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COMPLEX MOTOR DISTURBANCES IN A SEQUENTIAL DOUBLE LESION RAT MODEL OF STRIATONIGRAL DEGENERATION (MULTIPLE SYSTEM ATROPHY)

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Abstract—This study characterizes paw reaching, stepping and balance abnormalities in a double lesion rat model of striatonigral degeneration, the core pathology underlying levodopa unresponsive parkinsonism associated with multiple system atrophy. Extensive unilateral nigral or striatal lesions induced by 6-hydroxydopamine or quinolinic acid, respectively, produced a similarly marked contralateral paw reaching deficit without further deterioration following a secondary (complementary) lesion of ipsilateral striatum or substantia nigra. Contralateral stepping rates were reduced by unilateral 6-hydroxydopamine lesions without further deterioration following the secondary striatal lesion. In contrast, initial unilateral striatal quinolinic acid injections induced bilateral stepping deficits that significantly worsened contralaterally following the secondary nigral lesion. Contralateral sidefalling rates were significantly increased following primary nigral and striatal lesions. Secondary nigral but not secondary striatal lesions worsened contralateral sidefalling rates. Histological studies revealed subtotal (>90%) depletion of dopaminergic neurons in substantia nigra pars compacta and variable degrees of striatal degeneration depending on the lesion sequence. Animals prelesioned with 6-hydroxydopamine showed significantly larger residual striatal surface areas following the secondary striatal quinolinic acid lesion compared to animals with primary striatal quinolinic acid lesions ($P < 0.001$). These findings are in line with previous experimental studies demonstrating that striatal dopamine depletion confers neuroprotection against subsequent excitotoxic injury. Whether loss of dopaminergic neurons protects against the striatal disease process occurring in multiple system atrophy (Parkinson-type) remains to be elucidated.

In summary, this is the first experimental study to investigate spontaneous motor behaviour in a unilateral double lesion rat model. Our observations are consistent with a complex interaction of nigral and striatal lesions producing distinct behavioural and histological changes depending on the lesion sequence. Tests of forelimb akinesia and complex motor behaviour appear to provide a reliable tool that will be helpful for monitoring the effects of interventional strategies such as embryonic neuronal transplantation in the rat model of striatonigral degeneration. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: quinolinic acid, 6-hydroxydopamine, staircase test, balance test, stepping test, neuroprotection.

L-Dopa unresponsive Parkinsonism is widely considered the predominant motor disorder of multiple system atrophy (MSA).[52] The neuropathological changes underlying MSA-associated parkinsonism (MSA-P) are dominated by neuronal loss in anatomically related nigral and striatal pathways including afferent projections arising from the caudolateral substantia nigra pars compacta (SNc) and efferent striatopallidal projections originating in the dorsolateral posterior putamen. This characteristic pattern of neuronal degeneration was first referred to as striatonigral degeneration (SND) in 1960[65] and subsequently shown to affect anatomically related nigral and striatal projection neurons.[14,22,23,27,30,62] The pathogenesis of SND remains unsettled with conflicting evidence suggesting nigral or striatal disease onset.[62] In MSA-P a range of additional CNS regions appears to be affected by degenerative nerve cell loss, in particular spinal cord, brainstem and cerebellum.[71] This characteristic neuronal multiple system degeneration probably accounts for the frequent emergence of additional non-parkinsonian features in patients with advancing MSA-P, such as orthostatic hypotension, urinary incontinence, cerebellar ataxia and pyramidal signs.[71] In contrast to Parkinson's disease (PD) the majority of MSA-P patients exhibits a poor response to L-Dopa replacement therapy.[25] Consistent with this observation, progression of parkinsonian disability appears to be considerably faster in patients with MSA-P compared to PD, accounting for poorer survival.[4,68] Non-parkinsonian neurological deficits, if present, may also aggravate the disease course in patients with MSA-P, particularly in later stages of the disease.[4] Novel therapeutic strategies such as embryonic neuronal transplantation or neuroprotective approaches are urgently needed for patients with MSA-P.[67] Unfortunately, experimental models to address their safety and efficacy have been lacking until recently. Principally, stereotaxic injections of nigral (6-hydroxydopamine; 6-OHDA) and striatal (excitatory amino acid) neurotoxins may be employed to model the pathology of SND that underlies MSA-P.[51,69] Up to now, few studies attempted to combine nigral and striatal lesions assessing the resulting motor deficit by drug-induced circling behaviour.[2,17,35,37] Striatal lesions mediated by electrocoagulation,[35,37] radiofrequency[17] or excitotoxic insult[2]

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Abbreviations: AChE, acetylcholinesterase; DAB, diaminobenzidine tetrahydrochloride; GCI, glial cytoplasmic inclusion; GFAP, glial fibrillary acidic protein; HD, Huntington's disease; HE, hematoxylin–eosin; MFB, medial forebrain bundle; MSA, multiple system atrophy; MSA-P, MSA-Parkinson subtype; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; QA, quinolinic acid; SND, striatonigral degeneration; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

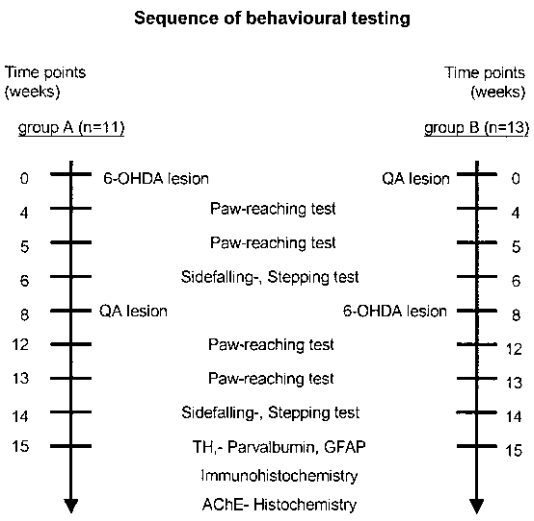

Fig. 1. Flow chart of stereotaxic surgery, behavioural testing and histological analysis.

were reported to diminish apomorphine-induced contralateral rotations in dopamine-depleted rats. Amphetamine rotation rates were either unaffected or reduced in animals with extensive damage extending to ventral striatum.[2] Recently, our group established a novel double lesion rat model of MSA-P by a sequential unilateral injection of 6-OHDA into the medial forebrain bundle and of quinolinic acid (QA) into the ipsilateral striatum.[69,70] Contralateral apomorphine-induced rotation rates were significantly reduced in 6-OHDA prelesioned rats following the secondary striatal QA injection. In contrast, amphetamine-induced rotation was not significantly altered by the secondary striatal lesion.

However, drug-induced rotational behaviour in dopamine depleted rats represents a poor measure of parkinsonian symptomatology.[36,44,58] Although robust and easy to quantify, drug-induced rotation behaviour is a functional test that has an unclear relation to the symptomatology of human PD. The most relevant aspect of dopamine agonist-induced turning is probably that it provides a behavioural measure of striatal dopamine receptor supersensitivity and agonist-induced dyskinesia in the animal model.

