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The effect of 6-hydroxydopamine lesions on the release of amino acids in the direct and indirect pathways of the basal ganglia: a dual microdialysis probe analysis

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Abstract

The loss of dopaminergic neurons of the substantia nigra in Parkinson's disease and in animal models of Parkinson's disease is associated with an imbalance in the activity of the so-called 'direct' and 'indirect' pathways of information flow through the basal ganglia. The aim of the present study was to determine whether the imbalance is reflected in changes in the release of GABA, aspartate and glutamate in the pathways using dual probe microdialysis in freely moving rats. Control and 6-hydroxydopamine-(6-OHDA)-lesioned rats were implanted with microdialysis probes in the neostriatum and substantia nigra or globus pallidus and the release of amino acids was analysed in the dialysates. Basal levels of amino acids were largely unaltered by the 6-OHDA lesion; however, the levels of GABA in the globus pallidus dialysates were significantly elevated in the lesioned rats, indicating an imbalance in favour of the indirect pathway. Administration of kainic acid to the neostriatum enhanced the release of GABA locally and in the distal probes in the substantia nigra and globus pallidus. In 6-OHDA-lesioned rats, stimulated release of GABA in the substantia nigra was abolished, indicating a reduction in transmission along the direct pathway. Thus, consistent with the direct–indirect pathway model of the basal ganglia, the 6-OHDA lesion results in an elevation of the basal release of GABA in the striatopallidal (indirect) pathway and a reduction in the evoked release of GABA in the striatonigral (direct) pathway. These imbalances may underlie, at least in part, the motor abnormalities of Parkinson's disease and in animal models of Parkinson's disease.

Introduction

Recent hypotheses of basal ganglia organization have proposed that information carried by the major input to the basal ganglia, the projection from the cortex to the neostriatum, is processed within the neostriatum and transmitted to the output nuclei of the basal ganglia, the entopeduncular nucleus (EP) (or internal segment of the globus pallidus in primates) and the substantia nigra *pars reticulata* (SNr), by direct and indirect pathways and thence to the ventral thalamus and/or subcortical premotor regions (Albin *et al*., 1989; Alexander & Crutcher, 1990; Alexander *et al*., 1990; Bergman *et al*., 1990; DeLong, 1990) (Fig. 7A). The direct pathway consists of a monosynaptic γ-aminobutyric acid (GABA)ergic projection from the neostriatum to the output nuclei, activation of which leads to inhibition of neurons in the output nuclei which in turn leads to disinhibition of the targets of the basal ganglia. Disinhibition of the targets of the basal ganglia is assumed to underlie behaviour that is associated with the basal ganglia (Chevalier & Deniau, 1990). In contrast, the indirect pathway consists of polysynaptic projections to the output nuclei via the globus pallidus (GP; external segment in primates) and subthalamic nucleus (STN). Activation of this pathway leads to increased firing of neurons in the output nuclei and thus greater inhibition of the targets of the basal ganglia, and is assumed attenuate behaviour that is associated with the basal ganglia (Albin *et al*., 1989; Chevalier & Deniau, 1990; Mink, 1996). A prediction of the direct–indirect pathways model is that the akinesia associated with Parkinson's disease is due to an imbalance in activity in the two pathways in favour of the indirect pathway, i.e. reduced activity in the direct pathway and/or increased activity in the indirect pathway (Albin *et al*., 1989; Bergman *et al*., 1990; DeLong, 1990). Indeed, in animal models of Parkinson's disease there are alterations in the levels of neuronal markers and physiological activity which are consistent with the concept of an imbalance in the activity of the direct and indirect pathways (Albin *et al*., 1989; Crossman, 1989, 1990; Bergman *et al*., 1990; DeLong, 1990; Gerfen *et al*., 1990; Aziz *et al*., 1991; Baik *et al*., 1995; Gerfen & Wilson, 1996; Wichmann & DeLong, 1996; Obeso *et al*., 2000).

Dual probe *in vivo* microdialysis is an approach that allows the activity of neuronal pathways to be monitored by measuring transmitter release at the site of origin and in the target regions of a projection following pharmacological manipulation of neurons giving rise to that projection. Using this approach we and others have demonstrated that stimulation of the neostriatum leads to the local release of GABA and other putative neurotransmitter amino acids as well as the release of GABA into the dialysate of distal probes in the substantia nigra (SN) and in the GP (Bianchi *et al*., 1994, 1998; Morari *et al*., 1996; Ferraro *et al*., 1998; Marti *et al*., 2000; Fattori *et al*., 2001). In the present experiment we have used dual probe *in vivo* microdialysis in freely

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moving rats to examine the basal release of GABA and other amino acids in the direct and indirect pathways of control rats and rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the dopaminergic neurons of the substantia nigra *pars compacta*. The objective was to test the hypothesis that altered activity in the direct and indirect pathways in parkinsonian animals is also reflected in alterations in the release of GABA in the pathways. Preliminary data relating to the work in this manuscript have been published in abstract form (Galeffi *et al*., 1997; Fattori *et al*., 2001; Hayes *et al*., 2001).

Materials and methods

6-Hydroxydopamine lesions

All the experiments involving laboratory animals were performed according to the Italian Guidelines for Animal Care (D.L. 116/92), which were also in accordance with European Communities Council Directives (86/609/ECC). Forty-eight male Wistar rats (175–200 g at the beginning of the experiments; Harlan-Nossan, Milano, Italy) received unilateral injections of 6-OHDA (5–7 μL of 3 mg/mL in 0.9% NaCl containing 0.2 mg/mL ascorbic acid). Under pentobarbitone anaesthesia (60 mg/kg, i.p.), the 6-OHDA was injected in the right medial forebrain bundle (co-ordinates: AP −4.5, L 0.9, DV 7.5 mm with respect to the Bregma and dural surface) at a rate of ≈1 μL/min using a 10-μL Hamilton microsyringe. The needle was left in place for 5–10 min and removed slowly. After 4 weeks the animals were weighed and challenged with d-amphetamine (5 mg/kg; i.p.) The number of times they rotated over a period of 30 min was noted. In those animals that performed 200 or more net ipsiversive rotations in the 30-min period, which is associated with a ≥90% loss of neostriatal dopamine (Hudson *et al*., 1993), were implanted with microdialysis probes 24–48 h later.

In vivo *microdialysis*

The successfully lesioned rats (220–300 g) and weight-matched controls were anaesthetized with chloral hydrate (400 mg/kg i.p.) and placed in a stereotaxic frame. One microdialysis cannula (concentric design) was implanted vertically in the right neostriatum (3-mm probe tip) and the second in the right SN for the striatonigral experiments or in the right GP for the striatopallidal experiments (1- and 2-mm probe tip, respectively). Stereotaxic co-ordinates relative to the Bregma were: AP 0.7, L 3.2, DV 5.5 mm for the neostriatum; AP −5.4, L 2.2, DV 8.9 mm for the SN and AP −1.0, L 3.2, DV 7.5 mm for the GP (Paxinos & Watson, 1986).

The microdialysis experiments were performed on freely moving rats ≈24 h later. The dialysis probes were perfused with artificial cerebrospinal fluids consisting of (in mM): NaCl, 140; KCl, 3; $CaCl_2$, 1.2; $MgCl_2$, 1.0; Na_2HPO_4, 1.2; NaH_2PO_4, 0.27; and glucose, 7.2 (pH 7.4) via polyethylene tubing (i.d. 0.38 mm) connected to a 1-mL syringe mounted on a microinfusion pump (CMA/100, CMA/Microdialysis A B, Stockholm, Sweden), at a rate of 2 μL/min. After a 60-min stabilization period, the dialysate samples were collected every 20 min. At least three samples were collected to measure the basal extracellular concentration of GABA, taurine, glutamate and aspartate under resting conditions before the administration of one of the two glutamate receptor agonists, kainic acid (KA) or α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA; Sigma-Aldrich, Milan, Italy). The drugs were administered at a concentration of 100 μM for 20 min (one fraction, defined as time 0) through the dialysis probe in the neostriatum. The dialysate samples were collected every 20 min for the next 3 h. Following this, 100 mM potassium chloride was administered to the neostriatum and either the substantia nigra or the globus pallidus for 20 min and three more samples were collected.

Measurements of amino acids

The content of GABA, taurine, glutamate and aspartate in the microdialysis perfusates was measured by high performance liquid chromatography with fluorimetric detection as described by Bianchi *et al*. (1999). Data on taurine will be presented elsewhere. Taurine levels, however, were used as a marker for the correct probe location in the GP, because very high KA- or AMPA-stimulated taurine release is a characteristic of the neostriatum but not the GP. Briefly, the amino acids were derived with mercaptoethanol and o-phthalaldehyde. The o-phthalaldehyde derivatives were then separated on a 5-μm reverse-phase Nucleosil C18 column (250 × 4 mm; Machery-Nagel, Duren, Germany) kept at room temperature, using a mobile phase consisting of methanol and potassium acetate (0.1 M, pH adjusted to 5.52 with glacial acetic acid) at a flow rate of 0.9 mL/min in a three linear step gradient (from 25 to 90% methanol). The dialysate samples were either analysed immediately or frozen and stored at −20 °C for up to 6 months before analysis (Bianchi *et al*., 1999).

