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Chronic L-DOPA treatment increases extracellular glutamate levels and GLT1 expression in the basal ganglia in a rat model of Parkinson's disease

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Abstract

There is growing experimental evidence for the implication of glutamate-mediated mechanisms both in the pathophysiology of Parkinson's disease and in the development of dyskinesias with long-term administration of L-3,4-dihydroxyphenylalanine (L-DOPA). However, the impact of this treatment on glutamate transmission in the basal ganglia has been poorly investigated. In this study, we examined the effects of 6-hydroxydopamine-induced lesion of nigral dopamine neurons with or without subsequent chronic L-DOPA treatment on several parameters of glutamate system function in the rat striatum and substantia nigra pars reticulata. All the lesioned animals treated with L-DOPA developed severe dyskinesias. Extracellular glutamate levels, measured by microdialysis in freely moving conditions, and gene expression of the glial glutamate transporter GLT1, assessed by *in situ* hybridization, were unaffected by dopamine lesion or L-DOPA treatment alone, but were both markedly increased on the lesion side of rats with subsequent L-DOPA treatment. No change in the expression of the vesicular glutamate transporters vGluT1 and vGluT2 was measured in striatum. These data show that chronic L-DOPA treatment leading to dyskinesias increases basal levels of glutamate function in basal ganglia. The L-DOPA-induced overexpression of GLT1 may represent a compensatory mechanism involving astrocytes to limit glutamate overactivity and subsequent toxic processes.

Introduction

It is long recognized that the loss of nigral dopamine neurons induces an abnormal activation of glutamate systems in the basal ganglia that is central to the pathophysiology of Parkinson's disease (PD; see for review Carlsson & Carlsson, 1990; Schmidt, 1998). Glutamate-mediated mechanisms are also thought to play a role in the development of dyskinesias with long-term administration of L-3,4-dihydroxyphenylalanine (L-DOPA), the most efficient treatment for PD. For instance, administration of *N*-methyl-D-aspartate (NMDA) or (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor antagonists alleviates L-DOPA-induced side-effects in animal models of PD (Papa & Chase, 1996; Marin *et al*., 2001). As cellular substrates, dyskinesias have been associated with changes at glutamate receptor level, such as a further increase in the phosphorylation of striatal NMDA receptor subunits (see Chase *et al*., 2000) in lesioned rats and upregulation of AMPA and NMDA receptors binding in MPTP monkeys (Calon *et al*., 2002). Moreover, consistent with their antidyskinetic potential, glutamate antagonists attenuate the L-DOPA-triggered further upregulation of striatal preproenkephalin (Perier *et al*., 2002) that has been associated, together with dynorphin overexpression, with the appearance of dyskinesias (Cenci *et al*., 1998; Henry *et al*., 1999; Calon *et al*., 2000; Calon & Di Paolo, 2002).

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There is converging evidence that dopamine denervation induces an increase in corticostriatal glutamate transmission (Lindefors & Ungerstedt, 1990; Calabresi *et al*., 1993; Ingham *et al*., 1998; Meshul *et al*., 1999). Microdialysis studies have suggested that dopamine lesion may also increase glutamate transmission in the basal ganglia output structures, substantia nigra pars reticulata (SNr; Abarca *et al*., 1995) and entopeduncular nucleus (EP; Biggs & Starr, 1997), presumably as a result of the abnormal activation of the subthalamic nucleus (see Hirsch *et al*., 2000). Paradoxically, the impact of chronic L-DOPA treatment inducing dyskinesias on glutamate transmission within these structures remains poorly understood. Available data from the striatum have shown increased sensitivity of NMDA receptor (Chase *et al*., 2000), selective increase in the expression of the glial glutamate transporter GLT1 (Lievens *et al*., 2001) that is responsible for the bulk of extracellular glutamate clearance and changes in the plasticity of corticostriatal synapses (Picconi *et al*., 2003). Whether or not these changes involving striatal neurons and glial cells result from changes in extracellular glutamate and are associated with modifications of presynaptic markers of glutamate synapses remains to be determined. Moreover, the involvement of glutamate systems in the L-DOPA-induced dyskinesias cannot be fully understood unless attention is provided to the changes in glutamate transmission in the output structures of the basal ganglia.

This study aimed at determining the effects of chronic L-DOPA administration inducing dyskinesias in rats with extensive lesion of nigral dopamine neurons on various parameters of glutamatergic transmission within basal ganglia. The parameters examined were:

(1) glutamate extracellular levels by dual-probe microdialysis in striatum and SNr in awake rats; (2) GLT1 mRNA expression by *in situ* hybridization in SNr and EP, and protein expression by immunoblotting and immunoautoradiography in SNr; and (3) striatal expression of the vesicular glutamate transporters vGluT1 and vGluT2 as presynaptic markers of glutamate afferents by immunoautoradiography.

Materials and methods

The animal experimental protocols performed in this study strictly conformed to the guidelines of the French agriculture and forestry ministry (decree 87-848) and were approved by the CNRS. Three separate series of experiments were conducted on male Wistar rats (250–280 g at the time of surgery; from Iffa Credo, L'Arbresle, France): series 1 for microdialysis; series 2 for *in situ* hybridization and vGluT immunoautoradiography; and series 3 for GLT1 immunoblotting. Each series comprised saline-injected controls (control), animals with L-DOPA treatment alone (L-DOPA) and animals with the dopamine lesion subsequently injected with saline [6-hydroxydopamine (6-OHDA) lesion] or with L-DOPA (6-OHDA lesion + L-DOPA). Controls of the dopamine lesion extent were based for the two first series on examination of [^{3}H]-mazindol binding to dopamine uptake sites and for the last one on high-pressure liquid chromatography (HPLC) measurement of tissular dopamine contents in the striatum, as described below. Analysis has been restricted to the dorsal striatum (corresponding to the putamen), i.e. excluding the more ventral part of the structure and nucleus accumbens. Only the animals showing extensive denervation using these criteria were processed for further morphological or biochemical studies. Further selection of the animals included for analysis of the microdialysis data was based on correct placement of the probes in both striatum and SNr, as assessed on Cresyl violet-stained sections. Series 1 (microdialysis) included $n = 8$ control animals, four treated with L-DOPA, eight with the 6-OHDA lesion and six with the 6-OHDA lesion + L-DOPA; series 2 (morphological and behavioural studies) comprised five to six animals in the control, 6-OHDA lesion and 6-OHDA lesion + L-DOPA groups and three–five animals in the L-DOPA group; and series 3 (immunoblotting) comprised four animals per condition.

Surgical procedures and L-DOPA treatment

Animals were anaesthetized with xylasine (15 mg/kg i.m.)/ketamine (50 mg/kg i.m.) and secured in a Kopf stereotaxic apparatus. Unilateral lesion of the dopamine neurons was performed by intranigral injection of 6-OHDA (Sigma, St. Quentin-Fallavier, France; 12 μg in 6 μL of 0.9% NaCl containing 1% ascorbic acid) into the right substantia nigra pars compacta (SNc) at the following coordinates from bregma: AP: −5.4 mm; L: −2.2 mm; H: −7.6 mm (from dura), according to the stereotaxic atlas of Paxinos & Watson (1986).

In the same surgical session, animals for microdialysis were stereotaxically implanted on the dopamine lesion side with two cannula guides CMA/11 (Carnegie Medicine, Stockholm, Sweden), one placed above the striatum (AP: +0.2 mm and L: +2.8 mm from bregma; DV: −3.3 mm from dura) and the other above the SNr (AP: −5.4 mm and L: −2.2 mm from bregma; DV: −7.4 mm from dura), according to the stereotaxic atlas of Paxinos & Watson (1986).

After a recovery period of 14 days, subsets of 6-OHDA-injected and control animals underwent i.p. injections of either saline or L-DOPA (100 mg/kg) plus benserazide (15 mg/kg) twice daily for 21 days. Twelve hours after the last injection, animals were either killed by decapitation (series 2 and 3) or processed for microdialysis (series 1). All studies were then conducted at 5 weeks post-lesion.

