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Selective Activation of Group III Metabotropic Glutamate Receptors by L-(+)-2-Amino-4-phosphonobutryic Acid Protects the Nigrostriatal System against 6-Hydroxydopamine Toxicity in Vivo

Anthony C. Vernon, Virginia Zbarsky, Krishna P. Datla, David T. Dexter, and Martin J. Croucher

Parkinson's Disease Research Group, Department of Cellular and Molecular Neuroscience, Faculty of Medicine, Imperial College London, Charing Cross Campus, London, United Kingdom

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ABSTRACT

Evidence from several studies suggests that the progressive degeneration of dopaminergic (DA) neurones of the substantia nigra pars compacta (SNc) in Parkinson's disease (PD) may in part be due to excessive release of glutamate from subthalamic projections onto nigral DA neurones. Previous in vitro studies have demonstrated that selective activation of Group III metabotropic glutamate receptors (mGluR) negatively modulates excitatory transmission in the SNc and is neuroprotective against glutamate-mediated toxicity. Consistent with this, we have reported preliminary data indicating that the selective group III mGluR agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) can also protect the nigrostriatal system against 6-hydroxydopamine (6-OHDA) toxicity in vivo. We have now extended these preliminary studies in this model and report here that both acute and subchronic intranigral injections of L-AP4 provide significant protection of the nigrostriatal system against 6-OHDA toxicity. This neuroprotection displays a bell-shaped profile with a clear concentration-dependent relationship. In contrast, when administered to animals 7 days post-6-OHDA lesioning, L-AP4 significantly protects the functionality but not the integrity of the nigrostriatal system. We further demonstrate that neuroprotection by L-AP4 in vivo is reversed by coadministration of the selective Group III mGluR antagonist (*RS*)-α-methylserine-*O*-phosphate, confirming a receptor-mediated mechanism of action. These data provide further compelling evidence that selective activation of Group III mGluR is neuroprotective in an in vivo experimental model of PD, a finding that may have important implications for the future treatment of this disease.

Parkinson's disease (PD) is a debilitating neurodegenerative disorder that afflicts 1% of people over 65 (Lang and Lozano, 1998a). Pathologically, the disease is characterized by a progressive degeneration of the dopaminergic (DA) neurones of the substantia nigra pars compacta (SNc). Currently, treatment of PD is focused on dopamine replacement therapy using the dopamine precursor levodopa (L-DOPA) and/or dopamine receptor agonists, which can provide dramatic amelioration of PD motor symptoms in the early stages of this disease (for review, see Schapira, 2005). However, prolonged use of these agents is associated with a long-term decline in efficacy and a number of severe motor and cognitive side effects (Olanow and Jankovic, 2005). Moreover, these treatments do nothing to halt the progression of nigral neuronal loss. Delaying or slowing the progression of PD would delay the need for L-DOPA therapy; therefore, the development of neuroprotective strategies may play an important role in preventing the onset and reducing the severity of L-DOPA-related adverse effects (Olanow and Jankovic,

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ABBREVIATIONS: PD, Parkinson's disease; DA, dopaminergic; SNc, substantia nigra pars compacta; L-DOPA, levodopa; STN, subthalamic nucleus; mGluR, metabotropic glutamate receptor; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; 6-OHDA, 6-hydroxydopamine; MSOP, (*RS*)-α-methylserine-*O*-phosphate; PBS, phosphate-buffered saline; TH-IR, tyrosine hydroxylase immunoreactivity; NGS, normal goat serum; DAB, 3′3-diaminobenzidine; NeuN, neuronal N; VTA, ventral tegmental area; MTN, medial terminal nucleus; AOI, area of interest; HPLC, high-performance liquid chromatography; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; ANOVA, analysis of variance; SNr, substantia nigra pars reticulata; LY379268, [(−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate]; PHCCC, *N*-phenyl-7-(hydroxymino)cyclopropa[*b*]chromen-1*a*-carboxamide; ECD, electrochemical detection.

2005). Despite extensive research, however, no current treatments for PD have clearly established neuroprotective effects and/or slow disease progression.

The degeneration of nigral DA neurones in PD is thought to result from a complex interplay of factors including oxidative stress, mitochondrial dysfunction, and defects in the ubiquitin-proteasome system (Jenner and Olanow, 1998). Interestingly, anatomical and electrophysiological evidence exists for a direct excitatory projection from the subthalamic nucleus (STN) to DA neurones in the SNc (Smith et al., 1990; Iribe et al., 1999). Considerable attention has focused on this pathway based on the discovery that the STN becomes overactive as a consequence of striatal dopamine depletion in PD, giving rise to the hypothesis that increased glutamate release onto nigral DA neurones may contribute to the continued degeneration of the nigrostriatal system (Rodriguez et al., 1998). Thus, pharmacological agents that negatively modulate glutamate transmission in the basal ganglia have been proposed as potential neuroprotective strategies for PD treatment (Nicoletti et al., 1996). In support of this, several studies have reported a neuroprotective action following blockade of ionotropic *N*-methyl-D-aspartate receptors in experimental models of Parkinsonism (Turski et al., 1991; Sonsalla et al., 1998; Blandini et al., 2001). However, the clinical usefulness of these compounds is limited by their association with severe cognitive and motor side effects (Lee et al., 1999).

Thus, recent research attention has focused on metabotropic glutamate receptors (mGluRs), which form part of the class C heptahelix G-protein-coupled receptor family and which by virtue of their differential expression and unique subcellular localizations play a number of key neuromodulatory roles in the mammalian brain (see Conn, 2003). To date, eight mGluR genes have been cloned and divided into three subtypes based on their sequence homology, signal transduction mechanisms, and pharmacological profiles (for review, see Conn and Pin, 1997). Group I (mGluR1 and 5) is primarily expressed in postsynaptic elements of neurones, where they positively modulate neuronal excitability. In contrast, Group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) mGluRs are presynaptically localized and negatively modulate the release of neurotransmitters, including glutamate (Cartmell and Schoepp, 2000). Interestingly, electrophysiological studies have shown that selective activation of Group III mGluR inhibits excitatory transmission onto nigral DA neurones (Wigmore and Lacey, 1998; Katayama et al., 2003). Furthermore, Valenti et al. (2005) have recently shown that activation of Group III mGluR, and in particular mGluR4, directly modulates glutamate transmission at STN:SNc synapses. This raises the possibility that selective activation of group III mGluR may protect nigral neurones against glutamate excitotoxicity and slow disease progression in PD. In support of this, selective activation of Group III mGluR has been reported to attenuate excitatory amino acid-mediated excitotoxic neuronal death both in vitro and in vivo (Lafon-Cazal et al., 1999b; Bruno et al., 2000). Furthermore, L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) has been reported recently to be neuroprotective in vitro against the pesticide toxin rotenone, which has been strongly implicated in the pathogenesis of PD (Jiang et al., 2006). We have reported previously preliminary data suggesting that L-AP4 is neuroprotective against 6-hydroxydopamine (6-OHDA) toxicity in vivo (Vernon et al., 2005). The aim of the current study was to extend these initial findings and characterize extensively the neuroprotective effects of Group III mGluR activation using a rodent 6-OHDA model of PD.

Materials and Methods

Animals

A total of 44 male Sprague-Dawley rats (Harlan UK Limited, Bicester, Oxon, UK) weighing 250 to 270g on the day of surgery were housed in groups of three at 21 ± 1°C on a 12-h light/dark cycle (lights on 7:00 AM, lights off at 7:00 PM). Standard rat chow and drinking water were available ad libitum throughout the study. All animal experiments were carried out in accordance with the Home Office Animals (Scientific procedures) Act, UK, 1986.

Stereotaxic Implantation of Guide Cannulae

Under isoflurane anesthesia (5% for induction, 1–3% for maintenance in oxygen, flow rate 2 l/min), rats were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) on a thermostatically controlled heated mat. Animals then received a 0.1-ml s.c. injection of Marcain (AstraZeneca, Chester, UK) for analgesia and 5 ml of 4% glucose in 0.18% saline administered by i.p. injection to prevent dehydration. A midline incision was made along the scalp, and the skin was retracted to expose the parietal bones for drilling. Stainless steel guide cannulae (26-gauge; Plastics One, Roanoke, VA) were stereotaxically implanted unilaterally 1 mm above the left SNc via a burr hole at the following coordinates: AP, −3.0 mm; ML, +2.5 mm (relative to bregma); and DV, − 7.6 mm (relative to dura), with the incisor bar set 5.0 mm above the interaural line (Paxinos and Watson, 1986). Cannulae were anchored in place using dental resin (Kemdent Simplex Rapid, Swindon, UK), and three screws were inserted into the skull. Indwelling cannulae were kept patent by the insertion of a stainless steel dummy cannula (26-gauge; Plastics One). Postsurgery, animals were placed in a heated recovery chamber and allowed to recover from the anesthetic. Postoperative care included individual caging and a mashed diet.

Induction of Substantia Nigra Lesions

After a minimum 10-day recovery period, unilateral lesions of the left SNc were created by infusion of 12 μg of 6-OHDA (free base; Sigma-Aldrich Ltd., Poole, UK) in 4 μl of 0.1% ascorbic acid saline solution in conscious animals. To avoid degradation, 6-OHDA was prepared fresh for each experiment, kept on ice, and protected from light. Infusions were made through a 26-gauge stainless steel injection cannula (Plastics One Ltd) inserted through and extending 1 mm below the tip of the indwelling guide cannula, attached with flexible tubing (Portex, Hythe, UK) to a 10-μl 700 series Hamilton syringe mounted on a motorized Harvard micropump (Harvard Apparatus, Edenbridge, UK). Infusions were made at a rate of 2 μl/min followed by a 2-min equilibration time, during which the needle remained in place. Sham-lesioned controls received identical surgery to lesioned animals but received intranigral infusions of 4 μl of 0.1 ascorbic acid saline instead of 6-OHDA, administered as described for 6-OHDA lesioning.