The levels of amino acid in the perfusate fractions were expressed as fmol of amino acid/μL of perfusate (nM). The basal value was obtained from the area under the concentration curve between −40 and 0 min (three fractions, normalized to the amount corresponding to one 20-min fraction; Table 1). The value for stimulated release (i.e. the release in response to the inclusion of kainic acid or AMPA in the neostriatal perfusate) was obtained from the area under the curve between 0 and 120 min (six fraction intervals). In the case of K^+ stimulation, net output was calculated as the area under the curve between time 0 (the 20-min fraction corresponding to K^+ perfusion) and 60 min (three fraction intervals), normalized to one 20-min fraction minus basal value (time 0 fraction). For statistical analysis net output values were used, i.e. the differences between stimulated and basal release, where

TABLE 1. Basal extracellular levels of GABA, aspartate and glutamate in the striatonigral and striatopallidal pathways

	Striatonigral pathway				Striatopallidal pathway			
	Neostriatum		Substantia nigra		Neostriatum		Globus pallidus	
Amino acid	Control ($n=15$)	Lesion ($n=17$)	Control ($n=16$)	Lesion ($n=18$)	Control ($n=18$)	Lesion ($n=9$)	Control ($n=21$)	Lesion ($n=11$)
GABA	20 ± 3	21 ± 2	23 ± 3	20 ± 2	26 ± 4	24 ± 3	17 ± 2	37 ± 7**
Aspartate	231 ± 32	252 ± 33	171 ± 19	275 ± 40*	232 ± 26	223 ± 33	251 ± 23	231 ± 28
Glutamate	396 ± 48	287 ± 55	411 ± 81	398 ± 87	325 ± 48	397 ± 82	236 ± 39	269 ± 44

Data are mean ±SEM of basal output (fmol/μL of perfusate, i.e. nM), i.e. the mean area under the concentration-time curve between −40 and 0 min, normalized to one time interval of 20 min. $^{**}P < 0.01$, $^{*}P < 0.05$ (lesion vs. control) as obtained by *post hoc* Fisher's LSD test after MANOVA (significant Interaction for aspartate, $F_{3,111} = 2.688$, $P < 0.05$ and GABA, $F_{3,117} = 5.521$, $p < 0.001$). *n* refers to the number of samples successfully analysed from 37 control and 29 6-OHDA-lesioned rats used for KA and AMPA stimulation in striatonigral and striatopallidal experiments.

TABLE 2. The effect of intrastriatal application of KA (100 μM) on the output of GABA, aspartate and glutamate from the striatonigral and striatopallidal pathways in control and 6-OHDA-lesioned rats

	Striatonigral pathway				Striatopallidal pathway			
	Neostriatum		Substantia nigra		Neostriatum		Globus pallidus	
Amino acid	Control	Lesion	Control	Lesion	Control	Lesion	Control	Lesion
GABA								
Mean ± SEM	196 ± 26	162 ± 59	165 ± 42	21 ± 17**	207 ± 46	212 ± 102	108 ± 25	172 ± 28
95% CI	137–256	23–301	68–262	−20 to +62	108–306	−51 to +475	54–161	104–239
(*n*)	(9)	(8)	(9)	(8)	(14)	(6)	(14)	(7)
Aspartate								
Mean ± SEM	1423 ± 317	2138 ± 703	1570 ± 355	2068 ± 476	1597 ± 494	1279 ± 283	1702 ± 540	1788 ± 328
95% CI	673–2173	330–3946	752–2387	971–3166	480–2713	551–2006	524–2880	1014–2563
(*n*)	(8)	(6)	(9)	(9)	(10)	(6)	(13)	(8)
Glutamate								
Mean ± SEM	1683 ± 379	1903 ± 508	1214 ± 453	1823 ± 541	1297 ± 249	2817 ± 793**	1244 ± 383	1612 ± 260
95% CI	810–2557	596–3210	170–2259	109–3271	722–1872	779–4856	390–2098	997–2273
(*n*)	(9)	(6)	(9)	(10)	(9)	(6)	(11)	(8)

Data are the mean net output ±SEM, i.e. the area obtained from the area under the concentration–time curve between 0 and 120 min (stimulated output) with the area under the curve between −40 and 0 min (basal output) subtracted and normalized to the time interval of 20 min. Concentration values were fmol/μL of perfusate (nM); 95% CI of the means are shown. For GABA release in the SN in lesioned animals, the 95% CI include zero, indicating that stimulated output is not significantly different from basal output. $^{**}P < 0.01$ (lesion vs. control) as obtained by *post hoc* Fisher's LSD test (MANOVA, Interaction $F_{3,60} = 3.452$, $P < 0.022$) indicates a highly significant difference vs. control. GABA release in the lesioned neostriatum of the striatopallidal experiment: in spite of the nonsignificant 95% CI, MANOVA indicated that it was not significantly different from control animals. For neostriatal glutamate in lesioned rats of the striatopallidal experiment, *P*-value *post hoc* Fisher's LSD test (MANOVA, Treatment $F_{1,63} = 5.374$, $P < 0.032$) indicates a significantly higher output than in controls. *n* refers to the number of samples successfully analysed from nine control and 10 6-OHDA-lesioned rats in the striatonigral experiment and 14 control and eight 6-OHDA-lesioned rats in the striatopallidal experiments.

basal values were normalized to the time interval of 120 min. For the construction of the graphs (Figs 2–6), mean concentration–time points were expressed as a percentage of their respective prestimulation value (time 0).

Statistical analysis

Data were evaluated by 95% confidence interval (CI) or analysis of variance of the actual areas. Stimulated output was considered to be significantly different from basal output when 95% CI of net output did not include zero. However, 95% CI only gave an indication of the variability shown individually by each experimental group. For a more detailed statistical analysis of differences between control and lesioned and striatonigral and striatopallidal groups, basal (Table 1) or stimulated data (net output; Tables 2 and 3) were analysed globally by analysis of variance (MANOVA, using the SPSS program for PC; IBM Clones Inc., Chicago, Illinois, USA) with two between-subject factors, Treatment (control and lesion) and Brain area (neostriatum and substantia nigra, neostriatum and globus pallidus). When the Treatment factor or the Interaction between the two factors was significant, the *post hoc* Fisher's LSD test was applied to verify the statistical significance of lesioned vs. control or striatonigral vs. striatopallidal groups.

Verification of probe site and tyrosine hydroxylase immunocytochemistry

One to two days following the dialysis session, the 6-OHDA-lesioned rats were deeply anaesthetized (pentobarbitone 60 mg/kg, i.p.) and perfused through the aorta with 100 mL 0.9% NaCl followed by 300 mL of a mixture of 3% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed from the skulls and stored in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) at 4 °C.

A vibrating microtome was used to cut 60-μm-thick frontal sections through the neostriatum, GP and SN. For some of the rats, sections of the SN were processed to reveal tyrosine hydroxylase (TH) immunoreactivity. After several washes in PBS the sections were incubated overnight at room temperature in PBS containing 0.1% Triton X-100 and a 1 : 1000 dilution of rabbit anti-TH antiserum (Eugene Tech, USA). The sections were then washed in PBS and incubated in the biotinylated goat antirabbit immunoglobulin (Vector Laboratories Ltd, Peterborough, UK; 1 : 100 dilution) for one hour. They were then washed again in PBS, and incubated in an avidin–biotin–peroxidase complex (Vector; 1 : 100 dilution in PBS) for 1 h. After several rinses in PBS and one in Tris buffer (0.05 M; pH 7.6), the immunoreactive sites were visualized by incubation in H_2O_2 (0.006%) in the presence of 3,3′ diaminobenzidine (0.025%; Sigma) in Tris buffer. After several washes, the sections of the neostriatum, GP and SN were mounted on gelatine-coated slides and stained with Cresyl Violet to determine the precise positions of the probe sites. The same histological protocol was used for the sections of control rats whose brains were fixed by immersion in 4% paraformaldehyde after decapitation under anaesthesia.

Results

Lesion and probe location

Sections of the mesencephalon of the lesioned animals which had been used for the dual probe dialysis study were subjected to immunocytochemistry for TH. Consistent with the fact that these rats had fulfilled the citerion for a successful lesion (i.e. performed at least 200 rotations in a 30-min period following amphetamine administration), there was a marked, but variable, loss of TH-immunoreactive perikarya and dendrites in the SN but with a relative sparing of the ventral tegmental area. There was no apparent loss of TH-positive neurons on the contralateral side. Quantitative analyses of the reduction in TH-positive neurons in the SN were not performed as the microdialysis probe track obviated accurate counts of TH-positive neurons.

