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# Embryonic ventral mesencephalic grafts improve levodopa-induced dyskinesia in a rat model of Parkinson's disease

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## Summary

**We investigated the role of dopamine neurons in the manifestation of levodopa-induced dyskinesia in a rat model of Parkinson's disease. Daily treatment with a subthreshold dose of levodopa gradually induced abnormal involuntary movements (AIM) in 6-hydroxydopamine-lesioned rats, which included stereotypy and contraversive rotation. After 4 weeks of levodopa treatment, rats with mild and severe AIM were assigned to two treatment subgroups. The graft subgroup received embryonic ventral mesencephalic tissue into the striatum, whilst the sham-graft subgroup received vehicle only. Rats continued to receive levodopa treatment for 3 months post-graft. Brain sections at the level of the basal ganglia were processed for autoradiography using a ligand for dopamine transporter, and *in situ* hybridization histochemistry for mRNAs encoding postsynaptic markers. Levodopa-induced AIM significantly improved in grafted rats. The severity of AIM correlated inversely with the density of dopamine nerve terminals in the striatum ($P < 0.001$), with almost no AIM when the density of dopamine nerve terminals was >10–20% of normal. Embryonic dopamine neuronal grafts normalized not only mRNA expression for preproenkephalin (PPE) in the indirect pathway, but also mRNA expression for prodynorphin (PDyn) in the direct pathway, which was upregulated by levodopa treatment. AIM scores correlated linearly with expression of PPE mRNA in the indirect pathway ($P < 0.001$) and also with PDyn mRNA in the direct pathway ($P < 0.001$). We conclude that embryonic dopamine neuronal grafts may improve levodopa-induced dyskinesia by restoring altered activities of postsynaptic neurons, resulting not only from dopamine denervation, but also from levodopa therapy, provided that the density of striatal dopaminergic nerve terminals is restored above a 'threshold' level.**

**Keywords**: Parkinson's disease; 6-OHDA; levodopa-induced dyskinesia; embryonic dopamine neuronal graft

**Abbreviations**: AIM = abnormal involuntary movements; GAD = glutamic acid decarboxylase; ISHH = *in situ* hybridization histochemistry; 6-OHDA = 6-hydroxydopamine; PDyn = prodynorphin; PPE = preproenkephalin; VM = ventral mesencephalic

## Introduction

Various types of dyskinesias may manifest in patients with Parkinson's disease. Certain dyskinesias are provoked by low or intermediate levels of dopaminergic stimulation (Marsden *et al*., 1982). However, the most common type of dyskinesia appears following administration of levodopa, in the form of chorea or dystonia, at peak dose or throughout the 'on' time. This levodopa-induced, peak dose dyskinesia presents in 30–80% of patients who are treated with levodopa, and increases in frequency and severity with progression of disease and duration of treatment (Marsden *et al*., 1982; Nutt, 1990). Since the early stage of the levodopa era, it has been recognized that levodopa-induced dyskinesia generally occurs in patients with a good therapeutic response to levodopa (Cotzias *et al*., 1967); it appears only after chronic treatment with levodopa, not with the first dose of levodopa; and it seems to appear earlier and be worse on the more severely affected side in asymmetrically affected patients (Mones *et al*., 1971; Kempster *et al*., 1989; Nutt, 1990). These clinical observations, in agreement with animal studies (Bedard *et al*., 1986; Clarke *et al*., 1989), indicate that both chronic levodopa therapy and nigrostriatal dopaminergic denervation play an important role in the pathogenesis of levodopa-induced dyskinesia.

In an earlier experiment, we studied the effects of levodopa

treatment in the rat with 6-hydroxydopamine (6-OHDA) lesions, in which the severity of dopaminergic denervation was static (Cenci *et al*., 1998). In this study, we explored the role of dopamine neurons in the pathogenesis of levodopa-induced dyskinesia using a grafting technique to increase the density of dopaminergic nerve terminals in the striatum. We report here our observations that suggest therapeutic effects of intrastriatal embryonic dopamine neuronal grafts on levodopa-induced dyskinesia.

## Material and methods

Female Sprague–Dawley rats, weighing 200–250 g at the time of lesion surgery, were given unilateral stereotaxic injections of 6-OHDA in the right ascending mesostriatal dopamine bundle. Two injections of 7.5 and 6 μg of free-base 6-OHDA (3 μg/μl in 0.02% ascorbate in saline) were given over 3–4 min under Equithesin anaesthesia [3 ml/kg body weight, intraperitoneally (i.p.)] at the following coordinates (in millimetres, relative to bregma and the dural surface): A = −4.4, L = 1.2, V = 7.8, tooth bar = −2.4; A = −4.0, L = 0.75, V = 8.0, tooth bar = +3.4. The toxin was infused at a rate of 1 μl/min, and the cannula was left in place for 3 min before being withdrawn. To determine lesion efficacy, rats were tested 1–2 weeks later with an automated rotometer following an injection of D-amphetamine (5 mg/kg, i.p.). Twenty-eight rats that displayed >7 ipsilateral rotations/min for 90 min were selected for the study.

### *Grafting*

#### *Pre-graft induction period*

Six to eight weeks after 6-OHDA lesions, the rats started to receive 8 mg/kg of L-dopa methyl ester (Sigma, St Louis, Mo., USA), mixed with 15 mg/kg of benserazide (kindly provided by Hoffmann-La Roche, Basel, Switzland), i.p. twice daily for the first 3 weeks. In the fourth week, the dose of L-dopa methyl ester was increased to 12 mg/kg (plus 15 mg/kg of benserazide) to increase the number of rats with severe abnormal involuntary movements (AIM). Behavioural tests for AIM were carried out twice a week. At the end of the induction period, 23 rats that had developed mild to severe AIM were divided into two groups based on their final AIM scores. Group A included 13 rats with severe AIM (AIM score >20), and group B included 10 rats with mild AIM (AIM score ⩽20). The rats in each group were assigned to the graft and the sham-graft subgroups, at random, subject to approximately equal average AIM scores in the two subgroups.

#### *Graft surgery*

The rats assigned to the graft subgroup received grafts of developing ventral mesencephalic (VM) tissue, obtained from 14-day-old rat embryos (crown–rump length 11 mm) of the same strain in the form of a cell suspension (Bjorklund *et al*., 1983). Each recipient rat received two 2 μl deposits of the graft suspension into the dopamine-denervated striatum at the following coordinates (in millimetres, relative to bregma and the dural surface, and with the tooth bar set 2.3 mm below the interaural line): (i) A = 1.6, L = 3.2, V = 4.5; (ii) A = 0.6, L = 3.5, V = 4.5. The rats assigned to the sham-graft subgroup received cell-free vehicle only. The identification numbers of the rats were changed by altering their earmarks during the grafting surgery. The examiner of their behaviour was blinded to the new identification numbers until the end of the experiment.

### *Post-graft period*

One week after the grafting surgery, treatment with levodopa was resumed with the same dose (12 mg/kg of L-dopa mesylate ester with 15 mg/kg of benserazide) but less frequently, twice weekly, for the following 12 weeks. The frequency of levodopa administration was reduced during the post-graft period to protect rats from developing excessive AIM, particularly masticatory dyskinesia, which could be self-mutilating. Behavioural measurements were carried out once a week. In the 13th week post-graft, rats were challenged by an increase in the dose or the frequency of the doses of levodopa. Rats in group B were given 24, 48, 96 and 192 mg/kg of levodopa (each mixed with 15 mg/kg of benserazide) every second day. Rats in group A were given 12 mg/kg of levodopa once daily during the 13th week. The rats were sacrificed for autoradiographic studies 3 days after the last dose of levodopa.

