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Cellular and behavioural effects of the adenosine A_{2a} receptor antagonist KW-6002 in a rat model of L-DOPA-induced dyskinesia

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Abstract

We have examined the ability of KW-6002, an adenosine A_{2a} antagonist, to modulate the dyskinetic effects of L-DOPA in 6-hydroxydopamine-lesioned rats. In animals rendered dyskinetic by a previous course of L-DOPA treatment, KW-6002 did not elicit any abnormal involuntary movements on its own, but failed to reduce the severity of dyskinesia when coadministered with L-DOPA. A second experiment was undertaken in order to study the effects of KW-6002 in L-DOPA-naïve rats. Thirty-five animals were allotted to four groups to receive a 21-day treatment with: (i) KW-6002 (10 mg/kg/day); (ii) L-DOPA (6 mg/kg/day) i.p.; (iii) KW-6002 plus L-DOPA (same doses as above) or (iv) vehicle. Chronic treatment with KW-6002-only produced a significant relief of motor disability in the rotarod test in the absence of any abnormal involuntary movements. Combined treatment with L-DOPA and KW-6002 improved rotarod performance to a significantly higher degree than did each of the two drugs alone. However, this combined treatment induced dyskinesia

to about the same degree as did L-DOPA alone. *In situ* hybridization histochemistry showed that KW-6002 treatment alone caused an approximately 20% reduction in the striatal levels of preproenkephalin mRNA, whereas neither the coadministration of KW-6002 and L-DOPA nor L-DOPA alone significantly altered the expression of this transcript in the dopamine-denervated striatum. Either alone or in combination with L-DOPA, KW-6002 did not have any modulatory effect on prodynorphin mRNA expression or FosB/ΔFosB-like immunoreactivity in the dopamine-denervated striatum.

These results show that monotreatment with an adenosine A_{2a} receptor antagonist can relieve motor disability without inducing behavioural and cellular signs of dyskinesia in rats with 6-hydroxydopamine lesions. Cotreatment with KW-6002 and L-DOPA potentiates the therapeutic effect but not the dyskinesia potential of the latter drug.

Keywords: indirect pathway, movement disorder, opioid, Parkinson's disease, striatonigral, striatopallidal.

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Pharmacological dopamine (DA) replacement with L-DOPA remains the cardinal treatment for Parkinson's disease. However, motor fluctuations and dyskinesias are experienced by the majority of patients after 5–6 years of L-DOPA pharmacotherapy (Obeso *et al.* 2000). The complications related to long-term L-DOPA pharmacotherapy have prompted a search for new, symptomatic or neuroprotective treatments to be used in Parkinson's disease. Much attention is presently being focused on drugs that block adenosine 2a (A_{2a}) receptors (for review see Jenner 2000; Fuxe *et al.* 2001), which are particularly enriched in striatal neurones of the 'indirect pathway' (for review see Fuxe *et al.* 1998; Svenningsson *et al.* 1999). Several features of these compounds make them very attractive as candidate antiparkinsonian agents. First, A_{2a} receptor inactivation has been shown to yield neuroprotective effects in experimental

models of dopaminergic neurotoxicity and neurodegeneration (Chen *et al.* 2001; Ikeda *et al.* 2002). Second, A_{2a} antagonists bear the promise of relieving parkinsonism without producing dyskinesia. When tested on marmosets previously intoxicated with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridin (MPTP), the adenosine A_{2a} antagonist

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Abbreviations used: AIM, abnormal involuntary movement; AUC, area under the curve; CPu, caudate-putamen; DA, dopamine; MPTP, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridin; OD, optical density; 6-OHDA, 6-hydroxydopamine; PDyn, prodynorphin; PPE, preproenkephalin.

KW-6002 produced a significant improvement in motor disability, which did not exhibit a loss of acute efficacy over at least 21 days of treatment. Moreover, the observed reduction in motor disability scores was not accompanied by manifestations of abnormal, excessive movement such as hyperlocomotion, stereotypies or dyskinesias (Kanda *et al.* 1998). In the same animal model, KW-6002 was found to synergistically enhance the antiparkinsonian action of low-dose L-DOPA or D2 receptor agonists (Kanda *et al.* 2000). These very encouraging data raise a hope that A_{2a} receptor antagonists will soon become available as either an alternative or an adjunct to L-DOPA for the treatment of Parkinson's disease. At the same time, these data call for further investigation on the extent to which A_{2a} receptor antagonists can modulate the effects of L-DOPA on both a behavioural and a cellular level.

Rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway are still the most extensively used animal model of parkinsonism. This model provides a cost-effective tool for pathophysiological studies and for a pre-clinical screening of candidate antiparkinsonian treatments (for review see Schwarting and Huston 1996; Cenci *et al.* 2002). A number of studies using unilaterally 6-OHDA-lesioned rats have reported an enhancing effect of A_{2a} receptor antagonists on L-DOPA-induced contralateral turning (Fenu *et al.* 1997; Koga *et al.* 2000; Pinna *et al.* 2001) and striatal c-Fos expression (Fenu *et al.* 1997). It is, however, difficult to provide an interpretation of turning behaviour and c-Fos expression in terms of their being representative of either the therapeutic or the dyskinetic action of L-DOPA.

In the present study we have taken advantage of recently validated rat models of dyskinesia and akinesia in order to examine the modulatory effect of KW-6002 on both the motor improvement and the adverse dyskinetic manifestations produced by L-DOPA. A rotarod test was used for an assessment of motor disability (Rozas and LaBandeira Garcia 1997; Rozas *et al.* 1997) and drug-induced motor improvements. A rat abnormal involuntary movements (AIMs) scale (Lundblad *et al.* 2002; Winkler *et al.* 2002) was used in order to rate dyskinetic motor manifestations. The cellular effects of chronic treatment with KW-6002 and L-DOPA (either alone or combined) were examined using markers of gene or protein expression which are strongly linked with dyskinesia in this rat model. Indeed, we have previously shown that the development of AIMs during chronic L-DOPA treatment is causally linked with an induction of Δ FosB-like proteins and prodynorphin (PDyn) mRNA in striatal neurones (Andersson *et al.* 1999; Andersson *et al.* 2001). Moreover, rats that develop dyskinesia show significantly higher striatal levels of preproenkephalin (PPE) gene expression than do non-dyskinetic animals receiving the same L-DOPA treatment (Cenci *et al.* 1998; Winkler *et al.* 2002).

Materials and methods

Subjects

The study was performed on female Sprague-Dawley rats (B & K Universal, Stockholm, Sweden; \approx 225 g body weight at the beginning of the experiment). The animals were housed under a 12-h light/dark cycle. The treatment of the animals and their conditions had been approved by the Malmö-Lund Ethical Committee on Animal Research.

Treatment groups and experimental design

The treatment regimens and number of animals in this study are summarized in Table 1. As shown in Table 1, the present study includes the following, distinct experiments.

Experiment 1. Effects of subchronic drug administration in 6-hydroxydopamine-lesioned rats rendered dyskinetic by previous L-DOPA treatment

This experiment comprised 19 rats with unilateral 6-OHDA lesions, which had developed maximally severe dyskinesia during a previous course of treatment with L-DOPA (4–6 mg/kg/day methyl L-DOPA i.p., combined with 12 mg/kg/day benserazide, \times 16 days). Two doses of KW-6002, i.e. 3 and 10 mg/kg/day p.o., were administered to these rats either alone or in combination with L-DOPA (4 mg/kg/day i.p. plus 12 mg/kg/day benserazide) according to a randomized, cross-over design (see below). The rats were tested for rotarod performance a total of eight times and for AIM ratings a total of four times over a period of 4 weeks. Drugs were given only on the days of behavioural testing, but the frequency of L-DOPA administration was maintained at one injection/day per 2–4 days/week. We have previously shown that this administration frequency is sufficient to maintain stable AIM scores over a period of months (Lee *et al.* 2000).