Neostriatal microdialysis probes were mainly located in the middle of the neostriatum (in dorsoventral and mediolateral planes) at a level ≈1 mm rostral to the GP. Nigral probes were placed in the middle third of the *pars reticulata* and those in the GP were in the

TABLE 3. The effect of intrastriatal AMPA application on the output of GABA, aspartate and glutamate from the striatonigral pathway in control and 6-OHDA-lesioned rats

	Striatonigral pathway			
	Neostriatum		Substantia nigra	
Amino acid	Control	Lesion	Control	Lesion
GABA				
Mean ± SEM	75 ± 24	56 ± 16	60 ± 18	7 ± 7*
95% CI	17–133	21–91	15–105	−9 to +23
(*n*)	(7)	(11)	(7)	(10)
Aspartate				
Mean ± SEM	681 ± 260	406 ± 132	635 ± 107	631 ± 271
95% CI	44–1318	112–700	372–897	5–1257
(*n*)	(7)	(11)	(7)	(9)
Glutamate				
Mean ± SEM	384 ± 118	250 ± 58	328 ± 118	274 ± 71
95% CI	82–686	120–381	40–615	110–439
(*n*)	(6)	(10)	(7)	(9)

Data are the mean net output ±SEM, i.e. the area obtained from the area under the concentration–time peak (stimulated output), with the area under the curve between −40 and 0 min (basal output) subtracted and normalized to the same time interval used to calculate stimulated output. The time interval for stimulated output was between 0 and 120 min, unless following the stimulation peak levels drifted below basal values (lesioned neostriatum 4 out of 11 for aspartate and 2 out of 10 for glutamate) when a shorter time interval, corresponding to return to basal value, was used. Concentration values were fmol/μL of perfusate (nM). 95% CI of the mean are shown. For GABA release in the SN in lesioned animals the 95% CI include zero, indicating that stimulated output is not significantly different from basal output. $^*P < 0.05$ (lesion vs. control) as obtained by *post hoc* Fisher's LSD test (MANOVA, Treatment: $F_{1,16} = 4.772$, $P < 0.044$) indicates a significant difference vs. control. Data were obtained from a group of seven control and 11 6-OHDA-lesioned rats, where *n* refers to the number of samples successfully analysed.

middle third in the rostrocaudal plane and the ventral third. There was a 100% success in the neostriatal placement of the probe, whereas there was a 30% and 20% failure in placing the probe within the SN and the GP, respectively. Misplacement of the probe within the SN was associated with an undetectable basal GABA output. Misplaced probes in the striatopallidal experiments were on the border of, or within, the neostriatum and were associated with very high KA- or AMPA-stimulated taurine release which is characteristic of the neostriatum but not the GP. Correct location of the probes was confirmed for 33 6-OHDA-lesioned rats (258 ± 4 g) and 37 weight-matched controls (250 ± 6 g). The location of the probes is summarized in Fig. 1.

Basal release of amino acids

Mean basal levels of GABA, aspartate and glutamate in the striatonigral and striatopallidal experiments in both control and 6-OHDA-lesioned rats were calculated from the three fractions (−40, −20 and 0 min) prior to stimulation with KA or AMPA (Table 1). In each region, i.e. the neostriatum, SN and GP, the output of the amino acids was stable for several hours (data not shown). For the most part, the basal output of the amino acids in the neostriatum was unaltered in the 6-OHDA-lesioned rats. However, the basal output of aspartate was

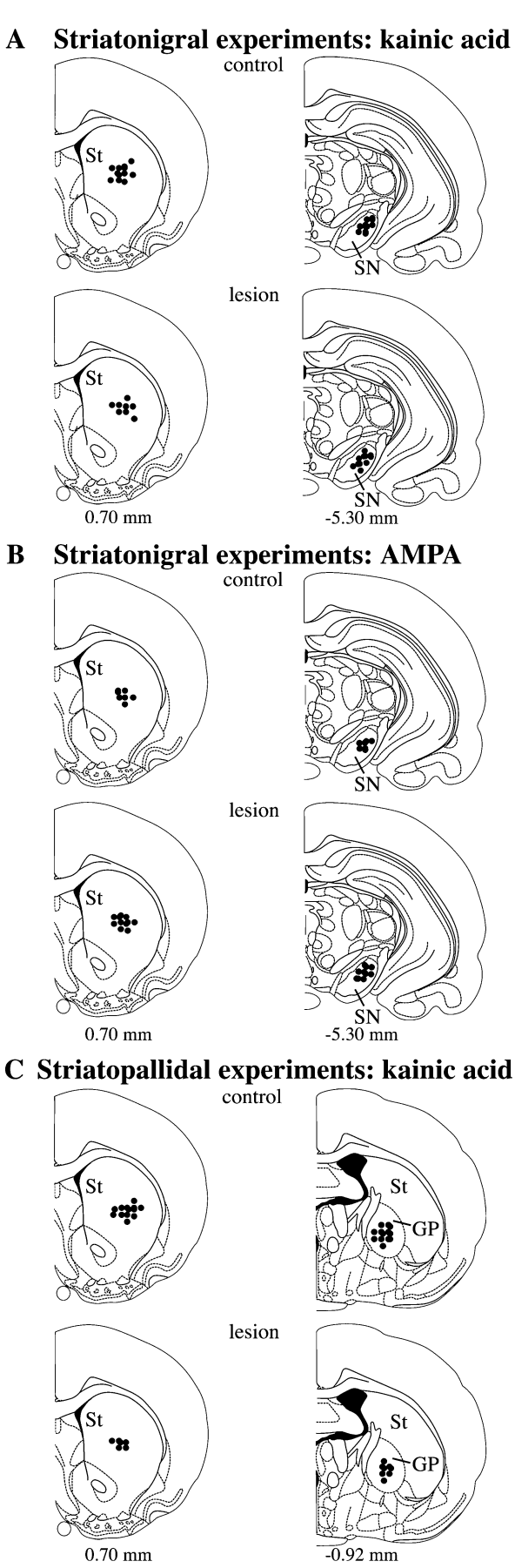

FIG. 1. Location of the microdialysis probes. The positions of the probes were identified by histological analysis and drawn onto diagrams taken from the atlas of Paxinos & Watson (1997). Each dot represents the position of the probe in one or more experiments for the control or 6-OHDA-lesioned rats (lesion) in the striatonigral (A,B) and the striatopallidal (C) experiments. The rostrocaudal positions varied slightly but have been compressed onto single diagrams for the sake of clarity. Numbers represent the distance in millimetres from Bregma. St, neostriatum; SN, substantia nigra; GP, globus pallidus.

significantly greater (161%) in the nigral dialysates of the 6-OHDA-lesioned rats than the control rats. Similarly, the basal output of GABA from the GP was significantly greater in the lesioned rats (218%). The basal release of aspartate and glutamate was 10 and 20 times greater than that of GABA, respectively.

Following stimulation of the neostriatum with 100 μM KA or AMPA (one fraction, 20 min), dialysate fractions were collected for up to 3 h and the concentrations of amino acids measured. The data were expressed in two ways: first, as plots of the concentration of the amino acids in each fraction over time (Figs 2–6), in which case the mean values were expressed as a percentage of their prestimulation level (time 0); second, as the mean net output (± SEM, 95% CI), i.e. mean area under the stimulated curve minus basal value (Tables 2 and 3). Although there were apparent differences when neostriatal data were expressed as percentages in the concentration–time plots, there were no significant differences between the striatonigral and the striatopallidal experiments when all mean net outputs were compared by analysis of variance.

GABA release

Stimulated release was considered statistically significant at $P < 0.05$ when mean net output values had 95% CI not including zero. Thus, consistent with previous observations (Bianchi *et al.*, 1994, 1998), inclusion of KA (100 μM) in the perfusate of the probe in the neostriatum induced a significant increase in the output of GABA from both the neostriatum and the ipsilateral SN (Fig. 2, Table 2). The magnitude of