Recently, a number of additional tests has been established to assess changes of spontaneous, i.e. non-drug-induced, motor behaviour in the unilateral PD model.

Tests of sensorimotor orientation[13] and so-called disengaged behaviour[55] are commonly used to assess complex motor responses to external stimuli—but they do not specifically test aspects of limb function. Skilled-paw reaching can be evaluated separately for either forelimb using the staircase test.[39] Schallert *et al*. introduced the stepping test, which is intended to pick up initiation deficits in the forelimbs, analogous to limb bradykinesia and start hesitation in parkinsonian patients.[54] Low-dose dopamine agonists as well as embryonic dopamine-rich grafts appear to be able to reverse contralateral stepping deficits in rats with unilateral 6-OHDA lesions.[44] Based on the stepping test manoeuvre, a balance test has been developed to assess balance adjustments to postural challenge in rodents.[74]

In the present experiment, we evaluated spontaneous motor behaviour such as paw reaching, stepping and sidefalling[39,44,74] in the double lesion MSA-P rat model. Since the disease process may be initiated at either the nigral or striatal level we compared two experimental paradigms with initial nigral or striatal lesions followed by complementary striatal and nigral lesions (nigrostriatal versus striatonigral lesion sequence).

EXPERIMENTAL PROCEDURES

Experimental design

The experiment comprised two groups of rats. Group A ($n = 11$) animals received initial unilateral nigral lesions followed by ipsilateral striatal lesions. Group B ($n = 13$) animals received initial unilateral striatal lesions followed by ipsilateral nigral lesions. Ten age- and sex-matched unlesioned rats served as control group for the behavioural paradigms. The sequence of lesion and behavioural testing is given in Fig. 1.

Animals and surgical procedure

A total of 34 male Wistar rats (Harlan Winkelmann, Germany), weighing 200–250 g at the beginning of the experiment, was used. The rats were maintained in a temperature- and humidity-controlled environment under a 12 h light/dark cycle with free access to food and water when not in experimental sessions. The following *in vivo* protocols were approved by the Federal Ministry of Science and Transport of Austria. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

To model nigral and striatal degeneration corresponding to human SND the animals received stereotaxic injections of either 5.5 μl 6-OHDA hydrobromide (3.6 μg/μl in 0.2 mg/ml L-ascorbate-saline; Sigma) into the left medial forebrain bundle (MFB) (group A, $n = 11$) or of 150 nmol QA (Sigma) dissolved in 0.01 M phosphate-buffered saline (PBS) with NaOH adjusted to pH 7.4) into left dorsolateral striatum (group B, $n = 13$). Anatomical coordinates were as follows (nose bar set at 3.3 mm below the intra-aural line). 6-OHDA injection: anterior = −4.52 mm, lateral = 1.4 mm, ventral = −8.4 mm, anterior = −4.16 mm, lateral = 1.8 mm, ventral = −8.4 mm; QA injection: anterior = 1.6 mm, lateral = 3.2 mm, ventral = −4.5 mm; anterior = 0.48 mm, lateral = 3.5 mm, ventral = −4.5 mm.[46] The rats were anaesthetized using halothane 2% (Halothan Hoechst, Z. Nr.: 11.812) and placed in a David Kopf small animal stereotaxic frame. The injections were made using a 21-gauge steel cannula connected to a 10 μl Hamilton syringe (6-OHDA injection) or a 5 μl Hamilton syringe (QA injection). 6-OHDA injections were delivered over 3 min and the cannula was left *in situ* for 5 min before being slowly retracted. QA was administrated over 1 min. The cannula remained in place for further 5 min to allow diffusion of the toxin, before being slowly withdrawn. The wound was then cleaned and sutured, and the rat allowed to recover. In order to achieve a complete lesion of nigral and striatal neurons, a complementary lesion of ipsilateral striatum (group A) or MFB (group B) was added eight weeks after the first lesion using the protocol outlined above.

Stepping test

Forelimb akinesia was assessed six weeks following lesion placement using a modified stepping test protocol.[44] In brief, the animals were held by the experimenter with one hand fixing the hindlimbs and slightly raising the hind part above the surface. One paw was touching the table, and was then moved slowly sideways (5 s for 1 m), first in the forehand and then in the backhand direction. The number of adjusting steps was counted for both paws in the backhand and forehand direction of movement. The sequence of testing was right paw forehand and backhand adjusting stepping, followed by left paw forehand and backhand directions. The test was repeated three times on three consecutive days, after an initial training period of three days prior to the first testing. Forehand adjusted stepping revealed no consistent differences between lesioned and healthy control animals (unpublished data). Analysis was therefore restricted to backhand adjusted stepping.

Balance test

Balance adjustments following postural challenge were also measured during the stepping test sessions.[74] The rats were held in the same position as described in the stepping test and, instead of

being moved sideways, tilted by the experimenter towards the side of the paw touching the table. This manoeuvre resulted in loss of balance and the ability of the rats to regain balance by forelimb movements was scored on a scale ranging from 0 to 3. Score 0 was given for a normal forelimb placement. When the forelimb movement was delayed but recovery of postural balance detected, score 1 was given. Score 2 represented a clear, yet insufficient, forelimb reaction, as evidenced by muscle contraction, but lack of success in recovering balance, and score 3 was given for no reaction of movement. The test was repeated three times a day on each side for three consecutive days after an initial training period of three days prior to the first testing.

Staircase test (paw reaching)

A modified version of the staircase test was used for evaluation of paw reaching behaviour four weeks following primary and secondary lesion placement.[39] Plexiglass test boxes (Dr E. Torres, MRC Cambridge Centre for Brain Repair) with a central platform and a removable staircase on each side were used. The apparatus is designed such that only the paw on the same side at each staircase can be used, thus providing a measure of independent forelimb use. For each test the animals were left in the test boxes for 15 min. The double staircase was filled with 7×2 chow pellets (Noyes precision food pellets, formula: P, purified rodent diet, size 45 mg; Sandown Scientific) on each side. After each test the number of pellets eaten (successfully retrieved pellets) and the number of pellets taken (touched but dropped) for each paw and the success rate (pellets eaten/pellets taken) were counted separately. After three days of food deprivation (12 g per animal per day) the animals were tested for 11 days. Full analysis was conducted only for the last five days.

Tissue fixation

At the completion of behavioural experiments, seven weeks following the second lesion, all animals were anaesthetized with 3 ml thiopental (1 g/40 ml a.d., Tyrol Pharma). The rats were perfused transcardially with 0.01 M PBS (pH = 7.4) for 2 min, followed by 4% paraformaldehyde (Merck) in PBS for 15 min. The brains were removed and placed in 4% paraformaldehyde for 24 h at 4°C. For dehydration they were then transferred to a 25% sucrose (Merck) solution in 0.1 M PBS at 4°C until they sank. The brains were frozen in methylbutan at −30°C for 2 min and stored at −80°C. Using a sledge microtome (mod. 2700-Frigocut, Reichert-Jung), 36 μm sections were taken from the genu of the corpus callosum (AP 1.7 mm) to the hippocampus (AP −1.8 mm) and from AP −4.16 to AP −6.72.[46] Sections were cut and stored in assorters in 0.25 M Tris buffer (pH 7.4) for immunohistochemistry. Sections for hematoxylin–eosin (HE) and acetylcholinesterase (AChE) stain were directly mounted on to gelatin-coated microscope slides.