Intranigral Drug Administration

L-AP4 and (*RS*)-α-methylserine-*O*-phosphate (MSOP) were dissolved in PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4) to produce a 5 mM stock solution, which was then serially diluted in PBS to produce the desired concentrations for intranigral injection, and the pH was adjusted to 7.4. Drugs were then aliquoted and frozen, and a fresh vial was used on each day of administration. In all treatment groups, intranigral injections (4 μl) of each mGluR ligand were made using a 26-gauge stainless steel injection cannula, inserted through the indwelling guide cannula as described above. Drug infusions were made over a period of 2 min, followed by an equilibration period of 2 min during which time the

needle remained in place. In all cases, control animals received an equivalent volume of PBS vehicle. Unless otherwise stated, L-AP4 was administered 1 h before the 6-OHDA neurotoxin.

Brain Tissue Preparation for Analysis of Integrity and Functionality of the Nigrostriatal Tract

In all treatment groups, 1 h after the final injection, animals were sacrificed by decapitation, and the brains were quickly dissected out onto a chilled platform. Using a rodent brain matrix, each brain was then cut coronally at the level of the infundibular stem (−4.16 mm from bregma) to produce a fore- and hindbrain block containing the corpus striatum and SNc, respectively. At this point, visual examination of needle tracts was made to confirm correct cannula placement. All animals that showed incorrect placement were excluded from the study. Hindbrain blocks were fixed in 4% paraformaldehyde for 5 days, cryoprotected in 30% sucrose for 24 to 48 h, and then stored desiccated at −80°C until subsequent cryostat sectioning. Serial coronal sections were cut on a cryostat (Bright Instruments, Cambridge, UK), maintained at −22°C, throughout the rostral to caudal extent of the SNc (−4.80 to 6.30 mm from bregma; Paxinos and Watson, 1986). Free-floating sections were collected and stored in 1 M PBS, pH 7.4, containing 0.05% sodium azide as a preservative until immunostaining. From the forebrain blocks, the left and right striata were quickly dissected out using the corpus callosum as a guide, snap-frozen, and stored at −80°C until biochemical analysis of monoamine content.

Histological Analysis of the Integrity of the Nigrostriatal Tract

Tyrosine Hydroxylase Immunostaining with Nissl Counterstain. To quantify the number of dopaminergic neurones in the SNc following 6-OHDA lesioning in vehicle- and drug-treated animals, immunostaining for tyrosine hydroxylase immunoreactivity (TH-IR), the rate-limiting enzyme in DA synthesis, was performed as described previously using a standard immunoperoxidase method (Vernon et al., 2005). In brief, free-floating sections were washed in 1 M PBS, pH 7.4 (3 × 5 min). Endogenous peroxidase activity was quenched by incubating sections in 1% hydrogen peroxide solution for 30 min followed by washing in 1 M PBS containing 0.1% Triton X-100 for 15 min. Nonspecific biding was blocked by incubating sections in 3% normal goat serum (NGS; MP Biomedicals, Eschwage, Germany) diluted in 1 M PBS for 60 min. Sections were then incubated with the TH primary antibody (rabbit polyclonal, AB151; Chemicon Europe, Chandlers Ford, UK) diluted 1:3000 in PBS plus 0.1% Triton and 3% NGS for 18 h at room temperature. Subsequently, sections were washed in PBS (3 × 5 min) and incubated with biotinylated secondary antibody (goat α-rabbit IgG; Vector Labs, Peterborough, UK) diluted 1:200 in 1 M PBS for 2 h followed by washing in PBS (3 × 5 min). Sections were incubated for a further hour in horseradish peroxidase conjugate (Vectastain Elite ABC Kit; Vector Labs), again followed by washing in 1 M PBS (3 × 5 min). Antibody binding was then visualized using a 3′3-diaminobenzidine (DAB) peroxidase staining kit (DAB peroxidase kit SK-4100; Vector Labs) as per the manufacturer's instructions, followed by washing in distilled water. Free-floating immunostained sections were mounted onto polylysine-coated slides (VWR International, Leicestershire, UK) and allowed to adhere by air-drying. Mounted sections were then counterstained in 0.1% cresyl-fast violet for 2 min and rinsed with tap water before dehydration through a series of graded alcohol and xylene solutions, followed by application of coverslips using DPX mounting medium. Negative controls were also performed in which 1 M PBS was substituted for the primary antibody. When this was done, no immunostaining was observed, confirming the specificity of the TH antibody.

Neuronal N Immunostaining. Because changes in TH-IR are only markers of cell phenotype, a reduction in this measure does not necessarily imply neuronal cell death. Therefore, for each TH-IR section from vehicle-treated animals, an adjacent section was immunostained for the specific neuronal marker neuronal N (NeuN). Immunostaining for NeuN was carried out using a standard immunoperoxidase method as described for TH above, using a mouse monoclonal NeuN antibody (MAB377; Chemicon Europe) diluted 1:2000 in 0.1% PBS-Triton and 3% NGS and biotinylated secondary antibody (goat α-mouse IgG; Vector Labs). Antibody binding was visualized using a DAB peroxidase kit with nickel enhancement as per the manufacturer's instructions (DAB peroxidase kit SK-4100; Vector Labs). As with TH, antibody specificity and optimal dilutions were determined by prior experiments.

Image Capture and Analysis

Quantitative Assessment of TH-IR and NeuN Cell Number. Images of nigral cells displaying robust TH-IR were captured using a JVC digital camera attached to Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) at ×100 magnification. Quantitative counting of the number of TH-IR cell bodies in the SNc was performed manually using Image Pro Plus version 5.0 image analysis software (Datacell, Finchampstead, UK). Initially, in all treatment groups, TH-IR cell bodies were counted in the contralateral and ipsilateral hemispheres of the brain at region B of the SNc (−5.30 mm from bregma) as defined by Carman et al. (1991) and with reference to a standard rat brain atlas (Paxinos and Watson, 1986). This region was chosen since the SNc and the nearby ventral tegmental area (VTA) are clearly separated by the medial terminal nucleus (MTN) of the accessory optic tract level. In each section, the region of the SNc was delineated by examination of the shape and distribution of TH-IR cells, at low magnification (×40) with reference to previous descriptions of these patterns (Carman et al., 1991) and to a standard rat brain atlas (Paxinos and Watson, 1986). Images were then subsequently captured at ×100 magnification for quantitative cell counting using Image Pro Plus version 5.0. On captured images, the SNc was defined by manually drawing an area of interest (AOI), and cell counting was then performed within that area only. Before analysis, operators were assessed for consistency of AOI drawing. Furthermore, the AOI drawn for each stereotaxic level of the SNc was "saved" such that it could be consistently applied to each section counted at each stereotaxic level. Only cells that were intact and displayed a robust TH-IR with a nucleus and a clear cytoplasm and axonal processes were counted. Cells that appeared severely deformed were not included (Yuan et al., 2005). For each animal, four to five adjacent sections were counted at level B, and these were pooled to give a mean cell count for each individual animal. These counts were further pooled to give a total mean cell count for the contralateral and ipsilateral hemispheres in each treatment group. In all cases, the lesion size was then expressed as a mean percentage loss compared with the contralateral side. This approach to cell counting has been used previously to ensure that comparable rostral to caudal levels of the SNc are sampled between animals (Vernon et al., 2005; Yuan et al., 2005), and the extent of cell loss at this point is reflective of cell loss throughout the entire structure (Yuan et al., 2005). Furthermore, this approach prevents errors in counting the numbers of cells residing in the SNc and VTA. As a further control, cell counts were reassessed by a second operator, blind to the treatment group in the same manner, and the data were compared for consistency.

Once this analysis was complete to rigorously assess loss or neuroprotection of TH-IR throughout the entire SNc structure, cell counting was performed in all treatment groups at the other stereotaxic regions of the SNc, defined A, C, D, and E (−4.80, −5.30, −5.80, and −6.30 mm from bregma, respectively) by Carman et al. (1991) to ensure complete sampling of the entire SNc structure. Quantitative counting of TH-IR cells was performed in the same manner as described above, using Image Pro Plus version 5.0 (Datacell), with care taken to clearly delineate the SNc from the nearby VTA by examination of the shape and distribution of TH-IR cells, at low magnification (×40) as described previously (Carman et al., 1991)

and with reference to the standard rat brain atlas (Paxinos and Watson, 1986). Images were then subsequently captured at ×100 magnification for quantitative cell counting using Image Pro Plus version 5.0 as described above. As before, the numbers of TH-IR cells were counted in four to five adjacent sections corresponding to each stereotaxic level of the SNc, and the data were pooled. A similar approach was used to quantitatively analyze the number of NeuN-IR cells in the SNc from vehicle-treated animals. In these sections, the SNc and VTA were defined according to the adjacent TH-IR section. As before, all counting was performed within a defined AOI initially at level B of the SNc and then across all stereotaxic SNc regions. Cell counts of NeuN-IR cells were made from four to five pairs of adjacent sections corresponding to each stereotaxic level of the SNc, and the data were pooled and expressed in the same manner as for TH-IR cell counts. Only cells that displayed a darkly stained nucleus and with a clear cytoplasm and axonal processes were counted, and cells that appeared severely deformed were not included. To further ensure an absence of bias in cell counting, all slides were coded and randomized to ensure that the operator was blind to the treatment group being counted, and cell counting was corroborated by a second operator also blinded to the treatment group. At the same time as cell counting was performed, representative photomicrographs of nigral cells displaying TH-IR or NeuN-IR were captured at ×40 magnification, using the same microscope apparatus and Image Pro Plus version 5.0 software (Datacell).