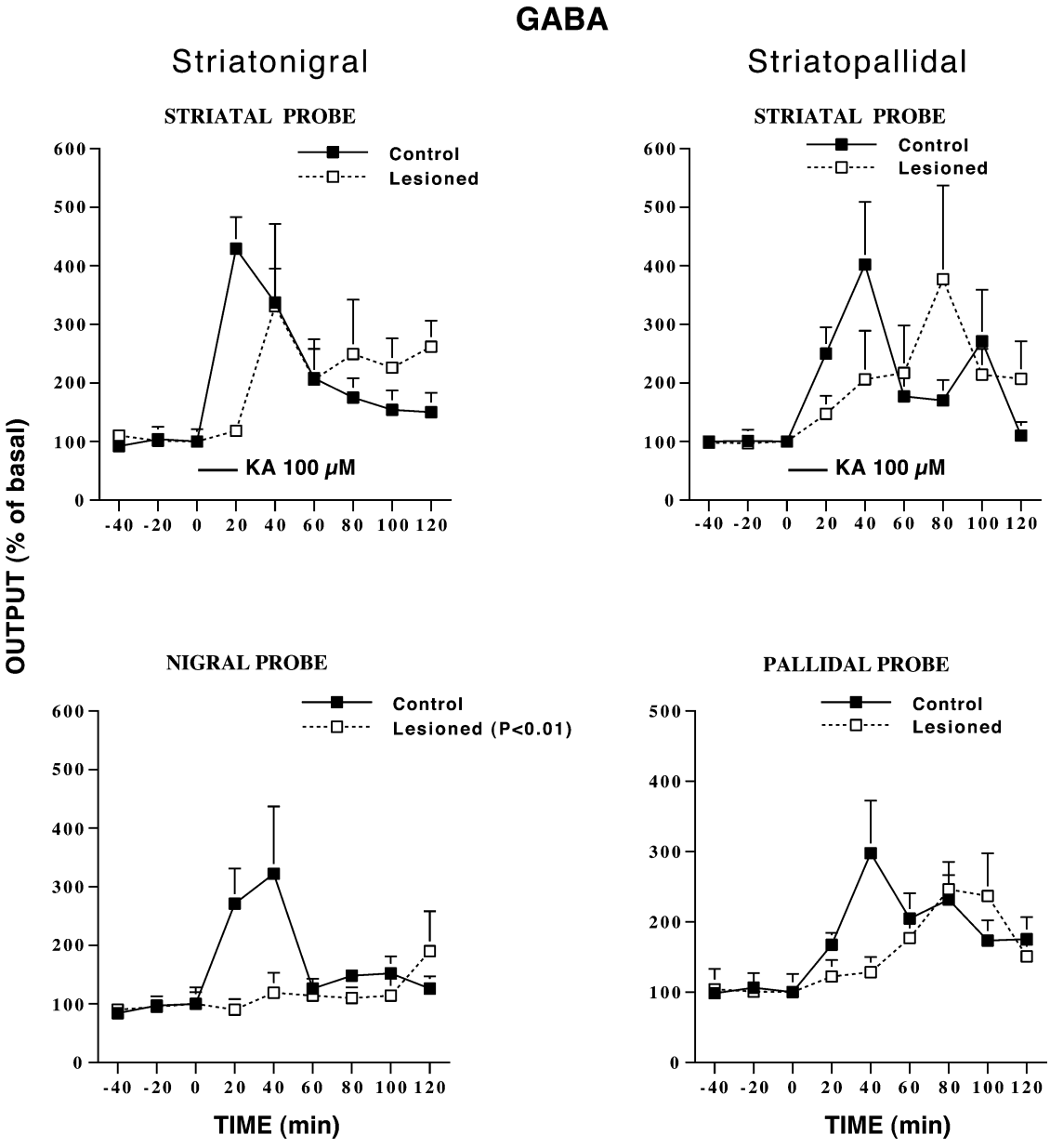

FIG. 2. Time courses of the release of endogenous GABA from the neostriatum, substantia nigra and globus pallidus in response to intrastriatal KA (100 μM) in control and 6-OHDA-lesioned rats. The panels on the left are from the striatonigral dual probe experiments and the panels on the right are from the striatopallidal dual probe experiments. Data points represent the mean concentration of GABA in each 20-min sample expressed as a percentage of the basal concentration at time 0. They are presented as the mean ±SEM of nine control and 10 lesioned rats in the striatonigral experiment and 14 control and eight lesioned rats in the striatopallidal experiment. The bar represents the time during which the KA was included in the neostriatal perfusate. *P*-value indicates statistical significance (lesioned vs. control) of net output based on analysis of variance followed by *post hoc* Fisher's LSD test (see Table 2).

the stimulated release of GABA was similar locally in the neostriatum and distally in the SN. Stimulation of the neostriatum with KA also resulted in a significant increase in the release of GABA from the pallidal probe. The results obtained with the 6-OHDA-lesioned rats showed a higher variability. The net stimulated output of GABA in the neostriatum of the lesioned animals, in both the striatonigral and striatopallidal experiments, was delayed but of the same magnitude as that observed in control animals; however, in the striatopallidal experiment net output did not reach statistical significance. In spite of the higher variability shown by this group, the analysis of variance indicated that net stimulated output of GABA in the neostriatum did not show any statistically significant difference when values from control and lesioned animals in both striatonigral and striatopallidal experiments were compared, indicating consistency between the two experiments. In contrast to this, the most prominent finding in the present study was that the stimulated output of GABA in the SN in response to KA perfusion in the neostriatum was abolished in the 6-OHDA-lesioned rats (Fig. 2, Table 2). Thus, the release of GABA was not significantly different from basal release but was highly significantly different from the release in nonlesioned rats (Table 2). There were no significant differences between the control and 6-OHDA-lesioned rats in the release of GABA in the GP following neostriatal KA stimulation.

Like the KA, addition of AMPA (100 μM) to the neostriatal perfusate caused a significant increase in the output of GABA locally in the neostriatum and a significant, but markedly delayed, increase (peak at 100 min) in the output of GABA in the distal probe in the SN (Fig. 3, Table 3). This delayed peak coincided with the presence of a late behavioural activation. In the striatopallidal experiment, there was no alteration in the release of GABA in the GP following AMPA administration to the neostriatum (data not shown). AMPA-evoked GABA release was ≈40% of that evoked by KA. The release of GABA from the neostriatum in the 6-OHDA-lesioned rats in response to neostriatal infusion of AMPA was not different from the control. However, as in the KA experiments, the distal release in SN was completely abolished in the 6-OHDA-lesioned rats (Fig. 3, Table 3). Thus, the release of GABA was not significantly different from basal release but was significantly different from the release in nonlesioned rats. The 6-OHDA lesion had no effect on the release of GABA in the GP following neostriatal AMPA stimulation (data not shown).

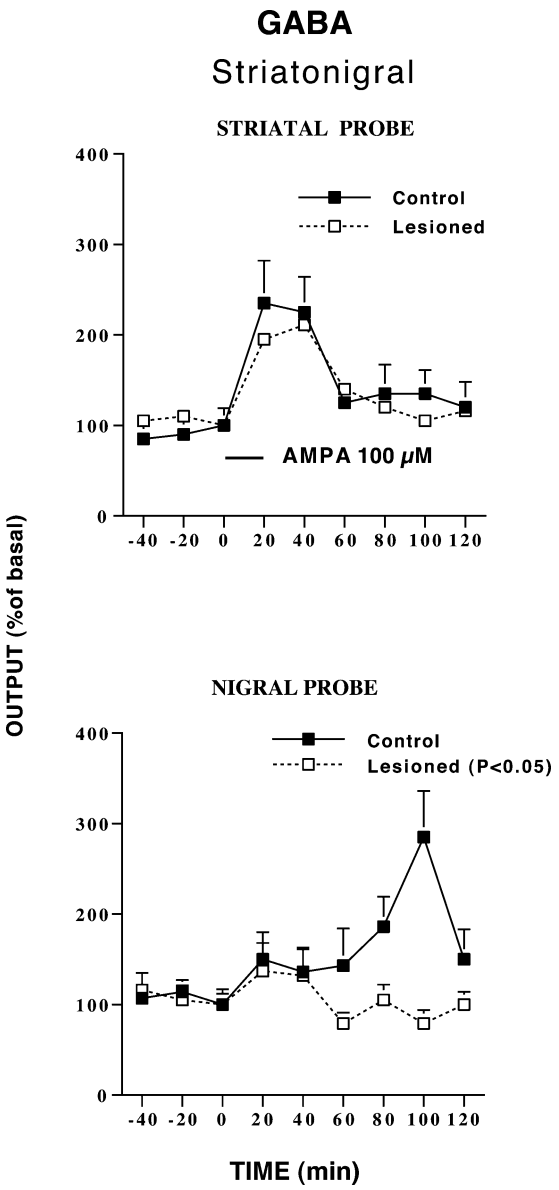

FIG. 3. Time courses of the release of endogenous GABA from the neostriatum and substantia nigra in response to intrastriatal AMPA (100 μM) in control and 6-OHDA-lesioned rats. Data points represent the mean concentration of amino acids in each 20-min sample expressed as a percentage of the basal concentration at time 0. They are presented as the mean ±SEM of seven control and 11 lesioned rats. The bar represents the time during which the AMPA was included in the neostriatal perfusate. *P*-value indicates statistical significance (lesioned vs. control) of net output based on analysis of variance followed by *post hoc* Fisher's LSD test (see Table 3).

Aspartate release

The intrastriatal application of KA induced a significant increase in the local output of aspartate from the neostriatal probe and a significant increase in the output from the distal probes in the SN, in confirmation of a previous study (Bianchi *et al*., 1998). Similarly, intrastriatal application of KA also induced a significant increase in the distal output of aspartate from the GP (Fig. 4, Table 2). In the 6-OHDA-lesioned rats, in both the striatonigral and striatopallidal experiments, the neostriatal release of aspartate in response to KA was similar in magnitude to that in the control rats but was delayed (Fig. 4). The net output from the distal probe in the SN was also not significantly different from that of the control rats. In the GP of 6-OHDA-lesioned rats the release had a similarly delayed time course to that of control animals but the magnitude of release was not significantly different from the control (Fig. 4, Table 2).