### *Behavioural measurements*

#### *AIM rating scale*

Levodopa-induced AIM, which include stereotypy and contraversive rotation, were quantified using the rat AIM rating scale (Table 1). Stereotypy was classified into three subtypes based on the topographic distribution: limb dyskinesia, axial dystonia, and masticatory dyskinesia. Suppressibility of levodopa-induced AIM was tested by challenging the rat with novel sensory input, such as touching the rat or tilting the cage. The rats were assessed before injection of levodopa and every 35 min for 140 min after injection of levodopa. The AIM score was a sum of all measurements from sequential assessments after injection of levodopa. The maximum attainable AIM score was 64.

### *Autoradiographic studies for ligand binding and* in situ *hybridization*

#### *Tissue preparation*

Three days after the last dose of levodopa, the animals were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and

**Table 1** *Rating scale for abnormal involuntary movements in the rat*

**Limb dyskinesia**: repetitive, rhythmic jerky movements or dystonic posturing of the forelimb on the side contralateral to the dopamine-denervated striatum
0 = Absent
1 = Increased appearance of isolated jerky movements of the contralateral forelimb
2 = Intermittent and frequent repetitive movements of the contralateral forelimb
3 = Continuous repetitive or dystonic movements; interrupted by sensory distraction
4 = Compulsive repetitive or dystonic movements; not interrupted by sensory distraction

**Axial dystonia**: lateral flexion and axial rotation of the neck and trunk towards the side contralateral to the dopamine-denervated striatum
0 = Absent
1 = Consistent contralateral rotation of the trunk in a bipedal sitting position
2 = Intermittent, contralateral flexion of the neck superimposed on consistent rotation of the trunk in a bipedal sitting position
3 = Continuous contralateral flexion and rotation of the neck and trunk; postural balance maintained
4 = Compulsive contractions of the neck and trunk; postural balance disturbed

**Masticatory dyskinesia**: repetitive chewing movements of the jaw ± tongue protrusion
0 = Absent
1 = Increased chewing movements; occasional
2 = Increased chewing movements; frequent
3 = Continuous chewing movements with small amplitude
4 = Compulsive and repetitive biting movements with large amplitude; frequently associated with tongue protrusion

**Contraversive rotation**: turning or rotating movements contralateral to the dopamine-denervated striatum
0 = Absent
1 = Contraversive turning with consistent directional bias; locomotor activity may be increased
2 = Intermittent, contraversive circular rotation
3 = Continuous contraversive rotation; interrupted by sensory distraction
4 = Compulsive contraversive rotation; not interrupted by sensory distraction

killed by decapitation. The brains were removed, frozen immediately on dry ice and stored at −80°C. Serial sections (16 μm thick) through the basal ganglia were cut coronally on a cryostat and mounted onto chrome alum-treated glass slides (six sections per slide). The slide-mounted sections were stored at –20°C.

### *Dopamine transporter autoradiography*

To assess the density of dopaminergic nerve terminals in the striatum, one series of sections was incubated with tritiated *N*-1-2-benzo(b)thiophenyl-cyclohexyl-piperidine ([$^3$H]BTCP) that binds to plasma membrane dopamine transporter (Vignon *et al*., 1988; Filloux *et al*., 1989). The slide-mounted sections were first washed for 20 min at 4°C in 50 mM Tris–HCl buffer, pH 7.0, containing 200 mM NaCl, and subsequently incubated for 90 min in the same buffer containing 1 nM [$^3$H]BTCP (specific activity 50 Ci/mmol; NEN DuPont, Boston, Mass., USA). Control sections were incubated with [$^3$H]BTCP in the presence of 100 μM cocaine to determine the non-specific binding. At the end of the incubation, the slide-mounted sections were washed four times for 6 min each time in ice-cold 50 mM Tris–HCl/200 mM NaCl buffer, dried under a cold air stream and apposed to tritium-sensitive film ($^3$H Hyperfilm, Amersham, Arlington Heights, Ill., USA) at 4°C for 6 weeks. The films were developed in Kodak D19, fixed and dried.

### *Preparation of probes for* in situ *hybridization histochemistry (ISHH)*

The probes were oligodeoxyribonucleotides (Scandinavian Gene Synthesis, Köping, Sweden) complementary to nucleotides 322–360 of the cloned preproenkephalin (PPE) cDNA (Howells *et al*., 1984), also known as PPE-A (Duty and Brotchie, 1997), nucleotides 934–982 of the cloned prodynorphin (PDyn, or proenkephalin B) gene (Civelli *et al*., 1985), and nucleotides coding for amino acids 389–405 of feline glutamic acid decarboxylase (GAD) (Kobayashi *et al*., 1987). Oligonucleotides (0.2 μM) were labelled at the 3′ end with 4 μM [α-$^{35}$S]dATP (>37 TBq/mmol; Amersham) using 25–30 U of terminal deoxynucleotidyltransferase (TdT; Amersham) for 2 h at 37°C. The labelled probes were purified on NENSORB 20 nucleic acid purification cartridges (NEN DuPont) to specific activities of $>10^9$ c.p.m./μg. Specificity of hybridization was determined by competition experiments where the $^{35}$S-labelled probe was diluted with an excess of either the same unlabelled oligonucleotide or an unlabelled unrelated oligonucleotide. The specificity of the oligonucleotide probes was determined by Northern blot

analysis, homology screens of the sequences in GenBank and comparisons of the anatomical distributions with previous studies attempting to localize the same mRNA transcripts.

### *Hybridization*

All solutions and buffers were prepared with distilled water treated with diethyl-pyrocarbonate (Sigma) to inhibit RNase activity and then autoclaved. The slide-mounted sections were air dried and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min, then rinsed three times in phosphate-buffered saline, 5 min each, before dehydration. The sections were dehydrated in a series of ascending concentrations of ethanol, and air dried. The slides were then incubated with the hybridization mixture, which comprised 50% formamide (deionized), 4× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 10 mg/ml of RNase-free bovine serum albumin), 1% sarcosyl, 10% dextran sulphate, 500 μg/ml sheared and denatured salmon sperm DNA, 200 mM dithiothreitol and $10^7$ c.p.m./ml of $^{35}$S-labelled oligonucleotide probe. Approximately 50 μl of hybridization cocktail was added to each section. Slides were coverslipped with parafilm and incubated overnight (16–18 h) at 42°C in a humid chamber. After hybridization, the parafilm coverslips were floated off in 1× SSC at 55°C, and the sections were given four washes (15 min each) in 1× SSC at 55°C, plus a final wash beginning at 55°C and cooling to room temperature. The slides were then rinsed twice in distilled water, dehydrated in ethanol as above and exposed to autoradiographic film (Bmax, Amersham) at 20°C for 10–21 days. The films were developed in Kodak D-19 for 4 min and fixed.

### *Quantitative image analysis*

Autoradiographic images were digitized and analysed by the image analysis program, Image (Wayne Rasband, NIMH). The optical density was converted to the intensity of radioactivity using $^{14}$C standards (Amersham) for ISHH or $^{3}$H standards (Amersham) for [$^{3}$H]BTCP binding exposed on the same film (Miller, 1991). Striatal expression of [$^{3}$H]BTCP binding or neuropeptide mRNA was analysed in 4–5 sections of the neostriatum, rostrocaudally at levels 10–18 according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986).