Experiment 2. Effects of chronic drug treatment in L-DOPA-naive rats

An additional batch of animals with unilateral 6-OHDA lesions ($n = 35$) was allotted to four treatment groups, which were balanced with respect to the rats' amphetamine-induced rotational rates. The groups received the following treatments: (i) KW-6002 (10 mg/kg/day p.o. and benserazide 12 mg/kg/day i.p.); (ii) L-DOPA (6 mg/kg/day i.p. plus 12 mg/kg/day benserazide) and methyl-cellulose vehicle p.o.; (iii) KW-6002 p.o. and L-DOPA + benserazide i.p., each at the same dose as above and (iv) benserazide i.p. and methyl-cellulose p.o. (control group). These treatments were given once daily for 21 days.

Drugs

L-DOPA methyl ester and benserazide-HCl were purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). They were dissolved in physiological saline and administered within 1 h at the volume of 1.0 mL/kg body weight by i.p. injection. L-DOPA was given 20 min before the start of each AIM-rating session and 30 min prior to rotarod testing. The doses of L-DOPA used in this study (4–6 mg/kg/day) are in the range used for the treatment of Parkinson's disease. In our hands, 6 mg/kg L-DOPA per injection (combined with 12–15 mg/kg benserazide) represents the therapeutic threshold dose, i.e. the lowest dose producing a significant relief

Table 1 Experimental groups and treatment regimens in the present study

	Treatment regimens	AIM incidence	Maximal AIM severity
Experiment 1			
L-DOPA-primed rats ($n = 19$)	KW-6002 3.0 or 10 mg/kg in methylcellulose (p.o.) and L-DOPA 4 mg/kg (i.p.) plus benserazide 12 mg/kg (i.p.) on the days of behavioural testing	19/19	11.5 \pm 0.8
Experiment 2			
Control group ($n = 7$)	Methylcellulose 5 mL/kg (p.o.) and benserazide 12 mg/kg (i.p.) \times 21 days	0/7	0 \pm 0.0
KW group ($n = 9$)	KW-6002 10 mg/kg in methylcellulose (p.o.) and benserazide 12 mg/kg (i.p.) \times 21 days	0/9	0 \pm 0.0
L-DOPA group ($n = 9$)	L-DOPA 6 mg/kg (i.p.) plus benserazide 12 mg/kg (i.p.) and methylcellulose 5 mL/kg (p.o.) \times 21 days	7/9	5.7 \pm 1.5
KW + L-DOPA group ($n = 10$)	KW-6002 10 mg/kg in methylcellulose (p.o.), L-DOPA 6 mg/kg (i.p.) plus benserazide 12 mg/kg (i.p.) \times 21 days	9/10	5.3 \pm 1.2

The incidence of abnormal involuntary movements (AIMs) is expressed as the number of dyskinetic animals/total number of animals in each group. The right-hand column shows the maximal AIM severity/monitoring period as group means \pm SEM. The maximum value possible is 16 (grade 4 on all four subtypes of AIMs, see Materials and methods).

of akinesia in this rat model of parkinsonism (Lundblad *et al.* 2002; Winkler *et al.* 2002; Lundblad and Cenci, unpublished).

KW-6002 was produced and kindly supplied by the Department of Medical Chemistry, H. Lundbeck A/S (Valby, Denmark). It was suspended in a solution of 0.5% methylcellulose/saline and administered in a final volume of 5.0 mL/kg body weight by oral gavage. When administered alone, KW-6002 was given 30 min before testing. When coadministered with L-DOPA, the compound was given 30 min prior to L-DOPA (i.e. 50 min before the start of AIM-rating sessions and 60 min before the start of rotarod tests). Before carrying out the actual testing experiments, all animals were habituated to the stress of p.o. methylcellulose vehicle administration once a day for 1 week. The doses of KW-6002 used in this study (3.0 and 10 mg/kg) are within the range producing a relief of parkinsonian motor disability in MPTP-intoxicated marmosets (Kanda *et al.* 1998).

Lesion surgery and amphetamine rotation

All the animals in this study were subjected to unilateral 6-OHDA lesions of the right ascending DA fibre bundle. The lesions were performed according to a standard procedure used in our laboratory (see, e.g. Cenci *et al.* 1998). Briefly, 6-OHDA-HCl (Sigma, St Louis, MO, USA) was dissolved in 0.02% ascorbate-saline at a concentration of 3 μ g/ μ L and injected into the right ascending DA fibre bundle at the following coordinates (in mm) relative to bregma and the dural surface: (1) A = -4.4, L = 1.2, V = 7.8, tooth bar at -2.4 (7.5 μ g deposit) and (2) A = -4.0, L = 0.75, V = 8.0, tooth bar at +3.4 (6 μ g deposit). At 2 weeks post-lesion, rats were tested for amphetamine-induced rotation (2.5 mg/kg d-amphetamine i.p.; 90 min testing) and only animals showing individual means > 5 full turns/min in the direction ipsilateral to the lesion were selected for

the study (Schmidt *et al.* 1982). This rotational score has been shown to correspond to > 90% depletion of DA fibre terminals in the striatum (Winkler *et al.* 2002).

Rotarod test

Four weeks after the amphetamine-induced rotation test, and before the onset of KW-6002 or L-DOPA treatment, all the animals in the present study were pre-trained on the rotarod apparatus (Rotamex 4/8; Columbus Instruments, Columbus, OH, USA) in order for them to reach a stable performance in this test. The training consisted of three sessions on three consecutive days, where each session included two separate testing trials. In the testing trials the animals were placed on the testing rod at an initial speed of 4 rpm. The rod speed then increased gradually to 44 rpm over 90 s. The animals were tapped on their tails several times in each session in order to help them maintain a high degree of alertness in the test. The time spent on the rod was recorded automatically for each animal and the average performance in the two consecutive trials was used for within-animal comparisons. Between the two testing trials, all animals were given a shorter session where the rod speed increased from 4 to 14 rpm over 25 s only. This enabled the animals to remain on the rod over the entire range of rotating speeds and was found to have a positive effect on the animals' willingness to perform in the test. This procedure was also repeated after the last testing trial. By the last training sessions all animals had reached a stable rotarod performance.

The drug treatment experiments were started 2 weeks after the last rotarod-training session. In experiment 1 (see Table 1), the rotarod test was performed 2 \times 2-day session/week. In each daily session, 50% of the animals were randomized to receive a given drug treatment, while the remaining 50% received the corresponding

vehicle. On the consecutive day of testing, the allocation to drug versus control treatment was switched (cross-over design). In experiment 2 (see Table 1), the rotarod test was executed a total of six sessions (two per week), each consisting of 3 × 90-s trials. (plus a shorter 'motivational' session in between trials and after the last trial). The results from the fifth testing session will not be reported in the following due to a technical failure (a transient reorganization of the animal facilities caused the rats to be visibly stressed, compromising the reliability of the behavioural data). The same acceleration speed and end speeds were used in this part of the study as in the training sessions. All testing was carried out by an investigator who was completely unaware of the rats' group membership (experimentally blinded) between 9 AM and 5 PM. Rotarod data are expressed as area under the curve (AUC), which was computed according to the formula:

$$\text{AUC} = \text{time on the rod(s)} \times [\text{time on the rod(s)} \times 0.44/2]$$

where 0.44 is the acceleration speed per s.