Behavioural observation

Qualitative examination showed that 100% of the 6-OHDA-lesioned rats had become dyskinetic by the end of the L-DOPA treatment. The quantification of abnormal involuntary movements (AIM) was performed on the animals of series 2 using the rating scale defined by Cenci *et al*. (1998), which scores four subtypes of AIMs, i.e. locomotive, axial, orolingual and forelimb dyskinesias, each on a scale from 0 to 4 (maximum score per monitoring period: 16). Individual animals were scored every 10 min, from 10 to 120 min after the injection of L-DOPA (12 monitoring periods of 1 min each). The maximum attainable AIM score over the assessment period after L-DOPA injection was 192. Four time points during the chronic treatment were examined: days 1 (corresponding to the first injection), 7, 14 and 21 (last injection).

Biochemical studies

In vivo *microdialysis and measurement of extracellular glutamate levels*

Dual-probe microdialysis (striatum and SNr in the same animal) was performed in freely moving animals. The objective was twofold: compare the basal levels of glutamate between experimental groups of animals and examine the acute effect of L-DOPA administration during the dialysis session. The microdialysis probes (CMA/11, Carnegie Medicine; length of the microdialysis membrane, 3 mm for the striatum and 1 mm for the SNr; molecular weight cut-off, 20 kDa; outer diameter, 0.5 mm) were lowered through the guide cannula so that the tip of the dialysing membrane reached DV: −6.3 mm for the striatum and DV: −8.4 mm (from dura) for the SNr. Probes were perfused with Ringer's solution (in mM: NaCl, 147; $CaCl_2$, 2.5; KCl, 4) at a constant flow rate (1 μL/min) using a CMA/102 microdialysis pump (Carnegie Medicine). The dialysates were collected every 20 min in tubes containing 10 μM ascorbic acid and 5 μM homoserine (standard for amino acid analysis), and were immediately frozen at −80 °C until analysis. Sample collection started 120 min after the onset of perfusion to achieve stable levels of amino acids, and six samples were collected for 120 min as basal values before the injection of either saline (control and 6-OHDA lesion groups) or L-DOPA at the dose used for the prior chronic treatment (L-DOPA and 6-OHDA lesion + L-DOPA groups). Sampling was then carried out for an additional period of 120 min (six samples). The determination of probe recovery (± 15%) was performed *in vitro* by perfusing standard solutions (37 °C) containing 10 μM amino acids before and after the dialysis session.

Measurement of glutamate levels in each collected sample was performed using HPLC coupled with a fluorimetric detection system (Waters, St. Quentin, France), including a precolumn derivatization with *o*-phthaldialdehyde/mercaptoethanol reagent and a C-18 (ODS2, 4.6 × 150 mm) Spherisorb column. A non-linear gradient delivered through a Waters 600 pump was used to separate derivatives (solvent A: 0.1 M potassium acetate with 25% methanol, pH 5.5; solvent B: 0.05 M potassium acetate and 60% methanol, pH 5.5). Samples (15 μL) were automatically injected (Waters 717 plus autosampler) and analysed using a Waters 474 detector. The limit of detection was 1.5 pmol/sample. Data were computed with WATERS MILLENIUM software (via a Waters bus SAT/IN module), and compound identification and peak quantification were achieved by comparison

with standard amino acid solution. If required, correction of the glutamate concentration was made with reference to the homoserine standard used to control for equal dialysate volume collection and injection.

The dialysate concentrations of glutamate were analysed statistically for the basal period using a two-way ANOVA with group as between-subjects factor and time as within-subject factor. No time effect being found during the period, the average of glutamate concentration per experimental group was then determined for each animal from the six samples. Data are means ± SEM of the values obtained for the *n* animals of each experimental group and are expressed as percentage of the corresponding mean control value. Statistical comparisons between groups were performed using a one-way ANOVA followed by Newman–Keuls test. Analysis of the effect of the acute L-DOPA or saline injection during the dialysis session was performed within each experimental group, separately, using repeated-measures ANOVA with two within-subject factors, time and treatment (basal/post-L-DOPA injection periods).

Measurement of tissular dopamine levels

Striatal tissue was homogenized in 0.1 M perchloric acid and supernatant kept for dopamine measurement by HPLC coupled with electrochemical detection, as described previously (Di Cara *et al*., 2001). In brief, the mobile phase consisted of 0.1 M sodium acetate, 0.17 mM octyl sulphate, 8% methanol, 0.7 mM EDTA, pH 4.5, and was delivered through a LC-10ADvp Shimadzu pump (Kyoto, Japan) into a C-18 (ODS2, 4.6 × 150 mm) Spherisorb column (Waters, Milford, MA, USA). Samples of 20 μL were injected and analysed using a Coulochem II, ESA detector (Chelmsford, MA, USA). The potential of the reference electrode was set at −50 mV and the working electrode at +280 mV. The limit of detection was 20 fmol/sample. Data were computed with WATERS MILLENIUM software (via a Waters bus SAT/IN module) running onto a PC system; compound identification and peak quantification were achieved by comparison to standard solution.

GLT1 Western blotting

Tissue samples were homogenized in a lysis buffer (Tris 50 mM, pH 7.5; EDTA 1 mM; Triton X-100 0.1%) with protease inhibitors (complete tablets, Roche, Meylan, France), sonicated and centrifuged for 15 min at 15 000 ***g*** at 4 °C. The supernatants were collected and their protein concentrations determined by the Bradford assay using bovine serum albumin as standard (Bradford, 1976). Laemmli buffer [Tris–HCl 1 M, pH 6.8; 2% sodium dodecyl sulphate (SDS); 20% glycerol; 5% beta mercaptoethanol; 0.05% bromophenol blue] was added to each sample (1/5, v/v). Samples of total protein (5 μg protein/lane) were electrophoresed through a stacking gel (10% SDS–polyacrylamide gel) and then transferred to nitrocellulose membrane (Protran BA 83, Schleicher and Shuell, Ecquevilly, France) with a semidry apparatus. Membranes were immersed for 1 h in a blocking buffer (5% non-fat dry milk; 0.1% Tween-20; 50 mM Tris saline buffer, TBS) and probed with rabbit anti-GLT1 polyclonal antibodies at 1 : 3000 (affinity-purified antibodies against a synthetic peptide corresponding to amino acid residues 562–573 of GLT1; kindly provided by G. Pietrini, Italy), and anti-actin (1 : 250, Sigma) antibodies diluted in 5% milk/TBS overnight at 4 °C. After washing, the blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1 : 10 000 in TBS milk 5% for 1 h. After three additional washes in TBS, the peroxidase signal was detected with an enzyme chemiluminescent kit Uptilight from Pierce (Interchim, Montluçon, France) and radio-films (Fuji, Clichy, France). Films were then digitized and quantified with the Scion image software (Frederick, Maryland, USA). Actin was used to control for equal protein loading. Only the OD of the immunoreactive bands at weight corresponding to GLT1 monomer (approximately 70 kDa) were quantified and corrected by actin. For each animal, the corrected OD values obtained in three separate experiments were averaged. Data are means ± SEM of the values obtained in the *n* animals and expressed as percentage of controls.

Morphological studies

Tissue preparation

The brains were quickly removed, frozen on dry ice and kept at −80 °C until cryostat sectioning. Frontal sections (10 μm) were cut at −20 °C at the level of striatum (AP: 10.2–8.6 mm), EP (AP: 6.7–5.7 mm) and SNr (AP: 4.2–3.2 mm), based on interaural coordinates from the stereotaxic atlas of Paxinos & Watson (1986), and thaw-mounted onto SuperFrost plus glass slides (Fisher Scientific, Elancourt, France). Tissue sections were stored at −80 °C until specific treatment. Adjacent sections were processed for GLT1 *in situ* hybridization histochemistry and vGluT immunoautoradiography. For each labelling, sections from the various experimental groups (at least three sections per animal) were run together in the same experimental session and exposed side by side on the same autoradiographic films.

[^{3}H]-Mazindol binding

The extent of the 6-OHDA-induced dopamine denervation was examined at striatal level by autoradiographic labelling of dopamine uptake sites using [^{3}H]-mazindol as a ligand (Javitch *et al*., 1985). In brief, after being air-dried and rinsed, striatal sections were incubated for 40 min at 4 °C with 15 nM of [^{3}H]-mazindol (NEN Life Science Products, Boston, MA, USA; specific activity: 17 Ci/mmole) in 50 mM Tris buffer containing 300 mM NaCl, 5 mM KCl and added with 0.3 μM of desipramine to avoid binding on noradrenalin transporters. After two rinses in the incubation buffer and one in cold distilled water, sections were rapidly dehydrated and exposed for 2 weeks to [^{3}H]-sensitive Hyperfilm (Amersham Pharmacia, Orsay, France).