Biochemical Analysis of Nigrostriatal Tract Functionality by Measurement of Striatal Monoamines Using HPLC-ECD

Striatal monoamine content was analyzed using HPLC-ECD, as described previously (Vernon et al., 2005). In brief, the left and right striata were thawed, weighed, and homogenized in 500 μl of ice-cold homogenization buffer (50 mM trichloroacetic acid, 0.5 mM EDTA) containing 0.5 pmol/ml 3,4-dihydroxybenzylamine hydrobromide as an internal standard. Striata were then homogenized for 20 s by sonication (Soniprep; Sanyo, Loughborough, UK) and placed on ice for 10 min to allow complete extraction of monoamines. Samples were then centrifuged at 13,000*g* at 4°C for 10 min (Heraeus Centrifuges, Newport Pagnell, UK). Samples were filtered (0.2-μm PTFE filter; Whatman, Maidstone, UK) and loaded onto an autosampler (Gina 50; Dionex, Camberley, UK) maintained at 5°C. Samples were then analyzed for DA, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) content using a phosphate buffer mobile phase (0.1 mM KH_2PO_4, 0.1 mM EDTA, 1 mM octyl sodium sulfonate, 10% methanol v/v, adjusted to pH 2.5 with orthophosphoric acid) at a flow rate of 0.9 ml/min on an Altex Ultrasphere 3 μm ODS column (4.6 mm × 7.5 cm; Beckman-Coulter, High Wycombe, UK). Samples were quantified by an electrochemical analytical cell (model 5011; ESA Analytical, Aylesbury, UK) attached to a Coulochem II electrochemical detector (ESA Analytical) with electrode one set at −0.20 mV and electrode two set at +0.34 mV with respect to the pallidium reference electrode. Striata from both control and mGluR agonist-treated groups were analyzed on the same day. All data analysis was performed using PC-based Chromeleon software (Dionex).

Compounds

L-AP4 and MSOP were obtained from Tocris Cookson Bioscience (Bristol, UK). All other reagents and compounds were obtained from Sigma-Aldrich Ltd. unless stated otherwise in the text. The chemical structures of L-AP4 and MSOP are shown in Figs. 1 and 5, respectively.

Statistical Analysis of Data

Individual comparisons were made using a paired or unpaired two-tailed Student's *t* test as appropriate. Where multiple compari-

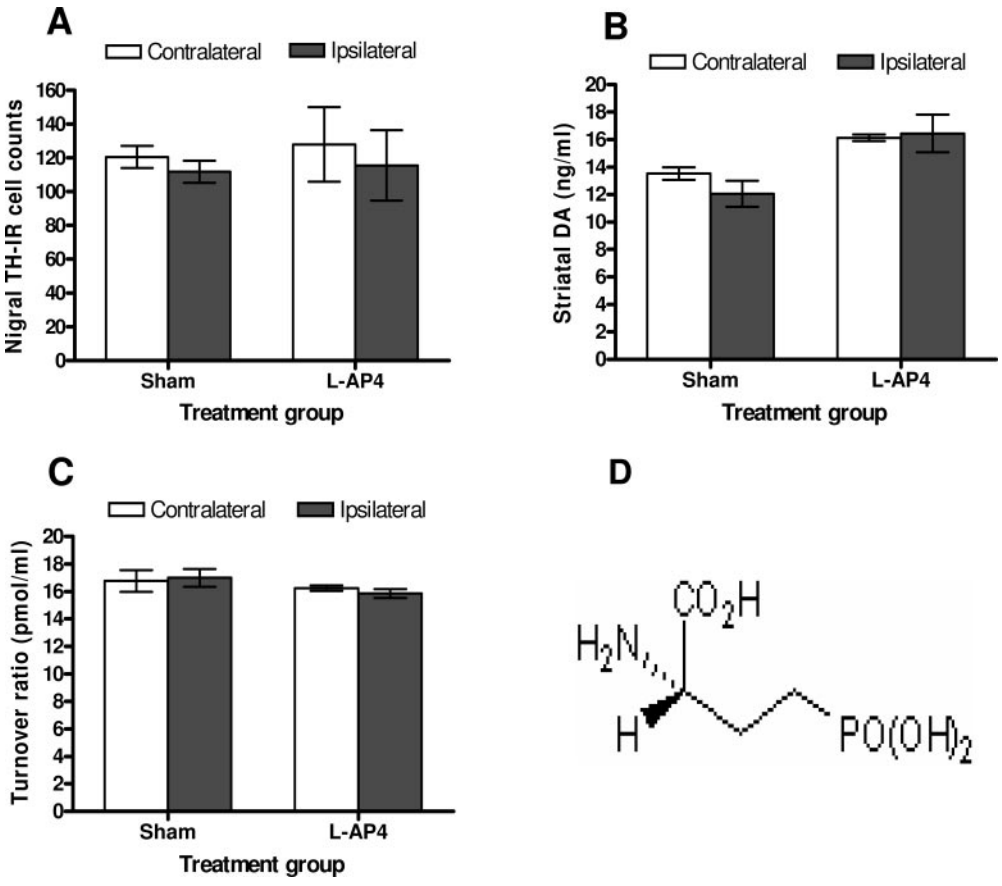

Fig. 1. A, mean number of nigral TH-IR cells in sham-lesioned control animals and sham-lesioned animals treated subchronically with 10 nmol in 4 μl of L-AP4. B, mean concentration of striatal dopamine in sham-lesioned animals and sham-lesioned animals treated subchronically with 10 nmol in 4 μl of L-AP4. C, mean dopamine turnover ratio in sham-lesioned animals and sham-lesioned animals treated subchronically with 10 nmol in 4 μl of L-AP4. D, chemical structure of L-AP4. Data shown are mean number of nigral TH-IR cells ± S.E.M. (A), mean striatal dopamine concentration in nanograms per milliliter ± S.E.M. (B), and mean dopamine turnover ratio ± S.E.M. (C).

sons were made, one-way ANOVA with post hoc Tukey's test for multiple comparisons was employed. Data for cell loss in vehicle and drug-treated groups are presented as the mean percentage change ± S.E.M.; mean cell numbers were also compared. Biochemical data for monoamines is presented as the mean concentration ± S.E.M. in nanograms per milliliter. In all figures, *n* values are shown in parentheses. Differences were considered statistically significant at $P < 0.05$, and all statistical data analysis was performed using GraphPad Prism version 4.00 software (GraphPad Software, San Diego, CA).

Results

Effects of Subchronic Intranigral L-AP4 Administration Alone on the Nigrostriatal System. In the current study, in all treatment groups, the mean number of nigral TH-IR cells in the SNc in the contralateral hemisphere of the brain was comparable and not significantly different. Likewise, in all treatment groups, the mean concentration of dopamine in the contralateral corpus striatum was comparable and not significantly different between treatment groups.

In sham-lesioned animals, no significant loss of TH-IR stained cells was observed in the SNc, nor were any significant changes observed in the levels of striatal dopamine, as shown in Fig. 1, A and B. Furthermore, no significant loss of striatal DOPAC or HVA was observed (Table 1), and no significant changes in striatal DA turnover were seen (Fig. 1C).

To investigate the effect of L-AP4 alone on the nigrostriatal system, sham-lesioned animals were treated subchronically (7 days) with 10 nmol/4 μl of L-AP4. Figure 1A shows clearly that subchronic intranigral treatment with L-AP4 results in no significant loss of nigral TH-IR cells in the ipsilateral SNc. Consistent with this, subchronic (7-day) intranigral treatment with 10 nmol/4 μl of L-AP4 in sham-lesioned animals had no significant effect on the concentration of dopamine in the ipsilateral corpus striatum, as shown in Fig. 1B. Moreover, no significant effects were observed on striatal concentrations of DOPAC or HVA between the ipsilateral and contralateral striata (Table 1), nor were any changes in striatal dopamine turnover observed, as clearly shown in Fig. 1C. To illustrate these data, representative photomicrographs of nigral TH-IR cells from both groups are shown in Fig. 2, A and B. Importantly, no gross behavioral abnormalities were observed following intranigral injections of L-AP4.

Subchronic Treatment with L-AP4 Protects the Nigrostriatal System against 6-OHDA Toxicity in Vivo. Infusion of 6-OHDA into the left SNc produced a significant reduction of nigral TH-IR cells (65.62 ± 1.61%; $P < 0.001$), which was mirrored by a significant reduction of the number of NeuN-IR cells in the SNc (73.31 ± 4.63% $P < 0.01$). The degree of both TH-IR and NeuN-IR cell loss is illustrated in representative photomicrographs, shown in Fig. 2, C and D. Coupled with these losses was a concomitant reduction in the mean concentration of striatal dopamine (12.58 ± 0.43 versus 6.42 ± 0.89 ng/ml, $P < 0.01$) comparing the intact and lesioned striata, as shown in Fig. 3C. Significant reductions were also observed in the levels of striatal DOPAC and HVA ($P < 0.01$, Table 1). Infusion of 6-OHDA caused a marked and significant increase in striatal dopamine metabolism comparing the ipsilateral and contralateral striata (14.23 ± 2.49 versus 10.53 ± 0.79 pmol/ml × 100; $P < 0.05$), as shown in Fig. 3D.

To investigate the neuroprotective potential of L-AP4, 6-OHDA-lesioned animals were intranigrally administered 2, 10, or 50 nmol of L-AP4 in an injection volume of 4 μl daily over a period of 7 days. In this treatment group, the initial injection of L-AP4 was made 1 h before the infusion of the 6-OHDA neurotoxin. Thus, on the 1st day of treatment, animals in this group received two injections of 4 μl each, giving a total volume of 8 μl. Subsequently, animals received a single unilateral injection daily of each concentration of L-AP4 in an injection volume of 4 μl for 7 consecutive days. To account for the possible dilution of toxin effects by solvent, an additional group of animals received intranigral injections of 4 μl of PBS 1 h before infusion of 6-OHDA. Infusion of PBS before 6-OHDA lesioning did not result in any significant effects on the 6-OHDA-induced loss of nigral TH-IR cells, depletion of striatal monoamines, or increases in striatal dopamine turnover (data not shown).

Intranigral administration of L-AP4 significantly attenuated the 6-OHDA-induced loss of TH-IR cells in the ipsilateral SNc, as indicated by one-way ANOVA, which revealed significant differences between L-AP4- and vehicle-treated groups ($F_{20.42}, P < 0.0001$). These data are in good agreement with our previous preliminary findings with this compound (Vernon et al., 2005). Indeed, post hoc analysis revealed that the mean percentage loss of TH-IR cells in the ipsilateral SNc was significantly reduced in animals treated with 2 and 10

TABLE 1
Mean striatal concentrations of dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) as measured by HPLC-ECD in the contralateral and ipsilateral striatum in each treatment group. Data shown are mean values in nanogram/milliliter wet weight tissue ± S.E.M.