### ***Statistical analysis***

In order to assess the effects of embryonic VM grafts during the post-graft period, the rat AIM scores were analysed on time and the treatment subgroup (grafted rats versus sham-grafted rats) in group A, and again in group B, using repeated measures analysis of variance. The last AIM scores during the pre-graft period were used as pre-graft baseline covariates. Effects of challenge tests (by higher or more frequent doses of levodopa) were evaluated by analysing AIM scores during the 13th week post-graft on the dose of levodopa and the treatment subgroup in group A, and again in group B, using repeated measures analysis of variance. The AIM scores in the 12th week post-graft were used as baseline covariates. To assess the effects of different doses of levodopa on the severity of AIM in the sham-grafted rats (in which the density of dopaminergic nerve terminals was not significantly different between groups A and B), the final post-graft AIM scores of group B (manifested by 192 mg/kg of levodopa) were compared with pre-graft baselines of the same group (manifested by 12 mg/kg of levodopa) by a paired *t*-test, and also with the final post-graft AIM scores of group A (manifested by 12 mg/kg of levodopa) by a two-sample *t*-test.

Signal intensity of [$^{3}$H]BTCP binding or mRNA hybridization on the autoradiographic films was expressed as a ratio of the value in the right striatum or globus pallidus (lesioned side) to the value in the left striatum or globus pallidus (unlesioned control side), respectively. The logarithmic transform of the right/left ratio of [$^{3}$H]BTCP binding was compared among four subgroups (grafted rats and sham-grafted rats in groups A and B) by analysis of variance, followed by Fisher's multiple comparisons. The logarithmic transforms of the right/left ratios of PPE, PDyn, $GAD_{67}$ mRNAs in the striatum or $GAD_{67}$ mRNA in the globus pallidus were analysed similarly. The relationship between AIM scores and a presynaptic marker was analysed by regression of AIM scores on the logarithm of [$^{3}$H]BTCP binding. The logarithmic transformations were successful in symmetrizing the data in all cases. Relationships between AIM scores and postsynaptic markers were analysed by regression of AIM scores on the expression of mRNAs. The data were expressed as the mean ± standard deviation unless specified otherwise. Statistical significance level was set at $P < 0.05$.

## Results

### ***Characterization of levodopa-induced AIM in a rat model of Parkinson's disease***

Pulsatile treatment with a subthreshold dose of levodopa gradually induced stereotypy or contraversive rotation in rats with unilateral 6-OHDA lesions, which were not observed in normal rats or in the 6-OHDA-lesioned rats that received vehicle only. Levodopa-induced stereotypy was observed only on the side contralateral to 6-OHDA lesions. Maneouvres for testing suppressibility of levodopa-induced movements evoked an arrest reaction in normal rats, but not in rats with severe levodopa-induced abnormal movements. Rats with mild to moderate levodopa-induced movements suppressed abnormal movements briefly, only to resume the same movements after pauses of variable length.

The motor pattern of each topographic subtype was highly stereotypic across individual rats as well as serial observations of the same rat. However, the proportion of each subtype was not uniform among individual rats. Furthermore, the manifestation of a motor pattern was influenced by the position of the rat: contraversive rotation was predominant in a quadrupedal position, whereas stereotypy was pre-

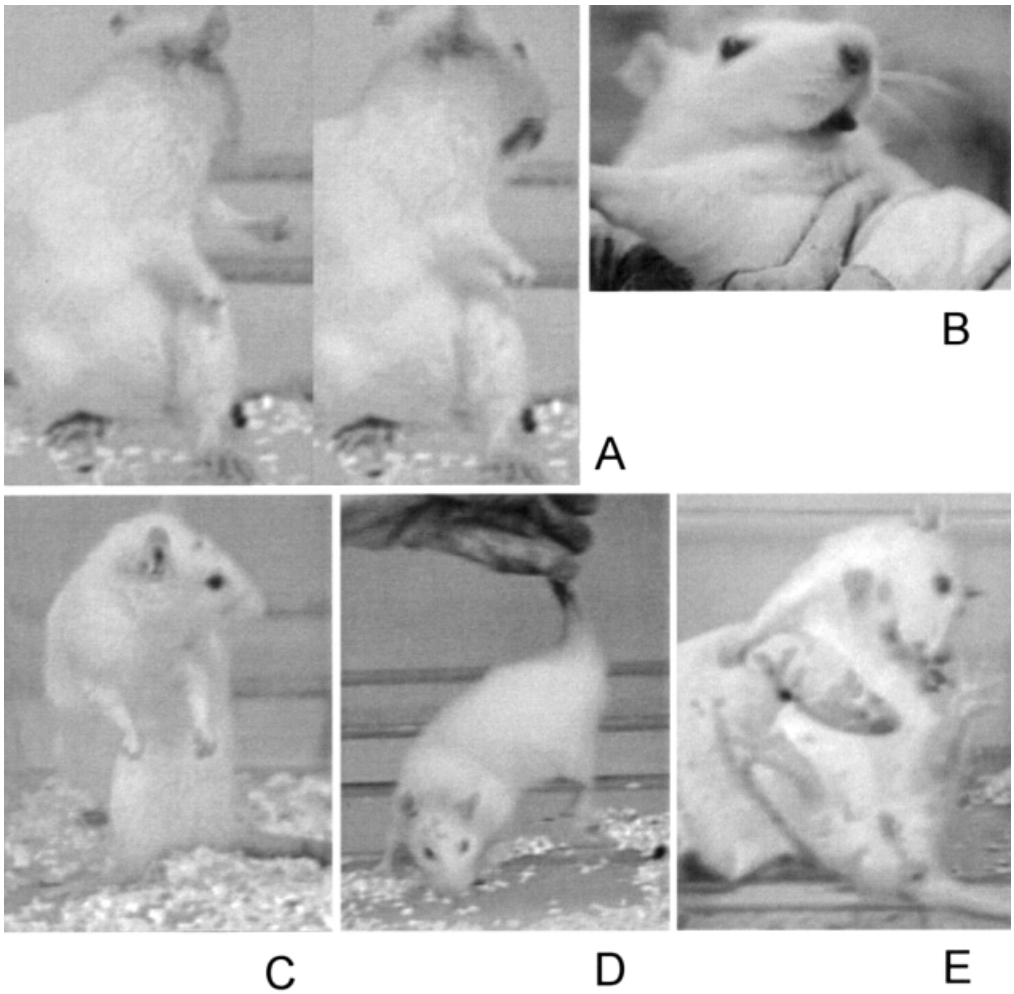


**Fig. 1** Abnormal involuntary movements (AIM) developed during the course of levodopa treatment in the rat. (**A**) Limb dyskinesia: sequential photographs of the same rat illustrate repetitive jerky movements of the left forelimb, contralateral to the dopamine-denervated striatum produced by 6-OHDA lesions. (**B**) Masticatory dyskinesia: repetitive chewing movements of the jaw in the absence of food, occasionally associated with repetitive protrusion of the tongue. (**C**) Axial dystonia: the rat was sitting (or standing) in a bipedal position with left lateral rotation of the neck and trunk, contralateral to the dopamine-denervated striatum. (**D**) Contraversive rotation: when the same rat was forced to put its forelimbs on the ground, it rotated to the left (contraversive to the dopamine-denervated striatum). (**E**) When the rat was lifted up, contraversive locomotor activity was replaced by contralateral limb dyskinesia (flexion of the left forelimb) and axial dystonia (left lateral flexion and rotation of axial muscles).

dominant in the bipedal sitting or standing position (Fig. 1). This mutually exclusive relationship of the two distinctive motor patterns was illustrated in the time–action curve of AIM subtypes, and of rotometer measurements (Fig. 2).