In situ *hybridization*

For GLT1, a synthetic radiolabelled DNA probe of 45 bases (5′-GGA CTGCGTCTTGGTCATTTCGATGTCTTCGTGCATTCGGTGTTG-3′) selected on the basis of the published sequence (Pines *et al*., 1992) was used. This probe was 3′-end-labelled by terminal deoxynucleotide transferase with [^{35}S]-dATP (> 1300 Ci/mmol; NEN Life Science Products). The probe was purified from enzyme and unincorporated nucleotides on Sephadex mini-spin columns (Roche).

Slide-mounted sections were postfixed in 3% paraformaldehyde for 5 min. Sections were sequentially rinsed twice in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 5 min and once in 2 × standard saline citrate (SSC; 1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate) for 10 min, incubated for 1 h in prehybridization buffer (2 × SSC and 1 × Denhardt's solution) and then for 10 min in 2 × SSC. Sections were acetylated (0.25% acetic anhydride in 0.1 M triethanolamine) for 10 min, dehydrated and delipided in graded series of ethanol solutions and chloroform. Sections were then covered with 35 μL of hybridization mixture containing the [^{35}S]-radiolabelled oligonucleotide probe (400 000 cpm/35 μL/section), 50% formamide, 1 × Denhardt's solution, 0.25 mg/mL yeast tRNA, 0.5 mg/mL

sheared salmon sperm DNA and 20 mM dithiothreitol, 10% dextran sulphate in 2 × SSC for 12 h at 42 °C in humid chambers. Sections were then successively washed in 1 × SSC at room temperature (10 + 15 + 45 min), in 1 × SSC at 45–50 °C (15 + 30 min) and in 0.1 × SSC at 45–50 °C (15 + 30 min). After a rapid dehydration in ethanol 95% and 100%, sections were apposed to Kodak Biomax MR1 (Eastman Kodak, Rochester, NY, USA) film during 5 days before developing and fixing. Sections were thereafter coated with Amersham LM1 autoradiographic emulsion and exposed at 4 °C for 1 month for GLT1 mRNA detection at cellular level. Exposed slides were developed in Kodak D-19 for 4 min at 13 °C and counterstained with toluidine blue.

Immunoautoradiography

The proteins vGluT1 and vGluT2 were detected by a radioimmunohistochemical method using [^{125}I]-radiolabelled secondary antibodies, as previously described (Herzog *et al*., 2001). The same method was used for GLT1 protein immunostaining. In brief, sections were fixed with 4% paraformaldehyde for 15 min and rinsed in 0.1 M PBS (pH 7.4) for 5 min. After preincubation in 0.1 M PBS solution containing 3% bovine serum albumin, 1% goat normal serum and 2 mM NaI for 1 h, sections were incubated overnight in the primary antibody diluted 1 : 10 000 for anti-vGluT1 or anti-vGluT2 [polyclonal rabbit antibodies kindly provided by S. El Mestikawy, France, production, characteristics and specificity have been extensively described by Herzog *et al*. (2001)] and 1 : 15 000 for anti-GLT1 (affinity-purified polyclonal rabbit antibodies against a synthetic peptide corresponding to amino acid residues 562–573 of GLT1; kindly provided by G. Pietrini, Italy). Sections were then incubated with [^{125}I]-labelled goat anti-rabbit IgG (0,25 μCi/mL, Amersham Pharmacia Biotech) for 2 h, rinsed three times in PBS and apposed to X-ray films (Kodak Biomax MS) for 5 days.

Data analysis and statistics

For *in situ* hybridization, sections were observed with an immersion 20 × objective of a microscope connected to a CCD camera (COHU, San Diego, USA) and the digitized images were transferred to the screen of a video monitor with a resulting magnification of 1000 ×. The GLT1 mRNA-expressing cells were first visualized using bright-field microscopy and quantification of labelling was then performed under dark-field epilumination, as illustrated in Fig. 1. Using the Visioscan image analysis system (BIOCOM, Les Ulis, France), the number of silver grains per cell was estimated by measuring OD with respect to a standard curve of a defined number of silver grains. A random sample of 30–50 labelled cells (showing at least 10 silver grains) per section and per brain side was quantified in three sections from each animal, and the mean number of silver grains per cell was determined.

For immunoautoradiography and [^{3}H]-mazindol binding, labelling was quantified by digitized image analysis from film autoradiograms using the Densirag software from BIOCOM, as reported previously (Salin *et al*., 2002). Grey levels were converted to OD using external standards (calibrated density step tablet; Kodak). The mean OD value was determined from three sections per animal after subtracting the background signal measured on each section by scanning the corpus callosum.

The data from the *n* animals per condition were then averaged and expressed as means ± SEM. Results are presented as percentage of the corresponding mean control value.

Statistical comparisons were performed for each anatomical region using a one-way ANOVA followed by Newman–Keuls test for multiple group comparison. A significance of $P < 0.05$ was required for rejection of the null hypothesis.

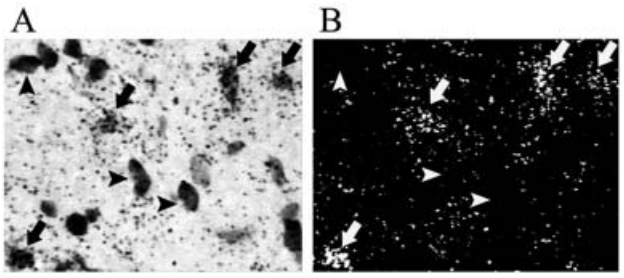

FIG. 1. Bright-field (A) and darkfield (B) photomicrographs of the same microscopic field illustrating GLT1 mRNA labelling in toluidine blue-counterstained sections of intact SNr. Arrows point at the GLT1-positive cells. Note that the cell bodies clearly identified as neurons (examples shown by arrowheads) do not show any hybridization signal.

Results

Behavioural observations

Animals with the lesion of nigral dopamine neurons had an ipsilateral bias in the head position, and some of them showed a weak tendency to ipsilateral rotation. All the 6-OHDA-lesioned rats became dyskinetic by the end of the L-DOPA treatment. Quantification of the AIM was done using the rating scale defined by Cenci *et al*. (1998). The time course examination of AIM rating/monitoring period after L-DOPA injection at days 1, 7, 14 and 21 of treatment is illustrated in Fig. 2A. To gain an overall picture of dyskinesias development, we examined the evolution of the total AIM score at each time point of treatment (Fig. 2B). A gradual increase of the scores was measured over time, the increase being more marked between day 1 and day 7 of treatment than thereafter. Repeated-measures ANOVA showed significant differences between testing sessions ($P = 0.0007$), and *post-hoc* comparison showed significant differences between the first and all the following injections. To characterize the interference of rotational behaviour with the dyskinetic behaviour, the locomotive AIM score was separately analysed. This score increased during the first week (day 1 : 11 ± 0.47; day 7 : 22.25 ± 2.22) and remained stable over the second and third week (day 14 : 23.25 ± 2.95; day 21 : 23 ± 3.36). Interestingly, expression of the contribution of locomotive AIM to total AIM as percentage reveals a decline over the treatment period (32.59% at the first injection, 26.25% on day 7, 21.42% on day 14 and 20.22% at day 21), which stresses the development of dyskinesias.

