within the treatment group was assessed via the Coefficient of error (CE) (Gundersen, 1988; West, 1993; Ma, 1995a,b, 1999a,b). THir neurons within the ventral tegmental area were excluded from this dissector analysis.

Statistical analysis

In the first experiment the dependent variables were evaluated using Two-way ANOVA with side (ipsi- vs. contralateral) and treatment group as factors. When individual group differences were examined, groups were compared using Newman Kuels' post hoc comparison following Oneway ANOVA. The ipsilateral and contralateral values were averaged for each animal and then tested using Oneway ANOVA where appropriate. In addition, Pearson correlation coefficients were generated between striatal biochemical measures and THir cell counts from the same animal. In the second experiment, the cell counts were evaluated using Two-way ANOVA.

Results

Preliminary study

Striatal DA levels were different among the three treatment groups ($F = 36.71$; $p < 0.001$) and between the ipsi- and contralateral sides ($F = 5.099$; $p < 0.05$) whereas no statistically significant interaction was observed ($F = 0.664$; Fig. 1A). In both 6-OHDA lesioned groups, post hoc analysis revealed that the contralateral DA levels were higher than the ipsilateral levels. Protein content was not affected by treatment, however (data not shown). Overall (average of left and right striata for each animal) DA levels were reduced by 68% in the 6-OHDA lesioned/saline treated animals relative to sham lesioned/saline treated controls whereas 6-OHDA lesioned/PPX treated animals exhibited only a 29% reduction. HVA levels were not altered by treatment due to high intragroup variability ($F = 0.613$; data not shown). DA activity (HVA/DA ratio) was, however, significantly altered and like DA, was affected by treatment ($F = 3.436$; $p < 0.05$) and by side ($F = 4.047$; $p < 0.05$;