## *Effects of embryonic dopamine neuronal grafts on levodopa-induced AIM*

### *Pre-graft induction period*

Among the 28 rats that received pulsatile treatment with levodopa, 23 developed mild to severe AIM by the end of the fourth week during the induction period. Five rats did not develop AIM. The onset of AIM was highly variable among individual rats (9.5 ± 7.9 days). The final AIM scores were also markedly variable. In group A, the mean final pre-graft AIM score was 40.9 ± 11.9 for the graft subgroup and 41.8 ± 10.2 for the sham-graft subgroup. There were no significant differences in the final pre-graft AIM score between the two treatment subgroups. In group B, the mean final pre-graft AIM score was 12.6 ± 3.8 for the graft subgroup and 14.0 ± 6.9 for the sham-graft subgroup, with no significant differences between the two treatment subgroups.

### *Post-graft period*

Figure 3 illustrates the temporal courses of AIM scores in each treatment subgroup before and after grafting embryonic

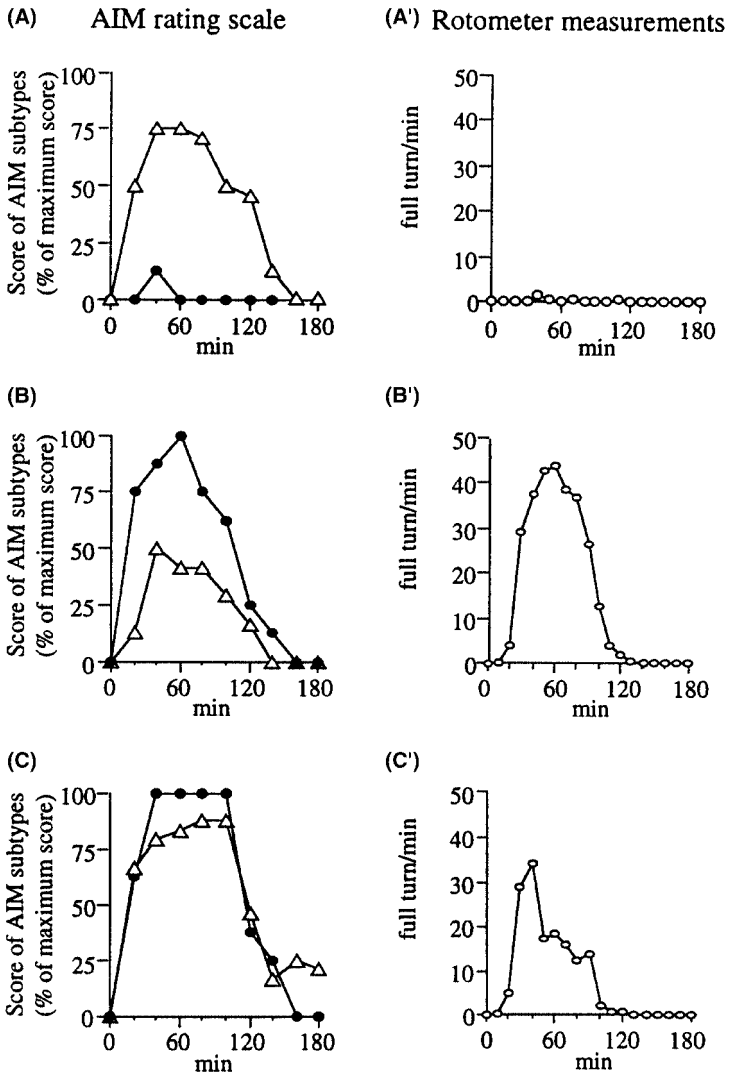


**Fig. 2** Time–action curves of AIM subtypes and automated rotometer measurements. Left column: *x*-axis, minutes after injection of levodopa; *y*-axis, severity of AIM subtypes, expressed as a percentage of maximum attainable score for each subtype. Right column: *x*-axis, minutes after injection of levodopa; *y*-axis, number of full turns in 10 min. Open triangle, stereotypy; filled circle, contraversive rotation; open circle, number of full turns for 10 min. (**A**) A rat with marked stereotypy and little contraversive rotation. (**A′**) Levodopa-induced stereotypy was not reflected in the rotometer measurements. (**B**) A rat with mild stereotypy and marked contraversive rotation. (**B′**) The same rat showed robust contraversive rotation with a unimodal peak in the time–action curve of rotometer measurements. (**C**) A rat with severe stereotypy and contraversive rotation. (**C′**) The same rat showed irregular, multimodal peaks in the time–action curve of the rotometer measurements.

VM tissue. Repeated measures ANOVA indicated a highly significant time × treatment interaction [$F(9,99) = 9.39$, $P < 0.0001$ for subgroup × time]. In particular, specific time points were compared between the two subgroups, adjusting for multiple comparisons. In the rats with severe pre-graft AIM (group A), the AIM scores became increasingly and significantly different between the graft and sham-graft treatment subgroups from the 5th week post-graft onwards [$F(1,11) = 8.09$, $P = 0.016$ for subgroups; $F(9,99) = 20.42$, $P < 0.0001$ for time]. In the 12th week post-graft, the mean AIM score was 24.7% of pre-graft baseline in the grafted rats, and 73.8% of pre-graft baseline in the sham-grafted rats. In the rats with mild pre-graft AIM (group B), AIM scores were significantly reduced in both grafted and sham-grafted rats during the 12 weeks after grafting ($P = 0.002$). There were, however, no significant differences between the grafted and sham-grafted rats. Challenge tests with higher doses of levodopa during the 13th week post-graft (group B) increased AIM scores significantly in both grafted and sham-grafted rats (to 7.8 ± 5.3 and 19.6 ± 12.2, respectively, $P =$

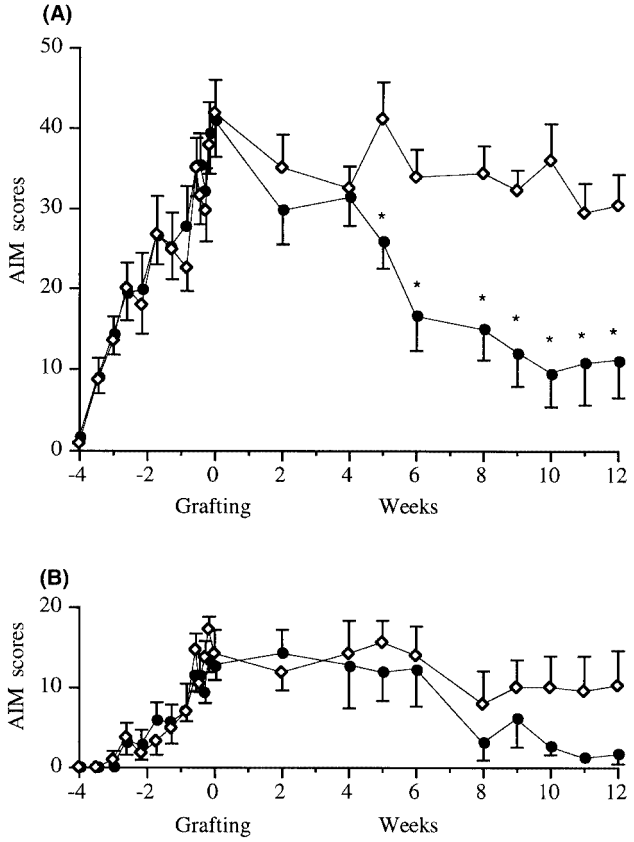


**Fig. 3** Time course of AIM scores of (**A**) group A and (**B**) group B during the pre- and post-graft periods. Symbols: filled circles = graft; open diamonds = sham graft. Rats received levodopa (12 mg/kg) once daily during the pre-graft induction period, and twice a week during the post-graft period. The frequency of levodopa treatment was reduced during the post-graft period to avoid excessive development of AIM, which could cause self-mutilation. In group A, the AIM scores became increasingly and significantly different between the graft and sham-graft subgroups from the 5th week post-graft onwards (*significantly different between the two treatment subgroups; error bar = 1 SEM).