The infusion of AMPA into the neostriatal probe caused a significant increase in the release of aspartate both locally from the neostriatum and from the distal probe in the SN (Fig. 5, Table 3) which was ≈50% of that elicited by KA. There was no significant difference between the control rats and the 6-OHDA rats. In the striatopallidal experiments there was no effect of neostriatal infusion of AMPA on the distal release in the GP (data not shown).

Glutamate release

The magnitude and time course of the release of glutamate in the neostriatum and SN following KA application to the neostriatum was similar to that described previously (Bianchi *et al*., 1998). Thus, there was a significant increase in glutamate release in the neostriatum and the SN (Fig. 6, Table 2). In the 6-OHDA-lesioned animals the magnitude of release from both probes were not significantly different from those of the control rats; however, a delay was observed in the neostriatum similar to that observed for aspartate. In the striatopallidal experiments, the infusion of KA into the neostriatum caused a significant increase in the release of glutamate both locally within the neostriatum

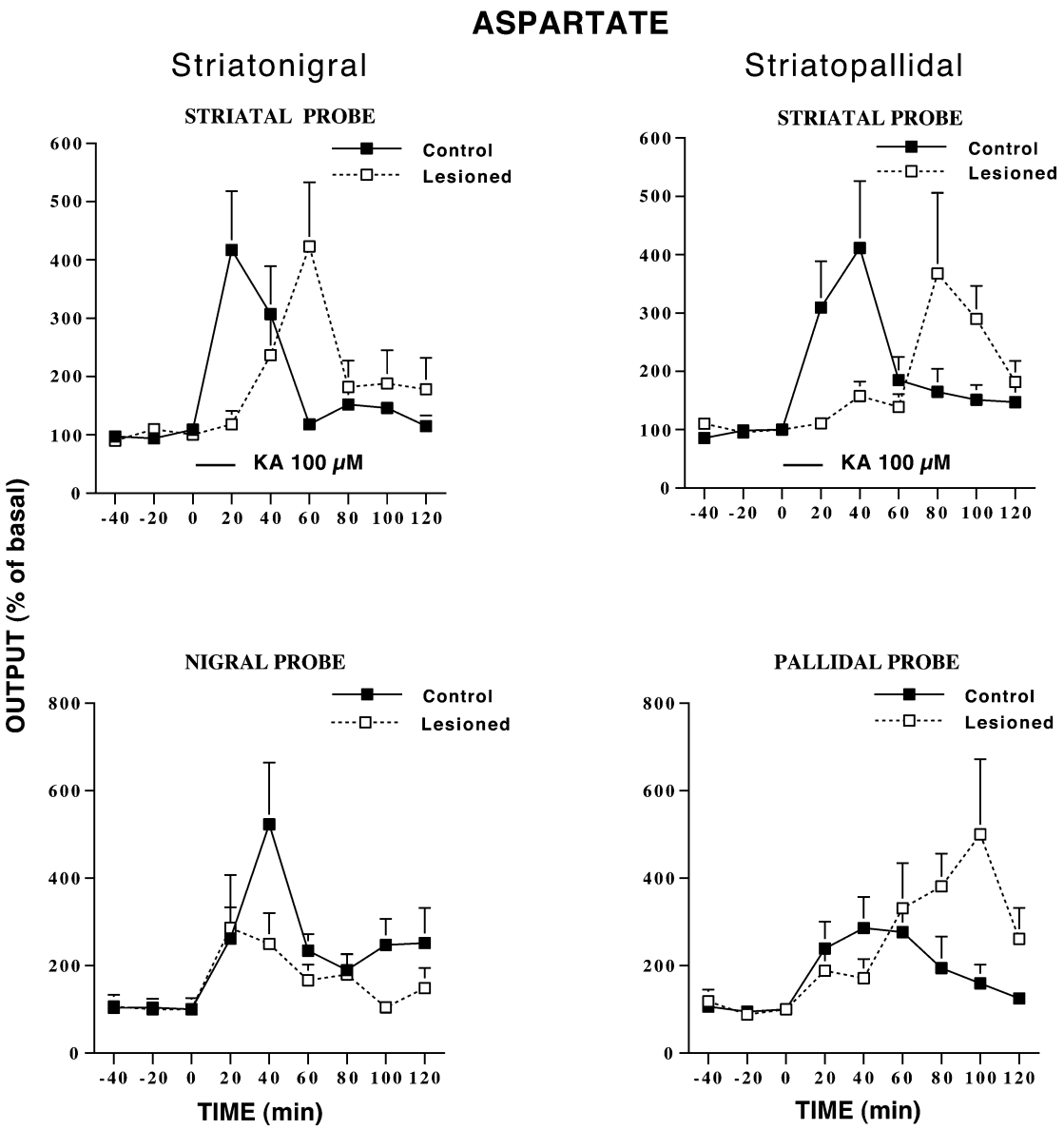

FIG. 4. Time courses of the release of endogenous aspartate from the neostriatum, substantia nigra and globus pallidus in response to intrastriatal KA (100 μM) in control and 6-OHDA-lesioned rats. The panels on the left are from the striatonigral dual probe experiments and the panels on the right are from the striatopallidal dual probe experiments. Data points represent the mean concentration of aspartate in each 20-min sample expressed as a percentage of the basal concentration at time 0. They are presented as the mean ±SEM of nine control and 10 lesioned rats in the striatonigral experiment and 14 control and eight lesioned rats in the striatopallidal experiment. The bar represents the time during which the KA was included in the neostriatal perfusate. Net output values are reported in Table 2.

and distally in the GP (Fig. 6, Table 2). In the 6-OHDA-lesioned rats the KA-induced release of glutamate in the neostriatum was again delayed but significantly greater than in control rats. The release in the GP, which also showed a delayed time course, was not significantly different in magnitude from the control rats (Fig. 6, Table 2).

The infusion of AMPA into the neostriatum caused a significant rise in the output of glutamate both in the local probe in the neostriatum and the distal probe in the SN (Fig. 5, Table 3). The responses in the 6-OHDA animals were not significantly different from those in the controls. In the striatopallidal experiments in both control and 6-OHDA-lesioned rats, there were no significant changes in the release of glutamate from the pallidal probes (data not shown).

K^+-evoked release

The local infusion of 100 mM K^+ (one fraction, 20 min) into the neostriatum, SN or GP induced a statistically significant net output of GABA, aspartate and glutamate in each of the areas examined (Table 4). Analysis of variance indicated that the K^+-evoked release of GABA in the neostriatum of the 6-OHDA rats was significantly increased in both the striatonigral (193%) and striatopallidal (225%) experiment. The 6-OHDA lesions did not affect the evoked GABA release in the SN, but induced a strong potentiation of the K^+-stimulated release of GABA in the GP (392%). The K^+-evoked release of aspartate in the 6-OHDA animals was not significantly different from control rats, but the K^+-evoked release of glutamate was significantly lower in the SN (64%).

Discussion

The present experiments were designed to address a specific hypothesis relating to the pathophysiology of the basal ganglia, namely that the imbalance in the direct and indirect pathways that has been reported to occur in Parkinson's disease and in animal models of