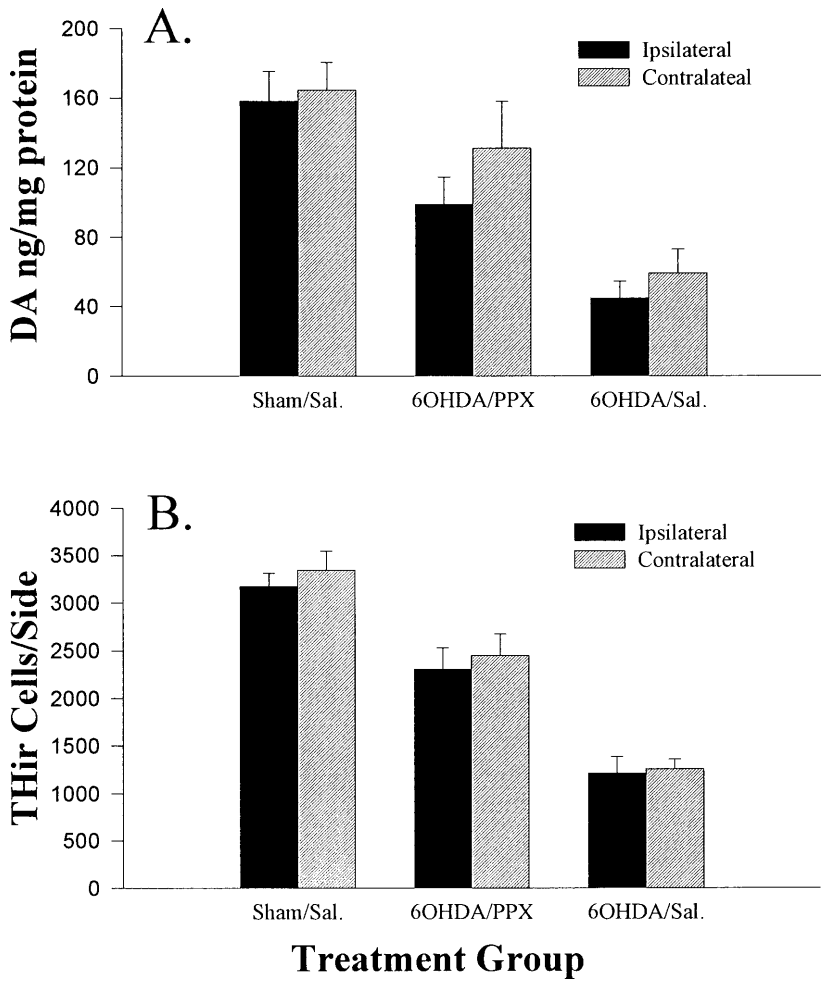

Fig. 1. The effects of PPX on striatal DA biochemistry (**A**) and substantia nigra THir cell counts (**B**) of 6-OHDA lesioned animals. 6-OHDA lesioned/saline treated animals (6OHDA/Sal.) exhibited reduced striatal DA levels on both sides and decreased THir cell counts relative to sham lesioned/saline treated (Sham/Sal.) controls. 6-OHDA lesioned/PPX treated (6OHDA/PPX) animals had increased DA levels and THir cell counts relative to 6-OHDA lesioned/saline (6OHDA/Sal.) treated animals. When ipsilateral and contralateral DA levels were compared, a statistically significant difference ($F = 5.099$; $p < 0.05$) was found in the striatal DA, but no asymmetry was observed in THir cell counts ($F = 0.425$). Values represent mean ± SD

data not shown). Overall (average of left and right striata for each animal) DA activity was increased by 210% in 6-OHDA lesioned/saline treated animals (0.121 ± 0.089) relative to sham lesioned/saline treated controls (0.039 ± 0.003) whereas activity was increased only by 161% in the 6-OHDA lesioned/PPX treated animals (0.102 ± 0.080). This increase in activity was primarily the result of increased activity in the contralateral hemisphere as reflected by the statistically significant interaction term ($F = 4.236$; $p < 0.05$) and the fact that no ipsilateral differences were detected within the three treatment groups using post hoc comparisons.

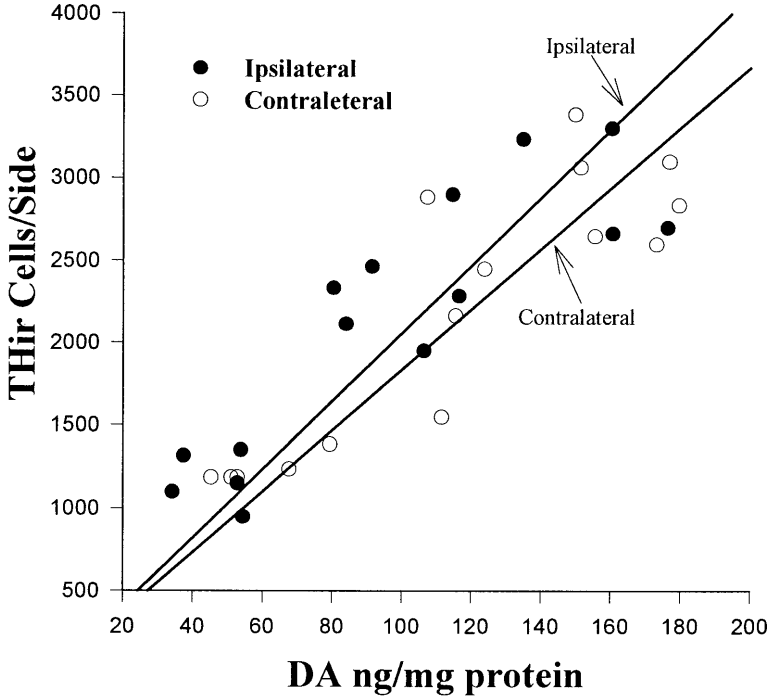

Fig. 2. Correlation between striatal DA levels and SN THir cell counts. Striatal DA and SN THir cell counts were highly correlated in these animals ($r^2 = 0.7385$; $p < 0.01$ for the contralateral side; $r^2 = 0.7913$; $p < 0.001$ for the ipsilateral side)

In contrast to the ipsilateral/contralateral asymmetries seen in the biochemical data, no such differences were seen in the nigral cell counts in each group ($F = 0.425$; Fig. 1B). Treatment did, however, significantly alter THir cell counts lateral to the accessory optic tract ($F = 37.242$; $p < 0.001$). Thus, overall the number of THir cell counts seen in 6-OHDA lesioned/saline treated animals was reduced by 62% relative to sham lesioned/saline treated controls whereas 6-OHDA lesioned/PPX treated animals exhibited only a 27% reduction. All three treatment groups were significantly different from one another ($p < 0.05$) when the overall cell counts from the ipsi-and contralateral sides were averaged and tested using Oneway ANOVA ($F = 21.09$; $p < 0.001$) with post-hoc comparisons. In the sham lesioned/saline treated animals the average estimated nigral THir cell count was 3,175 ($\pm$ 139) on the ipsilateral side, 3,346 ($\pm$ 202) on the contralateral side, and 6,521 ($\pm$ 281) in each animal based on Konigsmark's formula.