Controls of the microdialysis probe location and the dopamine lesion extent

Figure 3A and B illustrates the correct location of the microdialysis probes in the striatum and SNr, respectively, in the selected animals. No major tissue damage was observed in both structures. The animals with or without chronic L-DOPA treatment that received a unilateral injection of 6-OHDA showed an almost complete loss of [^{3}H]-mazindol binding in the ipsilateral striatum (Fig. 3, C1 and C2; Table 1). In series 3, striatal dopamine tissue levels in the dorsal striatum were dramatically reduced vs. controls after the lesion of nigral dopamine neurons with or without subsequent L-DOPA treatment (Table 1). No significant change in striatal [^{3}H]-mazindol binding or in striatal dopamine tissue levels was found in the side

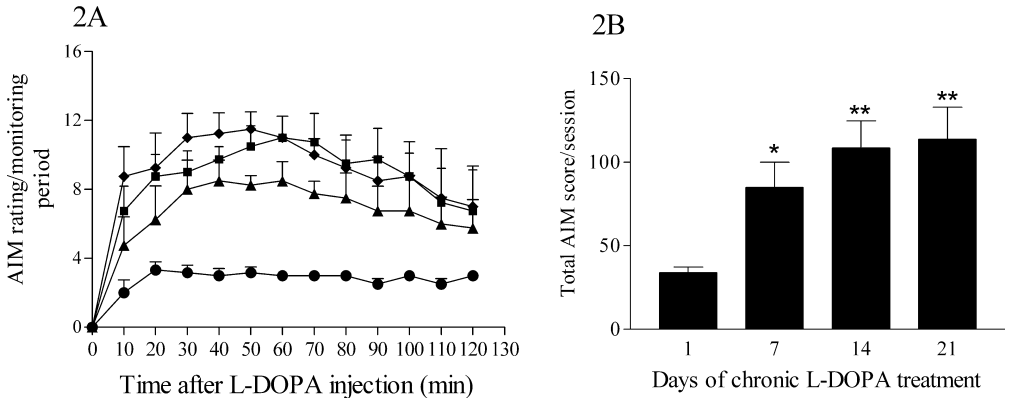

FIG. 2. Abnormal involuntary movements (AIM) development during the chronic L-3,4-dihydroxyphenylalanine (L-DOPA) treatment. AIM scores are expressed as the sum of the four AIM subtypes, each rated from 0 to 4. AIM scores were determined after injection of L-DOPA at different time points of the treatment: days 1, 7, 14 and 21. (A) Time course of AIM scores/monitoring period after a single injection of L-DOPA at the different time points of treatment (maximum attainable score per monitoring period: 16). (●) day 1; (▲) day 7; (■) day 14 and (◆) day 21 (B) Histograms representing the total AIM score per treatment time point (sum of the scores determined over the 12 monitoring periods after L-DOPA injection; maximum attainable score: 192). Statistical comparisons are performed using a repeated-measure ANOVA followed by Newman–Keuls test. $*P < 0.05$, $**P < 0.01$ compared with the score of the first injection.

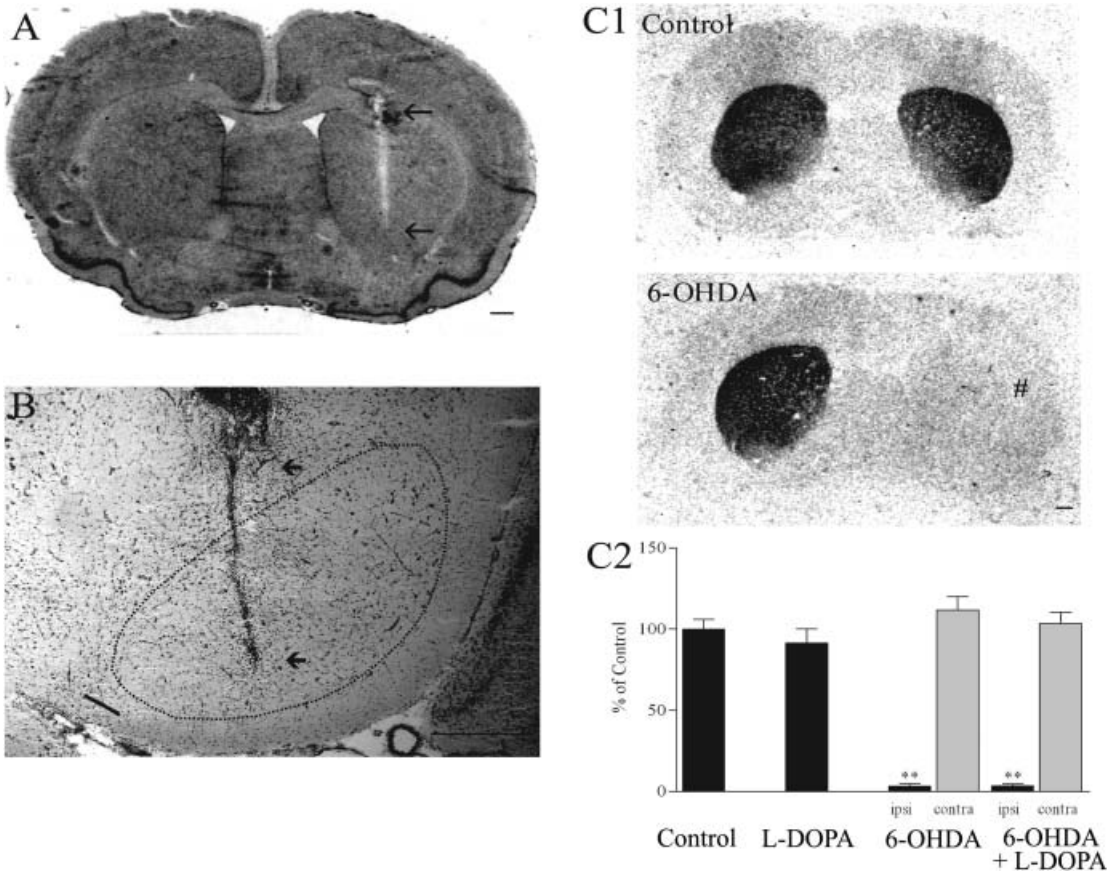

FIG. 3. Histological controls of the striatal dopamine denervation extent and the location of the microdialysis probes in the striatum and SNr. (A, B) Photomicrographs of Cresyl violet-stained sections at striatal (A) and nigral (B) levels illustrating the location of the microdialysis probes. Arrows delineate the probe length. Note in (B) that the probe is implanted in the rostral portion of the striatum and covers most of the dorsoventral extension of the structure. (C) Digitized autoradiographic images (C1) and quantitative analysis (C2) showing the effects of separate or combined unilateral 6-hydroxydopamine (6-OHDA) lesion and L-3,4-dihydroxyphenylalanine (L-DOPA) treatment on striatal [^{3}H]-mazindol binding to dopamine uptake sites. The data presented in the graphs are the means ± SEM of the optical density values determined from *n* animals per condition and are expressed as percentages of control ($n = 5$ controls, 5 L-DOPA, 8 6-OHDA lesion and 6 6-OHDA lesion + L-DOPA). Statistical comparisons are performed using a one-way ANOVA followed by Student's–Newman–Keuls test. $**P < 0.01$ compared with control values. # Side ipsilateral to surgery. Scale bar, 500 μm.

TABLE 1. Controls of the striatal dopamine denervation extent

	[^{3}H]-mazindol binding (%)		Tissue levels of dopamine (%)
	Series 1	Series 2	Series 3
6-OHDA	−96.31 ± 2.23	−97.19 ± 1.11	−96.36 ± 2.95
6-OHDA + L-DOPA	−95.66 ± 1.78	−97.68 ± 1.46	−95.18 ± 2.46

Data are the means ± SEM of the values determined from *n* animals per condition and are expressed as percent change from control.

contralateral to the lesion or in both brain sides in intact rats with L-DOPA treatment.

Extracellular glutamate levels in the striatum and SNr

The overall ANOVA analysis of basal extracellular glutamate levels showed no time effect but significant differences between groups in either striatum ($F_{5,15} = 0.41$, ns and $F_{3,23} = 37.89$, $P < 0.0001$, respectively) or SNr ($F_{5,15} = 0.94$, ns and $F_{3,22} = 12.38$, $P < 0.0001$); no significant time–group interaction was found in both structures. Basal levels of glutamate being stable in the various experimental groups of animals in either striatum or SNr (Fig. 4, A1 and B1), the data from the six samples per animal were averaged, and the mean value from the *n* animals per group was determined (Fig. 4, A2 and B2). Dopamine lesion or L-DOPA treatment alone did not induce significant change in basal extracellular glutamate levels in striatum (control: 0.57 ± 0.09 μM; L-DOPA treatment: 0.52 ± 0.11 μM; 6-OHDA lesion: 0.50 ± 0.06 μM) and SNr (control: 0.50 ± 0.1 μM; L-DOPA treatment: 0.59 ± 0.21 μM; 6-OHDA lesion: 0.46 ± 0.07 μM). By contrast, extracellular glutamate levels were significantly increased in the 6-OHDA lesion + L-DOPA treatment group compared with the other conditions ($P < 0.01$ according to Newman–Keuls test). The mean increase was of +225.04 ± 32.35% in the striatum and +174.1 ± 38.73% in the SNr on the lesion side vs. controls. Systemic injection of L-DOPA during the dialysis session at day 22 of treatment did not significantly modify extracellular glutamate levels in either striatum or SNr in both L-DOPA-treated unlesioned and 6-OHDA-lesioned rats (Fig. 5), the repeated-measures ANOVA analysis showing neither time nor treatment effect. However, note that there is a marked tendency towards increased levels after