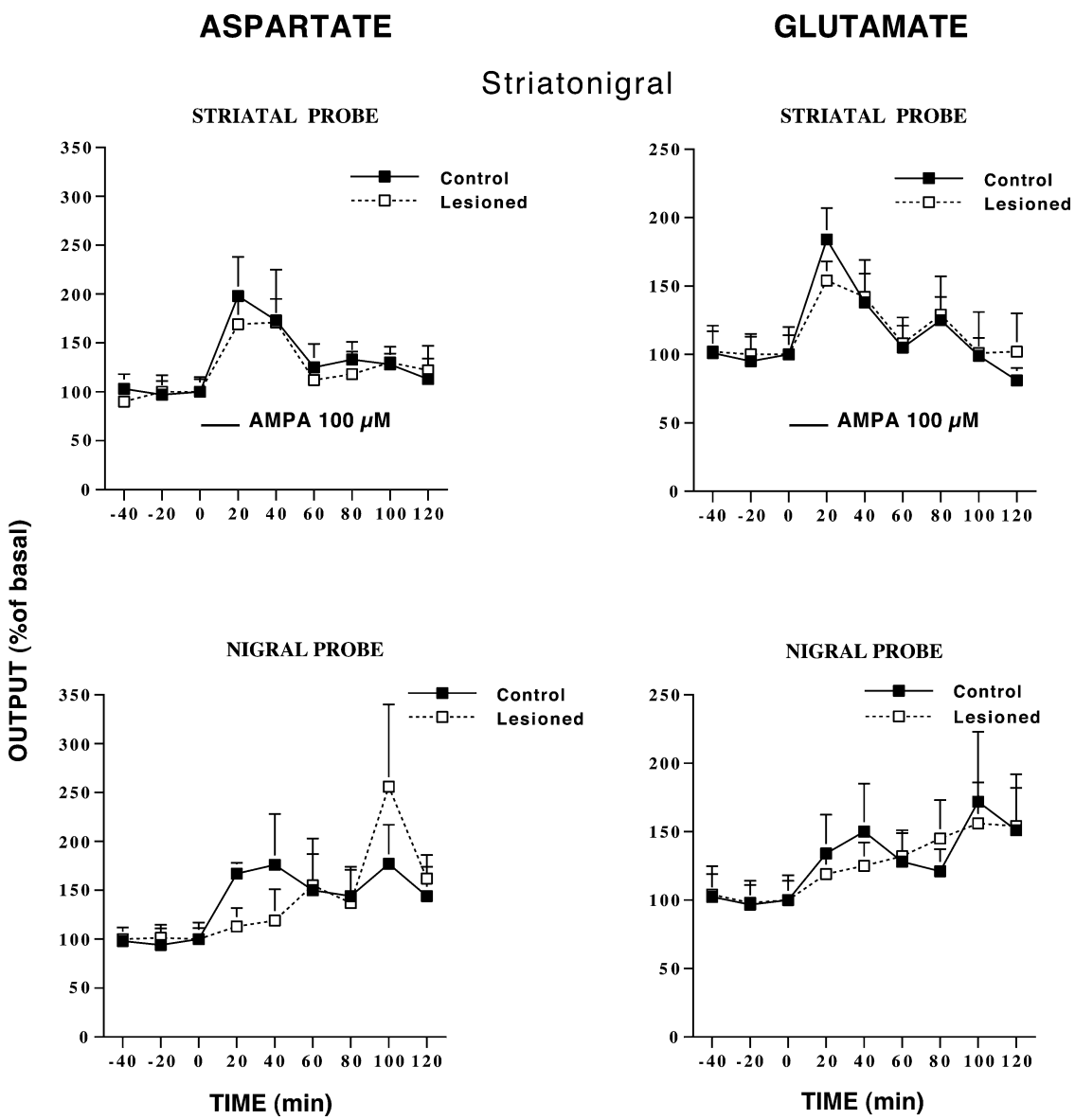

FIG. 5. Time courses of the release of endogenous aspartate (left panels) and glutamate (right panels) from the neostriatum and substantia nigra in response to intrastriatal AMPA (100 μM) in control and 6-OHDA-lesioned rats. Data points represent the mean concentration of amino acids in each 20-min sample expressed as a percentage of the basal concentration at time 0. They are presented as the mean ±SEM of seven control and 11 lesioned rats. The bar represents the time during which the AMPA was included in the neostriatal perfusate. Net output values are reported in Table 3.

Parkinson's disease, is reflected in an imbalance in the release of GABA in these pathways. To this end we performed dual probe microdialysis analyses *in vivo* to examine the release, primarily of GABA but also of other amino acids, in the direct (striatonigral) and the indirect (striatopallidal) pathways. The essential findings are that, indeed, there are imbalances in the release of GABA under both basal and stimulated conditions in the direct and indirect pathways of 6-OHDA-lesioned rats. Firstly, under basal conditions there was a significantly greater release of GABA in the GP, whereas the release was not altered in the SN (Fig. 7). Secondly, following stimulation of the two pathways by infusion of the glutamate receptor agonists, KA or AMPA, into the neostriatal microdialysis probe, the imbalance was evident as reduced release in the direct pathway (Fig. 7). Thus there were no changes in the enhanced release in the striatopallidal pathway but the enhanced release of GABA that occurs in the SN following stimulation of the neostriatum in control animals was abolished in the 6-OHDA-lesioned animals. Thirdly, GABA release in both the neostriatum and GP, but not in the SN, was much more sensitive to the local application of a high-potassium solution in the 6-OHDA-lesioned animals. Thus in these three situations the imbalance was in favour of the indirect pathway and is therefore consistent with the direct–indirect pathways model of basal ganglia function and dysfunction. Many studies of Parkinson's disease and animal models of Parkinson's disease have reported imbalances in the levels of peptides, neurotransmitter markers, receptors and metabolic activity that are consistent with a down-regulation of the direct pathway and up-regulation of the indirect pathway (for reviews see Gerfen, 2000; Hirsch *et al*., 2000; Obeso *et al*., 2000). Furthermore, changes in the physiological activity in the indirect pathway consistent with increased activity (and altered patterns of activity) have been reported to be associated with the loss of dopamine (for reviews see Wichmann & DeLong, 1996; Bergman *et al*., 1998). The present study now demonstrates alterations in the

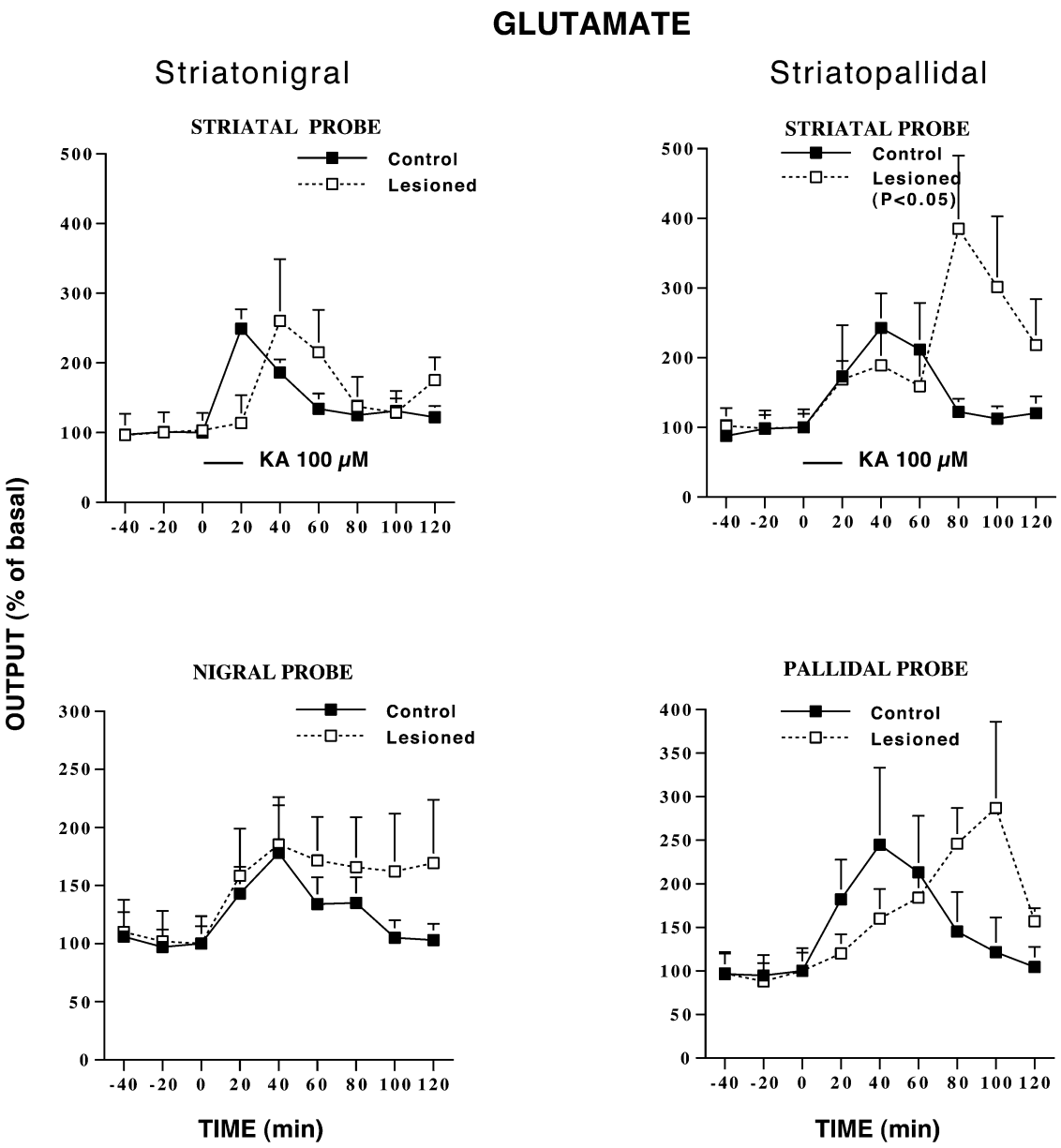

FIG. 6. Time courses of the release of endogenous glutamate from the neostriatum, substantia nigra and globus pallidus in response to intrastriatal KA (100 μM) in control and 6-OHDA-lesioned rats. The panels on the left are from the striatonigral dual probe experiments and the panels on the right are from the striatopallidal dual probe experiments. Data points represent the mean concentration of glutamate in each 20-min sample expressed as a percentage of the basal concentration at time 0. They are presented as the mean ±SEM of nine control and 10 lesioned rats in the striatonigral experiment and 14 control and eight lesioned rats in the striatopallidal experiment. The bar represents the time during which the kainic acid was included in the neostriatal perfusate. *P*-value of lesioned indicates a statistically significantly higher net output than control by *post hoc* Fisher's LSD test following analysis of variance (see Table 2).

release of GABA in the two pathways that are consistent with these findings.