Every eighth section from each series was taken and stained for Cresyl Violet, AChE, tyrosine hydroxylase (TH), parvalbumin and glial fibrillary acidic protein (GFAP) (see below). Nissl staining of cell bodies with Cresyl Violet was performed using a standard protocol.

Acetylcholinesterase

Activity of AChE enzyme was visualized according to Geneser-Jensen and Blackstad.[18] A series of sections of the striatum (1:8) was mounted on to gelatin-coated microscope slides. They were incubated overnight in Jensen–Blackstad AChE-medium at 4°C. After washing for 10 min with distilled water they were incubated for 10 min in 10% KFeCN at room temperature. The sections were further washed for 10 min in distilled water and differentiated in ascending alcohols, cleared in butylacetate and coverslipped with entellan.

Immunohistochemistry

A series of sections was processed for either free-floating TH, parvalbumin or GFAP immunohistochemistry. Following three rinses in 0.1 M PBS, endogenous peroxidase activity was quenched for 10 min in 0.3% H_2O_2–PBS. After rinsing in PBS, sections were pre-incubated in 10% normal horse serum (Sigma) for 5 min as blocking agent and transferred to either primary anti-rat TH rabbit antiserum (dilution 1:250) (Pel-Freeze Rogers, Arkansas); or primary anti-rat parvalbumin rabbit antiserum (dilution 1:2000) (Sigma); or primary anti-rat GFAP mouse-antiserum (dilution 1:100) (Boehringer Mannheim) in 0.1% Triton X-100-PBS. Following overnight incubation at room temperature, sections for TH immunoreactivity were rinsed in PBS (2×10 min) and incubated in biotinylated anti-rabbit immunoglobulin G raised in goat (dilution 1:200) (Vector) for 90 min, rinsed repeatedly and transferred to Vectastain ABC (Vector) solution for 1 h. Sections for parvalbumin and GFAP were incubated in biotinylated anti-mouse immunoglobulin G raised in horse (dilution 1:200) (Vector). 3.3′-Diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.1 M PBS, supplemented with 0.005% H_2O_2, served as chromogen in the subsequent visualization reaction. Sections were mounted on to gelatin-coated slides, left to dry overnight, counterstained with hematoxylin dehydrated in ascending alcohol concentrations and cleared in butylacetate. Coverslips were mounted on entellan.

Microscopic analysis

The extent of the striatal lesion and control side was measured by outlining the areas of normal AChE staining on each section, summing over all sections containing the striatum, using an Olympus BX60 microscope and computerized image analysis (Sony 3CCD video camera; Image Pro Plus software, Media Cybernetics, Silver Spring, USA). In order to accommodate any variation in shrinkage during processing, the data were analysed in terms of the ratio of striatal surface area of lesion side in per cent of intact side.[2] The extent of the nigral lesion was assessed according to Nakao *et al*.[41] by quantifying the average loss of TH-positive neurons determined at three levels of the SNc (rostral: AP − 4.8 mm; middle: AP −5.3 mm; caudal: AP −6.0 mm). On each section, the number of TH-immunostained neurons in SNc was counted bilaterally at 200× magnification with the aid of a superimposed grid. The numbers of TH-positive neurons were expressed as the mean number of counts per section calculated from these three sections.

The density of GFAP immunostaining was assessed in the lesioned and intact striatum using the computerized image analysis system mentioned above. Images of stained sections were captured at a magnification of 200× with the help of a CCD video-camera. Intensity values were calibrated prior to the measurement of optical densities (OD) of GFAP immunostaining [OD-min = 256 (white) and OD-max = 0 (black)]. Mean ODs were calculated from the available sections.

Statistical analysis

All data are expressed as mean ± S.D. Global comparisons of behavioural data (between and within groups) were made using two-way analysis of variance for repeated measures. Post hoc, pair-wise comparisons between groups were performed using LSD test. Bonferroni–Holm correction was applied when multiple comparisons were made within groups.[24] Two-tailed paired Student's *t*-tests were employed for morphological measures on the two sides of the brain and Student's *t*-tests for independent samples were used for comparison between the two lesioned groups. Pearson correlation and partial correlation analysis were used to specify the relationship between histological parameters and behavioural data. These results are expressed as first-order partial correlation coefficient (r) values ranking from −1 (indicating an inverse correlation) to + 1 (indicating a positive correlation), 0 value indicating no correlation. The level of statistical significance was set at $P < 0.05$. All analyses were performed using SPSS 8.0 statistical software.

RESULTS

Stepping test

Two-way ANOVA for repeated measures demonstrated significant effects of "group" ($F = 47.82$, $P < 0.001$), "time" ($F = 6.42$, $P < 0.05$) and "side" ($F = 89$, $P < 0.001$). When the differences between the sides were analysed in each group, significant impairment of contralateral backhand stepping in group A animals without further deterioration following the secondary lesion was detected (primary/secondary lesion time point: $t = 7.9$, $P < 0.001$/$t = 21.75$, $P < 0.001$) (Table 1). In contrast, no significant asymmetry was observed in group B animals following the primary striatal lesion. However, after

Table 1. Changes in stepping and sidefalling behaviour over time in lesioned and healthy control animals

	Primary lesion		Secondary lesion	
	Ipsilat. paw	Contralat. paw	Ipsilat. paw	Contralat. paw
Group A				
Stepping	15.8±4.6	4.9±2.7*‖††	14.8±1.4	4.3±2.2*††
Sidefalling	0.3±0.3	2.02±0.9*§††	0.2±0.2	1.9±0.5*††
Group B				
Stepping	10.6±2.2*††	10.8±3.7††	9.5±4.2§††	3.7±1.6*‡††
Sidefalling	0.5±0.3**	1.3±0.4*††	0.4±0.3¶	1.9±0.7*† ††
Normal	Ipsilat. + contralat. paw			
Stepping	15.6±2.7			
Sidefalling	0.1±0.1			

The values represent the means ± S.D. of sidefalling and stepping rates (backward direction of movement) of each animal group [nigrostriatal lesion sequence, $n = 13$ (group A); striatonigral lesion sequence $n = 11$ (group B), normal animals, $n = 10$]. *Significant reduction ($P < 0.001$) of ipsilateral side compared to contralateral side; †,‡significant reduction ($P < 0.01$ and $P < 0.001$, respectively) of stepping/sidefalling rates following primary and secondary lesion; §,‖significant reduction of stepping/sidefalling rates of group A and B animals ($P < 0.01$ and $P < 0.001$, respectively); ¶,**,††significant reduction ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively) of stepping/sidefalling rates of normal compared to lesioned animals.