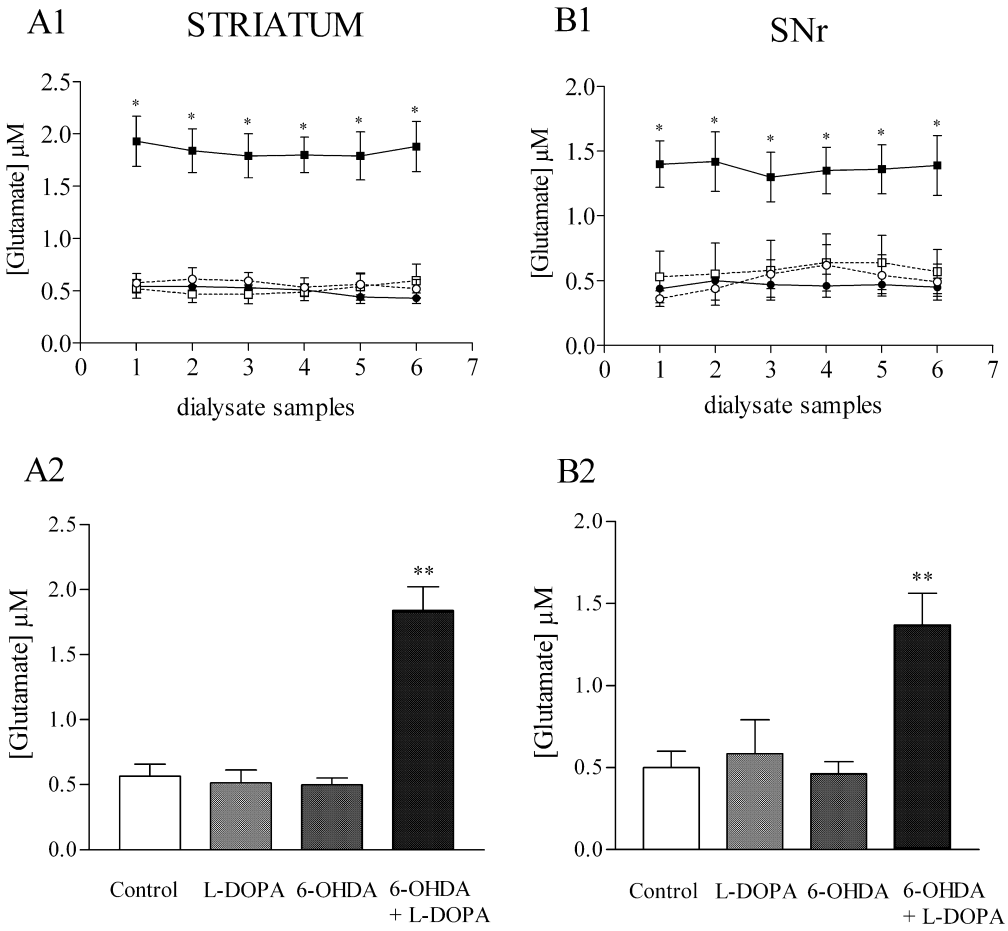

FIG. 4. Effects of dopamine denervation and L-3,4-dihydroxyphenylalanine (L-DOPA) treatment on extracellular glutamate levels. Time course of extracellular glutamate levels, measured by microdialysis in freely moving conditions, in the striatum (A1) and in the substantia nigra pars reticulata (SNr) (B1). Each point is the mean ± SEM determined from *n* animals per condition, (●) control; (□) L-DOPA; (○) 6-OHDA; and (■) 6-OHDA + L-DOPA. Statistical comparisons are performed using a two-way ANOVA, with time as within-factor and group as between-factor, followed by Newman–Keuls test; $^{**}P < 0.01$ compared with control values. Quantitative analysis of mean extracellular glutamate levels measured in the striatum (A2) and in the SNr (B2). The data presented in the graphs are the means ± SEM from *n* animals per condition of the averaged basal extracellular glutamate concentration determined from the six basal samples in individual animals ($n = 8$ controls, 4 L-DOPA, 8 6-OHDA lesion and 6 6-OHDA lesion + L-DOPA). Statistical comparisons are performed using a one-way ANOVA followed by Newman–Keuls test. $^{**}P < 0.01$ compared with control values.

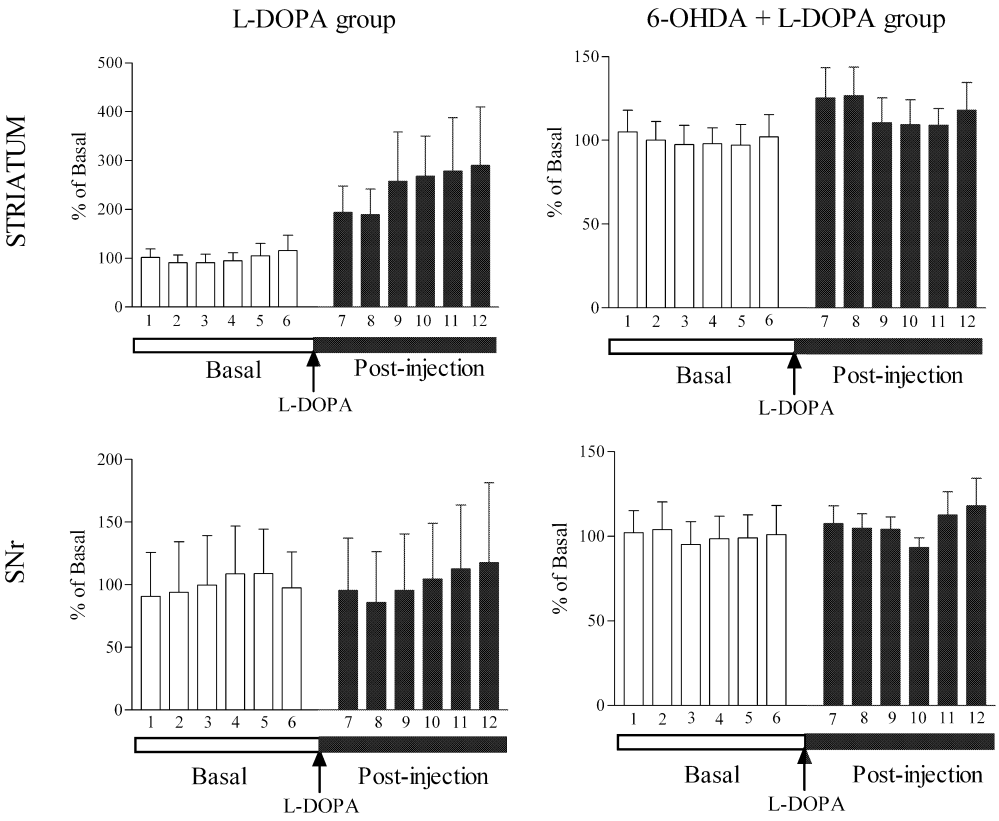

FIG. 5. Quantitative analysis of extracellular glutamate levels illustrating the effects of acute injection of L-3,4-dihydroxyphenylalanine (L-DOPA) (100 mg/kg, i.p.) at day 22 on treatment, during the microdialysis session, on extracellular glutamate levels in the L-DOPA-treated group with or without 6-hydroxydopamine (6-OHDA) lesion of nigral dopamine neurons. Data are the means ± SEM of extracellular glutamate concentrations in each sample pre- and post-injection determined from the *n* animals per group in the striatum and substantia nigra pars reticulata (SNr), and are expressed as percentage of the corresponding basal value (calculated as mean of the six basal samples). Repeated-measure ANOVA shows no significant treatment effect (basal/post-L-DOPA injection values) or difference between samples for each period.

L-DOPA administration in the striatum of unlesioned rats ($P = 0.1106$). Systemic injection of NaCl in unlesioned and 6-OHDA groups did not significantly modify extracellular glutamate levels in either striatum or SNr, discarding any stress effect (not illustrated).