Group (conc. nmol/μl)	DOPAC Contralateral	DOPAC Ipsilateral	HVA Contralateral	HVA Ipsilateral
		ng/ml		
Sham lesion	1.773 ± 0.175	1.632 ± 0.080	0.624 ± 0.067	0.513 ± 0.044
Sham + L-AP4 10	1.769 ± 0.081	1.805 ± 0.082	0.524 ± 0.036	0.511 ± 0.027
Drug vehicle	1.192 ± 0.070	0.516 ± 0.393*	0.399 ± 0.021	0.206 ± 0.023*
L-AP4 2	4.880 ± 0.280	3.770 ± 0.230*†	1.191 ± 0.124	0.835 ± 0.044*†
L-AP4 10	1.430 ± 0.180	0.970 ± 0.220*†	0.468 ± 0.040	0.322 ± 0.061*†
L-AP4 50	1.370 ± 0.170	0.560 ± 0.230*	0.390 ± 0.046	0.205 ± 0.040*
L-AP4 10 (acute)	1.360 ± 0.140	1.000 ± 0.070*†	0.377 ± 0.031	0.333 ± 0.042*†
MSOP 50	1.190 ± 0.007	0.726 ± 0.077*	0.415 ± 0.027	0.259 ± 0.016*
MSOP 50+ L-AP4 10	1.353 ± 0.153	0.918 ± 0.170*	0.363 ± 0.071	0.231 ± 0.071*
Drug vehicle (postlesion)	0.997 ± 0.074	0.868 ± 0.057*	0.261 ± 0.031	0.170 ± 0.026*
L-AP4 10 (postlesion)	1.377 ± 0.112	0.286 ± 0.085*	0.430 ± 0.022	0.143 ± 0.034*

* $P < 0.05$ contralateral vs. ipsilateral striata; † $P < 0.05$ ipsilateral striata of drug vs. vehicle-treated.

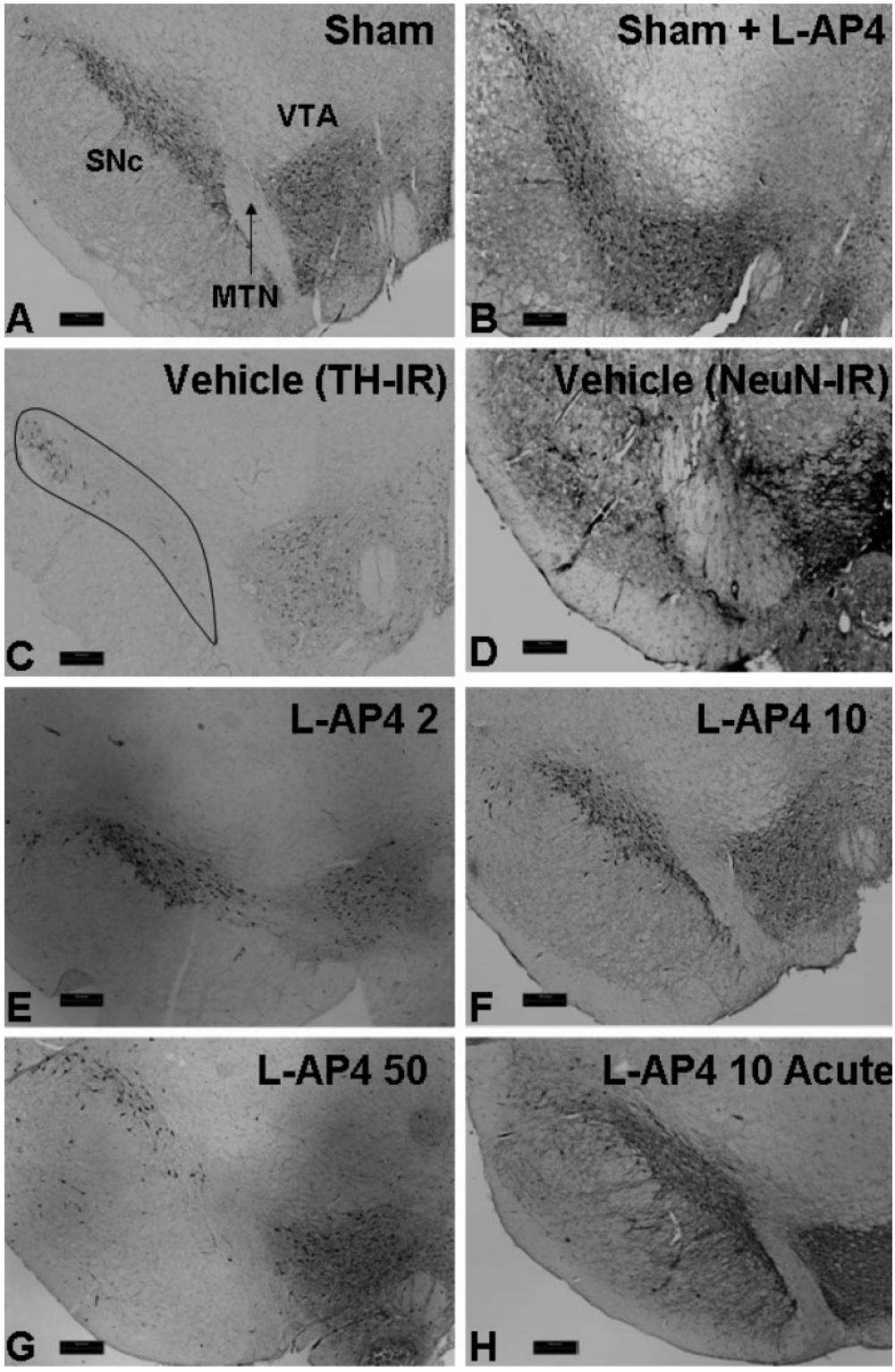

Fig. 2. Representative photomicrographs of nigral TH-IR cells from individual treatment groups (A–C and E–H) and NeuN-IR cells from vehicle-treated animals (D), corresponding to level B of the SNc (−5.33 mm from bregma) at ×40 magnification. Scale bar, 250 μm. An example of a defined AOI for quantitative cell counting is illustrated in B. SNc, VTA, and MTN are highlighted in A.

nmol/4 μl of L-AP4 (2 nmol/4 μl, 47.6 ± 3.7%, $P < 0.05$; 10 nmol/4 μl, 33.9 ± 4.2%, $P < 0.001$; Fig. 3A). Unexpectedly, the mean percentage loss of TH-IR cells in animals treated with 50 nmol/4 μl of L-AP4 was not significantly different from that observed in vehicle-treated animals (60.6 ± 3.2% versus 65.62 ± 1.61% $P > 0.05$; Fig. 3A). These data indicate that L-AP4 exhibits a bell-shaped neuroprotective profile in this model, with maximal protection of TH-IR cells achieved at 10 nmol/4 μl of L-AP4.

One-way ANOVA analysis indicated no significant differences in neuroprotection of TH-IR cells across the different stereotaxic regions of the SNc (A–E) for any L-AP4-treated group ($F_{0.624}$, $P = 0.651$). However, the mean percentage loss of TH-IR cells was significantly different from vehicle-treated animals in all regions of the SNc in animals treated with 2 and 10 nmol/4 μl of L-AP4 as shown in Fig. 3B, suggesting substantial protection of TH-IR cells throughout the extent of the SNc by L-AP4. In animals treated with 50 nmol/4 μl of

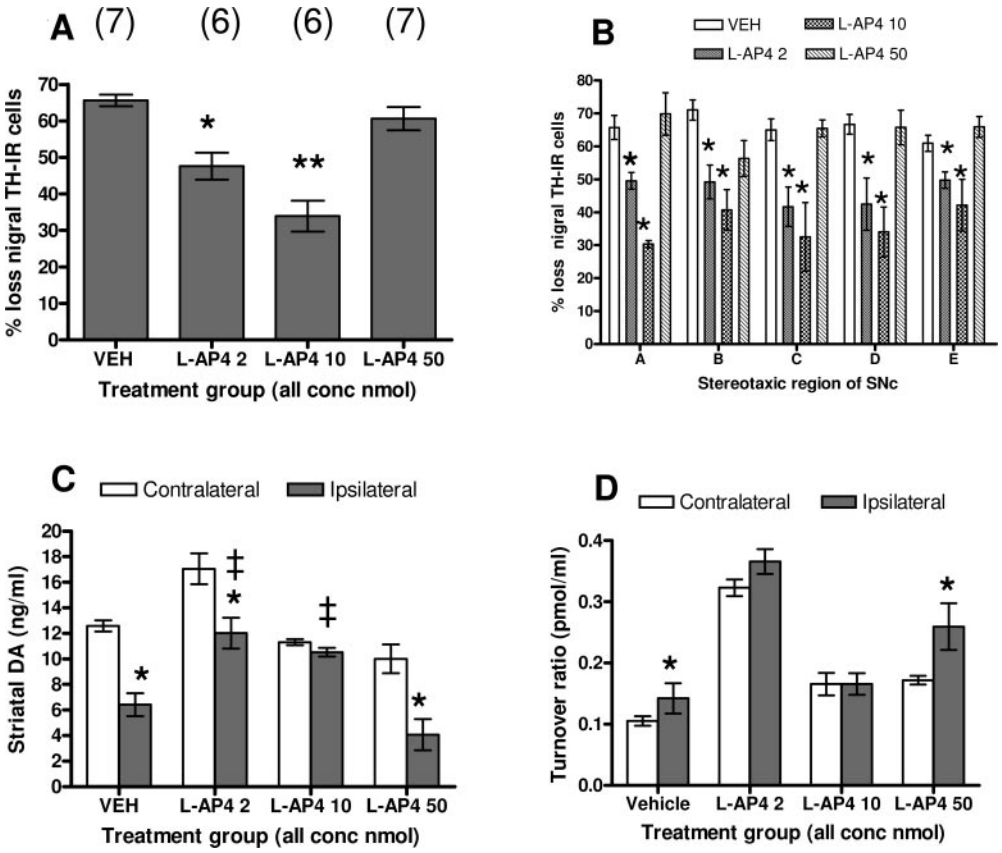

Fig. 3. A, subchronic treatment with L-AP4 reduces the mean percentage loss of nigral TH-IR cells following 6-OHDA lesioning in a concentration-dependent manner. B, mean percentage loss of nigral TH-IR cells is reduced in all regions of the SNc compared with vehicle-treated animals in the 2 and 10 nmol of L-AP4 groups but not the 50 nmol-treated group. No regional differences in the neuroprotection of nigral TH-IR cells were observed across the different stereotaxic regions of the SNc at any concentration of L-AP4 tested. Data shown are mean percentage cell loss ± S.E.M. *, $P < 0.05$, vehicle versus L-AP4-treated; **, $P < 0.01$, vehicle versus L-AP4-treated. C, subchronic treatment with 2 and 10 nmol of L-AP4 prevents the 6-OHDA-induced loss of striatal DA; and D, blocks increases in mean striatal DA turnover in a concentration-dependent manner. Data shown are mean striatal DA in nanograms per milliliter ± S.E.M. and mean turnover ratio ± S.E.M. *, $P < 0.01$, contralateral versus ipsilateral striata; ‡, $P < 0.01$, ipsilateral striata vehicle versus L-AP4-treated. *n* values are shown in parentheses.