the complementary nigral lesion group B animals exhibited marked stepping asymmetry with significant contralateral impairment ($t = 5.51$, $P < 0.001$). Direct comparison of group A and B animals revealed a significantly greater contralateral stepping deficit following primary nigral compared to striatal lesions ($P < 0.001$). Conversely, ipsilateral stepping was significantly reduced in animals with primary striatal compared to primary nigral lesions ($P < 0.001$). Following the secondary lesion, contralateral stepping deficits were comparable in group A and B animals. However, ipsilateral stepping rates in 6-OHDA prelesioned group A animals remained significantly elevated following the secondary lesion compared to QA prelesioned group B animals ($P < 0.01$). In comparison to healthy animals, group A animals exhibited significant stepping deficits contralaterally to the initial nigral and subsequent striatal lesion ($P < 0.001$). In contrast, adjusted backhand stepping was significantly impaired bilaterally in group B animals following both lesion time points (primary lesion time point: $P < 0.001$, secondary lesion time point: $P < 0.001$).

Balance test

Two-way ANOVA for repeated measures demonstrated significant "group" ($F = 58.89$, $P < 0.001$) and "side" ($F = 147.86$, $P < 0.001$) effects.

In both animal groups there was a significant impairment of contralateral postural balance after the initial lesion (group A: $t = 7.9$, $P < 0.001$; group B: $t = 8.34$, $P < 0.001$). This asymmetry remained unchanged after the secondary lesion (Table 1).

Following the primary lesion contralateral sidefalling rates were significantly higher in group A animals compared to group B animals ($P < 0.01$). Following the secondary lesion there was no significant difference of contralateral sidefalling rates between group A and B animals. Although in group B animals ANOVA for repeated measures revealed no significant influence by parameter "time", Bonferroni–Holm correction exhibited significant balance deficits contralaterally to the initial striatal and subsequent nigral lesion ($t = 2.2$, $P < 0.05$). In comparison to healthy animals group A animals exhibited significantly increased contralateral sidefalling rates following primary and secondary lesion placement ($P < 0.001$); however, ipsilateral postural balance was preserved at both lesion time points. In contrast, postural balance was significantly impaired bilaterally in group B compared to control animals following primary and secondary lesions ($P < 0.05$).

Staircase test (paw reaching)

For the parameter "eaten", two-way ANOVA for repeated measures demonstrated significant effects of group ($F = 29.26$, $P < 0.001$) and "side" ($F = 41.3$, $P < 0.001$). For the parameter "taken", two-way ANOVA for repeated measures demonstrated significant effects of "group" ($F = 17.08$, $P < 0.001$) and "side" ($F = 55.12$, $P < 0.001$).

As summarized in Table 2 there was a significant reduction in the mean number of food pellets eaten and taken with the contralateral paw compared to the ipsilateral paw following primary and secondary lesions in both group A and B animals (group A: primary lesion time point for pellets taken/eaten: $t = 7.76$, $P < 0.001/t = 7.18$, $P < 0.001$; secondary lesion time point for pellets taken/eaten: $t = 5.43$, $P < 0.001/t = 5.6$, $P < 0.001$; group B: primary lesion time point for pellets taken/eaten: $t = 2.42$, $P < 0.05/t = 3.51$, $P < 0.01$; secondary lesion time point for pellets taken/eaten: $t = 5.48/P < 0.001$, $t = 5.9/P < 0.001$). Contralateral compared to ipsilateral success rates were also significantly reduced in both experimental groups (primary lesion time point: group A, $t = 2.67/P < 0.05$; group B, $t = 4.37/P < 0.01$; secondary lesion time point: group A, $t = 3.67/P < 0.01$; group B, $t = 4.9/P < 0.001$). There was no significant deterioration in primary contralateral paw-reaching deficits following the second complementary lesion in group A. The magnitude of ipsilateral and contralateral paw-reaching impairments measured by mean number of food pellets eaten and taken as well as success rate was not significantly different between group A and B animals at any time point. Compared to healthy animals paw reaching was significantly impaired bilaterally in both animal groups following primary and secondary lesion placement (Table 2).

Table 2. Summary of paw-reaching performances with the ipsilateral and contralateral paw in normal and lesioned animals

	Primary lesion		Secondary lesion	
	Ipsilat. paw	Contralat. paw	Ipsilat. paw	Contralat. paw
Group A				
Eaten	5.3 ± 2.1§	1.8 ± 0.9*,¶	4.9 ± 2.3‖	1.5 ± 0.7*¶
Taken	9 ± 2.6§	4.2 ± 0.8‡¶	8.7 ± 3.2§	4.5 ± 1.6*¶
% Success	0.6 ± 0.2	0.4 ± 0.2*¶	0.6 ± 0.1§	0.4 ± 0.2†¶
Group B				
Eaten	4.2 ± 3.4‖	1.5 ± 1.8†¶	4.7 ± 2.3‖	1.6 ± 1.4‡¶
Taken	7.2 ± 4.3‖	4.9 ± 2.7‡‖	8 ± 3.7‖	3.8 ± 2.4‡¶
% Success	0.5 ± 0.2§	0.3 ± 0.23†¶	0.6 ± 0.1§	0.4 ± 0.1‡¶
Normal				
Eaten	8.5 ± 2.5	8.6 ± 2.4		
Taken	12.1 ± 3	11.4 ± 2.5		
% Success	0.7 ± 0.1	0.8 ± 0.1		

The values represent the means ± S.D. of pellets "eaten" and "taken" of each animal group [nigrostriatal lesion sequence, $n = 13$ (group A), striatonigral lesion sequence $n = 11$ (group B), normal animals, $n = 10$]. *,†,‡Significant reduction ($P < 0.05$; $P < 0.01$ and $P < 0.001$, respectively, of ipsilateral side compared to contralateral side; §,‖,¶significant reduction ($P < 0.05$; $P < 0.01$ and $P < 0.001$, respectively) of paw-reaching test scores of normal compared to lesioned animals. % Success: percentage of "taken" pellets that were eaten.

Histology

All animals had severe unilateral nigrostriatal lesions with more than 90% loss of TH-immunoreactive neurons in SNc (group A, $t = 15.17/P < 0.001$; group B, $t = 37.05/P < 0.001$) and no significant differences were apparent between the two experimental groups (Table 3, Fig. 2).