Striatal DA and SN THir cell counts were highly correlated in these animals ($r^2 = 0.7385$; $p < 0.01$ for the contralateral side; $r^2 = 0.7913$; $p < 0.001$ for the ipsilateral side; Fig. 2). However, a significant correlation coefficient was only detected on the side ipsilateral to the i.c.v. injection when THir cell counts and DA activity were evaluated ($r^2 = 0.3179$; $p = 0.230$ for the contralateral side; $r^2 = 0.5596$; $p < 0.05$ for the ipsilateral side).

Stereological study

The results from the stereological assessments were similar to those seen in the preliminary study although the THir cell counts in the SN were significantly higher (Table 1). Overall visualization of the mesencephalons of these animals revealed graded differences in cell loss at the level of the accessory optic tract. 6-OHDA lesioned/saline treated animals exhibited the most profound cell loss in the lateral and inferior regions of the SN (Fig. 3D) that was apparently attenuated by PPX treatment (Fig. 3B). Sham lesioned/PPX treated animals appeared to exhibit increased fiber density in the more inferior regions of the substantia nigra (Fig. 3C) relative to the pattern seen in sham lesioned/saline treated controls (Fig. 3A). The perfusion fixation procedure yielded readily visible individual THir cell bodies in the SN typical of that seen in the rat (Fig. 4A and 4B) and appropriate for stereological assessment.

As was true in the preliminary study, ipsilateral/contralateral differences in THir cell counts were not observed among the treatment groups ($F = 2.519$; $p = 0.120$) whereas the effect of treatment was highly significant ($F = 5.410$; $p < 0.005$). When the values for the ipsilateral and contralateral sides were combined, the 6-OHDA lesioned/saline treated animals exhibited an overall 47% reduction in THir cell counts in the SN relative to sham lesioned/saline treated animals whereas the 6-OHDA lesioned/PPX treated animals exhibited a reduction of only 26% (Fig. 5). The THir cell counts in sham lesioned/PPX-treated animals were similar to those seen in sham lesioned/saline treated controls.