L-AP4, the lesion size was not significantly different from that observed in vehicle-treated animals in any SNc region (Fig. 3B). To illustrate the magnitude of the neuroprotection by L-AP4, a representative photomicrograph of TH-IR cells in the SNc from animals treated with each concentration of L-AP4 is shown in Fig. 2, E, F, and G, respectively. However, despite this robust protection of nigral TH-IR cells, it is important to note that a small but significant loss of TH-IR cells was still observed when comparing the mean TH-IR cell number in the contralateral and ipsilateral hemispheres of the brain within each treatment group ($P < 0.01$, data not shown).

Subchronic intranigral administration of L-AP4 also significantly attenuated the 6-OHDA-induced reduction of striatal dopamine concentration in the ipsilateral striatum (ANOVA $F_{10.24}$, $P < 0.0001$), as clearly illustrated in Fig. 3C. In good agreement with the TH-IR data, post hoc analysis showed that striatal dopamine depletion was only significantly reduced in the lesioned striata compared with vehicle-treated animals in animals treated with 2 and 10 nmol/4 μl of L-AP4 (2 nmol/4 μl, 14.89 ± 1.21 versus 6.42 ± 0.89 ng/ml, $P < 0.05$; 10 nmol/4 μl, 10.52 ± 0.34 versus 6.42 ± 0.89 ng/ml, $P < 0.01$; Fig. 3C). The restoration of striatal dopamine was maximal in animals treated with 10 nmol/4 μl of L-AP4 because no significant difference was found between the mean dopamine concentration in the ipsilateral and contralateral striata in this treatment group (11.31 ± 0.23 versus 10.52 ± 0.34 ng/ml; $P > 0.05$). It should be noted, however, that a significant loss of striatal dopamine was still observed comparing the ipsilateral and contralateral striata within the 2 nmol/4 μl of L-AP4-treated group (17.96 ± 0.83 versus 14.89 ± 1.21 ng/ml; $P < 0.05$). Thus, 6-OHDA still induces a loss of striatal dopamine in these animals, but this is markedly and significantly reduced compared with the depletion seen in vehicle-treated animals. In animals treated with 50 nmol of L-AP4, the mean striatal dopamine concentration in the lesioned striata was not significantly different from vehicle-treated animals (4.06 ± 0.89 versus 6.42 ± 0.89 ng/ml; $P > 0.05$; Fig. 3C), in good agreement with the data collected for nigral TH-IR cells. Similar results were observed for striatal concentrations of DOPAC and HVA in all the L-AP4-treated groups (Table 1).

In terms of dopamine metabolism, treatment with 2 and 10 nmol/4 μl of L-AP4 completely blocked the increase in the (DOPAC +HVA)/DA ratio observed in vehicle-treated animals. These data suggest that the neuroprotective effects of L-AP4 on striatal monoamines are not due to increases in metabolic activity by the surviving nigral TH-IR cells. In contrast, in the 50 nmol/4 μl-treated group, a significant increase in dopamine turnover was observed in the ipsilateral striata compared with the contralateral side (25.94 ± 3.18 versus 17.18 ± 0.70 pmol/ml × 100; $P < 0.05$; Fig. 3D). This increase is similar to that observed in vehicle-treated animals and is consistent with the absence of a neuroprotective effect at this concentration of L-AP4. Because neuroprotection by L-AP4 appears to be maximal in animals treated with 10 nmol/4 μl of L-AP4, this concentration was used in all subsequent experiments.

Acute Treatment with L-AP4 Protects the Nigrostriatal System against 6-OHDA Toxicity in Vivo. G-protein-coupled receptor functions, including mGluR functions, are tightly linked to receptor membrane expression and trafficking, which are regulated by G-protein receptor kinases and second messenger-dependent kinases, respectively

(Mundell et al., 2004). This has been well characterized for Group I mGluR, but much less is known about desensitization and internalization of Group III mGluR. Previous in situ studies have shown that Group III mGluR as a whole undergo heterologous desensitization through a protein kinase A and C-dependent mechanism (Cai et al., 2001). In support of this, the reversal of akinesia following i.c.v. administration of L-AP4 in reserpine-treated rats is markedly reduced following a second injection of L-AP4 (MacInnes et al., 2004). These data suggest that Group III mGluR-mediated responses undergo rapid heterologous desensitization in vivo. Thus, it may be hypothesized that acute treatments with L-AP4 might provide similar or indeed greater neuroprotection than more chronic ones. To investigate this possibility, animals were treated acutely with 10 nmol in 4 μl of L-AP4. As before, animals received an initial injection of L-AP4 1 h before infusion of the 6-OHDA neurotoxin. Thus, on the 1st day of treatment, animals in this group received two injections of 4 μl each, giving a total volume of 8 μl. Subsequently, animals received a single unilateral injection daily of 10 nmol of L-AP4 in an injection volume of 4 μl for 3 consecutive days. Animals were then allowed to survive for a further 4 days before being sacrificed, and the brain tissue was collected for analysis. Figure 4A clearly shows that acute treatment (3 days) with 10 nmol/4 μl of L-AP4 also significantly attenuates the 6-OHDA-induced loss of nigral TH-IR cells (23.76 $\pm$ 9.98% versus 65.62 $\pm$ 1.61%; $P < 0.001$), and this is not significantly different from that observed following subchronic treatment (23.76 $\pm$ 9.98% versus 33.89 $\pm$ 4.2%, respectively; $P > 0.05$). Consistent with the subchronic data, no regional differences were observed in neuroprotection of TH-IR cells in any region of the SNc (A–E) following acute treatment with L-AP4 ($F_{0.060}$, $P = 0.953$), and acute treatment with L-AP4 significantly reduced the mean percentage loss of nigral TH-IR cells compared with vehicle-treated animals in every region of the SNc, as illustrated in Fig. 4B. The degree of neuroprotection of TH-IR cells is illustrated in representative photomicrographs shown in Fig. 2H. Acute treatment with 10 nmol/4 μl of L-AP4 also significantly attenuated the reduction of striatal dopamine in the ipsilateral striata compared with vehicle-treated animals (12.03 $\pm$ 0.28 versus 6.42 $\pm$ 0.89 ng/ml, respectively; $P < 0.01$), and this also was not significantly different from subchronically treated animals, as shown in Fig. 4C (12.03 $\pm$ 0.28 versus 10.52 $\pm$ 0.34 ng/ml, respectively; $P > 0.05$). Acute treatment with 10 nmol of L-AP4 significantly reduced the fall in striatal DOPAC and HVA induced by 6-OHDA; again, this was not significantly different from that observed in subchronically treated animals (Table 1). Furthermore, acute treatment with L-AP4 also completely blocked the 6-OHDA-induced increases in striatal dopamine turnover, similar to that observed following subchronic treatment with this agonist (Fig. 4D).

Neuroprotection of the Nigrostriatal System following L-AP4 Treatment Is Reversed by Coadministration with MSOP in 6-OHDA-Lesioned Animals. To investigate whether the neuroprotection of the nigrostriatal system by L-AP4 observed in the current study is mediated through Group III mGluR, 6-OHDA-lesioned animals received subchronic intranigral injections of 10 nmol of L-AP4 coadmin-