The QA lesions were easily identifiable in all animals. AChE histochemistry revealed a significant reduction in striatal surface area on lesioned side in both animal groups (group A: mean reduction 56% ($t = 15.05/P < 0.001$); group B: mean reduction 85% ($t = 7.98/P < 0.001$) (Table 4, Fig. 3). Remaining relative striatal surface areas were significantly larger in animals with initial nigral compared to striatal lesions ($t = 7.58/P < 0.001$). Parvalbumin-positive neurons were significantly depleted ipsilateral to the lesion in both animal groups (group A: mean reduction 74%, $t = 7.58/P < 0.001$; group B: mean reduction 81%, $t = 9.23/P < 0.001$). The number of parvalbumin-positive neurons tended to be higher in animals with initial nigral lesions compared to animals receiving primary striatal lesions ($t = 2.11/P = 0.05$) (Table 4). However, even animals with large striatal lesions occasionally revealed parvalbumin-positive neurons adjacent to the margin of the lesion.

GFAP immunoreactivity was significantly increased in the residual ipsilateral compared to contralateral striatum (group A; $P < 0.01$; group B; $P < 0.001$) (Table 5). There was a significant increase in GFAP immunoreactivity in animals with initial nigral lesions compared to animals receiving primary striatal lesions ($t = 3.01/P < 0.01$).

Histological–behavioural correlations

Both ipsilateral stepping and sidefalling behaviour correlated with the loss of striatal surface area ($r = 0.42$, $P < 0.05$ for stepping behaviour with the variable number of nigral dopaminergic neurons held constant, $r = 0.44$, $P < 0.05$ for stepping behaviour with the variable number of parvalbumin neuronal cells held constant, $r = 0.46$, $P < 0.05$ for sidefalling behaviour with the variable number of nigral dopaminergic neurons held constant, $r = 0.37$, $P < 0.08$ for sidefalling behaviour with the variable number of parvalbumin neurons held constant). Neither ipsilateral stepping nor balance behaviour was correlated with parvalbumin cell counts or number of nigral dopaminergic neurons.

DISCUSSION

Degeneration of the dopaminergic nigrostriatal and GABAergic striatopallidal pathways is the central pathological event in MSA-P, a condition unique to the human CNS and clinically characterized by progressive levodopa-unresponsive parkinsonism.[11,68] This disorder has been mimicked experimentally in rats using sequential unilateral stereotaxic injections of 6-OHDA into the MFB followed by intrastriatal administration of QA.[51,69] The MSA-P double lesion rat model is morphologically characterized by neuronal degeneration in SNc and ipsilateral striatum, thus combining key neuropathological features of widely used PD and HD animal models. Double-lesioned MSA-P rats exhibit a characteristic pattern of drug-induced motor asymmetry characterized by ipsiversive amphetamine-induced rotation and absent rotational response to apomorphine. These rotational responses appear to be distinct from those observed in PD or HD lesion models.[70] PD rat models are commonly generated by a complete 6-OHDA-induced MFB lesion resulting in high rates of ipsiversive amphetamine and contraversive apomorphine-induced rotation.[58] This type of lesion, which is associated with a >90% loss of nigral dopamine neurons and >97% reduction in total striatal dopamine content, may be likened to the extensive neuropathology of advanced PD.[29,56] Large excitotoxic striatal lesions mimicking HD neuropathology have been reported to induce ipsiversive rotation asymmetry to both amphetamine and apomorphine.[12,29,56,57] However, contraversive rotation after either toxin in response to apomorphine has also been observed.[43]

Table 3. Average cell counts of tyrosinhydroxylase-positive neurons in substantia nigra pars compacta of double-lesioned group A and B animals

	SN pars compacta ip.	SN pars compacta co.	N pars compacta (%)†
Group A	8.33±6.69*	190.67 ± 36	4.42±3.31
Group B	8.33±8.34*	163.42 ± 13.51	5.05±5.09

Values represent the mean ± S.D. per three sections in group A and group B animals as described.
*Significant reduction ($P < 0.001$) of ipsilateral side (ip.) compared to contralateral side (co.) in group A and B animals.
†TH-positive neurons of ipsilateral side in per cent of contralateral side.

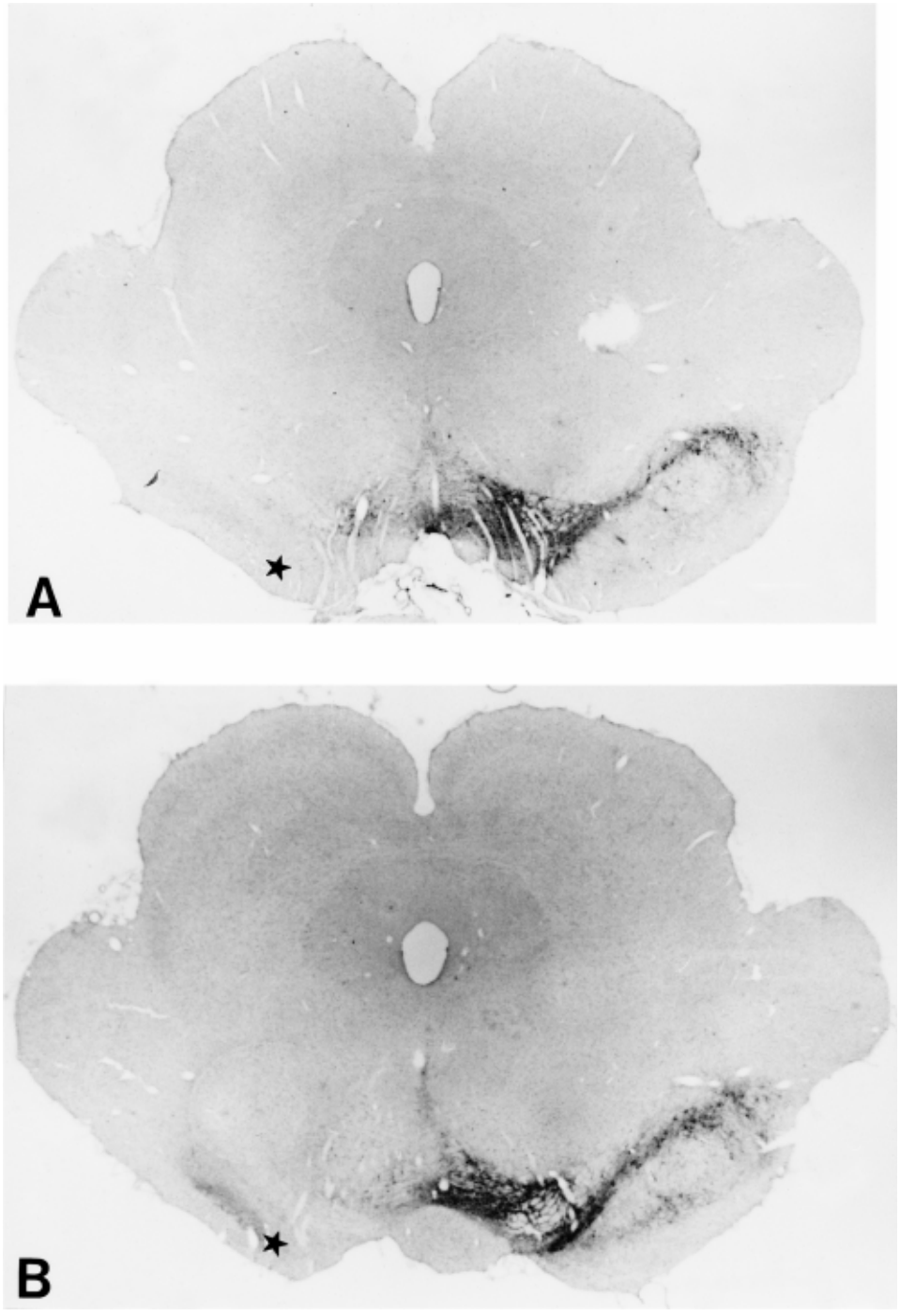

Fig. 2. Photomicrographs of two sections through the sampled area of the substantia nigra, processed for TH immunohistochemistry, from animals subjected to the nigrostriatal lesion sequence (A) and striatonigral lesion sequence (B). Asterisk denotes lesioned side. All animals had severe unilateral nigrostriatal lesions with more than 90% loss of TH-immunoreactive neurons in SNc, and no significant group differences were apparent between the two experimental groups.