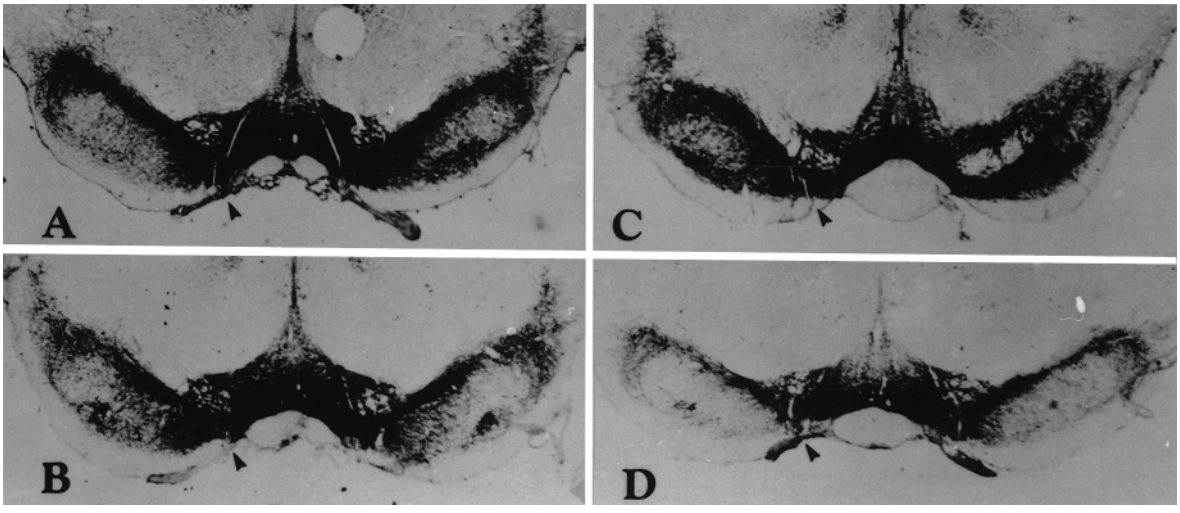

Fig. 3. Pattern of TH staining of substantia nigra sections at level of the accessory optic tract. **A** From sham lesioned/PPX treated animal; **B** from 6-OHDA lesioned/PPX treated animal; **C** from sham lesioned/saline treated animal; **D** from 6-OHDA lesioned/saline treated animal. The decrease in TH immunoreactivity occurred mainly in the lateral and inferior regions of the substantia nigra (**D**) and PPX appeared to attenuate this loss in both regions (**B**)

Table 1. THir cell counts, THir cell densities, and average nuclear volumes assessed using unbiased stereology

Treatment group	THir Cells			Density			Volume		
	Ipsilateral	Contralateral	Total	Ipsilateral	Contralateral	Averaged	Ipsilateral	Contralateral	Total
Sham/Sal.	11,523 ± 2,945	10,768 ± 1,723	22,291 ± 3,672	3,517 ± 578	3,499 ± 661	3,508 ± 346	3.26 ± 0.42	3.11 ± 0.38	6.37 ± 0.79
Sham/PPX	11,591 ± 4,029	11,961 ± 5,106	23,552 ± 8,038	3,053 ± 854	3,524 ± 525	3,288 ± 611	3.76 ± 0.44	3.73 ± 0.40	7.50 ± 0.83
6OHDA/Sal.	5,584 ± 679	6,215 ± 1,631	11,799 ± 1,397	1,927 ± 289	1,956 ± 484	1,942 ± 204	2.91 ± 0.28	3.17 ± 0.28	6.08 ± 0.53
6OHDA/PPX	6,878 ± 1,693	9,703 ± 1,073	16,581 ± 2,467	2,148 ± 501	2,940 ± 713	2,544 ± 597	3.25 ± 0.45	3.31 ± 0.49	6.56 ± 0.91

Values reflect group mean ± s.d.