0.007 for both subgroups), without significant differences between the two treatment subgroups. In the sham-grafted rats, the final post-graft AIM scores, which were manifested by 192 mg/kg of levodopa, were not significantly different from the final pre-graft AIM scores that were manifested by 12 mg/kg of levodopa ($P = 0.64$). In contrast to group B, daily administration of the same dose of levodopa (12 mg/kg) did not change AIM scores significantly in group A (9.0 ± 9.9 for grafted rats, 32.3 ± 10.8 for sham-grafted rats). The final post-graft AIM scores in the sham-grafted rats of group A were still significantly greater than those of group B ($P = 0.049$), despite the fact that the latter were manifested by a 16-fold higher dose of levodopa than the former.

### *Effects of dopaminergic neuronal grafts on presynaptic and postsynaptic markers*

Figure 4 illustrates autoradiographic images of [$^3$H]BTCP binding and *in situ* hybridization for mRNA encoding PPE, $GAD_{67}$ or PDyn in the striatal or pallidal sections. Results of quantitative analysis for presynaptic and postsynaptic markers are summarized in Table 2. In group A, the mean density of striatal [$^3$H]BTCP binding was significantly greater in the grafted rats than in the sham-grafted rats ($P < 0.001$). Analysis of group B showed similar results ($P < 0.001$). [$^3$H]BTCP binding in the sham-grafted rats did not show significant differences between groups A and B, contrary to the final post-graft AIM scores ($P = 0.81$). The mean expression of PPE mRNA was significantly less in the grafted rats than in the sham-grafted rats in group A ($P < 0.001$). Group B showed analogous results ($P < 0.001$). The mean expression of PDyn mRNA was significantly less in grafted rats than in sham-grafted rats ($P < 0.001$ for both groups A and B). The mean expression of $GAD_{67}$ mRNA in the striatum (measured over the entire caudate nucleus and putamen, rostral to the globus pallidus) or $GAD_{67}$ mRNA in the globus pallidus was also significantly less in the grafted rats compared with the sham-grafted rats (striatal $GAD_{67}$ mRNA, $P < 0.001$ for both groups A and B; pallidal $GAD_{67}$ mRNA, $P = 0.009$ for group A, $P = 0.006$ for group B).

### *Relationship of the severity of levodopa-induced AIM to presynaptic or postsynaptic markers*

In group A, the final post-graft AIM scores correlated inversely with the logarithm of [$^3$H]BTCP binding ($P < 0.001$, Fig. 5A). AIM scores were markedly reduced when [$^3$H]BTCP binding was >10–20% of that of the control side. Analysis of group B showed similar findings (Fig. 5B). The magnitude of increase in AIM scores of group B during the challenge test correlated inversely with the logarithm of [$^3$H]BTCP binding ($P = 0.016$, Fig. 5C).

Expression of PPE mRNA correlated inversely with the logarithm of [$^3$H]BTCP binding ($P < 0.001$, Fig. 6A). PPE mRNA expression was normalized when [$^3$H]BTCP binding was >10–20% of that of control. Analysis of PDyn mRNA and striatal $GAD_{67}$ mRNA showed similar findings ($P < 0.001$ for both, Fig. 6B and C). Expression of $GAD_{67}$ mRNA in the globus pallidus also correlated inversely with the logarithm of [$^3$H]BTCP binding ($P = 0.002$, Fig. 6D).

The final post-graft AIM scores in group A showed significant linear correlation with PPE mRNA, PDyn mRNA, striatal $GAD_{67}$ mRNA and $GAD_{67}$ mRNA in the globus pallidus (Fig. 7). Analysis of the final post-graft AIM scores in group B also showed significant correlation with mRNA encoding PPE, PDyn, $GAD_{67}$ in the striatum or $GAD_{67}$ in the globus pallidus (Fig. 7).

## Discussion

### *Levodopa-induced AIM of the rat*

Pulsatile treatment with a low dose of levodopa gradually induced stereotypy and contraversive rotation in the

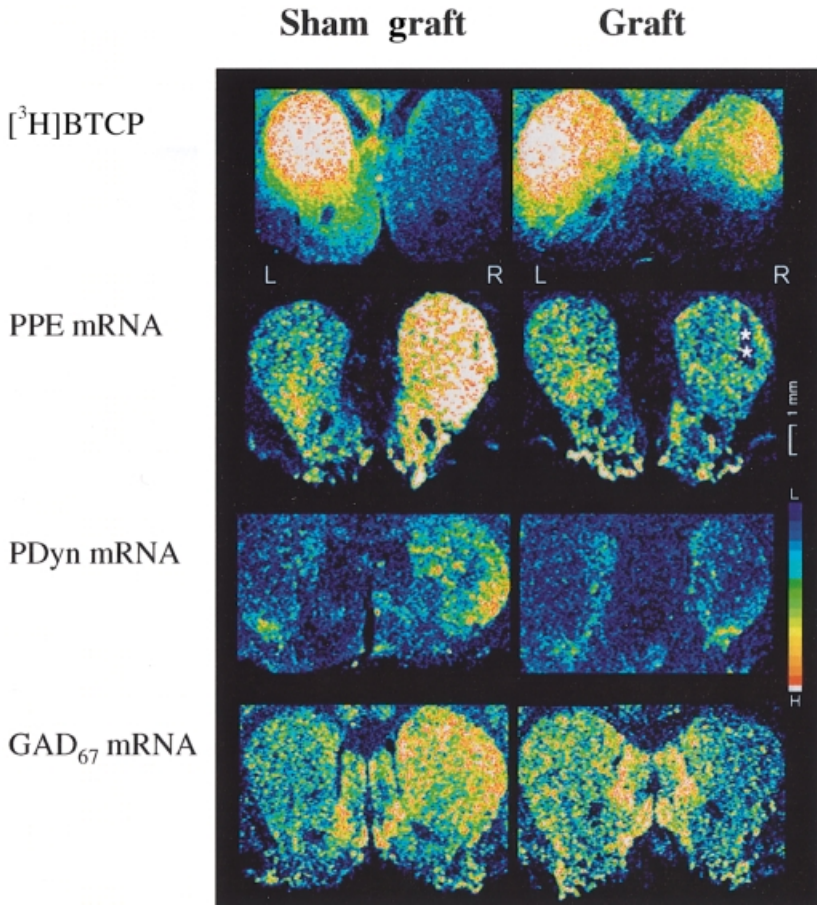


**Fig. 4** Autoradiographic images of [$^3$H]BTCP binding, and hybridization signals of PPE mRNA (preproenkephalin), PDyn mRNA (prodynorphin) and $GAD_{67}$ mRNA (glutamic acid decarboxylase) in the striatum. Left column, striatal sections of sham-grafted rats; right column, striatal sections of grafted rats. First row: survival of dopaminergic neuronal grafts in the host striatum was evaluated by measuring the density of dopaminergic nerve terminals using [$^3$H]BTCP, a marker for dopamine transporter. Embryonic dopamine neuronal grafts were shown in the background of dopamine-denervated host striatum. Second, third and fourth rows: expression of mRNA encoding PPE, PDyn or $GAD_{67}$, respectively, was upregulated in the sham-grafted striatum (left column) and normalized in the grafted striatum (right column). *A needle tract, L = left striatum, R = right striatum.