These differences in response may be related to either the size or locus of the lesions. Indeed, a recent investigation into the effect of unilateral ibotenic acid lesions in the dorsal striatum of the rat demonstrated distinct drug-induced rotational responses depending on posterior versus anterior lesion placement.[16]

Consistent with previous experimental studies investigating rodent PD or HD rat models the ipsiversive amphetamine-induced rotation that can be observed in the unilateral double lesion MSA-P rat model is likely to result from stimulated dopamine release on the intact side.[51,58,67] The reduced or absent response to apomorphine that is present following a secondary striatal lesion in dopamine-depleted MSA-P rats correlates with the striatal lesion volume, i.e. loss of dopamine receptor-bearing striatal neurons.[2] Although robust and easy to quantify, drug-induced rotation is a functional test that has an unclear relation to the symptomatology of human MSA-P. The most relevant aspect of drug-induced behaviour in double-lesioned rats is probably the lack of circling in response to apomorphine which appears to be analogous to the lack of therapeutic benefit seen in more than 90% of MSA-P patients receiving dopaminergic replacement therapy.

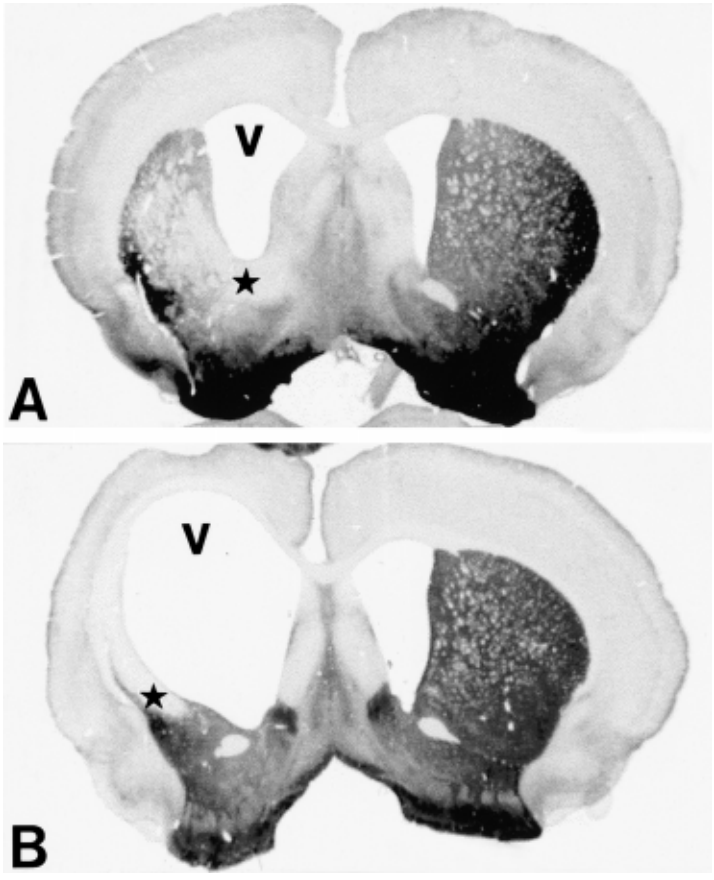

Fig. 3. AChE staining demonstrating the extent of the striatal lesion in one animal of group A and group B. Asterisk denotes lesioned side. The remaining striatal surface area appears larger in the group A animal (A) compared to the group B animal (B). V, lateral ventricle.

There is a clear need for studying non-drug induced motoric behaviour in the MSA-P rat model. In the present study we therefore evaluated complex motor deficits reflecting limb akinesia and deficits of complex motor function in rats subjected to a unilateral double lesion of the nigrostriatal and striatonigral projection.

Behavioural abnormalities in the double lesion multiple system atrophy-Parkinson subtype model

Stepping test. The stepping test was introduced by Schallert *et al*. and modified by the group of Olsson[44,54] using the unilateral 6-OHDA PD rat model. Complete unilateral dopamine denervation induces marked long-lasting deficits in forelimb adjusting stepping on the side contralateral to the lesion, while the changes on the ipsilateral side are subtle or absent.[9,29,44] In our hands, backhand adjusted stepping proved to be the most reliable measure across animals and test sessions. Unlike previous investigators we were unable to evaluate forehand adjusted stepping due to considerable variation and marked observer dependency.[29,44] Consistent with previous reports contralateral backhand adjusted stepping rates were markedly reduced in group A animals subjected to unilateral dopamine denervation.[44] There was no further reduction in contralateral stepping rates following the secondary striatal lesion, suggesting that a maximal functional deficit had already been established by the preceding subtotal striatal dopamine denervation. This assumption is supported by previous studies reporting progressive deficits of forelimb akinesia that correlated with increasing dopamine denervation in lateral striatum of rats subjected to striatal 6-OHDA lesions.[29,74] Interestingly, forelimb stepping deficits (unlike paw-reaching deficits) resulting from complete lesions of the rodent nigrostriatal projection may be substantially improved by tonic activation of striatal dopamine receptors using embryonic mesencephalic grafts or dopamine agonists.[44] In contrast to severe contralateral forelimb akinesia and in line with literature reports, ipsilateral stepping performance was maintained at control level in group A animals following nigral and striatal lesion placement.[29,44]

To our knowledge there have been no previous studies on the effects of striatal lesions on the stepping test. Group B animals exhibited a characteristic pattern of stepping impairment that was distinct from group A animals and that

Table 4. Residual striatal surface area and cell counts of parvalbumin-positive neurons in double-lesioned animals

	Ipsilateral striatum	Contralateral striatum	Striatum (%)§
Residual striatal surface area (mm^2)			
Group A	5.08±1.18*	11.56±0.6	44.06±10.61
Group B	1.84±0.79*‡	12.92±4.78	15.27±7.37‡
Number of parvalbumin-positive neurons per section			
Group A	16.89±12.11*	66.07±23.5	25.67±14.85
Group B	10.85±12.46*†	52.10±17.61	19.29±14.59

Values represent the mean ± S.D. per six sections in group A and group B animals as described. *Significant reduction between the ipsilateral and contralateral side ($P < 0.001$); †,‡significant reduction between group A and B animals ($P < 0.05$ and $P < 0.001$, respectively); §residual striatal surface area of ipsilateral side in per cent of contralateral side.