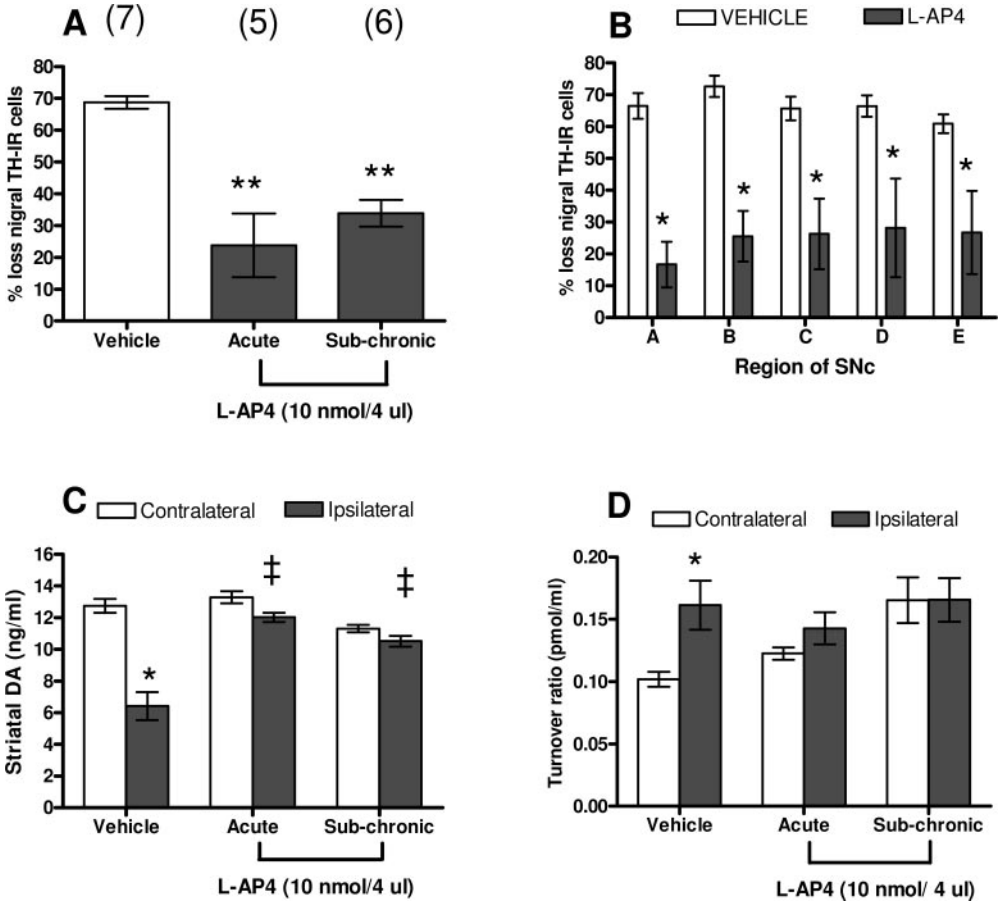

Fig. 4. A, acute treatment with 10 nmol of L-AP4 significantly reduces the mean percentage loss of nigral TH-IR cells compared with vehicle-treated animals. B, no regional differences in neuroprotection of nigral TH-IR cells are observed following acute L-AP4 treatment, and the mean percentage loss of TH-IR cells is significantly reduced compared with vehicle-treated animals in all regions of the SNc. Data shown are mean percentage loss nigral TH-IR cells $\pm$ S.E.M. *, $P < 0.05$, L-AP4 versus vehicle-treated; **, $P < 0.01$, L-AP4 versus vehicle-treated. C, acute treatment with L-AP4 prevents dopamine depletion in the ipsilateral striatum. D, acute treatment with L-AP4 blocks 6-OHDA-induced increases in mean striatal dopamine turnover. Data shown are nanograms per milliliter mean striatal dopamine and mean striatal turnover ratio $\pm$ S.E.M. *, $P < 0.01$, contralateral versus ipsilateral striata; ‡, $P < 0.01$, ipsilateral striata L-AP4 versus vehicle-treated. *n* values are shown in parentheses.

istered with 50 nmol of the selective Group III mGluR antagonist MSOP in an injection volume of 4 μl. In a separate group, MSOP was administered alone to determine the effect of Group III mGluR blockade in vivo on the lesion induced by 6-OHDA. In these experiments, MSOP and L-AP4 were coadministered in a final injection volume of 4 μl, whereas MSOP was administered alone in an injection volume of 4 μl. As before, drug injections were made 1 h before infusion of the 6-OHDA neurotoxin. Thus, on the 1st day of treatment, animals in these groups received two injections of 4 μl each, giving a total volume of 8 μl. Subsequently, animals received a single unilateral injection daily of either L-AP4 + MSOP or MSOP alone in an injection volume of 4 μl for 7 consecutive days.

Interestingly, Fig. 5A shows that in 6-OHDA-lesioned animals, subchronic intranigral administration of 50 nmol/4 μl of MSOP alone slightly enhanced the mean percentage loss of nigral TH-IR cells induced by 6-OHDA (65.62 $\pm$ 1.61% versus 77.23 $\pm$ 3.42%, $P > 0.01$). Treatment with MSOP also appeared to slightly enhance the 6-OHDA-induced depletion of striatal dopamine in the ipsilateral striata (4.36 $\pm$ 1.07 versus 6.42 $\pm$ 0.89 ng/ml), but this failed to reach statistical significance, as shown in Fig. 5B. Similar results were observed for the 6-OHDA-induced depletion of DOPAC and HVA levels in the ipsilateral striatum (see Table 1). Moreover, administration of MSOP did not block the 6-OHDA-induced increases in striatal dopamine turnover seen following 6-OHDA lesioning, as shown in Fig. 5C.

In subsequent drug studies, coadministration of MSOP with L-AP4 led to a complete reversal of the previously observed neuroprotective effects of L-AP4 on nigral TH-IR cells (80.5 $\pm$ 2.1% versus 33.9 $\pm$ 4.2%; $P < 0.01$; Fig. 5A). Consistent with our data for MSOP alone, coadministration of MSOP with L-AP4 slightly enhanced the 6-OHDA lesion such that the mean percentage loss of nigral TH-IR cells was significantly greater than that observed in vehicle-treated animals (65.62 $\pm$ 1.61% versus 80.5 $\pm$ 2.1%; $P < 0.05$; Fig. 5A). To illustrate this, representative photomicrographs of TH-IR in the SNc from each treatment group are shown in Fig. 6, A–D, respectively. Likewise, the prevention of striatal dopamine depletion in the ipsilateral striata previously observed with L-AP4 alone was fully reversed by coadministration of MSOP (4.87 $\pm$ 1.32 versus 4.36 $\pm$ 1.07 ng/ml; $P > 0.05$, Fig. 5B). However, the presence of MSOP did not significantly enhance the depletion of striatal dopamine (4.87 $\pm$ 1.32 versus 6.42 $\pm$ 0.89 ng/ml; $P > 0.05$). Moreover, the increase in striatal dopamine metabolism, which was blocked by L-AP4 alone, was completely reversed by coadministration with MSOP, as shown in Fig. 5C.

Subchronic Intranigral Treatment with L-AP4 Slows 6-OHDA-Mediated Nigrostriatal Degeneration in Animals Already Undergoing Nigrostriatal Degeneration. The neuroprotective effect of L-AP4 may depend on its administration before lesioning with 6-OHDA. Therefore, to investigate whether L-AP4 is neuroprotective or can "rescue" nigrostriatal neurones following 6-OHDA lesioning, animals were lesioned with 6-OHDA and left for 7 days to allow degeneration of the nigrostriatal tract to proceed. After 7 days, animals were then treated subchronically (7 days) with 10 nmol/4 μl of L-AP4. As before, animals received single unilateral injections of L-AP4 in a final injection volume of 4 μl for 7 consecutive days. Figure 7A shows that in animals treated with drug vehicle starting 7 days after the infusion of 6-OHDA into the SNc, there is a significantly increased loss of nigral TH-IR cells compared with that seen in vehicle-treated animals sacrificed 7 days post-6-OHDA lesioning

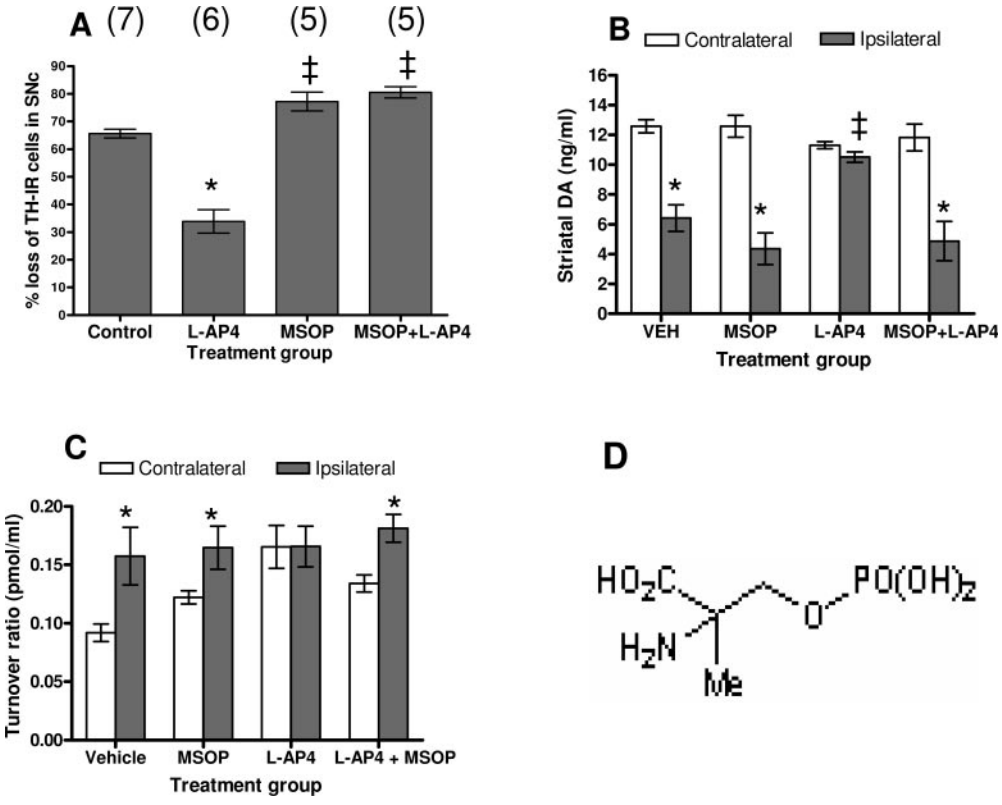

Fig. 5. A, coadministration of 50 nmol of MSOP with 10 nmol of L-AP4 abolishes the neuroprotection of nigral TH-IR cells by L-AP4 and enhances the lesion generated by 6-OHDA. MSOP alone enhances 6-OHDA-induced loss of nigral TH-IR. Data shown are mean percentage loss of TH-IR cells $\pm$ S.E.M. ‡, $P < 0.05$, mean percentage loss TH-IR in L-AP4 + MSOP or MSOP versus vehicle-treated; *, $P < 0.05$, L-AP4 versus vehicle-treated. B, coadministration of 50 nmol of MSOP with 10 nmol of L-AP4 similarly abolishes the prevention of dopamine depletion in the ipsilateral striatum by L-AP4. MSOP alone does not affect 6-OHDA-induced dopamine depletion. Data shown are mean striatal dopamine in nanograms per milliliter $\pm$ S.E.M. *, $P < 0.01$, contralateral versus ipsilateral striatum; ‡, $P < 0.01$, ipsilateral striatum of L-AP4 versus vehicle-treated. C, similarly, MSOP coadministered with L-AP4 abolishes the blockade of increases in striatal dopamine turnover produced by L-AP4 alone. Data shown are mean striatal turnover ratio $\pm$ S.E.M. *, $P < 0.05$, ipsilateral versus contralateral striatum. *n* values are shown in parentheses. D, chemical structure of MSOP.