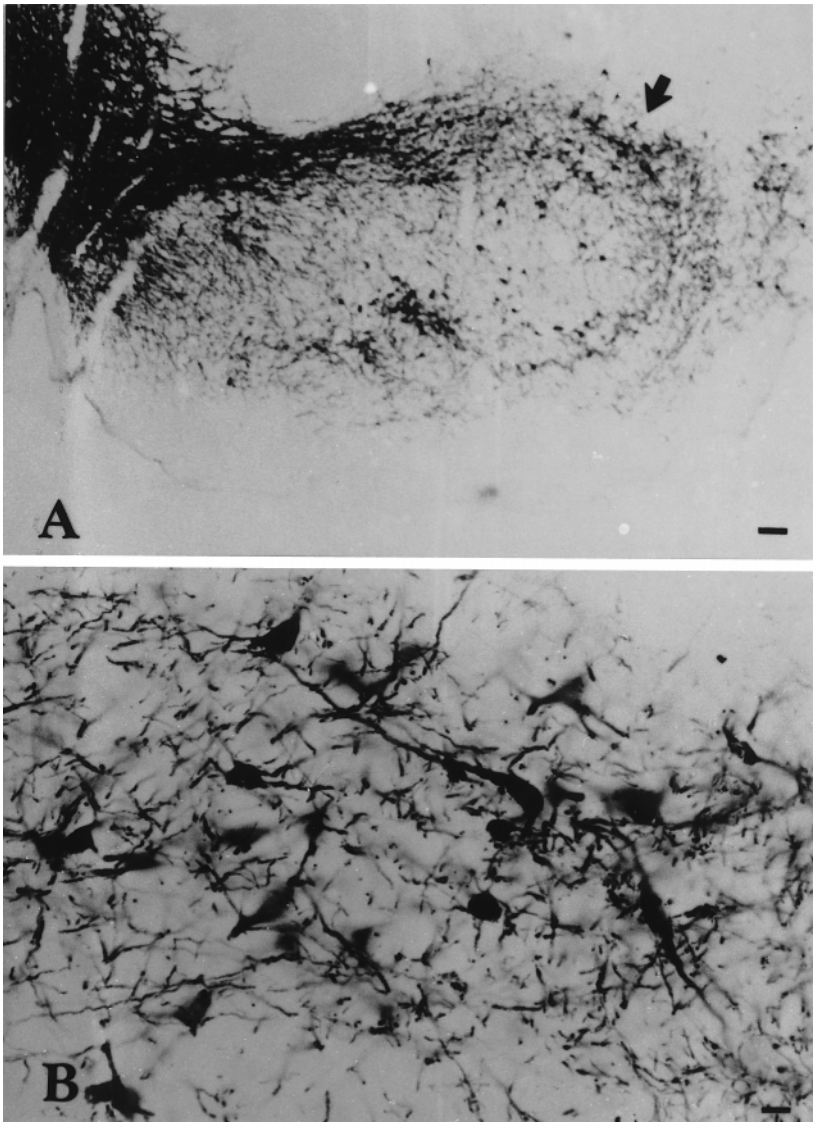

Fig. 4. Morphology of THir cells in the perfusion fixed substantia nigra. This close look at the substantia nigra of animals from 6-OHDA lesioned/PPX treated group in stereological study demonstrates that THir staining of these sections yielded clear THir cell bodies and processes. Panel **B** is a magnified area in panel **A** indicated by the arrow (bar = 48 μm on panel **A** and bar = 12 μm in panel **B**)

The results of THir neuron density (THir cells per cubic mm) among the four groups were similar to the total cell counts ($F_{group} = 6.216$: $p < 0.001$; $F_{side} = 2.140$; $p = 0.646$; Table 1). Thus, the THir cell density in the 6-OHDA lesioned/saline treated animals was 43% lower then that of the sham lesioned/saline-treated controls whereas the density observed in the 6-OHDA lesioned/PPX treated animals exhibited only a 26% reduction. The density observed in the sham lesioned/PPX treated animals and sham lesioned/saline

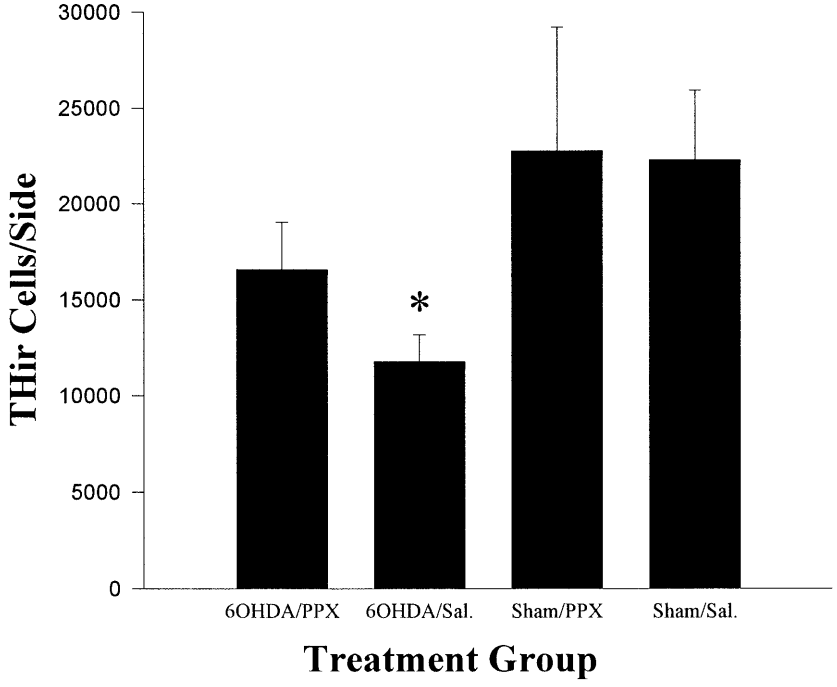

Fig. 5. The effect of 6-OHDA on THir cell counts in the substantia nigra in perfusion fixed animals. 6-OHDA lesioned/saline treated animals (6OHDA/Sal.) exhibited an overall 47% reduction in THir cell counts (ipsi-lateral plus contra-lateral sides) in the substantia nigra relative to sham lesioned/saline treated controls (Sham/Sal.). In comparison, 6-OHDA lesioned/PPX treated animals (60HDA/PPX) exhibited only a 26% loss of THir cells. Post-hoc comparisons revealed that 6-OHDA lesioned/PPX treated group was significantly different from the 6-OHDA lesioned/saline treated group (*, $p < 0.05$) indicating a neuroprotective effect of PPX against 6-OHDA toxicity