**Table 2** *Results of autoradiographic studies*

| | Group A | | Group B | |
|---|---|---|---|---|
| | Graft | Sham-graft | Graft | Sham-graft |
| Number | 7 | 6 | 5 | 5 |
| Striatal [$^3$H]BTCP | 19.07 ± 8.66* | 4.99 ± 2.36 | 22.20 ± 13.09* | 4.68 ± 2.48 |
| Striatal PPE mRNA | 112.79 ± 31.50* | 197.77 ± 16.20 | 109.18 ± 13.58* | 170.95 ± 20.41 |
| Striatal PDyn mRNA | 111.31 ± 25.44* | 170.97 ± 24.87 | 107.58 ± 18.74* | 170.84 ± 34.92 |
| Striatal $GAD_{67}$ mRNA | 114.17 ± 24.86* | 180.53 ± 7.46 | 106.94 ± 9.87* | 168.81 ± 19.91 |
| Pallidal $GAD_{67}$ mRNA | 108.79 ± 6.73* | 118.78 ± 7.57 | 110.92 ± 5.71* | 123.11 ± 2.15 |

Analysis was carried out using logarithmic transforms of the variables (see Statistical analysis). However, data presented above are in untransformed values (mean ± SEM; *significant difference from the sham-graft subgroup).

6-OHDA-lesioned rats. The involuntary nature of these levodopa-induced abnormal movements was inferred by their self-injuring tendency, and by their inability to suppress these abnormal movements when challenged by novel sensory stimuli (Walters *et al*., 1990). Levodopa-induced stereotypy was classified into three subtypes based on the topographic distribution. Similar to levodopa-induced dyskinesia in patients with Parkinson's disease (Marsden *et al*., 1982; Hughes *et al*., 1994), the relative proportion of each topographic subtype varied considerably among individual rats,

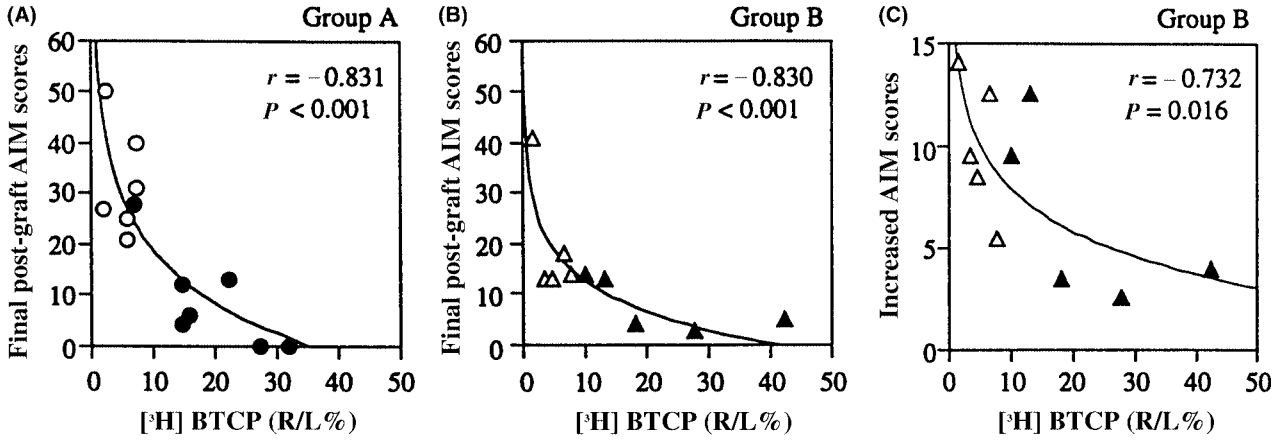


**Fig. 5** Scatter plots displaying the relationship between the severity of levodopa-induced AIM and presynaptic dopaminergic nerve terminals. Final post-graft AIM scores were regressed on the logarithm of [$^3$H]BTCP binding in group A (**A**) and again in group B (**B**). Open circle and open triangle: sham-grafted rats in groups A and B, respectively. Filled circle and filled triangle: grafted rats in groups A and B, respectively. The severity of levodopa-induced AIM sharply decreased when the density of dopaminergic nerve terminals was >10–20% of that of the unlesioned control side. (**C**) The magnitude of the increase in AIM scores by challenging tests correlated inversely with the logarithm of [$^3$H]BTCP binding. Analysis by Spearman rank correlation yielded analogous results.

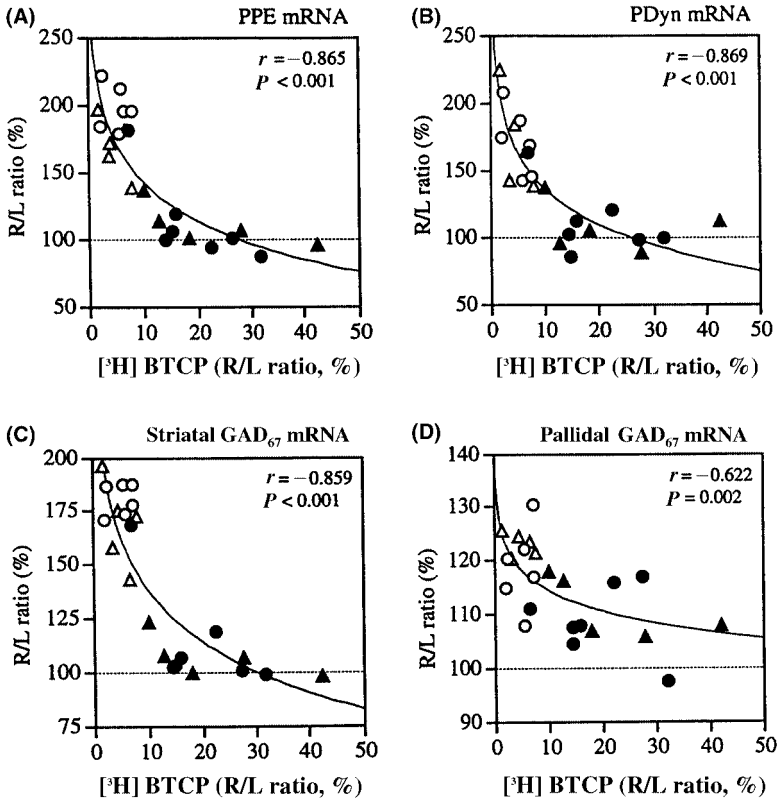


**Fig. 6** Scatter plots illustrating the relationship between the presynapitc and postsynaptic markers. Expression of mRNA encoding PPE, $GAD_{67}$ and PDyn in the striatum, or $GAD_{67}$ in the globus pallidus was regressed on the logarithm of [$^3$H]BTCP binding. Open circle, sham-grafted rats in group A; filled circle, grafted rats in group A; open triangle, sham-grafted rats in group B; filled triangle, grafted rats in group B.

yet it was consistent through serial observations in the same rat. Earlier studies have shown that stereotypy is mediated primarily through the neostriatum and the globus pallidus, whereas the locomotor activity also involves the nucleus accumbens and ventral pallidum (Kelly *et al.*, 1975; Scheel-Kruger, 1984; Sharp *et al.*, 1987). Microinjection of a dopamine agonist into the ventrolateral striatum generates orofacial or limb stereotypy (Kelley *et al.*, 1989; Amalric and Koob, 1993; Dickson *et al.*, 1994). Thus, it seems that the basal ganglia are topographically organized in the rat, as