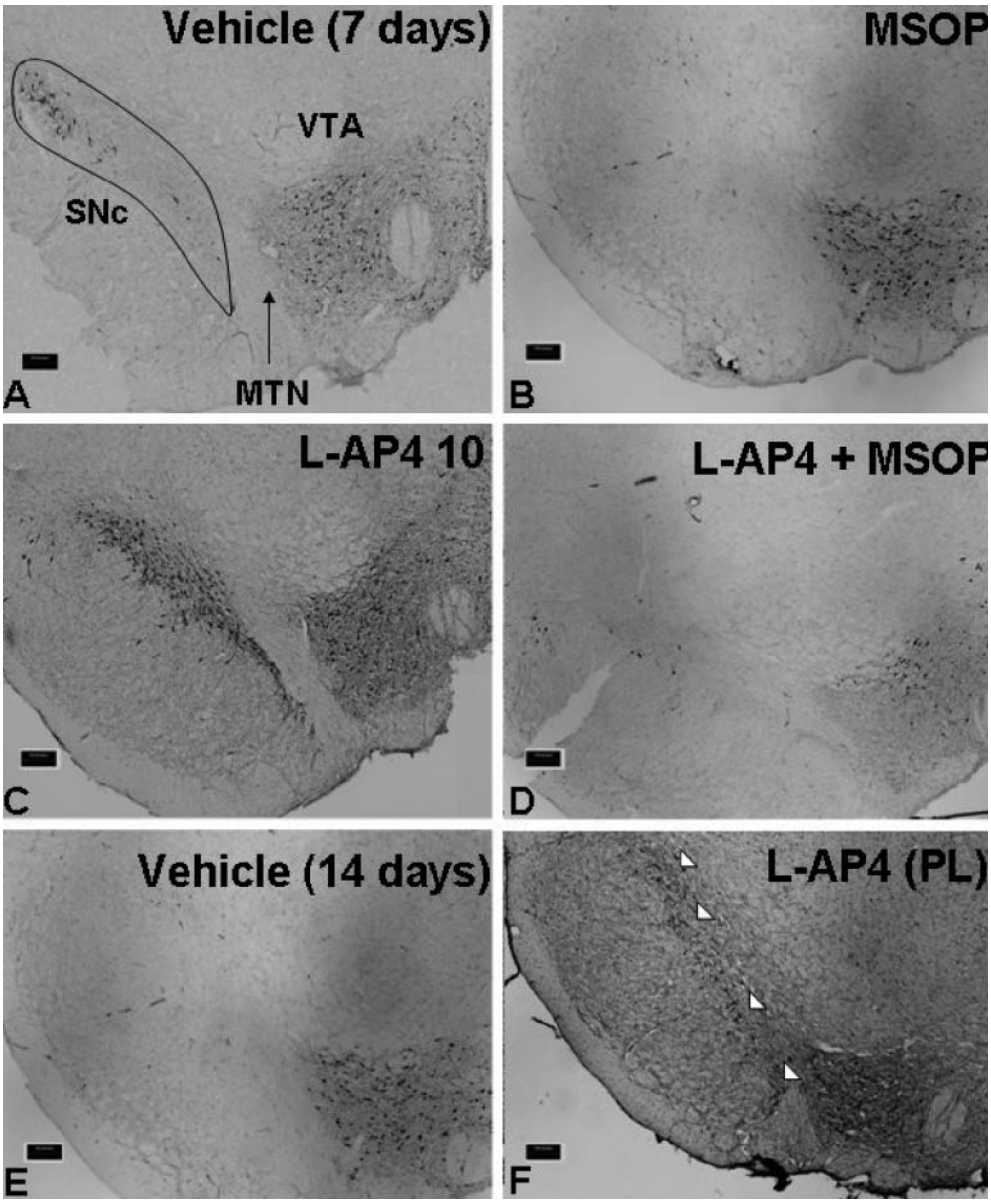

Fig. 6. Representative photomicrographs of nigral TH-IR cells from individual treatment groups (A–F) corresponding to level B of the SNc (−5.33 mm from bregma) at ×40 magnification. Scale bar, 250 μm. An example of a defined AOI for quantitative cell counting is illustrated in A. SNc, VTA, and MTN are highlighted in A. Arrowheads in F, surviving TH-IR neuron cell bodies in the SNc following postlesion (PL) treatment with L-AP4 (10 nmol in 4 μl).

(77.20 ± 1.55% versus 68.75 ± 1.95%; $P < 0.01$; Fig. 7A) and an enhanced depletion of striatal dopamine (2.079 ± 0.85 versus 6.42 ± 0.89 ng/ml; $P < 0.001$; Fig. 7C) and DOPAC and HVA ($P < 0.01$, Table 1). Furthermore, there is a very significant increase in striatal dopamine turnover in these animals, as shown in Fig. 7D. These data suggest the presence of a continued progressive lesion of the nigrostriatal system after the initial insult, consistent with previous observations in this model (Stanic et al., 2003). In animals treated subchronically with intranigral injections of 10 nmol/4 μl of L-AP4 7 days after 6-OHDA administration, the mean percentage loss of nigral TH-IR cells was found not to be significantly different from that observed in vehicle-treated animals, although a trend toward increased neuroprotection was observed (71.57 ± 9.10 versus 77.20 ± 1.55%; $P > 0.05$; Fig. 7A). Furthermore, in all regions of the SNc, the mean percentage loss of nigral TH-IR cells was not significantly different from vehicle-treated animals as shown in Fig. 7B. Importantly, the small sample size of this group must be considered when interpreting these data. Interestingly however, the mean percentage loss of nigral TH-IR in L-AP4-treated animals is not significantly different from that observed in animals sacrificed 7 days post-6-OHDA lesion, the time point at which drug or vehicle treatment started (71.57 ± 9.10% versus 65.62 ± 1.61%; $P > 0.05$; Fig. 7A). To illustrate, the representative photomicrographs are shown in Fig. 6, E and F. In contrast, the depletion of striatal dopamine in the ipsilateral striatum was significantly reduced in L-AP4-treated animals compared with vehicle-treated controls (8.067 ± 0.81 versus 2.079 ± 0.85 ng/ml; $P < 0.001$; Fig. 7C), and the depletion of dopamine in L-AP4-treated animals was not significantly different from that observed in vehicle-treated animals sacrificed 7 days post-6-OHDA-lesion (8.067 ± 0.81 versus 6.42 ± 0.89 ng/ml; $P > 0.05$; Fig. 7C). Similar effects were observed with the mean concentrations of DOPAC and HVA (Table 1). There was, however, a signif-

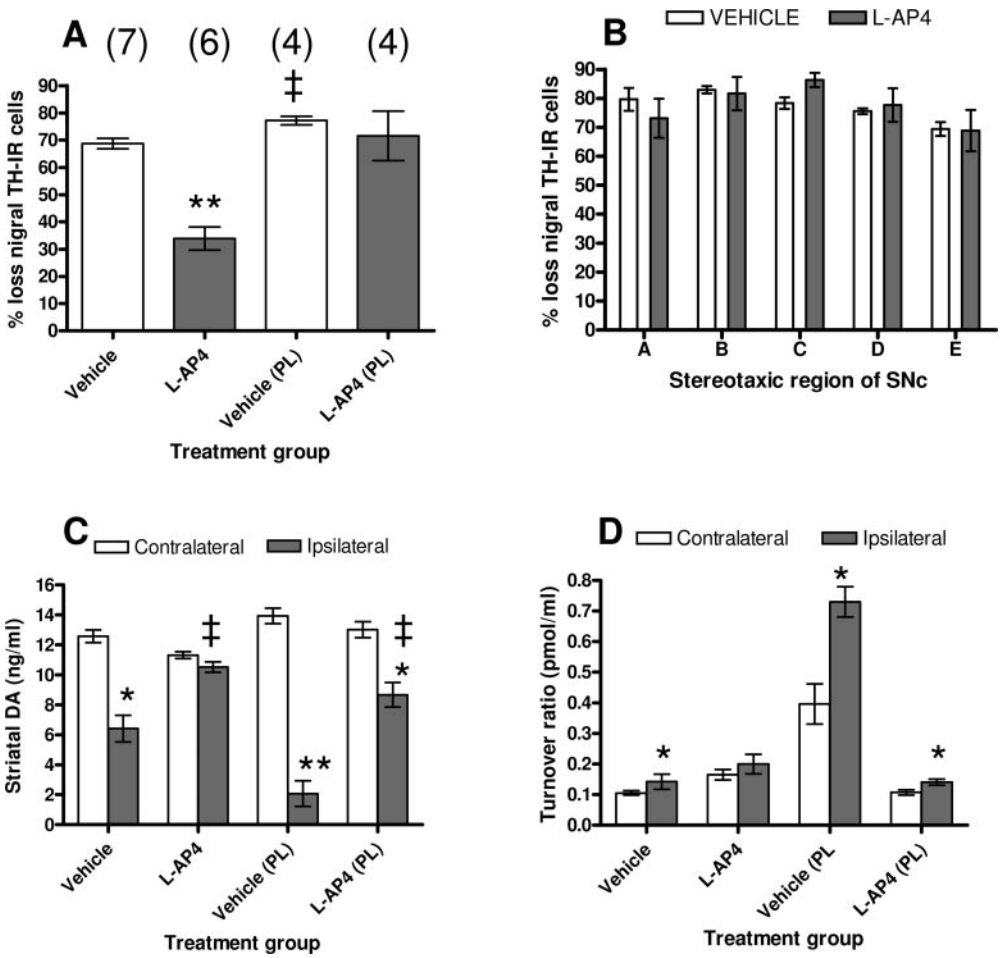

Fig. 7. A, subchronic treatment with 10 nmol in 4 μl of L-AP4 administered 7 days postinfusion of 6-OHDA into the SNc does not significantly protect nigral TH-IR cells against 6-OHDA toxicity. B, mean percentage loss of nigral TH-IR cells in L-AP4-treated animals is not significantly different from vehicle-treated animals in any stereotaxic region of the SNc. C, L-AP4 prevents the further depletion of dopamine, compared with vehicle-treated animals, which may in part be explained by an increase in striatal dopamine turnover (D). PL, postlesion treatment groups. A and B, data shown are mean percentage loss of nigral TH-IR cells ± S.E.M. **, $P < 0.01$, L-AP4 versus vehicle-treated; ‡, $P < 0.05$, 14- versus 7-day vehicle-treated. C, mean concentration of striatal dopamine in nanograms per milliliter ± S.E.M. *, $P < 0.05$; **, $P < 0.01$, contralateral versus ipsilateral striatum; ‡, $P < 0.05$, ipsilateral striatum of L-AP4 versus vehicle-treated. D, mean striatal dopamine turnover ratio ± S.E.M. *, $P < 0.05$, contralateral versus ipsilateral striatum. *n* values are shown in parentheses.

icant increase in striatal dopamine turnover in the L-AP4-treated animals as shown in Fig. 7D, although this was not as marked as observed in vehicle-treated animals.

