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Dopamine D_3 receptor stimulation underlies the development of L-DOPA-induced dyskinesia in animal models of Parkinson's disease ☆

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ABSTRACT

Development of L-DOPA-induced dyskinesia (LID) remains a major problem in the long-term treatment of Parkinson's disease (PD). Sensitization to L-DOPA correlates with ectopic expression of D_3 dopamine receptors in the striatum, implicating D_3 receptors in development of LID.

We demonstrate that the selective D_3 antagonist S33084 abolishes development of LID over 30 days in MPTP-lesioned marmosets without effecting the anti-parkinsonian actions of L-DOPA. Furthermore, following a 14 day washout, when challenged with L-DOPA in the absence of S33084, these animals continued to exhibit reduced LID. In the 6-OHDA-lesioned rat, S33084 similarly attenuated development of behavioural sensitization to L-DOPA. Additionally, L-DOPA–induced elevations in striatal pre-proenkephalin-A (PPE-A) (but not PPE-B, phospho[Thr^{34}]DARPP-32, D_1, and D_2 receptor mRNA or D_3 receptor levels) were reduced in S33084 treated animals.

Our data suggest a role for D_3 receptors in the development of LID and suggest that initiating L-DOPA treatment with a D_3 antagonist may reduce the development of LID in PD.

Introduction

L-3,4-dihydroxyphenylalanine (L-DOPA) remains the most effective symptomatic therapy for PD (Lang and Lozano, 1998). However, the development of L-DOPA-induced dyskinesia (LID), following long-term treatment, remains a major problem (Fabbrini et al., 2007; Obeso et al., 2000). Current treatment options for LID are limited. Thus prevention, involving early use of dopamine receptor agonists, is the main approach to management (Rascol et al., 2000). However, re-evaluation of clinical trials revealed this strategy merely delays the onset of LID and does not prevent it (Constantinescu et al., 2007; Rascol et al., 2006). Moreover, dopamine agonists are less effective anti-parkinsonian agents than L-DOPA (Constantinescu et al., 2007; Rascol et al., 2000, 2006). Thus, it would be preferable to employ L-DOPA, as the most effective anti-parkinsonian drug available, but prevent the development of LID.

Studies in both the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated non-human primate and 6-OHDA-lesioned rat models of PD have implicated several molecular mechanisms in the development, expression and maintenance of sensitization to L-DOPA (Bezard et al., 2001; Cenci, 2007; Cenci and Lundblad, 2006; Jenner, 2008) including the proposal that the dopamine D_3 receptor may play a role in LID (Bezard et al., 2003; Bordet et al., 1997; Guigoni et al., 2005).

D_3 receptors comprise <1% of dopamine receptors in the caudate-putamen (Levesque et al., 1992; Sokoloff et al., 1990). However, in 6-OHDA-lesioned rats and MPTP-lesioned primates, sensitization to L-DOPA correlates with a progressive ectopic expression of D_3 receptors (Bordet et al., 1997; Guigoni et al., 2005). Though these data implicate the D_3 receptor, they do not indicate whether they are a cause of, or a response to LID, nor whether D_3 receptor signalling underlies the development of LID or solely its expression once established. Initial support of an involvement in the expression of established LID was provided by reports that the D_3 receptor partial agonist BP897 can reduce established LID in monkeys (Bezard et al., 2003; Hsu et al., 2004) and that D_3 receptor knockdown can reduce L-DOPA-sensitized behaviours in rats (van Kampen and Stoessl, 2003). However, uncertainty regarding the selectivity of BP897 (Visanji et al., 2006b) and the observation that anti-dyskinetic effects of BP897 are accompanied by a reduction in the anti-parkinsonian actions of L-DOPA (Hsu et al., 2004) question these conclusions. Furthermore, the demonstration that the selective D_3 receptor antagonist S33084 has no anti-dyskinetic actions in monkeys with established LID suggests that D_3 receptors have no role in the expression of LID (Silverdale et al., 2004). In addition to differences in selectivity between BP897 and S33084, it has been suggested that these discrepancies might result from differences in the expression of D_3 receptors between different models, as elevations in striatal D_3 receptors are not consistently seen (Hurley et al., 1996; Quik et al., 2000). To date, while striatal D_3

☆ D_3 receptors and development of dyskinesia.

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receptor levels have been reported as being slightly decreased in the brains of PD patients assessed post-mortem (Piggott et al., 1999), the relationship between these changes and the presence, or absence, of dyskinesia has not been reported.

The role of D_3 receptors in the development of sensitization to L-DOPA is unknown. We investigated the effect of the D_3 selective antagonist S33084 (Millan et al., 2000a,b) on the development of LID in MPTP-lesioned marmosets and potential underlying mechanisms in 6-OHDA-lesioned rats.

Materials and methods

MPTP-lesioned non human primate model of PD

Twelve female marmosets (*Callithrix jacchus*), weight 366 ± 14 g, age 35.4 ± 0.5 months (Worldwide Primates Inc, Miami, FL, USA), were treated with MPTP hydrochloride (2.0 mg/kg s.c. for 5 consecutive days) (Sigma-Aldrich, St Louis, MO, USA-Aldrich, St Louis, MO, USA), resulting in a stable parkinsonian syndrome, characterized by bradykinesia, hunched posture and a reduced range of movement, as previously described (Fox et al., 2002; Henry et al., 2001). Animals used in accordance with approved local institution protocol (UHN 02/053) and the regulations defined by the Canadian Council on Animal Care. All efforts were made to reduce animal numbers used to the minimum required for valid statistical analysis. The animals were kept in controlled housing conditions, in groups of 2–3, with constant temperature (25 °C), relative humidity (50%) and 12 h light/dark cycle (08.00 lights on). The animals had free access to chow, fresh fruit supplements and water. The housing environment was enriched with auditory and tactile stimuli. Following the MPTP administration, the animals were allowed to recover for 12 weeks to allow parkinsonian symptoms to stabilize.