treated animals were not statistically different from one another. Interestingly, the volumes of the SN were affected by treatment group ($F = 7.759$: $p < 0.001$) whereas left-right asymmetries were not observed ($F = 0.604$: $p = 0.749$). Post hoc comparisons following Oneway ANOVA ($F = 3.441$) on the combined ipsilateral-contralateral sides revealed that the volumes of SN of the sham lesioned/PPX treated animals were significantly increased relative to the other three groups ($p < 0.05$). The co-efficient of error (CE) for each measurement in THir cell counts, volume of SN, and density of THir cells showed a range from 0.03 to 0.08 indicating only a minium variation in the stereological analysis.

Discussion

The present study demonstrated that PPX treatment attenuated the loss of THir cells in the SN as well as the reduction of striatal DA induced by i.c.v. 6-OHDA infusion. This suggests that PPX possesses neuroprotective effects extending earlier in vitro studies where PPX was shown to attenuate the levodopa-induced cell loss seen in mesencephalic cultures (Carvey, 1997a)

and hybrid mesencephalic cell line cultures (Zou, 1997). The present findings also compliment previous in vivo studies where PPX was shown to attenuate the DA loss produced by high-dosages of methamphetamine (Hall, 1996).

Central to the interpretation of the present data is whether or not i.c.v. 6-OHDA killed DA neurons or suppressed TH expression in the cells, both of which would result in the observed decreases in THir cell counts. This has become an important issue in the field of neuroprotection. Glial cell line-derived neurotrophic factor (GDNF) infusion into rats recovered from 6-OHDA infusion was shown to increase THir cell counts in the substantia nigra (Bowenkamp, 1996) and previous studies suggested that mesencephalic cells other than DA neurons (cells were positive for l-dopa but negative for DA) could express TH (Mons, 1989). This raises the possibility that the 6-OHDA-induced loss of THir cells in the present study was a consequence of dopaminergic neuron phenotype suppression or alteration rather than cell death and the present data cannot discriminate between these two possibilities. This problem might be overcome had fluorogold injections into the striatum been performed prior to 6-OHDA treatment or if nissl stains in adjacent THir sections of the nigra, or assessment of other dopaminergic phenotypic markers such as dopa-decarboxylase and the DA transporter been collected.

Regardless, two different methods of cell count assessment were performed in the current study and both revealed similar results. In the preliminary study where a post-fixation was performed, we assessed the number of THir cell bodies in the mesencephalon lateral to the accessory optic tract. The cell counts there were reduced on average 62% by 6-OHDA relative to the cell counts seen in sham lesioned/saline treated controls. This agrees well with the 64% reduction we observed in a previous study where the same method was used (Carvey, 1991). In this preliminary study, PPX was shown to significantly attenuate that cell loss. However, since only those mesencephalic sections that included the accessory optic tract were evaluated, it was possible that the attenuation in cell loss observed did not reflect the entire rostral caudal extent of the SN, even though the relative cell count technique used in the preliminary study is a widely accepted technique for quantification of biological samples (Guillery, 1997). In addition, it could be argued that stereological cell counts, which better control for variations in cell counts due to nuances in tissue fixation or tissue shrinkage are needed to better assess the effects of 6-OHDA and possible rescue by PPX (Coggeshall, 1996). However, the second study employed traditional stereological procedure, yet yielded similar results. Thus, 6-OHDA lesioned/saline treated animals exhibited a 47% loss in THir cell bodies in the SN whereas the cell counts in the 6-OHDA lesioned/PPX treated animals were only 26% reduced. Although there was a slight discrepancy in the magnitude of the THir cell count reductions between the two cell assessment techniques, both produced similar trends enhancing the reliability of the effect observed. Moreover, since the density differences within these groups mimicked the cell count data, it is concluded that the changes seen were not the result of tissue artifact, fixation shrinkage, or incomplete antibody penetration (Ma, 1996a). In addition, these results sug-

gest that evaluation of THir cell counts using a simple sampling procedure at the level of the accessory optic tract may produce results consistent with those gathered from a time-intensive stereological procedure when simple group comparison data is being gathered.