Abnormal involuntary movement ratings

Quantification of the AIMs induced by L-DOPA was carried out as extensively described in previous papers (Lee *et al.* 2000; Lundblad *et al.* 2002; Winkler *et al.* 2002). Animals were tested for L-DOPA-induced AIMs twice per week in both experiment 1 and 2 by an experimentally blinded investigator. Briefly, rats were observed individually for 1 min every 20 min during the 3 h that followed an injection of L-DOPA (observations were performed during the light hours, i.e. between 9 a.m. and 5 p.m.). Dyskinetic movements were classified based on their topographic distribution into four subtypes: (i) axial AIMs, i.e. twisted posture or choreiform twisting of the neck and upper body toward the side contralateral to the lesion; (ii) forelimb AIMs, i.e. jerky movements or dystonic posturing of the contralateral forelimb, and/or purposeless grabbing movement of the contralateral paw; (iii) orolingual AIMs, i.e. empty jaw movements and contralateral tongue protrusion and (iv) locomotive AIMs, i.e. increased locomotion with contralateral side bias. Each AIM subtype was rated on a severity scale from 0 to 4 (1 = occasional, 2 = frequent, 3 = continuous but interrupted by sensory distraction and 4 = continuous, severe, not interrupted by sensory distraction) in each monitoring period. Since locomotive AIMs differ greatly from the other three AIM subtypes in terms of their pharmacological features and anatomical substrate (Andersson *et al.* 1999; Lundblad *et al.* 2002; Winkler *et al.* 2002), they will be presented and analysed as a separate item. By contrast, axial, forelimb and orolingual AIMs will be presented together as a sum of scores per session.

Tissue preparation

Two days after the last drug administration, the animals were deeply anaesthetized with sodium pentobarbitone (240 mg/kg i.p.; Apoteksbolaget AB, Lund, Sweden) and killed by decapitation. Brains were rapidly extracted, frozen on powdered dry-ice and stored at -80°C. Coronal sections through the striatum were cut on a cryostat at 16-µm thickness and thaw-mounted onto microscope slides (SuperFrost Plus; Menzel Gläser, Braunschweig, Germany for immunohistochemistry and Superfrost End; Electron Microscopy Sciences, Washington, PA, USA for *in situ* hybridization). The slides were air-dried and stored at -20°C.

Immunohistochemistry

One series of sections per animal was fixed by 10-min immersion in phosphate-buffered 4% paraformaldehyde (pH 7.4) and then rinsed three times in potassium phosphate-buffered saline. The sections were pre-incubated for 1 h in 3% bovine serum albumin (Sigma Aldrich Sweden AB) and 12% avidin-blocking solution (Avidin Biotin Blocking Kit; Vector Laboratories, Burlingame, CA, USA). After a brief rinse, the sections were incubated overnight at 4°C with an affinity-purified rabbit polyclonal antiserum raised against an N-terminal peptide which is common to full-length FosB and ΔFosB (Santa-Cruz Biotechnology, Santa Cruz, CA, USA). The immunostaining obtained with this antibody will be referred to as FosB/ΔFosB immunoreactivity in the following. The specificity of this antibody has been extensively documented in previous studies (Andersson *et al.* 1999; Andersson *et al.* 2001; Westin *et al.* 2001). The primary antibody was diluted at 0.15 µg/mL in 1% bovine serum albumin and 12% biotin-blocking solution (Avidin Biotin Blocking Kit; Vector Laboratories). Detection of the tissue-bound antibodies was carried out using a standard peroxidase-based method (Vectastain Elite ABC Kit; Vector Laboratories) as described in Andersson *et al.* (1999).

In situ hybridization histochemistry

In situ hybridization histochemistry was performed using synthetic oligomers complementary to PPE and PDyn mRNA, which have been extensively characterized in previous papers (Cenci *et al.* 1998; Andersson *et al.* 1999; Johansson *et al.* 2001; Westin *et al.* 2001; Winkler *et al.* 2002). Oligonucleotides (0.2 µM) were labelled at the 3' end with 4 µM [α -³⁵S] dATP (> 37 TBq/mmol; Amersham Pharmacia Biotech, Bucks, UK) using 15 U of terminal deoxynucleotidyltransferase (Amersham Pharmacia Biotech) for 2 h at 37°C. The labelled probes were purified by spin-column chromatography (Chroma Spin Columns; Clontech Laboratories, Palo Alto, CA, USA) to specific activities of > 10⁹ cpm/µg. Sections were air dried for 10 min and then fixed in phosphate-buffered 4% paraformaldehyde (pH 7.4). The sections were rinsed for 3 × 4 min in potassium phosphate-buffered saline, dehydrated in 70 and 95% ethanol (2 min each) and air dried again for 10 min. Sections 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 and 10 mg/mL RNase-free bovine serum albumin), 1% sarcosyl, 10% dextran sulphate, 500 µg/mL sheared and denatured salmon sperm DNA, 25 µL/mL tRNA, 200 mM dithiothreitol, 0.2 mg/mL heparin and 10⁷ cpm/mL ³⁵S-labelled oligonucleotide probe. Forty microlitres of hybridization cocktail were added to each section (i.e. 240 µL/slide). Slides were coverslipped with parafilm and incubated for 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 4 × 15 min washes in 1 × SSC at 55°C, plus a final wash beginning at 55°C and cooling down to room temperature. The slides were then rinsed twice in distilled water, dehydrated in 70 and 95% ethanol (2 min each) and exposed to imaging plates (FujiFilm Sweden AB, Stockholm, Sweden) for 1 h (PPE) or 16 h (PDyn). To obtain publication-quality pictures, the sections were also exposed to autoradiographic film (β-max; Amersham Pharmacia Biotech, Bucks, UK) for 2–10 days at -20°C. The films were developed in D-19 (Eastman Kodak

Company, New Haven, CT, USA) and fixed in Snabbfix (Scanfors A/S, Skovlunde, Denmark). In order to confirm that the signal on the films corresponded to specific cellular labelling, sections were dipped in photographic K.5 emulsion (ILFORD Imaging, Knutsford, UK), diluted 1 : 1 with distilled water, exposed for 2–7 weeks at -20°C and developed in D-19 at 18°C . The sections were then fixed, counterstained with cresyl violet and coverslipped with DPX mountant (BDH laboratories supplies, Poole, UK).

Image analysis

Image analysis was performed by an investigator who was kept completely unaware of the identity of the animals and their behavioural performance.

Phosphorimager plates

For a quantification of PPE or PDyn mRNA levels, imaging plates were scanned in a phosphorimager (BAS-5000; Fujifilm Sweden AB) and analysed using the software (Tina Adobe 2.10; Fujifilm). The photostimulated luminescence (PSL) emitted by the hybridized sections was calibrated against radiolabelled standards (^{14}C -microscales; Amersham Pharmacia Biotech), which had been coexposed with the sections. Measurements of PPE and PDyn mRNA levels were carried out on three coronal sections per animal spanning the mid part of the caudate-putamen (CPu), i.e. between levels $+1.60$ and -0.48 mm relative to bregma (according to Paxinos and Watson 1997). This portion of the CPu is highly innervated by the somatosensory and primary motor cortex in the rat (McGeorge and Faull 1989; Kincaid and Wilson 1996) and exhibits pronounced molecular changes following chronic dyskinesigenic treatment with L-DOPA (Andersson *et al.* 1999). The hybridization signal measured on both the DA-denervated and the intact side of the CPu was expressed as PSL/ mm^2 after subtraction of the tissue background (as determined on the corpus callosum in each section).