Discussion

In this study, a rodent 6-OHDA model of PD was employed to investigate the potential neuroprotective effects of selective activation of Group III mGluR by the Group III mGluR agonist L-AP4. Consistent with our previous preliminary observations, we report that intranigral administration of L-AP4 is significantly neuroprotective against 6-OHDA toxicity in vivo in a clear concentration-dependent manner. Furthermore, this neuroprotective effect is observed following either acute or subchronic treatment and is reversed by coapplication of the selective Group III mGluR antagonist MSOP, indicating a Group III mGluR-mediated mechanism of action.

These data are consistent with previous reports of a neuroprotective action of L-AP4 against glutamate-mediated toxicity both in vitro and in vivo (Lafon-Cazal et al., 1999a; Bruno et al., 2000) and very recent data reported by Jiang et al. (2006), who demonstrated that L-AP4 is neuroprotective against rotenone toxicity in vitro, providing evidence for a neuroprotective effect of selective Group III mGluR activation in an experimental model of PD. To our knowledge, the current study is the first to document such an action for L-AP4 in an experimental model of PD in vivo. Theoretically, the decrease in nigral TH-IR cells and the neuroprotective effects of L-AP4 could be due to transient atrophy of cells and down-regulation of TH expression rather than true cell death and neuroprotection. We have addressed this possibility by staining sections adjacent to those stained for TH from vehicle-treated lesioned animals for the specific neuronal marker NeuN. From these sections, we observed that infusion of 6-OHDA into the SNc produces a similar reduction of NeuN-IR cells compared with TH-IR cells (65.62 ± 1.61% versus 73.31 ± 4.63%). Thus, the observed loss of TH-IR cells can be attributed to cell death and not simple down-regulation of TH in atrophic neurones. Accordingly, the observed neuroprotective effects of L-AP4 cannot be explained by TH up-regulation. Importantly, L-AP4 treatment also attenuated the 6-OHDA-induced depletion of striatal dopamine and its metabolites, which indicates preservation of axonal projections. The observation that L-AP4 also blocks 6-OHDA-induced increases in striatal dopamine turnover suggests that the prevention of dopamine depletion by L-AP4 is due to neuroprotection of dopamine terminals rather than increased dopamine metabolism by the surviving nigral TH-IR cells. Furthermore, because of the short time between 6-OHDA lesioning and HPLC measurements (7 days post-6-OHDA lesion), it is unlikely that the preservation of striatal dopamine could be explained by sprouting of the remaining axonal projections, which has been shown to occur 4 weeks after 6-OHDA lesioning (Stanic et al., 2003). Taken together, these data suggest a true neuroprotective effect of L-AP4 in vivo.

The neuroprotective action of L-AP4 shows a bell-shaped

profile with an absence of neuroprotection at higher concentrations. Interestingly, similar findings have been reported in vitro against *N*-methyl-D-aspartate toxicity in striatal GABAergic neuronal cultures (Lafon-Cazal et al., 1999b). Indeed, high concentrations of L-AP4 enhanced *N*-methyl-D-aspartate toxicity, which was suggested to be due to inhibition of GABA release by activation of presynaptic mGluR7 removing the hyperpolarizing influence of GABA on striatal neurones, thus sensitizing them to excitotoxicity (Lafon-Cazal et al., 1999b). It is noteworthy that a similar role for presynaptic group III mGluR as negative modulators of GABA release in both the SNc (Giustizieri et al., 2005) and SNr (Wittmann et al., 2001, 2002) has been reported, consistent with data reporting localization of mGluR7 receptor protein at symmetric (inhibitory) synapses in the SNr (Kosinski et al., 1999). Moreover, synaptically released GABA in the both the SNc and the SNr is implicated in the control of dopaminergic cell firing (Wittmann et al., 2001, 2002; Giustizieri et al., 2005). Thus, it is conceivable that when high concentrations of L-AP4 are administered intranigrally, GABA transmission is inhibited by activation of presynaptic mGluR7 on SNr GABAergic neurones forming axo-axonic synapses on glutamatergic STN axon terminals, thereby enhancing glutamatergic transmission onto nigral neurones. This is anatomically feasible because the SNc sends dendrites deep into the SNr (Iribe et al., 1999), and STN axon terminals are known to terminate on dopaminergic dendrites in the SNr (Smith et al., 1990). However, responses mediated by Group III mGluR are sensitive to dopamine depletion, which seems to reduce the ability of L-AP4 to inhibit GABA release in vitro (Wittmann et al., 2002). Importantly, inhibition of excitatory transmission in the SNr by L-AP4 was not affected by dopamine depletion (Wittmann et al., 2002). Therefore, in the Parkinsonian brain, inhibition of GABA release by activation of Group III mGluR on GABAergic nigral neurones may be reduced by dopamine depletion. Importantly, the extent of the lesion seems to critically determine the function of the mGluR subtypes involved. In the current study, the level of dopamine depletion induced by 6-OHDA (<70%) may not sufficiently affect mGluR7-mediated inhibition of GABA release by high concentrations of L-AP4; therefore, perturbation of GABAergic inputs to nigral dopaminergic neurones may still explain the absence of neuroprotection. Interestingly, 6-OHDA toxicity is not enhanced by high concentrations of L-AP4, as observed in vitro (Lafon-Cazal et al., 1999b). Speculatively, this could be attributed to the Group III mGluR-mediated reduction of glutamate release balancing out reductions in GABA transmission.

Postlesion treatment with L-AP4 did not significantly protect nigral TH-IR cells, although a trend toward increased protection was observed. This may be explained in terms of the time course of DA neuronal death in the 6-OHDA model. Evidence suggests this occurs in two phases, an initial rapid phase of cell death, followed by a progressive loss of the remaining neurones over time (Stanic et al., 2003). Thus, 7 days post-6-OHDA lesion may be too late to provide significant protection of nigral TH-IR cells because the majority of cell death may have already occurred. Interestingly, postlesion treatment with L-AP4 significantly prevents further depletion of dopamine in the ipsilateral striatum, which is difficult to reconcile with the absence of protection of nigral TH-IR cells. Thus, it cannot be definitively confirmed that L-AP4 is neuroprotective when administered postlesion, although perhaps if L-AP4 treatment were started earlier, significant neuroprotection may be observed. However, these data are consistent with a previous study in the same model in which the selective Group II mGluR agonist LY379268 was reported to provide a small, but not significant, protection of nigral TH-IR cells in animals undergoing nigrostriatal degeneration, which was associated with reduced but significantly increased dopamine turnover (Murray et al., 2002).

Importantly, the neuroprotection mediated by L-AP4 is completely reversed by coadministration with the selective Group III mGluR antagonist MSOP, which indicates that this neuroprotection is mediated through a Group III mGluR-dependent mechanism, further supporting a critical role of this class of mGluR. However, since L-AP4 is a broad-spectrum agonist with activity at all Group III mGluR subtypes (Schoepp et al., 1999), we cannot definitively state the Group III mGluR subtypes through which L-AP4 exerts this effect. Based on the high potency with which L-AP4 inhibits excitatory transmission in the SNc in vitro, mGluR4 and mGluR8 are likely candidates (Valenti et al., 2005). Recent studies in vitro suggest that mGluR4 but not mGluR8 mediates this effect (Valenti et al., 2005). This is interesting when considering the potential mechanisms of neuroprotection by L-AP4. Neuroprotection following activation of Group III mGluR is suggested to result from presynaptic inhibition of glutamate release (Valenti et al., 2003). In support of this, L-AP4 has been reported to inhibit evoked EPSCs at synapses onto midbrain dopaminergic neurones, with a clear presynaptic mechanism of action (Wigmore and Lacey, 1998; Katayama et al., 2003). However, previous studies have also reported a significant contribution of glial cells to neuroprotection induced by mGluR agonists (Bruno et al., 1998). Both astroglia and microglia are known to express functional Group III mGluR; thus, speculatively, activation of either mGluR4 or 8 expressed on these cell types may contribute to neuroprotection by L-AP4 in our model in which significant gliosis occurs in response to 6-OHDA-induced cell death. In support of this concept, activation of astroglial and microglial Group III mGluR has been shown recently to be neuroprotective in vitro (Taylor et al., 2003; Yao et al., 2005). Thus, it will be important in future studies to define the exact subtype of Group III mGluR that mediates neuroprotection by L-AP4 in vivo. Interestingly, evidence from in vitro studies suggests that positive allosteric modulators selective for mGluR4 are also neuroprotective in vitro (Maj et al., 2003). This is interesting since the use of direct mGluR agonists like L-AP4 as a chronic treatment for PD is likely to be limited by adverse effects associated with excessive activation of the receptor, including receptor desensitization and loss of the neuronal activity dependence of receptor activation by pulsatile release of neurotransmitter (Conn, 2003). In conclusion, the findings presented herein, together with complementary data published during the course of the present study suggesting that the mGluR4-selective positive allosteric modulator PHCCC is neuroprotective against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced nigrostriatal damage in mice (Battaglia et al., 2006), provide compelling evidence that selective activation of Group III mGluR is neuroprotective in vivo in experimental models of parkinsonism, which may have implications for the future treatment of PD.

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Address correspondence to: Dr. Martin J. Croucher, Faculty of Medicine, Sir Alexander Fleming Building, Imperial College London, London SW7 2AZ, UK. E-mail: m.croucher@imperial.ac.uk