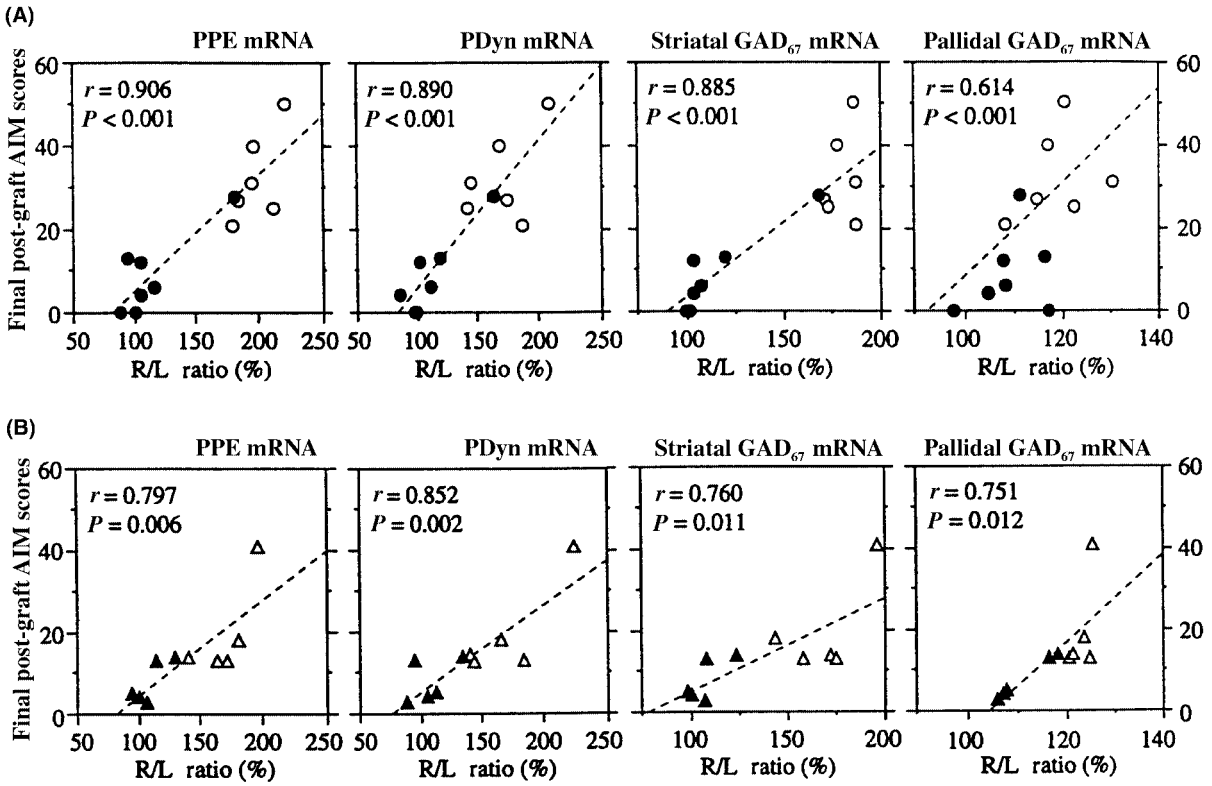


**Fig. 7** Scatter plots illustrating the relationship between the severity of levodopa-induced AIM and postsynaptic markers: (**A**) group A; (**B**) group B. The final AIM scores correlated linearly with PPE, PDyn and $GAD_{67}$ mRNAs in the striatum, and $GAD_{67}$ mRNA in the globus pallidus. Spearman rank correlation analysis yielded analogous results. Open circle, sham-grafted rats in group A; filled circle, grafted rats in group A; open triangle, sham-grafted rats in group B; filled triangle, grafted rats in group B.

has been observed in primates (DeLong *et al.*, 1984; Alexander *et al.*, 1986). Assessment of both topographic subtypes of stereotypy and locomotor activity, therefore, would enhance the sensitivity of detecting behavioural changes resulting from pathology in the neostriatum.

A rotometer has been used traditionally for measurement of drug-induced rotation in rats (Ungerstedt and Arbuthnott, 1970). We have demonstrated a competitive relationship between stereotypy and contraversive locomotor activity for the behavioural expression, as described previously (Koob *et al.*, 1984; Rebec and Bashore, 1984; Sharp *et al.*, 1987). Although rats are quadrupedal animals using both forelimbs and hindlimbs for locomotor activity, we observed that they use forelimbs for handling food in a bipedal sitting position. Hence, the forelimbs are involved in two different motor programmes, i.e. locomotor and feeding activities, which cannot be executed simultaneously. These behavioural characteristics of normal rats resemble the position-dependent appearance of stereotypy or locomotor activity in levodopa-treated rats with 6-OHDA lesions. Increased locomotor activity with contraversive directional bias in the rat with unilateral 6-OHDA lesions, which was induced by repetitive levodopa treatment, was expressed predominantly in the quadrupedal position, involving both forelimbs. In contrast, the limb dyskinesia was expressed predominantly in the bipedal sitting or standing position, involving only one forelimb on the side contralateral to the dopamine-denervated striatum. This position-dependent switching of the predominant motor pattern explains inconsistent, multiple peaks in the time–action curve of rotometer measurements in the rats that have both severe stereotypy and contraversive locomotor activity (as shown in Fig. 2C). In order to measure the maximum intensity of stereotypy or locomotor activity, rats were forced into a bipedal sitting or standing position for expression of stereotypy, and into a quadrupedal position for expression of locomotor activity. This study shows that stereotypy and contraversive locomotor activity, induced by repetitive levodopa treatment, share the same characteristics as levodopa-induced dyskinesia in patients with Parkinson's disease. Both are abnormal involuntary movements, and appear during the course of levodopa treatment. Both show similar time courses of development: a peak-dose response after administration of levodopa, and gradual enhancement of the peak-dose response with repetitive levodopa treatment. Thus, we consider levodopa-induced stereotypy and contraversive locomotor activity to be a rat version of levodopa-induced, peak-dose dyskinesia.

## *Effects of embryonic dopamine neuronal grafts*

### *On presynaptic markers*

In the grafted rats, the mean density of dopaminergic nerve terminals measured by [$^3$H]BTCP binding was 21.0% (range:

6.72–42.17%) of the contralateral control side, significantly greater than the 4.8% mean of the control in the sham-grafted rats. Earlier studies have shown that 6-OHDA lesions produce ~97–99% reduction of striatal tissue dopamine (Schmidt *et al*., 1982), and that embryonic VM grafts increased striatal tissue dopamine on average to 13–18% of normal, with the highest individual values reaching ~50% (Schmidt *et al*., 1983). Thus, the ~15% increase of dopaminergic nerve terminals by embryonic VM grafts in this study is comparable with previous results in the rat.