FosB/ Δ FosB immunoreactivity

In order to count FosB/ Δ FosB-positive cells in the striatum, sample areas (0.54 mm^2 on the section) were selected through a $20\times$ objective in an AX-70 microscope (Olympus Optical Co., Tokyo, Japan) under bright-field illumination and digitized by a ProgRes videocamera (Kontron Elektronik, Munich, Germany). Camera setting and lighting conditions were optimized in order to avoid signal saturation and kept rigorously constant for all sections. Immunoreactive neurones were counted using the freeware *NIH Image, 1.61* (downloadable from rsbweb.nih.gov) in two sample areas in the medial and lateral CPu at mid-striatal levels (bregma $+0.3$ and -0.3 mm according to Paxinos and Watson 1997). Measurements were carried out on both the DA-denervated and the intact side of the CPu. In addition to counting labelled cells in a sample area, NIH Image software provides information on the number of pixels (above background threshold) and optical density (OD) value relative to each counted object. These parameters were used in order to provide an estimate of the amount of staining over single FosB/ Δ FosB-like immunoreactive cells, according to the formula:

$$\text{area covered by the cell} \times \text{average OD value/unit area.}$$

Statistical analysis

Behavioural data requiring paired comparisons on a single time point (experiment 1) were analysed using Student's (two-tailed)

t-test. Behavioural data which had been collected on several testing sessions during the chronic drug treatment period (experiment 2) were analysed using repeated measures analysis of variance (ANOVA) where time (testing session) and group (treatment) were entered as independent variables. Cell counts and *in situ* hybridization data were compared using a two-factor ANOVA with side (contralateral vs. ipsilateral to the lesion) and group as independent variables. All *post-hoc* comparisons were carried out using the Tukey 'honestly significant difference' method. Statistical significance was set at $p < 0.05$. Data will be expressed as group means \pm SEM.

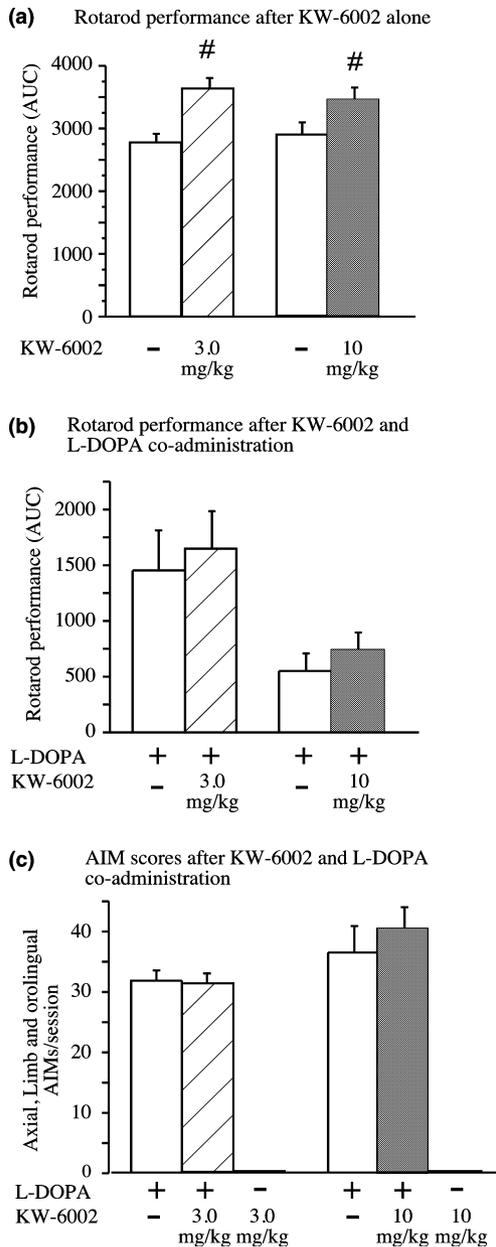
Results

Behavioural effects of KW-6002 in rats with severe dyskinesia established by prior treatment with L-DOPA

In the first experiment, we tested the ability of therapeutic doses of KW-6002 to modulate the effects of L-DOPA in severely dyskinetic rats. These animals provide a model of a very advanced and complicated stage of Parkinson's disease, in which the therapeutic window for L-DOPA is lost (Mouradian *et al.* 1989). The rats in this experiment exhibited a significant relief of motor disability when administered with KW-6002 alone. Indeed, both 3.0 and 10.0 mg/kg of KW-6002 produced a significant improvement in rotarod performance when administered without L-DOPA (≈ 20 – 30% increase in AUC value; $p < 0.05$ vs. vehicle; Fig. 1a). The higher dose tested (10 mg/kg) did not produce an additional benefit over the lower dose of KW-6002 (cf. hatched and closed bars in Fig. 1a). The motor improvement induced by the adenosine A_{2a} antagonist was not accompanied by any AIMs (Fig. 1c). Administration of a dose of L-DOPA that is slightly below the therapeutic threshold value in the rat (i.e. 4 mg/kg L-DOPA combined with 12 mg/kg benserazide; cf. Winkler *et al.* 2002) not only did not improve but actually disrupted the rats' rotarod performance (cf. open bars in Fig. 1a and b; note the different scaling on the *y*-axis). This paradoxical effect of L-DOPA was due to its pronounced dyskinetic effect in all the animals in this experiment (Table 1). Indeed, L-DOPA would otherwise ameliorate the performance of 6-OHDA-lesioned rats in the rotarod test (see below). Coadministration of L-DOPA and either 3.0 or 10 mg/kg KW-6002 did not result in a significant improvement in rotarod performance compared with L-DOPA alone (Fig. 1b). This lack of improvement was matched by a failure of KW-6002 to significantly relieve the severity of L-DOPA-induced AIMs in the same animals (Fig. 1c).

Behavioural effects of chronic KW-6002 treatment in L-DOPA-naive rats

In a second experiment, L-DOPA-naive rats were treated chronically with the higher dose of KW-6002 (10 mg/kg), which was administered either alone or together with 6 mg/kg



L-DOPA (combined with 12 mg/kg benserazide) for 21 days. Two additional groups of animals were treated with either L-DOPA alone (same dose and regimen as above) or the methylcellulose vehicle plus benserazide for a comparison.

Abnormal involuntary movement ratings

The development of dyskinesia during the course of chronic drug treatment differed greatly among the groups (Fig. 2a; $p < 0.0001$ for treatment effect, $p < 0.0001$ for time effect and $p < 0.0001$ for treatment and time interaction on a repeated measures ANOVA). Indeed, chronic treatment with either KW-6002 plus L-DOPA or L-DOPA

Fig. 1 In rats rendered dyskinetic by previous treatment with L-DOPA, monotreatment with KW-6002 relieves motor disability without producing dyskinesia. However, the severity of L-DOPA-induced abnormal involuntary movements (AIMs) is not reduced when the adenosine A_{2a} antagonist is coadministered with L-DOPA. In this experiment L-DOPA was given at a dose of 4 mg/kg/injection (combined with 12 mg/kg benserazide). (a) Rotarod performance after KW alone; (b) rotarod performance after cotreatment with L-DOPA and KW-6002; (c) effects of cotreatment with L-DOPA and KW-6002 on AIM scores. As shown in (b), the animals' rotarod performance under L-DOPA declined during the course of this experiment (compare the open bars on the right- and left-hand side). This decline was paralleled by a worsening of L-DOPA-induced dyskinesia during the course of the experiment (compare the open bars on the right- and left-hand side of Fig. 1(c)). $\#p < 0.05$ versus vehicle (methylcellulose 5 mL/kg p.o.); $n = 19$ (paired t -test). AUC, Area under the curve.

alone produced increasingly severe AIMs affecting trunk, limb and orolingual muscles. The incidence and severity of dyskinesia were similar in these two groups (Table 1), both of which differed significantly from the controls and the KW-6002-only group in all testing sessions (Fig. 2a). Neither the KW-6002-only group nor the control group showed any type of AIMs during the chronic treatment period. Locomotive AIM scores were significantly higher in animals treated with L-DOPA, alone or in combination with KW-6002, compared with both KW-6002-only and control treatment (Fig. 2b; $p < 0.001$ for treatment effect, $p < 0.001$ for time effect and $p < 0.006$ for time and treatment interaction). Rats cotreated with KW-6002 and L-DOPA tended to show higher locomotive AIM scores than animals receiving L-DOPA only (Fig. 2b). It is worth pointing out that locomotive AIM scores as such are unable to distinguish between the effects of an increased motor activation and those of dyskinesia (Lundblad *et al.* 2002). The data in Fig. 2(b) cannot, therefore, be interpreted as indicating that KW-6002 may potentiate the dyskinetic effects of L-DOPA.