GLT1 mRNA expression in the striatum and the output structures of basal ganglia

Striatal GLT1 mRNA levels assessed on film autoradiograms were unchanged after dopamine lesion or L-DOPA treatment, but significantly increased after the lesion and subsequent L-DOPA treatment, this increase being more marked in the dorsolateral part (142.31 ± 9.62% of control; $P < 0.01$ according to Newman–Keuls test) than the ventromedial part of the striatum (120.68 ± 8.98% of control; $P < 0.05$). In SNr and EP, analysis of *in situ* hybridization signal was performed at cellular scale using microautoradiography. Neither L-DOPA treatment nor dopamine lesion alone induced significant change in GLT1 mRNA levels in these structures vs. controls (Fig. 6). By contrast, animals with the lesion treated with L-DOPA showed a dramatic increase in GLT1 mRNA expression in both structures (Fig. 6, A1 and B1), further illustrated by the shift towards the right of the frequency distribution of labelling per cell (Fig. 6, A2 and B2). Quantitative analysis (Fig. 6, A3 and B3) showed that GLT1 transcript levels were 194.55 ± 7.98% of controls ($P < 0.01$ according to Newman–Keuls test) in the SNr and 460.37 ± 94.51% of control in the EP ipsilateral to the lesion. In the side contralateral to the lesion, GLT1 mRNA levels were not modified in SNr but significantly increased in the EP (219.91 ± 50.96% of control; $P < 0.01$ according to Newman–Keuls test).

GLT1 protein expression in the SNr

In the animals with the dopamine lesion or the L-DOPA treatment alone, there was no significant change in nigral GLT1 protein expression compared with control values as assessed by Western blotting (Fig. 7, A1 and B1) and immunoautoradiography (Fig. 7, A2 and B2). In contrast, animals with the unilateral dopamine lesion treated with L-DOPA showed a significant increase in GLT1 protein levels in the ipsilateral SNr vs. controls (+20% by immunoblotting and +31% by immunoautoradiography; $P < 0.01$ according to Newman–Keuls test).

VGluT (vGluT1 and vGluT2) protein expression in the striatum

No significant change in vGluT1 and vGluT2 protein levels was measured in the striatum whatever the experimental condition examined in comparison with controls (Fig. 8).

Discussion

This study demonstrates that chronic treatment with repeated administration of L-DOPA producing dyskinesias in rats with dopamine denervation causes an increase in extracellular glutamate levels in the

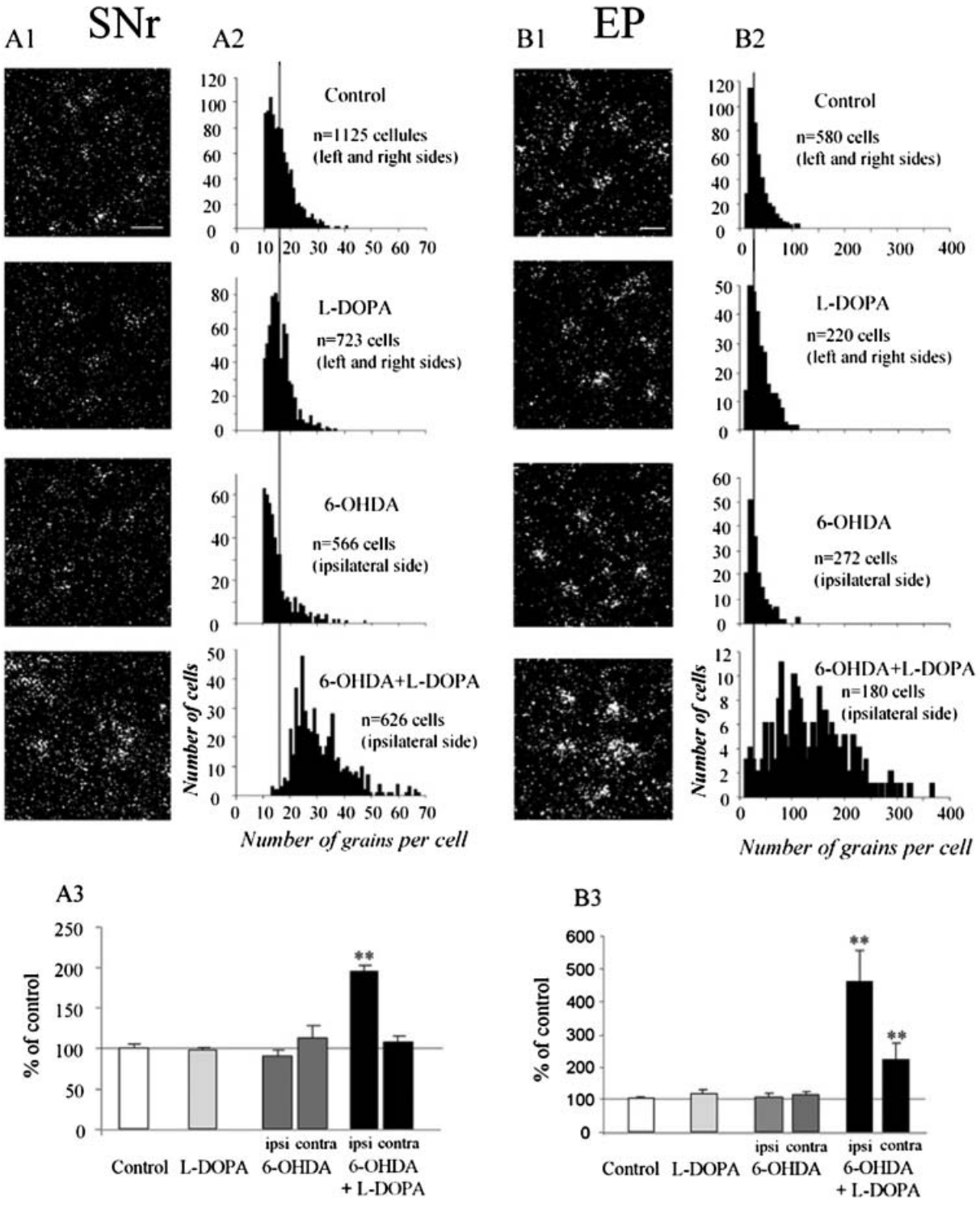

FIG. 6. Effects of chronic L-3,4-dihydroxyphenylalanine (L-DOPA) treatment and of 6-hydroxydopamine (6-OHDA)-induced lesion of nigral dopamine neurons on GLT1 mRNA expression in the substantia nigra pars reticulata (SNr) and entopeduncular nucleus (EP). Sections were processed for *in situ* hybridization with [^{35}S]-radiolabelled GLT1 oligoprobe and for emulsion autoradiography. Photomicrographs taken under darkfield epi-illumination (A1 and B1) and histograms of frequency distribution of labelling (A2 and B2) illustrating the effects in the side ipsilateral to the lesion. Histograms representing the means ± SEM of the number of silver grains per cell determined from *n* animals per condition ($n = 5$ controls, 3 L-DOPA, 5 6-OHDA lesion and 5 6-OHDA lesion + L-DOPA) on the side ipsilateral (ipsi) and contralateral (contra) to the lesion in SNr and EP (A3 and B3). Data are expressed as percentages of control. Scale bar, 25 μm. Statistical comparisons are performed using a one-way ANOVA followed by Newman–Keuls test. $^{**}P < 0.01$ compared with control values.

striatum and SNr, providing additional neurochemical support for the involvement of glutamate-mediated mechanisms in the long-term effects of DOPA-therapy. This treatment also results in increased expression of the glial glutamate transporter GLT1 in the striatum and output structures of the basal ganglia, suggesting widespread adaptive changes in astrocyte glutamate metabolism.