Table 5. The density of GFAP-positive cells

	Ipsilateral striatum	Contralateral striatum	Striatum (%)‖
GFAP			
Group A	80.59±30.24*	151.99 ± 23.74	55 ± 22.34
Group B	45.69±20.69†§	134.06 ± 26	34.63 ± 15.29‡

Values represent the mean OD ± S.D. per striatal surface area in group A ($n = 10$) and group B ($n = 10$) animals as described. *,†Significant reduction between the ipsilateral and contralateral side ($P < 0.01$ and $P < 0.001$, respectively); ‡,§significant reduction between group A and B ($P < 0.05$ and $P < 0.001$, respectively); ‖residual striatal surface area of ipsilateral side in percent of contralateral side. GFAP, Glial fibrillary acidic protein.

may have been related to more extensive striatal pathology. Indeed, consistent with previous reports, the remaining striatal surface area was approximately 64% smaller in group A animals with primary dopamine denervation compared to group B animals with primary striatal lesions.[6] Primary unilateral intrastriatal QA injections in group B animals induced moderate bilateral stepping deficits that significantly worsened contralaterally following the secondary nigral lesion. The stepping test therefore appeared to be a sensitive behavioural tool measuring accumulating functional deficits in group B animals. In contrast to group A animals, significant ipsilateral stepping deficits occurred in group B animals following primary and secondary lesions of the striatonigral and nigrostriatal projection, respectively. Indeed, the loss of striatal surface area correlated significantly with ipsilateral stepping performance. Such ipsilateral impairments are likely to result from bilateral distribution of information in the output pathways of the basal ganglia.[19,26] In addition, input pathways to the striatum are not completely lateralized.[15,31] The absence of ipsilateral stepping deficits in group A animals suggests that functional deficits resulting from unilateral removal of dopaminergic nigrostriatal afferents are predominantly mediated by ipsilateral uncrossed striatal output pathways. In contrast, behavioural impairment induced by unilateral striatal destruction appears to be mediated by contralateral and ipsilateral output pathways provided the lesion is marked. In line with this interpretation, ipsilateral deficits were absent in group A animals with moderate rather than severe striatal degeneration. The preservation of ipsilateral stepping performance in group A animals may represent a behavioural index of dopamine denervation-induced striatal protection against subsequent excitotoxic damage.

Balance test. This is the first study to investigate balance deficits in rodents with sequential nigral and striatal lesions. In general, the observed balance deficits paralleled impairments of stepping test performance. Previous investigators have demonstrated a marked inability of rats receiving a unilateral 6-OHDA MFB lesion to regain balance following postural challenge.[44,73,74] Further evidence for the ability of striatal dopamine denervation to induce deficits of balance adjustments was recently presented by Winkler *et al.*, who reported a significant correlation of balance test scores and striatal TH-immunoreactive fibre density in rats with striatal 6-OHDA lesions.[74] Consistent with these observations, primary 6-OHDA MFB lesions in group A animals induced marked contralateral balance deficits with increased sidefalling rates which remained unchanged following the placement of additional striatal lesions. The lack of deterioration of contralateral imbalance in group A animals following completion of the lesion sequence parallels the stepping test performance and suggests that the functional deficit resulting from initial complete dopamine denervation had already been maximal. Ipsilateral regaining of balance was unaffected by the primary nigral and secondary striatal lesion. The preserved ipsilateral balance test performance in group A animals following intrastriatal QA administration may be accounted for by the incomplete striatal lesion.

Effects of striatal lesions on balance control have not been studied previously. Following placement of primary striatal lesions, group B animals exhibited bilateral balance deficits that were more marked contralaterally. Ipsilateral balance deficits correlated with the degree of the striatal lesion as measured by loss of surface area. These observations parallel the stepping test results and support the functional relevance of bilateral basal ganglia projections in rodents as reviewed above. The secondary nigral lesion augmented contralateral sidefalling rates significantly in group B animals, whereas ipsilateral balance deficits remained unchanged. Therefore, the balance test provided a robust behavioural index of accumulating axial deficits in group B, but not group A animals. The clinical relevance of these findings will be discussed below.

Staircase test (paw reaching). Previous studies on 6-OHDA lesions in rodents have shown that significant impairments in contralateral skilled paw use do not appear until 80–90% of striatal dopamine and 60–80% of TH-positive neurons in SNc are lost.[3,29,34,36,42,73] Furthermore, moderate deficits were also observed with the ipsilateral paw, suggesting that limb use is controlled by uncrossed as well as crossed descending pathways.[3,58,72] Consistent with these observations, primary nigral lesions in group A animals induced marked bilateral impairments of skilled paw use that were more pronounced on the side contralateral to the lesion. Similar to the results of forelimb akinesia and balance tests, additional striatal lesions failed to augment pre-existing functional deficits in group A animals. Considering the evolution of stepping and balance deficits discussed above we therefore propose that in group A animals contralateral paw-reaching deficits were largely determined by the initial nigral lesion resulting in marked loss of dopaminergic neurons.

Impairments of skilled forelimb use have been reported by several groups in rodents subjected to lesions of the lateral striatum.[13,38,47,48,72] More recently, Fricker *et al.*[16] observed that rats receiving ibotenic acid lesions of dorsolateral striatum showed a marked impairment of contra- more than ipsilateral paw use on the staircase test, while animals with medial striatal lesions showed no significant difference compared to unoperated control animals.[16] In the present series of experiments, group B animals exhibited bilateral paw-reaching deficits following intrastriatal QA administration. These deficits were more marked contralaterally and failed to significantly deteriorate following the secondary 6-OHDA lesion.[13,38] Paw-reaching performance appeared to be more sensitive to QA-induced striatal degeneration than stepping behaviour or postural imbalance. We propose that this differential effect on motor behaviour is consistent with the known functional heterogeneity of the striatum. Unlike forelimb stepping or regaining of balance, paw-reaching

behaviour has a major motivational component that appears to be mediated by ventral striatopallidal projections.[72] In most of group B animals lesion extension towards the ventral striatum was observed and may have reduced the behavioural drive to reach for food pellets, thus partly accounting for the pronounced effects of QA-induced striatal lesions on paw reaching. Furthermore, the integrity of the sensorimotor cortex was disrupted in some group B animals. This additional cortical lesion may have also contributed towards the observed paw-reaching impairment.[38,49,72]

Although the remaining striatal surface area was relatively spared in group A compared to group B animals impairments of contra- and ipsilateral skilled forelimb use were not significantly different between both groups following completion of the lesion protocol. The similar magnitude of ipsi- and contralateral paw-reaching deficits in double-lesioned group A and group B animals suggests that both primary nigral and striatal lesions resulted in maximal functional deficits that were unaffected by further lesioning of corresponding striatal (group A) and nigral (group B) projection pathways. The staircase test therefore failed to detect progressive behavioural deficits associated with accumulating structural deficits in both group A and group B lesion paradigms. However, the lesions that were generated in the present study were considerable, corresponding to advanced SND. It remains to be established whether smaller double lesions of SNc and striatum mimicking mild or moderate SND may result in progressive (i.e. additive) impairments of complex motor function that may be detected using the paw-reaching test.