Rotarod

Each of the drug treatments under investigation (i.e. KW-6002, L-DOPA and KW-6002 + L-DOPA) produced a significant improvement in rotarod performance above the levels measured in the control group (Fig. 2c; $p = 0.002$ for treatment effect, $p = 0.041$ for time effect and $p = 0.151$ for time and treatment interaction). KW-6002 alone produced a stable 80–90% increase in the AUC value (a function of the time spent on the rod at different turning speeds), which was significantly larger than control levels in all of the testing sessions. L-DOPA produced an effect of similar extent, which was however, more variable among animals and testing sessions ($p < 0.05$ vs. controls in testing sessions 2, 4 and 6 only). The combined treatment with KW-6002 and L-DOPA initially improved

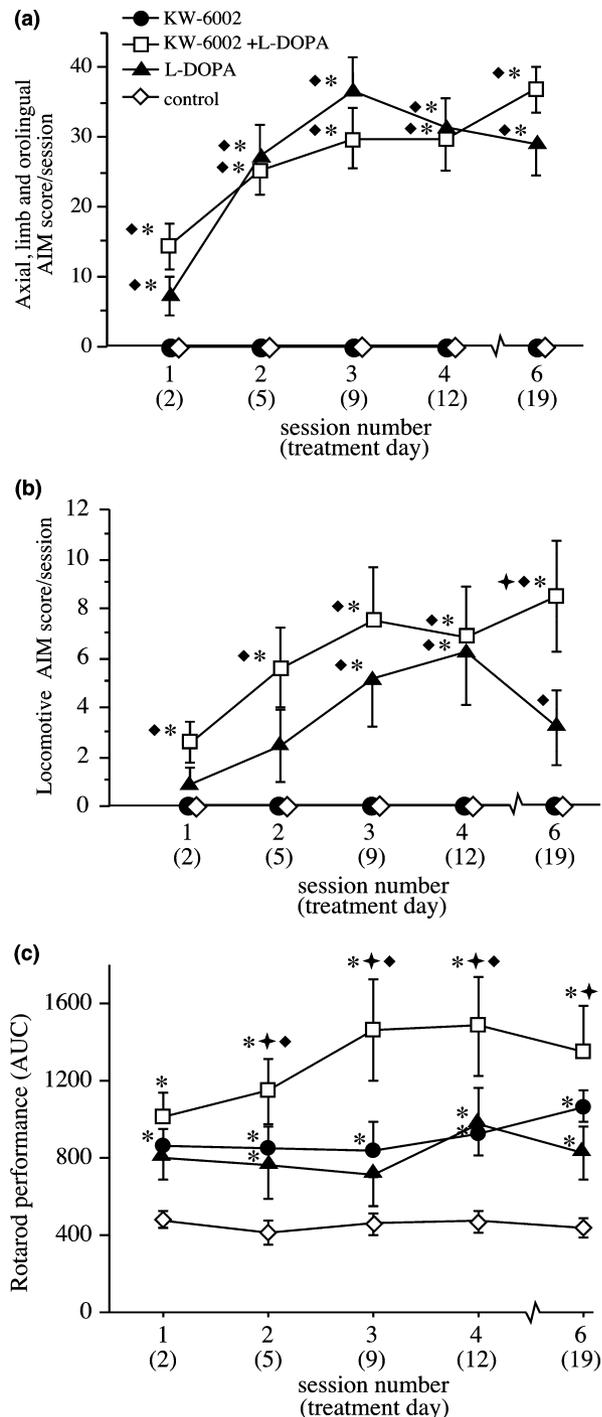


Fig. 2 Effects of chronic treatment with KW-6002 (10 mg/kg/day p.o.) and L-DOPA (6 mg/kg/day i.p. plus 12 mg/kg benserazide), alone or combined, in previously L-DOPA-naïve animals. The chronic treatment spanned over 21 days during which dyskinesia ratings and rotarod performance were measured a total of six times. (a) Time course of axial, limb and orolingual abnormal involuntary movement (AIM) scores; (b) locomotive AIM scores; (c) rotarod performance. The discontinuity on the x-axis indicates exclusion of the fifth testing session from the statistical analysis due to technical failure (see Materials and methods). $p < 0.05$: *versus control group; †versus L-DOPA group; ♦versus KW-6002 group; $n = 7-10$ per group, as specified in Table 1 (repeated measures ANOVA and *post-hoc* Tukey test). AUC, Area under the curve. ●, KW-6002; □, KW-6002 + L-DOPA; ▲, L-DOPA; ◇, control.

Effects of chronic KW-6002 treatment on striatal levels of opioid precursor mRNAs

Accumulating evidence attests to an association between L-DOPA-induced dyskinesia and striatal up-regulation of opioid precursor mRNA (see, e.g. Brotchie *et al.* 1998; Cenci *et al.* 1998; Zeng *et al.* 2000; Winkler *et al.* 2002). We thus compared the striatal expression of transcripts encoding for PPE (PPE-A) and PDyn (PPE-B) in rats chronically treated with KW-6002, L-DOPA or KW-6002 + L-DOPA.

Preproenkephalin mRNA

Striatal up-regulation of PPE mRNA after DA depletion has been widely documented in the literature. Accordingly, we found PPE mRNA levels to be significantly larger in the DA-denervated CPu than on the contralateral intact side in all the treatment groups (Fig. 3a; $p < 0.0001$ for side-effect, $p = 0.02$ for treatment effect and $p = 0.26$ for side and treatment interaction on a two-factor ANOVA; see autoradiographs in Figs 4a-d). However, animals receiving mono-treatment with KW-6002 showed a significant 20% reduction in PPE mRNA levels in the DA-denervated striatum compared with both the control group and the L-DOPA-only group ($p < 0.05$; Fig. 3a). A tendency towards lower levels of this transcript was also seen in the KW-6002 + L-DOPA group but this effect did not reach statistical significance. Treatment with L-DOPA alone neither reduced nor exacerbated the up-regulation of PPE mRNA seen in the DA-denervated CPu (non-significant difference vs. controls; Fig. 3a). The levels of PPE mRNA in the intact CPu did not differ significantly between any of the treatment groups.

Prodynorphin mRNA

As reported in a number of previous studies, DA denervation causes down-regulation of PDyn mRNA in the CPu, while L-DOPA has an inductive effect on the same transcript (see, e.g. Cenci *et al.* 1998; Andersson *et al.* 1999; Johansson *et al.* 2001; Fredduzzi *et al.* 2002). As expected, PDyn mRNA levels were significantly reduced ipsilaterally to the

the animals' performance to about the same extent as did each of the two drugs alone (see session 1 in Fig. 2c; $p < 0.05$ vs. controls). However, the KW-6002 + L-DOPA group showed a further improvement in rotarod performance over time, which reached its peak levels on testing sessions 3 and 4 (AUC increase by 223% above controls; $p < 0.05$ vs. all the other groups).