It is important to note that values in excess of 10,000 cells/animal should be seen when SN cell assessments are reported (German, 1993). In the preliminary study where only three sections were counted, application of Konigsmark's equation ($Nt = Ns \times [St/Ss]$) produced total cell counts under 10,000 (mean sham lesioned/saline treated control cell counts were 6,521 ($\pm$281) cells). These low cell counts probably reflect reduced cellular fixation and antibody penetration associated with the post-fixation procedure. In contrast, the stereological study estimated 22,291 $\pm$ 3,672 cells in the SN of the sham lesioned/saline treated controls. This is in excellent agreement with the values reported by German and Manaye (1993) who reported an average value of 20,912, but higher than those reported by Anden et al. (3,500 cells) (Anden, 1966) and Hedreen and Chalmers (7,300 cells) (Hedreen, 1972). It is important to note that in both experiments, the SN THir cell count reduction observed were equal on both sides in agreement with our previous study (Carvey, 1996). The bilateral loss of cells suggests that even though the 6-OHDA was infused unilaterally, it distributed equally throughout the ventricular system and gained access to DA terminals and cell bodies where it was taken up by DA neurons.

The biochemical data collected in the preliminary studied complemented the THir cell count data. Striatal DA levels in the striatum of the post-fixed animals were highly correlated with the THir cell counts from the same animals on both the ipsilateral and contralateral sides of the brain suggesting that even though the THir cell counts were assessed in only one region of the SN, the cell count data was indicative of DA levels throughout the entire striatum. Similar to the cell count data, striatal DA levels in the 6-OHDA lesioned/saline treated animals were reduced 68% relative to sham lesioned/saline treated controls while PPX treatment attenuated that DA loss (29% decrease). However, unlike the THir cell counts, ANOVA revealed ipsilateral/contralateral differences in DA. Thus, in both 6-OHDA lesioned groups, the DA levels in the striatum contralateral to the infusion were higher regardless of treatment history. This suggests that the ipsilateral infusion of 6-OHDA had a more profound effect on the striatum adjacent to the infused toxin. A similar asymmetry was seen in DA activity ([HVA]/[DA]); more elevated on the contralateral side. Since increased DA activity is generally considered a compensatory response to reductions in dopaminergic function (Hollerman, 1990) the contralateral increases observed might suggest that a greater compensatory reserve existed. This would then suggest that the DA cells on the contralateral side were not damaged as much by the 6-OHDA as were the cells on the ipsilateral side. Since the THir cell loss from these animals was symmetric, it appears that various degrees of dysfunction may accompany inhibition of the THir phenotype. Although this may be the case, such asymmetries do not appear to reflect an increase in PPX's ability to rescue cells since it did not appear to rescue cells on the contralateral side

more so than on the ipsilateral side. Regardless, the apparent dissociation between the cell counts and the biochemical indices of DA suggests that assessment of both striatal biochemistry and THir cell counts provides greater insight into the effects of the 6-OHDA lesion.

Possible mechanisms of PPX's neuroprotective actions

All of the in vitro and in vivo systems in which PPX was neuroprotective involve increased DA or DA neurotoxin exposure, which are thought to kill cells through increased free radical production (Olanow, 1992; Fahn, 1992). It is thus possible that PPX's neuroprotective effect involves attenuation of oxidant stress. Indeed, PPX is an excellent free radical scavenger and has been shown to reduce the production of reactive oxygen species (Hall, 1996; Cassarino, 1998; Kitamura, 1998). However, we have shown that although the inactive stereoisomer (+ enantiomer) of PPX is partially neuroprotective, it is not as effective, especially at sub-micromolar ranges where its free radical scavenging effects are not pronounced, as the D_2/D_3 full agonist active isomer (− enantiomer). Moreover, D_3 antagonists dose-dependently attenuate the neuroprotective action of PPX implicating its DA agonist action in its neuroprotective effect (Ling, 1999). In addition, Cassarino et al. (1998) have recently demonstrated that PPX inhibited opening of the mitochondrial transition pore in response to MPP+ and Kitamura et al. (1998) have shown that PPX selectively increases the production of the antiapoptotic protein Bcl-2, both of which could produce neuroprotective effects. We have also shown that PPX increases the production of a 35 kDa soluble protein in DA neurons and that mesencephalic extracts from rats chronically treated with PPX enhanced the growth of DA neurons in culture suggesting increased trophic activity (Ling, 1998). These neuroprotective actions of PPX, especially in regard to increased neurotrophic actions, are particularly interesting since the unbiased stereological data revealed an increase in the volume of the substantia nigra of sham lesioned/PPX treated animals relative to controls. This is consistent with traditional neurotrophic effects such as increased sprouting. Unfortunately, in the preliminary study a sham lesioned/PPX treated group was not assessed so it is impossible to determine if the SN volume increase observed in the stereological study could be associated with an increase in DA. Regardless of the mechanism(s) involved, however, the present data strongly suggest that PPX is neuroprotective to the DA neuron phenotype in 6-OHDA treated rats. Since other DA agonists have also been shown to possess neuroprotective actions (Gassen, 1998), whether or not PPX is more potent in this regard needs to be established.