Histological characterization of the striatonigral double lesion

Anatomical analysis of the striatal lesions in both animal groups indicated a significant reduction of remaining striatal surface area compared to the contralateral side. Furthermore, a significantly greater reduction of striatal surface area was observed in animals with primary striatal compared to nigral lesions. These data are consistent with the concept that the intact nigrostriatal pathway potentiates the vulnerability of striatum to excitotoxic damage.[6] In line with this interpretation, parvalbumin cell counts tended to be less markedly reduced in group A animals with initial striatal dopamine denervation. Furthermore, GFAP immunohistochemistry revealed significant attenuation of astroglial activation in double-lesioned striatum of group A compared to group B animals. Previous studies revealed a decreased vulnerability of striatum against ischemic damage following lesioning of substantia nigra.[10,21] Several mechanisms have been proposed to explain the modulatory influence of dopamine on striatal damage produced by cerebral ischemia or injections of excitotoxic amino acids. Globus *et al.* found that the striatal concentration of dopamine increases 500-fold on the intact side but not on the SNc prelesioned side during transient forebrain ischemia.[20] The deleterious effect of dopamine may be attributable to free radicals that are formed as a consequence of dopamine oxidation.[60] Dopamine has also been shown to have a direct neurotoxic effect in cultured neurons.[45,53] There is good evidence that under physiological conditions, the dopaminergic nigrostriatal pathway exerts a modulatory presynaptic action on corticostriatal glutamatergic transmission, counteracting increasing glutamatergic activity.[28] Following unilateral lesioning of SNc, glutamate and aspartate levels derived from ipsilateral striatum were markedly increased.[63] This increased activity of excitatory corticostriatal pathways might induce a gradual postsynaptic glutamate receptor down-regulation resulting in relative protection from excitotoxic insults.[5,6]

6-OHDA lesioning of the MFB resulted in more than 90% reduction of ipsilateral TH-immunoreactive neurons in SNc without significant differences in both experimental groups. These findings contrast with the observations of Venero *et al.* indicating that intrastriatal QA injections protect against 6-OHDA-induced lesions of the dopaminergic nigrostriatal system. The missing neuroprotective effect of nigral neurons following striatal QA lesioning in our study could be partially explained by methodological differences. Venero *et al.* employed striatal rather than MFB 6-OHDA injections and they analysed neurochemical alterations of dopamine metabolisms instead of TH cell counts.[66]

In contrast to the present findings, MSA-P is characterized by severe striatal degeneration in areas of marked dopamine depletion.[14,30] Therefore, non-dopaminergic factors such as oligodendroglial inclusions containing alpha synuclein and microglial activation may contribute to striatal degeneration in MSA-P.[7,8,32,33,40,50,59,61,64,75]

Clinical relevance

In the present study we characterized for the first time complex motoric deficits in a unilateral double lesion rat model of MSA-P. Since it is unknown whether the disease process of SND starts at a nigral or striatal level or both in our experiment we compared the behavioural consequence of a nigrostriatal versus striatonigral lesion sequence. The neuropathological analysis revealed that distinct double lesion patterns were obtained by simple alternation of toxin administration. Animals receiving primary 6-OHDA and secondary QA injections exhibited nigral predominant SND-like pathology, whereas animals receiving primary QA and secondary 6-OHDA injections showed striatal predominant SND-like pathology. The complex motor deficits that were observed in our double lesion model included severe bilateral paw-reaching impairments that were more marked contralateral to the lesion. Remarkably, primary nigral or striatal lesions produced similar paw-reaching deficits; furthermore, there was no deterioration following the secondary (i.e. striatal or nigral) lesion.[62] These findings reflect the present understanding of pathophysiological mechanisms underlying parkinsonism in MSA-P. Clinicopathological and functional imaging studies demonstrated that both nigral and striatal pathology appear to contribute towards parkinsonism in MSA-P, although their relative importance remains unknown.[1] Correspondingly, nigral and striatal lesions were able to induce marked bilateral, yet asymmetric reaching deficits in our animals and there was no further deterioration following completion of the lesion sequence in both paradigms.

However, the paw-reaching paradigm has been criticized recently because it is rather complex and appears to involve poorly defined motivational aspects.[44] Stepping and regaining of balance have been shown to monitor deficits of motor initiation in the forelimbs without significant intrusion of motivational drive analogous to limb akinesia and dysequilibrium, both of which frequently accompany the clinical syndrome of MSA-P.[44] In our study, primary or secondary nigral lesions were more powerful than striatal lesions in

inducing severe contralateral deficits, i.e. poor stepping and increased falling rates. This observation is in agreement with previous investigators demonstrating reversal of impaired stepping and regaining of balance mediated by dopamine-rich embryonic grafts implanted into the striatum of 6-OHDA-treated rats.[44] There are no published studies reporting stepping and balance test results in rodents receiving striatal lesions. Stepping test performance was only moderately affected and there was no asymmetry compared to balance or paw-reaching testing. This observation may be explained by the lesion placement as well as functional heterogeneity of rodent striatum. Paw reaching appears to be represented in dorsal posterolateral striatum whereas stepping and balance are sensitive to dopaminergic denervation of centrolateral striatum.[16,74] Our striatal QA lesions were targeted at the dorsolateral posterior striatum and may have therefore relatively spared central parts that were still innervated by dopaminergic afferents prior to the secondary 6-OHDA lesion. It remains to be established whether central striatal lesion placements may result in more severe deficits of stepping and balance function. Following the secondary 6-OHDA injection QA-prelesioned animals displayed a significant behavioural deterioration, with more marked contralateral stepping deficits and increased sidefalling rates. We propose that this behavioural deterioration represents a functional measure of removal of dopaminergic afferents in spared striatum or extrastriatal regions such as nucleus accumbens. Due to their differential sensitivity to nigral versus dorsolateral striatal lesions, both stepping and balance tests appear to be reliable tools to measure progressive functional deficits arising from sequential striatonigral, but not nigrostriatal double lesions in a rat model of MSA-P.

CONCLUSIONS

Our observations are consistent with a complex biological and functional interaction of nigral and striatal lesions that produces variable behavioural and histological changes depending on the sequential double lesion paradigm. Tests of complex motor behaviour appear to provide a reliable tool that will be helpful for monitoring the effects of interventional strategies such as embryonic neuronal transplantation in the MSA-P rat model.

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