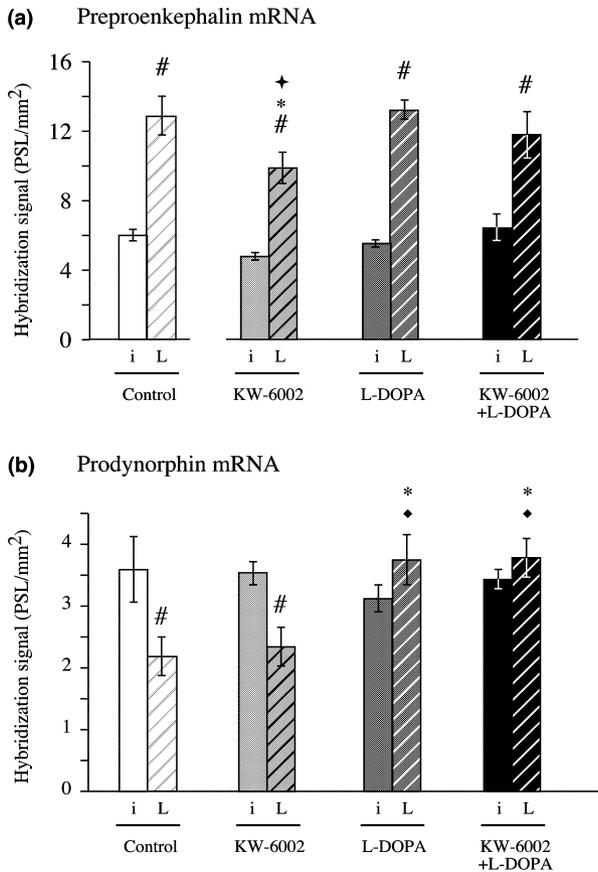


Fig. 3 Effects of the different drug treatments in the chronic-treatment study (experiment 2) on the levels of (a) preproenkephalin mRNA and (b) prodynorphin mRNA in the 6-hydroxydopamine-lesioned caudate-putamen (Cpu) (L) and the contralateral, intact side (i). $p < 0.05$: #versus intact CPu within the same group; *versus dopamine (DA)-denervated CPu in the control group; ♦versus DA-denervated CPu in the KW-6002 group; †versus DA-denervated CPu in the L-DOPA group; $n = 7-10$ per group, as specified in Table 1 (two-factor ANOVA and *post-hoc* Tukey test). PSL, Photostimulated luminescence.

lesion in the control animals, but were markedly elevated on the lesioned side after treatment with L-DOPA (Fig. 3b; $p = 0.072$ for side-effect, $p = 0.065$ for treatment effect and $p = 0.001$ for side and treatment interaction; see autoradiographs in Figs 4e-h). Chronic administration of KW-6002 did not have any inductive or modulatory effect on the PDyn transcript. On one hand, animals treated with KW-6002 alone did not differ from the control group (cf. Figs 4e and f), both groups showing an approx. 40% reduction in PDyn mRNA levels on the DA-denervated versus the intact side ($p < 0.05$ for within-group side comparisons; Fig. 3b). On the other hand, animals treated with KW-6002 + L-DOPA did not differ from the rats receiving L-DOPA only (cf. Figs 4g and h). Indeed, all the animals exposed to L-DOPA showed an up-regulation of PDyn mRNA in the DA-denervated CPu by

approx. 70% above control levels, irrespective of KW-6002 coadministration (Fig. 3b).

Effects of chronic KW-6002 treatment on striatal levels of FosB/ Δ FosB immunoreactivity

Δ FosB-like proteins are induced in the striatum by both DA-denervating lesions (Doucet *et al.* 1996; Cenci *et al.* 1999) and chronic L-DOPA treatment (Andersson *et al.* 1999). However, denervation-induced FosB/ Δ FosB induction occurs mainly in the medial part of the CPu in rats, whereas the inductive effect of L-DOPA on this marker can be seen in all striatal regions (Cenci *et al.* 1999). Moreover, the intensity of FosB/ Δ FosB-like immunoreactivity over single cells is much lower after DA denervation alone than after a subsequent, dyskinesigenic treatment with L-DOPA (Cenci *et al.* 1999). Because of these previous data, quantitative analysis of FosB/ Δ FosB-like immunoreactivity was carried out separately in the medial and the lateral part of the CPu. Moreover, group comparisons were carried out with respect to both the number of FosB/ Δ FosB-immunoreactive cells and the average amount of staining per cell in each region.

In the medial CPu, the four treatment groups under investigation showed an average twofold increase in the number of immunoreactive cells on the DA-denervated side versus the intact side (Fig. 5a; $p < 0.0001$ for side-effect on a two-factor ANOVA). The number of FosB/ Δ FosB-immunoreactive cells in the DA-denervated striatum did not differ between the treatment groups ($p = 0.73$ for treatment effect and $p = 0.73$ for treatment and side interaction on a two-factor ANOVA). However, the amount of staining per cell was significantly elevated by about 45% above control levels in the two groups that received L-DOPA (Fig. 5c; $p < 0.0001$ for side-effect, $p = 0.0002$ for treatment effect and $p = 0.001$ for side and treatment interaction on a two-factor ANOVA). Animals treated with KW-6002-only tended to show lower levels of cellular immunostaining for FosB/ Δ FosB than controls, but this effect did not reach significance (Fig. 5c).

In the lateral CPu the number of FosB/ Δ FosB-immunoreactive cells was significantly increased in all treatment groups on the side ipsilateral to the lesion relative to the intact side (Fig. 5b; $p < 0.0001$ for side-effect). Moreover, important group differences were found in this region on the DA-denervated side (Fig. 5b; $p < 0.0001$ for side and treatment interaction and $p = 0.0002$ for treatment effect). Indeed, both the animals treated with L-DOPA-only and those treated with L-DOPA and KW-6002 showed an over two-fold increase in the number of immunoreactive neurones above control values ($p < 0.05$ vs. control group). The number of FosB/ Δ FosB-positive cells was similar in both of the groups administered with L-DOPA, irrespective of KW-6002 coadministration. The animals treated with KW-6002-only showed a slight trend towards a lower