Since we have shown that PPX dose-dependently increases the production of a mesencephalic trophic activity both in cultures (Carvey, 1997a, 1997c) and in animals (Carvey, 1997b) it is possible that the attenuation of THir cell loss observed here is a consequence of increased neurotrophic activity (Ling, 1998). It could be argued that if PPX was acting through a trophic mechanism, it should have increased the THir cell counts in sham lesioned/PPX treated

animals which received 28 treatments with PPX but no 6-OHDA. This was not the case since the non-lesioned animals treated with PPX exhibited an 8% non-significant decrease in THir cell counts relative to the sham lesioned/saline treated animals. It is possible that the trophic action hypothesized to occur as a result of exposure to PPX could not increase THir cell counts relative to control because of a ceiling effect. Thus, PPX might reduce the loss of cells that are being challenged by oxidants or toxins but is unable to increase the actual number of cell bodies in a normal intact animal.

Although an increase in trophic activity could be responsible for the attenuation in THir cell loss observed, other mechanisms might also be involved. 6-OHDA is known to gain access to the DA neuron via the DA transporter (DAT) (Jonsson, 1970). If PPX possessed affinity for the DAT it would have reduced the amount of 6-OHDA taken up by the DA neurons and thereby attenuated THir cell loss. However, PPX concentrations as high as 10 μM have been shown to be ineffective at blocking DA uptake into striatal synaptosomes (Piercey, personal communication). It could also be argued that increased synaptic DA would compete with 6-OHDA for access to the DAT but among DA agonists, PPX is one of the most potent inhibitors of DA release (Piercey, 1996; Kreiss, 1995). Therefore, if anything, PPX's reduction of striatal DA would have potentiated 6-OHDA-induced THir cell loss. Because PPX possesses antioxidant actions, it is possible that the attenuation in THir cell loss observed was the result of PPX decreasing the oxidant stress produced by 6-OHDA infusion. Other DA agonists such as pergolide, bromocriptine, and apomorphine, have been shown to possess free radical scavenging activity and protect cells from oxidant insults like PPX (Nishibayashi, 1996; Asanuma, 1995; Gassen, 1996; Gassen, 1998). However, PPX produces significant antioxidant activity at concentrations $> 5\,\mu M$ (Von Voigtlander, personal communication) and whether or not such concentrations are achieved in the extracellular space of the brain is unknown. Alternatively, since PPX has high affinity for both the D_2 and the D_3 receptors that are found both in the striatum and SN (Levant, 1997; Moragan, 1997), it is possible that the concentrations of PPX in the vicinity of the DA neuron were higher than the general extracellular space. Such a "site-directed" antioxidant action might explain the attenuation of THir cell loss observed here.

The data presented here are consistent with the notion that PPX possesses DA neuroprotective effects. The mechanism through which this occurs is unknown at this time although its ability to induce an increase in the production of DA autotrophic factor as well as its ability to act as an anti-oxidant may be involved. Regardless of the mechanism through which its neuroprotective action occurs, its ability to attenuate the DA neuron loss produced by several neurotoxins both in vitro as well as in vivo suggests that it might slow the progression of Parkinson's disease.

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Authors' address: P. M. Carvey, Ph.D., Department of Pharmacology, Rush Presbyterian St. Luke's Medical Center, 2242 West Harrison Street (Suite 260), Chicago, IL 60612, U.S.A., e-mail: pcarvey@rush.edu