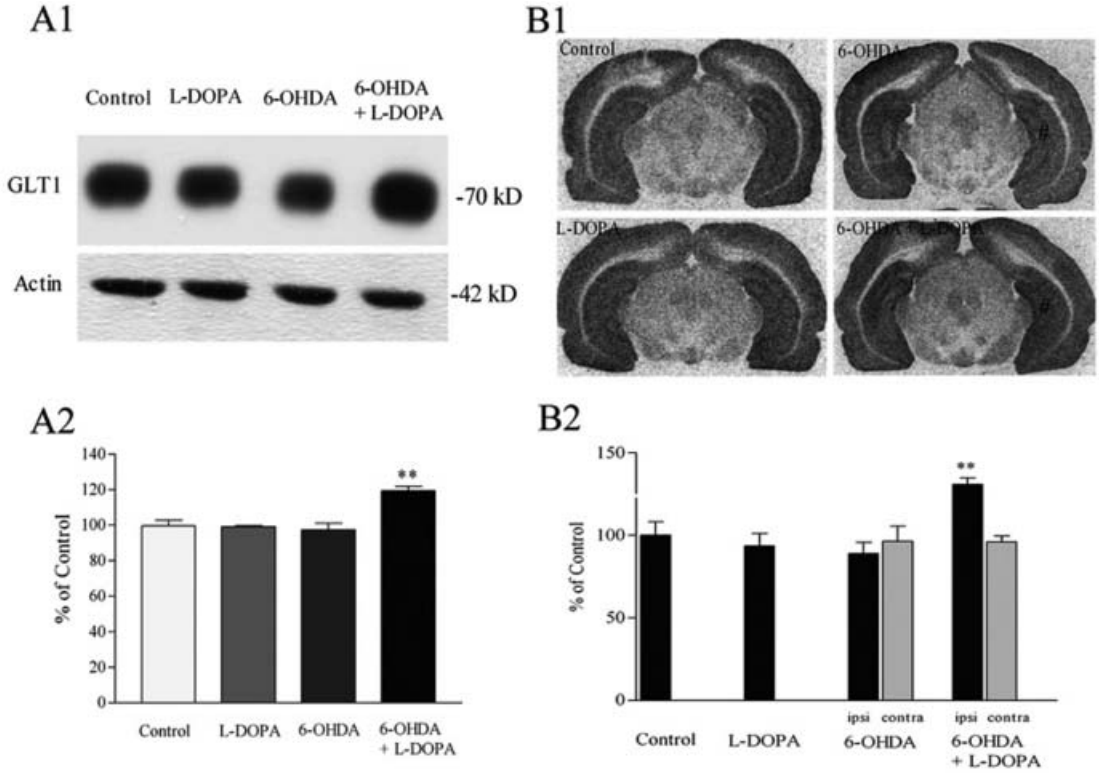

FIG. 7. Effects of separate or combined unilateral 6-hydroxydopamine (6-OHDA) lesion and L-3,4-dihydroxyphenylalanine (L-DOPA) treatment on glutamate transporter GLT1 protein expression in the SNr assessed by Western blotting (A1 and B1) and immunoautoradiography (A2 and B2). The data presented in the graphs are the means ± SEM of the OD values determined from *n* animals per condition and are expressed as percentages of control (blotting experiments: $n = 4$ animals per condition; immunoautoradiography: $n = 5$ controls, 5 L-DOPA, 8 6-OHDA lesion and 6 6-OHDA lesion + L-DOPA). Statistical comparisons are performed using a one-way ANOVA followed by Newman–Keuls test. $^{**}P < 0.01$ compared with control values. #Side ipsilateral to surgery.

There is still an unexplained controversy as to the effects of dopamine lesion on extracellular glutamate levels in the striatum as well as in SNr, the available reports showing either no change (Luo *et al*., 2002; Bianchi *et al*., 2003; Corsi *et al*., 2003), as found here, or increased levels (Lindefors & Ungerstedt, 1990; Abarca *et al*., 1995; Meshul *et al*., 1999; Jonkers *et al*., 2002). Although attempts have been made to explain this variability by the post-lesion delay examined (Meshul *et al*., 1999), this is unlikely to be the only factor involved, as both types of responses have been reported at similar time points. Among the other potentially interfering factors are the dopamine denervation extent and topography, linked to the lesional procedure used (6-OHDA injection in median forebrain bundle vs. SNc) and the use or not of dopamine agonist injection for animal selection on the basis of rotational behaviour. The lack of changes in extracellular glutamate, as observed here, appears as inconsistent with the converging data demonstrating that dopamine denervation induces a reactive increase in corticostriatal glutamate transmission (Calabresi *et al*., 1993; Marti *et al*., 1999; Gubellini *et al*., 2002) and an abnormal activation of the subthalamic nucleus (see Hirsch *et al*., 2000), predicting increased glutamatergic outflow from this nucleus to basal ganglia output structures. However, there is accumulating evidence that extracellular glutamate levels measured by microdialysis arise in the larger proportion from non-vesicular release (Herrera-Marschitz *et al*., 1996; Timmerman & Westerink, 1997; Baker *et al*., 2002; Del Arco *et al*., 2003) and strongly depend on the balance between release processes and glutamate transport activity (Herrera-Marschitz *et al*., 1996), so that increase in synaptically released glutamate could be masked by more general changes in non-vesicular release or glutamate uptake. In this connection, we previously reported that glutamate uptake activity, as well as gene expression of the three main glutamate transporters, GLT1, glutamate/aspartate transporter and excitatory amino acid carrier 1, are unchanged in the dopamine denervated striatum (Kerkerian *et al*., 1987; Lievens *et al*., 2001). Here, we extended this study by showing that, under similar lesion conditions, GLT1 expression is not affected either in SNr or EP, suggesting no major change in glutamate clearance in these structures. We also provide evidence for a lack of change in the expression of the vesicular glutamate transporters vGluT1 and vGluT2 in the striatum that have been both localized to synaptic vesicles in axonal terminals forming asymmetrical synaptic contacts, a feature of excitatory amino acid-containing striatal projections (Bellocchio *et al*., 1998; Herzog *et al*., 2001). Altogether, these data suggest that the reactive increase in glutamate transmission in parkinsonian state is not related to glutamate transporter dysfunction or to dysregulation of presynaptic vesicular glutamate transporter expression. A possible explanation is that this glutamate hyperactivity may be underlain primarily by the changes occurring at glutamate receptor level on target cells, such as the increase in striatal NMDA receptor phosphorylation state (see Chase *et al*., 2000) or changes in glutamate receptor gene expression (Rodriguez-Puertas *et al*., 1999).

It has been previously reported that acute injection of L-DOPA increases extracellular glutamate levels in the striatum both in intact and dopamine-lesioned rats; however, the underlying mechanisms are presumably different, as blockade of NMDA receptor suppresses the increase in lesioned rats but not in intact rats (Jonkers *et al*., 2002). To date, the effects of chronic administration of L-DOPA remain

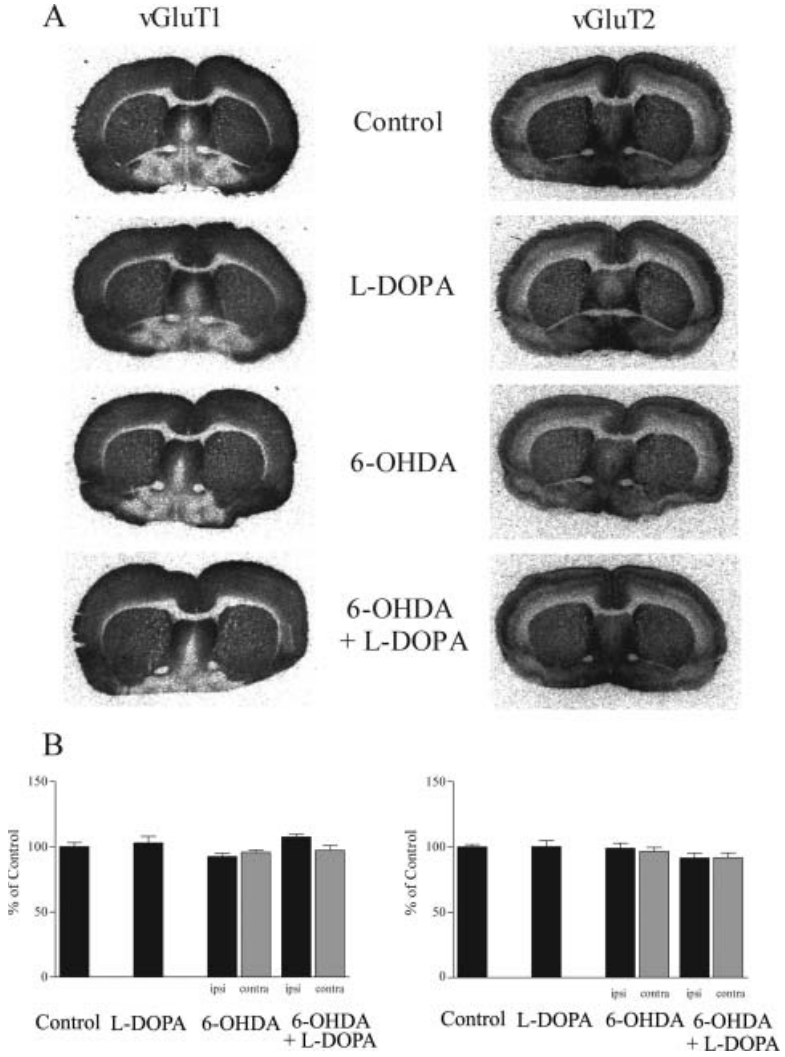

FIG. 8. Digitized autoradiographic images (A) and quantitative analysis (B) showing the effects of dopamine denervation with or without subsequent chronic L-3,4-dihydroxyphenylalanine (L-DOPA) treatment on expression of vesicular glutamate transporter VGluT1 and vGluT2 proteins in the striatum. The data presented in the graphs are the means ± SEM of the OD values determined from *n* animals per condition and are expressed as percentages of control ($n = 5$ controls, 5 L-DOPA, 8 6-OHDA lesion and 6 6-OHDA lesion + L-DOPA). Statistical comparisons are performed using a one-way ANOVA followed by Newman–Keuls test. #Side ipsilateral to surgery.