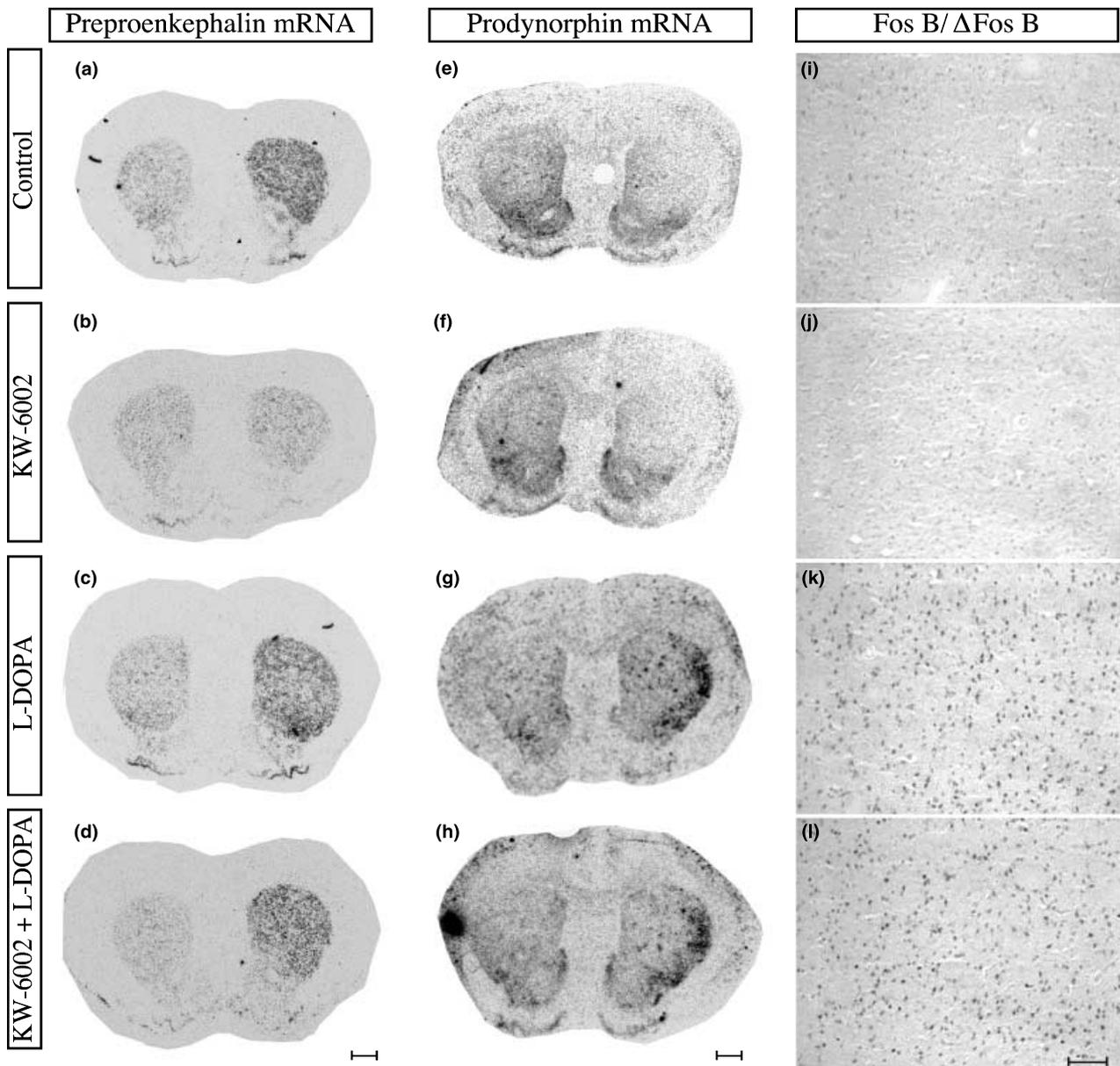


Fig. 4 Distribution patterns of (a)–(d) preproenkephalin mRNA and (e)–(h) prodynorphin mRNA in sections through the mid striatum. The dopamine (DA)-denervated caudate-putamen (Cpu) is shown to the right in all sections. The autoradiographs represent typical cases in the chronic treatment study (experiment 2); (a) and (e), 6-hydroxydopamine-lesioned (vehicle-treated) controls; (b) and (f), rats treated with

KW-6002-only; (c) and (g), rats treated with L-DOPA-only; (d) and (h), combined treatment with KW-6002 and L-DOPA. Photomicrographs in (i)–(l) show FosB/ΔFosB-immunoreactive cells in the DA-denervated CPu (lateral part) in the same cases. Scale bar, 1 mm in (a)–(h) and 100 μm in (i)–(l).

number of immunoreactive cells, but the difference from controls did not reach significance (Fig. 5b).

An analysis of the OD values on the counted cells showed about 60% higher levels of FosB/ΔFosB immunostaining per cell in the animals treated with L-DOPA or L-DOPA + KW-6002 compared with the control group ($p < 0.0001$ for side-effect, $p = 0.0037$ for treatment effect and $p = 0.009$ for side and treatment interaction on a

two-factor ANOVA; Fig. 5d). By contrast, animals treated with KW-6002-only did not differ significantly from controls (Fig. 5d).

Altogether, these data show that treatment with KW-6002 alone did not have any inductive effect on ΔFosB-like transcription factors in the DA-denervated CPu and that cotreatment with KW-6002 and L-DOPA did not attenuate the inductive effect of the latter compound on these proteins.

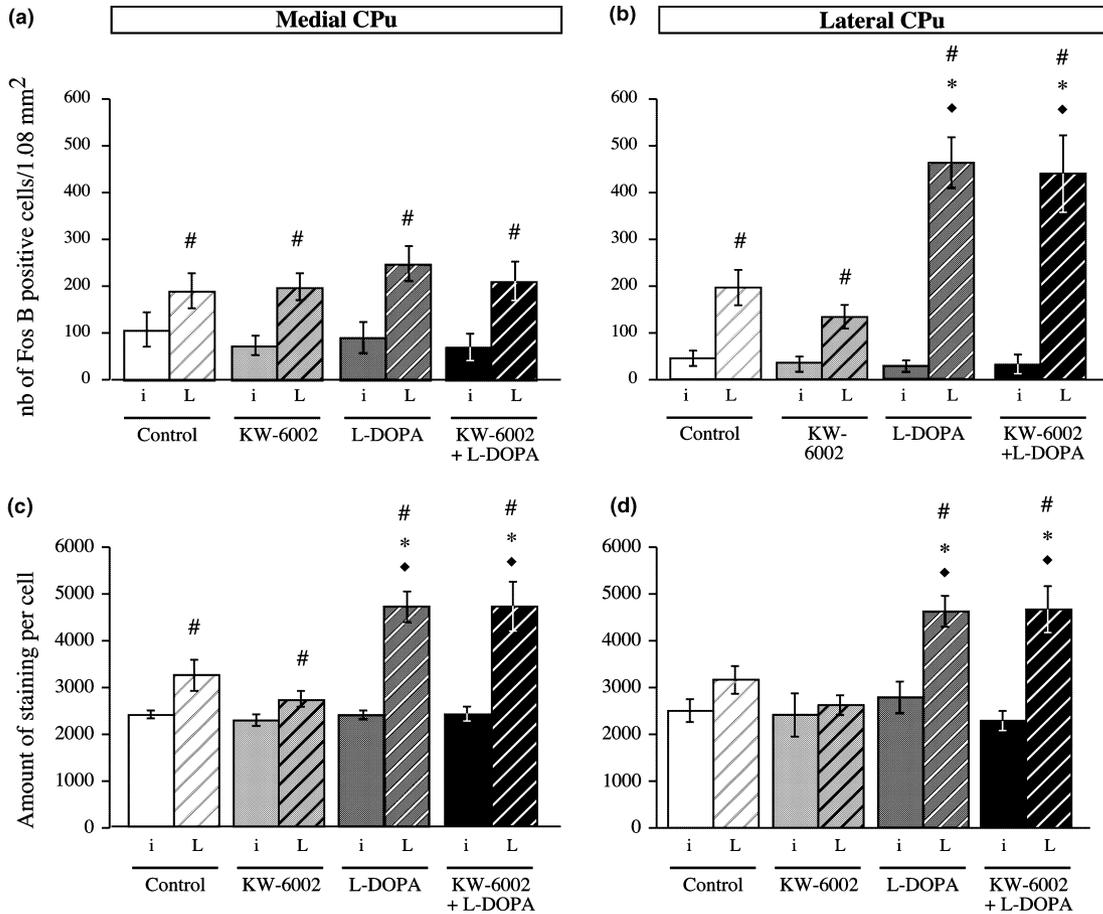


Fig. 5 (a) and (b) Number of FosB/ΔFosB-immunoreactive cells and (c) and (d) amount of immunostaining per cell in the medial caudate-putamen (Cpu) (a and c) or in the lateral part of the same structure (b and d). Measurements were carried out in the four treatment groups comprised in experiment 2 (see captions below the x-axis; cf. Table 1) both within the 6-hydroxydopamine-lesioned CPU (L) and in the con-

tralateral, intact side (i). In (c) and (d), the amount of staining per cell was calculated according to the formula: [area of the cell] × [optical density/unit area]. $p < 0.05$: #versus the intact CPU within the same group, *versus dopamine (DA)-denervated CPU in the control group; ♦versus DA-denervated CPU in the KW-6002 group; $n = 7-10$ per group, as specified in Table 1 (two-factor ANOVA and *post-hoc* Tukey test).