Basal release of GABA in the direct and indirect pathways

The present experiments were carried out on 'freely moving', behaving rats. One would predict that during normal behaviour, the direct and indirect pathways would be functioning appropriately and playing their roles in the control of movement and behaviour in general. Thus the 'basal release' in these experiments, unlike that in anaesthetized animals, is a reflection of ongoing activity in the striatofugal pathways. In this situation we observed an increase in the basal release of GABA in the GP in the 6-OHDA-treated rats, which presumably reflects the overactivity which is predicted by the direct–indirect pathway model (Fig. 7). Similar findings have been made by others in behaving (Robertson *et al*., 1991; Ochi *et al*., 2000) but not anaesthetized (Segovia *et al*., 1986; Tossman *et al*., 1986) animals. In contrast to the observations in the striatopallidal experiments, the basal release of GABA in the SN was not altered in the 6-OHDA-lesioned animals. This finding is consistent with some studies in anaesthetized and freely moving animals (Lindefors *et al*., 1989; Sarre *et al*., 2001) but contradicts another, where increased release of GABA was observed in the SN in anaesthetized rats (You *et al*., 1994), although it is not clear what the contribution of the direct pathway is to the basal release of GABA in the SN (Bianchi *et al*., 1994, 1998). It should be remembered, however, that the source of the GABA in the GP and in the SN is not solely neostriatal neurons giving rise to the indirect or direct pathways. Thus in the GP, in addition to terminals derived from neostriatal neurons of the indirect pathway, the local collaterals of GP

TABLE 4. The effect of local application of K^+ (100 mM) on the net output of GABA, aspartate and glutamate from the neostriatum, substantia nigra and globus pallidus in control and 6-OHDA-lesioned rats

	Striatonigral pathway				Striatopallidal pathway			
	Neostriatum		Substantia nigra		Neostriatum		Globus pallidus	
Amino acid	Control	Lesion	Control	Lesion	Control	Lesion	Control	Lesion
GABA								
Mean ± SEM	247 ± 64	476 ± 34**	617 ± 114	506 ± 72	183 ± 29	412 ± 73**	214 ± 35	839 ± 179**
(*n*)	(15)	(16)	(12)	(15)	(16)	(8)	(21)	(9)
Aspartate								
Mean ± SEM	214 ± 62	218 ± 60	242 ± 53	186 ± 30	137 ± 37	92 ± 21	183 ± 31	148 ± 29
(*n*)	(12)	(17)	(10)	(15)	(11)	(7)	(16)	(7)
Glutamate								
Mean ± SEM	285 ± 52	266 ± 49	493 ± 108	309 ± 75**	207 ± 40	164 ± 33	228 ± 57	196 ± 46
(*n*)	(13)	(15)	(11)	(17)	(15)	(8)	(18)	(8)

Data are the mean net output ±SEM, i.e. the area obtained from the area under the K^+-stimulated concentration–time curve, normalized to one time interval of 20 min, with basal output subtracted (concentration in the fraction preceding K^+ stimulation). Concentration values were fmol/μL of perfusate (nM). $^{**}P < 0.01$ (lesion vs. control) as obtained by *post hoc* Fisher's LSD test (MANOVA: GABA Treatment $F_{1,35} = 46.551$, $P < 0.0001$, Brain area $F_{3,105} = 10.907$, $P < 0.0001$ and Interaction $F_{3,105} = 15{,}238$, $P < 0.0001$; glutamate Treatment $F_{1,33} = 4.104$, $P < 0.05$, Brain area $F_{3,99} = 8.20$, $P < 0.0001$). *n* refers to the number of animals where local K^+ stimulation could be performed and successfully analysed at the end of each experiment.

neurons themselves will contribute to the detected GABA. Furthermore, all neostriatal neurons which give rise to projections to basal ganglia output nuclei, i.e. neurons of the direct pathway, also give rise to collaterals to the GP (Kawaguchi *et al*., 1990; Wu *et al*., 2000). Under basal conditions it may, in fact, be that the major source of GABA is the collaterals of pallidal neurons as they have a high resting discharge rate and, in Parkinson's disease and in animal models of Parkinson's disease, their activity is increased (for references see Bevan *et al*., 2002). Similarly, the sources of GABA in the SN include the terminals of neostriatal neurons constituting the direct pathway, terminals of neurons of the GP and collaterals of GABAergic output neurons of the SNr. It is difficult to judge the relative contribution of each set of terminals, although it is likely that under basal conditions, despite providing the majority of terminals in the SN, neostriatal terminals are not the major source of GABA because of their low resting discharge rate. The levels of GABA detected in the microdialysates are likely to reflect the release from pallidonigral terminals and from the collaterals of SNr neurons as both classes of neurons have a high resting discharge rate. In the 6-OHDA-lesioned animals the presumably enhanced release from pallidonigral terminals is likely to

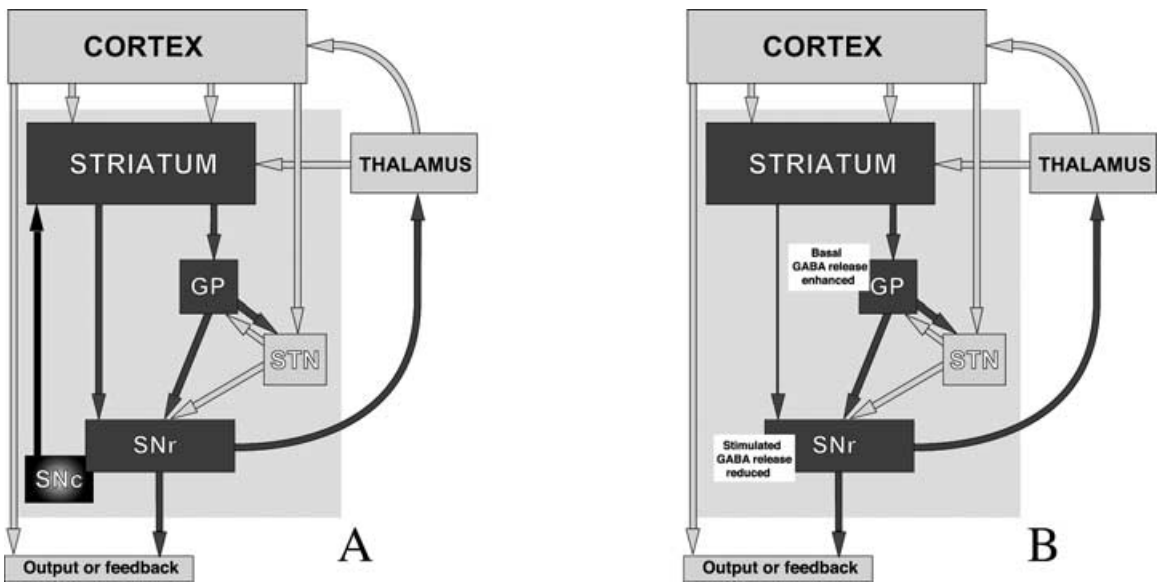

FIG. 7. Simplified block diagram of basal ganglia circuitry. (A) Simplified block diagram of basal ganglia (within grey box) illustrating the major GABAergic (dark boxes and arrows) and glutamatergic (light boxes and arrows) connections. The projection from the neostriatum (striatum) to the substantia nigra constitutes the direct pathway. The projection from the neostriatum to the substantia nigra which includes the globus pallidus and the subthalamic nucleus constitutes the indirect pathway. The major sources of GABA collected in the striatal probes are the local collaterals of striatonigral and striatopallidal neurons and local GABAergic interneurons. Glutamate and aspartate are likely to be derived from the corticostriatal and the thalamostriatal afferents. The major source of GABA in the substantia nigra and globus pallidus is likely to be the terminals of striatal projection neurons and the local axon collaterals of nigral and pallidal neurons, respectively. The major source of glutamate/aspartate is likely to be the terminals of neurons of the subthalamic nucleus. (B) Following lesion of the dopaminergic nigrostriatal pathway there is a marked reduction in the release of GABA in the substantia nigra following stimulation of the neostriatum. Although stimulated release of GABA in the globus pallidus was unaltered in lesioned animals there was an enhanced basal release of GABA. GP, globus pallidus; SNr, substantia nigra *pars reticulata*; STN, subthalamic nucleus.

reduce the release from the collaterals of SNr neurons; the net effect in these animals was no change in levels of GABA detected.