### *On postsynaptic markers*

Nigrostriatal dopaminergic denervation upregulates expression of PPE mRNA and $GAD_{67}$ mRNA, but not PDyn mRNA in the rat with 6-OHDA lesions (Normand *et al*., 1988; Gerfen *et al*., 1990; Li *et al*., 1990; Chesselet *et al*., 1993). Repetitive levodopa treatment upregulates PDyn mRNA (Cenci *et al*., 1998). Consistent with these earlier observations, the sham-grafted striatum showed upregulation of PPE mRNA, $GAD_{67}$ mRNA and PDyn mRNA. In contrast, the grafted striatum showed near-normalized levels of PPE mRNA, $GAD_{67}$ mRNA and PDyn mRNA, in agreement with previous reports (Cadet *et al*., 1991; Bal *et al*., 1993; Mendez *et al*., 1993; Roy *et al*., 1995; Cenci *et al*., 1997). Correlation analysis has shown that PPE mRNA was normalized when the density of dopaminergic nerve terminals was >10–20% of that of control. These observations are in keeping with previous studies in the rat with partial 6-OHDA lesions (Chritin *et al*., 1996). Levodopa-induced upregulation of PDyn mRNA was normalized in the grafted striatum when the density of dopaminergic nerve terminals was >10–20% of that of control. These observations indicate that upregulation of PDyn mRNA (which is induced by repetitive levodopa treatment) requires a certain level of dopamine terminal loss, as in the case of upregulation of PPE mRNA (that is induced by dopaminergic denervation). In the sham-grafted rats, expression of pallidal $GAD_{67}$ mRNA was upregulated, in agreement with previous reports (Kincaid *et al*., 1992; Delfs *et al*., 1995). In the grafted rats, expression of pallidal $GAD_{67}$ mRNA was near normal, suggesting that the grafted dopaminergic neurons in the striatum normalized upregulated expression of $GAD_{67}$ mRNA in the globus pallidus. These observations are consistent with recent reports that intrastriatal VM grafts normalize neuronal activity not only in the target area, but also in remote areas, the globus pallidus and output nuclei of the basal ganglia (Nakao *et al*., 1998). Taken together, our data show that dopamine neuronal grafts normalize PPE mRNA and $GAD_{67}$ mRNA in the indirect pathway and PDyn mRNA in the direct pathway if the density of dopaminergic nerve terminals is greater than a 'threshold' level (which is 10–20% of normal, measured by [$^3$H]BTCP).

### *On motor behaviours*

AIM scores in the grafted rats began to fall during the second month post-graft. The delayed functional effects of dopaminergic neuronal grafts have been observed in the rat (Dunnett *et al*., 1983, 1987; Blunt *et al*., 1991) and in patients with Parkinson's disease (Lindvall *et al*., 1992; Kordower *et al*., 1995; Defer *et al*., 1996; Kopyov *et al*., 1996). There was also a modest, but significant reduction of AIM scores in the sham-grafted rats. This reduction may result from reduced frequency of levodopa administration during the post-graft period. Anatomical studies have not consistently shown evidence for graft-induced axonal sprouting in the host striatum (Kordower *et al*., 1995, 1998). The inverse correlation between the final AIM scores and the density of surviving dopaminergic nerve terminals suggests that dopaminergic neuronal grafts predominantly contributed to the improvement of levodopa-induced AIM during the post-graft period. Previous studies have also shown that the functional effects of grafts are dependent on the number of grafted dopamine neurons (Lindvall, 1994; Kopyov *et al*., 1997).

## ***Factors influencing the severity of levodopa-induced AIM***

### *Presynaptic dopaminergic nerve terminals*

In the previous section, we have shown that the severity of AIM was inversely correlated with the density of surviving dopaminergic nerve terminals. Levodopa-induced AIM almost disappeared when the density of dopaminergic nerve terminals was >10–20% of that of control. These observations are consistent with the view that dopaminergic denervation is required for the manifestation of levodopa-induced dyskinesia, as has been observed in primates (Boyce *et al*., 1990) and in patients with Parkinson's disease (Nutt, 1990).

### *The dose of exogenous levodopa*

Challenge tests (in group B) showed that higher doses of levodopa increased the severity of AIM in both sham-grafted and grafted rats. The magnitude of increased AIM scores correlated inversely with the density of surviving dopaminergic nerve terminals. Thus, our data suggest that the severity of levodopa-induced AIM is an outcome of interplay between the dose of levodopa and the density of surviving dopaminergic nerve terminals. These observations are consistent with the concept that dopaminergic neuronal loss lowers the threshold for the expression of levodopa-induced dyskinesia (Nutt, 1990). However, in sham-grafted rats, the final post-graft AIM scores remained significantly lower in group B (which received 192 mg/kg of levodopa) than in group A (which received 12 mg/kg of levodopa). As the density of dopaminergic nerve terminals was not significantly different between groups A and B, these observations suggest that the severity of levodopa-induced AIM is also determined by other factors in addition to the dose of levodopa and the density of dopaminergic nerve terminals.

*Role of neurotransmitters in postsynaptic neurons*

Consistent with our previous observations (Cenci *et al*., 1998) as well as those of others (Brotchie, 1999), the severity of AIM correlated with the expression of PPE mRNA in the indirect pathway, and also with the expression of PDyn mRNA in the direct pathway. The mRNAs are changed in a manner that precedes and is proportional to the changes in product peptide levels (Li *et al*., 1988; Gerfen *et al*., 1990; Engber *et al*., 1991), suggesting that expression of mRNA may reflect the rate of synthesis of corresponding product peptide. Enkephalin inhibits the release of excitatory amino acids from corticostriate fibres in the striatum (Jiang and North, 1992), and inhibits the release of GABA in the globus pallidus (Dewar *et al*., 1987; Maneuf *et al*., 1994). Thus, enkephalin in striatopallidal neurons simulates the action of D2 receptor agonists (Marin and Chase, 1995). In primates, injection of a GABA antagonist into the external globus pallidus induces dyskinesia in the monkey (Crossman *et al*., 1984; Matsumura *et al*., 1995). These observations suggest that the overactivity of enkephalin, which is induced by dopaminergic denervation, may be related to levodopa-induced dyskinesia. On the other hand, microinjection of dynorphin or a κ-opioid agonist into the substantia nigra reduces overactive glutamatergic transmission in the output nuclei of the basal ganglia (Maneuf *et al*., 1995) and suppresses neuronal activity in the substantia nigra pars reticulata (Lavin and Garcia-Munoz, 1986; Thompson and Walker, 1990). In the rat, microinjection of a κ-opioid agonist into the substantia nigra pars reticulata improves experimental parkinsonism, and induces contraversive rotation (Matsumoto *et al*., 1988). Upregulated expression of PDyn mRNA in the striatum correlated with the enhancement of the contraversive rotational response (Duty and Brotchie, 1997). These observations as well as ours suggest that the enhanced activity of dynorphin in the striatonigral neurons may contribute to the development of dyskinesia during levodopa treatment. Taken together, these observations suggest that levodopa-induced AIM are manifested by a synergistic action of neurotransmitters in the indirect and the direct pathways, which are upregulated by dopaminergic denervation and levodopa treatment; and that embryonic dopamine neuronal grafts may improve levodopa-induced AIM through normalization of postsynaptic neuronal activities that were altered by dopaminergic denervation and levodopa treatment. This view is consistent with clinical observations that levodopa-induced dyskinesia requires chronic levodopa therapy and dopaminergic denervation (Nutt, 1990).

In summary, using a novel quantitative rating scale for levodopa-induced AIM in the rat, we have demonstrated that embryonic VM grafts improved levodopa-induced AIM. Clinical observations, however, have not been consistent with regard to the therapeutic effects of foetal dopaminergic neuronal grafts on levodopa-induced dyskinesia (Peschanski *et al*., 1994; Kordower *et al*., 1998). This inconsistency may result from variable viability of the grafts derived from different transplantation techniques (Kordower *et al*., 1998; Lindvall, 1998). Indeed, post-graft improvement of the uptake rate constant ($K_i$) in [$^{18}$F]DOPA/PET varies substantially in different studies, ranging from none to 100% of the pre-graft $K_i$ (Lindvall *et al*., 1989, 1994; D. Eidelberg and J. Stoessl, personal communication). Observations in this rodent study suggest that the severity of levodopa-induced dyskinesia is determined by multiple factors: the density of presynaptic dopaminergic nerve terminals, the dose of levodopa and the activity of postsynaptic neurons in the direct and indirect pathways. Foetal dopamine neuronal grafts may improve levodopa-induced dyskinesia by normalization of the activity of neurotransmitters in postsynaptic neurons, provided that the density of striatal dopaminergic nerve terminals is restored above a 'threshold' level.

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