unknown. We here show that chronic treatment with repeated injection of L-DOPA for 21 days also increases extracellular glutamate levels in the striatum of hemi-parkinsonian rats that have developed dyskinesias but not in intact rats, and demonstrate similar changes in SNr. The differential response elicited by chronic L-DOPA treatment in intact and lesioned rats confirms that different mechanisms are involved in the action of L-DOPA on glutamate function depending on the integrity of the nigrostriatal system. The observation of increased extracellular glutamate in chronically treated rats with the lesion provides additional support to the concept that intermittent DOPA therapy leads to abnormal glutamatergic transmission in the striatum, and suggests that such abnormal transmission may also occur in output structures of the basal ganglia.

Whether or not extracellular glutamate levels vary during acute injection of L-DOPA under chronic intermittent treatment is an interesting matter of concern in terms of the pathophysiology of L-DOPA-induced dyskinesias. In intact rats chronically treated with L-DOPA, acute L-DOPA administration during the dialysis session induced a clear tendency of increase in extracellular glutamate in the striatum, approximating the increase produced by acute L-DOPA injection in naïve rats (Jonkers *et al*., 2002), although it did not reach statistical significance in our study. This suggests a relative preservation of a pulsatile effect of L-DOPA on extracellular glutamate in intact striatum. No change in extracellular glutamate is measured under this condition in SNr of intact rats, showing specific regulatory mechanisms in the striatum consistent with a local action. Interestingly, we found that, after 3 weeks of treatment, acute injection of L-DOPA during the dialysis session in 6-OHDA-lesioned rats did not significantly modify the levels of extracellular glutamate in the striatum or SNr, suggesting that extracellular glutamate remained at steady-state high levels within the 12 h separating the L-DOPA injections in both structures. The non-pulsatile character of the increase in extracellular glutamate contrasts with the short half-life of L-DOPA and this drug-triggered pulsatile stimulation of dopamine receptor that is considered as a main factor for the development of dyskinesias (see for review Baas, 2000; Olanow & Obeso, 2000). Therefore, the increase in extracellular glutamate levels cannot be directly related to the transient expression of dyskinesias, but may be indirectly involved by

modifying the adaptive properties of glutamate synapse functioning and its interactions with other neurotransmitter systems. For instance, it has been proposed that changes in extracellular glutamate could be an index of volume transmission-mediated action involving extrasynaptic receptors with functional properties different from those located at the synapse and producing specific electrophysiological and neurochemical effects (see Del Arco *et al*., 2003). In this connection, it has been recently reported that dyskinetic rats, contrary to control and non-dyskinetic rats, did not show striatal depotentiation after long-term potentiation induction (Picconi *et al*., 2003).

Further investigating the possible origin of the L-DOPA-induced raise in extracellular glutamate, we found no change in the expression of vesicular transporters in the striatum. This does not suffice to exclude a contribution of increased release from glutamate terminals unless more is known about the relationship between vesicular release capacities and expression or activity of these vesicular transporters. Interestingly, increased extracellular glutamate in several pathological conditions has been associated with dysfunction of glutamate transporters, and particularly of GLT1 that is responsible for the bulk of extracellular glutamate clearance in the brain (see Danbolt, 2001). This is unlikely to be the case in our experimental conditions, as we found that GLT1 mRNA and protein expression are not decreased but are paradoxically increased parallel to L-DOPA-triggered raise in extracellular glutamate both in the striatum and SNr of dopamine-depleted rats. In SNr, the increase in protein expression appears as modest compared with the increase in GLT1 mRNA levels. Among the possible explanations, different sensitivity of the methods, differential regulation at transcriptional vs. translational level, this apparent mismatch could be due to an underestimation of the changes when analysis is performed at structural scale. For instance, prior analysis of GLT1 mRNA in SNr on film autoradiograms (not shown) showed lower changes (+40%) than analysis at cellular level (+94%). A large overexpression of GLT1 transcripts is also measured in EP of L-DOPA-treated lesioned rats. Intriguingly, overexpression in EP is observed in both hemispheres, which is difficult to explain, but can be compared with several studies in the literature showing bilateral changes in glutamate function in this nucleus after unilateral dopamine denervation (Biggs & Starr, 1997; Biggs *et al*., 1997). It then could be that L-DOPA treatment unmasks contralateral changes in glutamate function in EP through pathways that remain to be elucidated. Altogether, these data demonstrate that chronic L-DOPA treatment in parkinsonian state produces an overexpression of GLT1 both in the main input and output structures of the basal ganglia. The observation of increases in both extracellular glutamate and GLT1 is puzzling. A possible explanation is that GLT1 overexpression is part of a compensatory mechanism by which astrocytes may prevent neurotoxic effects of L-DOPA. *In vitro*, L-DOPA has been reported to induce neuronal death, involving both auto-oxidative processes and glutamate-mediated mechanisms, in glia-poor cultures of rat striatum (Cheng *et al*., 1996; Maeda *et al*., 1997), but there is no evidence for loss of striatal neurons *in vivo*. It could be that GLT1 overexpression limits the raise in extracellular glutamate and subsequent excitotoxic processes. It also could be that the increases in both extracellular glutamate and GLT1 are linked through a common mechanism of cell defence against L-DOPA-induced oxidative stress. Studies in cultured glial cells have shown that L-DOPA enhances cellular content of glutathione (GSH) (van Muiswinkel *et al*., 2000), a tripeptide with crucial antioxidant function. Now, GSH levels are highly dependent on the function of glutamate transporters (Reichelt *et al*., 1997; Re *et al*., 2003): transported glutamate is directly inserted into GSH and its export drives the entry of cysteine by the cysteine–glutamate antiporter x_c^-. This antiporter being responsible for most of the basal extracellular glutamate measured by microdialysis (Jabaudon *et al*., 1999; Baker *et al*., 2002; see Del Arco *et al*., 2003), the L-DOPA-induced elevation in both extracellular glutamate and GLT1 expression could reflect increased activity of the exchanger, sustained by an increase in transported glutamate. Such adaptive changes in glutamate metabolism involving glial cells may be essential for neuron survival, especially knowing that astrocytes release GSH for supplying neurons with GSH precursors (see Dringen & Hirrlinger, 2003).

In conclusion, this study provides strong evidence that chronic administration of L-DOPA triggers persistent adaptive changes in glutamate function in structures of the basal ganglia that are central to the expression of motor disorders. These changes are dependent on the integrity of the nigrostriatal dopamine system, as they are observed in animals with the dopamine denervation but not in intact rats, and may both underlie functional changes in glutamate transmission and contribute to protective mechanisms against cytotoxic processes resulting from glutamate/dopamine imbalance.

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Abbreviations

AIM, abnormal involuntary movements; AMPA, (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EP, entopeduncular nucleus; GLT1, glutamate transporter 1; GSH, glutathione; HPLC, high-pressure liquid chromatography; NMDA, *N*-methyl-D-aspartate; 6-OHDA, 6-hydroxydopamine; L-DOPA, L-3,4-dihydroxyphenylalanine; PBS, phosphate-buffered saline; PD, Parkinson's disease; SDS, sodium dodecyl sulphate; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; SSC, standard saline citrate; TBS, Tris-buffered saline; vGluT, vesicular glutamate transporter.

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