Stimulated release of GABA in the direct and indirect pathways

In contrast to the basal release, in which there is likely to be minimal stimulation of striatofugal pathways, the infusion of KA into the neostriatum is likely to lead to a massive stimulation of striatofugal pathways. In this situation there was an enhanced local release of amino acids in the neostriatum and enhanced release in the distal probes located in the SN or GP. Under these conditions it is likely that, because of the massive activation of striatofugal neurons and their large number of terminals in their target regions, the major source of released GABA was the terminals of neostriatal neurons giving rise to the direct and indirect pathway, respectively. These findings are consistent with our previous studies in which we demonstrated the dependence of both local and distal release of amino acids on receptor activation in the neostriatum and the propagation of action potentials (Bianchi *et al*., 1994, 1998), and are consistent with the known anatomy of the basal ganglia (see Smith *et al*., 1998).

In the 6-OHDA-lesioned rats the KA-enhanced release of amino acids was largely unaltered in the neostriatum and GP but both the KA- and AMPA-enhanced release of GABA in the SN was abolished (Fig. 7B). The abolition of the release of GABA in the distal probe in the SN following the 6-OHDA lesions was unlikely to be due to a nonspecific reduced responsiveness of striatonigral neurons as enhanced release of GABA still occurred in response to glutamate receptor stimulation within the neostriatum after the 6-OHDA lesions. However, local stimulation of terminals in the SN (which would include the terminals of neostriatal neurons) with a high concentration of potassium ions still produced a profound release of GABA in the SN. Furthermore, in 6-OHDA-lesioned rats, the striatonigral pathway is known to be intact as electrical stimulation of the pathway *in vivo* leads to inhibition of neurons in the SNr (MacLeod *et al*., 1990). Our findings suggest therefore that following depletion of dopamine there is an altered responsiveness of neostriatal neurons of the direct pathway to AMPA/KA receptor stimulation. The failure of neostriatal stimulation to elicit release of GABA in the SN following 6-OHDA lesions may of course be, in part, a consequence of the reduced levels of dopamine locally within the SN itself.

Potassium-stimulated release of GABA

Elevation of the extracellular concentration of potassium ions has been the most frequently used depolarizing method for the local stimulation of neurotransmitter release from *in vitro* and *in vivo* brain preparations. The information provided by analysis of released amino acids following the local application of K^+, however, is limited to the generalized level of excitability of the exposed tissue. This is a rather nonselective stimulation, which simply indicates that depolarization of neural structures, glial as well as neuronal, can result in release of neurotransmitter, but tells us little about the nature and origin of the release (Di Chiara, 1990; Bernath, 1991).

In the present study potassium stimulation was mainly used as a control at the end of each experiment to ensure that transmitter release mechanisms were intact (Bianchi *et al*., 1998). This was particularly important for the striatonigral experiments, when the 6-OHDA lesion abolished the distal amino acid release in the SN evoked by intrastriatal application of KA. In these cases, as pointed out above, the ability of local K^+ stimulation to induce GABA release in the SN acted as a control for the specificity of the effects of the 6-OHDA lesions. In the event, the local K^+ stimulation provided additional information on differences in excitability induced by the 6-OHDA lesion. In agreement with previous data a higher sensitivity to local K^+ stimulation of GABA release was observed in both neostriatum (Tossman *et al*., 1986; Lindefors *et al*., 1989; Campbell *et al*., 1993) and GP (Segovia *et al*., 1986; Robertson *et al*., 1991). This, together with the new findings of a lack of change of GABA release and a lower sensitivity of glutamate release to K^+ stimulation in the SN, is all consistent with the direct–indirect basal ganglia model.

Release characteristics of aspartate and glutamate

As glutamate is the neurotransmitter of the neurons of the subthalamic nucleus, which are critical components of the indirect pathway, and of excitatory afferents from the cortex and thalamus to the neostriatum and other regions of the basal ganglia, we also examined the release of this amino acid and aspartate in these experiments. Consistent with previous data (Bianchi *et al*., 1998), KA stimulation evoked a release of glutamate and of aspartate locally in the neostriatum and in distal probes in the SN and GP. Similarly, the present data provide the first demonstration of AMPA-evoked release of endogenous glutamate and aspartate locally in the neostriatum and distally in the SN, although Patel *et al*. (2001) have reported AMPA-evoked release of preloaded radiolabelled glutamate in the neostriatum. Both KA- and AMPA-evoked release of glutamate and aspartate were largely unaffected by the 6-OHDA lesion, as was, in agreement with Abarca & Bustos (1999), the basal release of glutamate. However, variable and time dependent effects on basal glutamate levels following 6-OHDA treatment in the neostriatum have been found by others (Tossman *et al*., 1986; Reid *et al*., 1990; Marti *et al*., 1999; Meshul *et al*., 1999). The observation of an enhanced basal level of aspartate, but not glutamate, in the SN in 6-OHDA-lesioned rats is in agreement with the finding of a greater increase of aspartate release than glutamate in the EP of 6-OHDA-lesioned behaving rats (Biggs & Starr, 1997; Biggs *et al*., 1997). In contrast, a decreased responsiveness to high potassium stimulation in the SN of 6-OHDA-lesioned rats was observed for glutamate but not aspartate. The unknown source of aspartate and the fact that some of these differences were not reflected by the levels of glutamate, or *vice versa*, makes it difficult to interpret these observations. Despite these difficulties in interpretation, the findings with glutamate and aspartate act as good controls for the altered release of GABA in the SN following the 6-OHDA lesions. Thus, the abolition of GABA release in the SN in the 6-OHDA animals following stimulation of the neostriatum was an effect selective for GABA, as the enhanced release of glutamate and aspartate were not altered by the lesion.

Functional implications

The implication of our findings in the 6-OHDA-lesioned animals is that, under conditions of minimal stimulation of striatofugal pathways (i.e. basal release), the striatopallidal pathway is in some way upregulated and, following maximal stimulation of striatofugal pathways, the striatonigral pathway is downregulated. It may, of course, be that in the basal conditions neurons of the direct pathway are also downregulated but that their contribution to the detectable GABA in the SN is only minimal under these conditions (see above). These findings are thus consistent with the direct–indirect pathways model of basal ganglia physiology and pathophysiology (Albin *et al*., 1989; DeLong, 1990). Thus reduced activity, or downregulation, of the direct pathway and increased activity or upregulation of the indirect pathway are likely to have a similar behavioural effect, i.e. reduced motor activity. Furthermore, the decreased local responsiveness of the release of glutamate to high potassium stimulation observed in the SN of 6-OHDA-lesioned rats, on the other hand, is in keeping with the reported down-regulation of glutamate receptors in EP/SNr as a consequence of hyperactivity of subthalamo-nigral pathway (Difazio *et al*., 1992; Porter *et al*., 1994; Wullner *et al*., 1994).

The molecular mechanisms underlying these changes are at present unclear, although it is clear that dopamine receptor stimulation, and hence depletion of dopamine by 6-OHDA, can alter the expression, trafficking and responsiveness of excitatory amino acid receptors. Thus dopamine receptor stimulation alters the state of phosphorylation, and hence function, of subunits of the *N*-methyl-D-aspartate (NMDA) and AMPA receptors (Snyder *et al*., 2000; Dunah & Standaert, 2001) and 6-OHDA lesions have been shown to lead to an upregulation (Samuel *et al*., 1990; Ulas *et al*., 1994; Wullner *et al*., 1994; Tremblay *et al*., 1995; Ganguly & Keefe, 2001), altered subunit composition and trafficking (Dunah *et al*., 2000), or increased responsiveness of NMDA receptors (Marti *et al*., 1999, 2000) in the neostriatum. The same lesions have also been variously reported to have no effect on the level of mRNAs for AMPA receptor subunits (Bernard *et al*., 1996) and AMPA binding (Errami & Nieoullon, 1988; Porter *et al*., 1994), an upregulation of GluR1 and GluR2 mRNA (Tremblay *et al*., 1995) and GluR1 protein (Betarbet *et al*., 2000) or a transient decrease in AMPA and KA binding (Wullner *et al*., 1994). Thus although the mechanism of altered responsiveness to KA/AMPA receptor stimulation in 6-OHDA-lesioned rats remains to be established, it presumably relates to the loss of the modulatory effect of dopamine receptors on excitatory amino acid transmission in the neostriatum and the differential effect on the direct and indirect pathways presumably relate to the differential distribution of dopamine receptors.

In conclusion, the findings of the present study demonstrate that, in the unilateral 6-OHDA model of Parkinson's disease in the rat, consistent with the direct–indirect pathways model of basal ganglia, there is an imbalance in the release of GABA in favour of the indirect pathway both in basal conditions and when the striatofugal pathways are stimulated. These imbalances in GABA release may, in part, underlie the motor abnormalities in Parkinson's disease and in animal models of Parkinson's disease.

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Abbreviations

6-OHDA, 6-hydroxydopamine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole; CI, confidence interval; EP, entopeduncular nucleus; GABA, γ-aminobutyric acid; GP, globus pallidus; KA, kainic acid; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline (0.01 M pH 7.4); SN, substantia nigra; SNr, substantia nigra *pars reticulata*; STN, subthalamic nucleus; TH, tyrosine hydroxylase.

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