Discussion

The present study has examined the motor and cellular effects of KW-6002, an adenosine A_{2a} receptor antagonist, in a rat model of Parkinson's disease and L-DOPA-induced dyskinesia. On a behavioural level, our data confirm some essential features related to the antiparkinsonian action of A_{2a} receptor antagonists, which were first described in MPTP-intoxicated marmosets (Kanda *et al.* 1998, 2000). These features can be summarized as follows: (i) chronic administration of A_{2a} antagonists produces a relief of motor disability (as assessed in this study using a rotarod test), which does not exhibit tolerance over at least 21 days of treatment; (ii) this improvement is not accompanied by manifestations of abnormal, excessive movement (as evaluated in this study using ratings of axial, limb, orolingual and locomotive AIMs); (iii) even in animals rendered dyskinetic by previous L-DOPA treatment, administration of A_{2a}

antagonists does not provoke any AIMs and (iv) the severity of fully established AIMs, however, is not attenuated when this category of compounds is coadministered with L-DOPA, as compared with L-DOPA treatment alone. Rather than revealing novel features of the compound under investigation, these data verify the validity of the rat Parkinson model for a pre-clinical screening of candidate antiparkinsonian drugs. The present study does in addition contribute novel data on the effects of combining pharmacological adenosine A_{2a} receptor blockade with L-DOPA treatment in previously drug-naïve animals (in the studies by Kanda and colleagues KW-6002 had been administered to L-DOPA-primed animals).

In agreement with data obtained from MPTP-lesioned marmosets (Kanda *et al.* 2000), our results show that a combined treatment with L-DOPA and KW-6002 produces an additional therapeutic benefit over L-DOPA alone on measures of motor disability. Moreover, our results show

that this additional benefit augments gradually over 21 days of KW-6002 + L-DOPA cotreatment (i.e. the time span tested in this study). Indeed, while the effects on rotarod performance did not differ between drug treatments on the first testing session, the integral of time spent on the rod versus turning speed (AUC value) was about 60% larger in animals treated with KW-6002 + L-DOPA, compared with L-DOPA alone, by the end of the experiment. This gradual motor improvement is likely to result from beneficial adaptive responses induced by the combined treatment at the molecular and cellular level. The nature of these plastic responses remains to be established. However, our data allow us to rule out a normalizing effect of the combined KW-6002 + L-DOPA treatment on the striatal up-regulation of PPE mRNA, which had been induced by the DA-denervating lesion. This molecular response has been considered a marker of the hyperactivity of the 'indirect pathway' neurones that is associated with Parkinson's disease (for review see Richardson *et al.* 1997). In our study the levels of PPE mRNA in the DA-denervated striatum were significantly reduced following chronic treatment with KW-6002 alone, but not after combined administration of KW-6002 and L-DOPA, which yielded a greater antiparkinsonian effect in the rotarod test. Since the drug treatment producing less motor improvement had a stronger normalizing effect on PPE mRNA levels, it is unlikely that the up-regulation of this transcript plays a causal role in the pathogenesis of parkinsonian motor disability. This interpretation is in agreement with studies performed in MPTP-intoxicated monkeys, describing a temporal dissociation between the development of parkinsonian motor features and the elevation of striatal PPE mRNA levels after the neurotoxic lesion (Schneider *et al.* 1999; Bezdard *et al.* 2001).

With regard to dyskinesia, our data show that pharmacological A_{2a} receptor antagonism cannot prevent the development of L-DOPA-induced AIMs even when the combined treatment is administered *de novo*. Indeed, AIM scores did not differ between *de novo* KW-6002 + L-DOPA cotreatment compared with L-DOPA treatment alone (experiment 2). Since this experiment was performed in L-DOPA-naïve animals, the lack of an antidyskinetic effect by KW-6002 cannot be ascribed to possible priming phenomena induced by a previous exposure to L-DOPA. Fredduzzi *et al.* (2002) have shown that genetic inactivation of A_{2a} receptors prevents the sensitization of contralateral rotation and motor stereotypies induced by chronic L-DOPA treatment in unilaterally 6-OHDA-lesioned mice. These results have led the authors to conclude that A_{2a} receptors play an essential role in the maladaptive molecular plasticity at the basis of L-DOPA-induced dyskinesia. Since the extent of A_{2a} receptor inactivation obtained by pharmacological blockade is not as complete as that produced by gene ablation techniques, our results do not allow us to rule out the conclusions of

Fredduzzi *et al.* (2002). However, our data allow us to predict that cotreatment with A_{2a} receptor antagonists and L-DOPA will not prevent the occurrence of dyskinesia if the latter drug is given at full, therapeutic doses to severely DA-denervated subjects. Nevertheless, A_{2a} antagonists may reduce the incidence of dyskinesia in Parkinson's disease by diminishing the dose requirement for L-DOPA. Indeed, previous studies have shown that cotreatment with L-DOPA and adenosine A_{2a} receptor antagonists lowers the dose of L-DOPA needed to produce the same amount of motor activation (Kanda *et al.* 2000; Pinna *et al.* 2001). These previous findings are supported by the rotarod data obtained in experiment 2, which show a significant potentiation of the therapeutic effect of L-DOPA by KW-6002 cotreatment.

The failure of KW-6002 to prevent L-DOPA-induced AIMs was accompanied by a similar failure to block the up-regulation of PDyn mRNA and FosB/ΔFosB immunoreactivity induced by L-DOPA in the DA-denervated striatum. A lack of inductive effect of A_{2a} antagonists on the PDyn transcript has recently been reported by Carta *et al.* (2002). However, this is the first study to examine the modulatory effect of an A_{2a} antagonist on the up-regulation of both PDyn mRNA and FosB/ΔFosB-related proteins induced by a suprathreshold dose of L-DOPA. As shown in previous studies (Cenci *et al.* 1998; Andersson *et al.* 1999, 2001; Doucet *et al.* 1996), the up-regulation of these markers is closely associated with dyskinesia development and occurs in neurones of the 'direct pathway'. On the other hand, A_{2a} receptors are expressed in striatal neurones of the 'indirect' pathway, where they oppose the effects of D2-type DA-receptors (for review see Richardson *et al.* 1997; Fuxe *et al.* 1998; Svenningsson *et al.* 1999).

Although A_{2a} receptors are located on the 'indirect pathway' neurones, drugs acting on these receptors may affect virtually any type of cell within the striatum by causing changes in GABA and acetylcholine release (Mori *et al.* 1996; Kurokawa *et al.* 1996). Despite these multiple, potential mechanisms of action, an A_{2a} receptor antagonist did not affect the changes in PDyn mRNA and FosB/ΔFosB expression which were induced by chronic L-DOPA treatment in dyskinetic animals. Thus, the results of the present study favour a pathophysiological model whereby the gradual development of dyskinesia during chronic L-DOPA treatment is due to molecular changes occurring at the level of 'direct pathway' neurones (e.g. *fosB* induction; Andersson *et al.* 1999). As long as it cannot prevent such changes, pharmacological A_{2a} receptor blockade does not attenuate the dyskinesias induced by a concomitant treatment with L-DOPA. However, A_{2a} receptor antagonists can relieve parkinsonism (without producing dyskinesia) by counteracting the hyperactivity of 'indirect pathway' neurones, which results from DA depletion and lack of DA (D2) receptor stimulation (Albin *et al.* 1989). By doing this, A_{2a} receptor antagonists can

restore a functional balance between the two striatal output pathways, that act as a push-pull system to increase or decrease movement.

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