Following this stabilization period, animals were acclimatized to handling and placed into the study observation cages (0.8 m × 0.8 m × 0.7 m) with a branch, a dish of fruit and a water bottle, for 2 h every 1–2 days over a 2 week period. Animals were divided into 2 groups. One group ($N = 6$) received L-DOPA as Prolopa® p.o. (15 mg/kg L-DOPA and 3.75 mg/kg benserazide) (Hoffman-La Roche, Mississauga, ON, Canada) and vehicle. The second group ($N = 6$) received L-DOPA and S33084 [(3aR,9bS)-N-[4-(8-cyano-1,3a,4,9b-tetrahydro-3H-benzopyrano[3,4-c]pyrrole-2-yl)-butyl]-(4-phenyl)benzamide] (2.5 mg/kg p.o.) (IDR Servier, Paris, France). Treatments were administered twice-daily (9:00 and 15:00) for 30 days (days 1–30). At the end of this 30 day period, all animals received twice daily treatment with L-DOPA (15 mg/kg and 3.75 mg/kg benserazide) only, for a total of 30 days (days 31–60).

Behavioural assessment for parkinsonian disability and LID were assessed following morning administration of drug; animals were immediately placed singly into the observation cages. The behaviour was then recorded for a period of 4 h, using a digital video camera connected to a DVD recorder, for *post hoc* assessment. Animals were undisturbed for the duration of the recording. Parkinsonian disability and LID were scored, by an observer blinded to both the treatment group and day of treatment, twice an hour in 10 min time intervals for 4 h, using scales as previously described (Fox et al., 2002; Visanji et al., 2006a). Peak dose scores were defined as the period of maximal reversal of parkinsonian disability (times 40 min–110 min post L-DOPA dose). Parkinsonian disability was scored using the following scale: range of movement (0–9), bradykinesia (0–3), posture (0–1), and attention (0–1); in all cases 0 represents absent and the higher the score the more severe the behaviour. The score assigned in each time period was the most prevalent activity. A total parkinsonian disability score was defined using the formula [(range of movement × 1) + (bradykinesia × 3) + (posture × 9) + (attention × 9)]), maximum score per 10 min time frame = 36. In this score each component receives a weighting such that each contributes equally to the total score thus balancing the components of the scale so as to avoid biasing the total score towards any individual component symptom. LID was rated using a LID disability rating scale as previously described (Fox et al., 2002; Visanji et al., 2006a). Chorea and dystonia were rated separately (score 0–4), the higher the score, the more disabling and the most disabling score used, maximum possible score per 10 min = 4. Behavioural assessments were performed on pre-treatment, and days 1, 7, 14, 30, 33, 44 and 60.

6-OHDA lesioned rat model of PD

Male, Sprague-Dawley rats (250–275 g, Charles River, Saint-Constant, QC, Canada) were housed, with access to food and water *ad libitum*, in a temperature and humidity controlled environment (temperature 19–21 °C; humidity 55%) subject to a 12-hour light/dark cycle (lights on 0700). All procedures were carried out in accordance with the Institution's Animal Care Committee (Permit UHN 821.7) and all efforts were made to reduce animal numbers used to the minimum required for valid statistical analysis and to refine techniques to reduce suffering. Following 30 min pre-treatment with pargyline (5 mg/kg i.p. Sigma-Aldrich, St Louis, MO, USA) and desipramine (25 mg/kg i.p. Sigma-Aldrich, St Louis, MO, USA), and under general anaesthesia (4% isofluorane in 95% O_2, 5% CO_2), animals received stereotaxic injection of 6-OHDA (12.5 µg in 2.5 µl 0.1% ascorbate, Sigma-Aldrich, St Louis, MO, USA) into the right medial forebrain bundle at the following co-ordinates, according to Paxinos and Watson (Paxinos and Watson, 1986): AP −2.8 mm, LM 2.0 mm, DV −9.0 mm from Bregma and skull surface, with the incisor bar set at 3.3 mm below ear bars.

Drug treatment

Following 14 days recovery, animals were divided into 6 treatment groups (each $N = 8–10$). Thus animals were administered either L-DOPA/benserazide (15/3.75 mg/kg) (Sigma-Aldrich, St Louis, MO, USA), or vehicle i.p. in combination with either vehicle, S33084 (0.63 mg/kg) or raclopride (0.16 mg/kg Tocris Bioscience, Ellisville, MO, USA) s.c. Animals were treated twice daily, 6 h apart, for 21 consecutive days.

Behavioural analysis

On days 1, 5, 9, 13, 17 and 21, immediately following the first of the day's drug treatment, animals were placed in hemispheric rotometers (MedAssociates, St Albans, VT, USA) and their rotational activity in response to treatment was recorded for 3 h. Animals were then given a 24 h washout of all drugs prior to assessing their rotational response to an L-DOPA/benserazide challenge on day 22. On day 23, animals received their original treatment and were sacrificed 1 h later, when L-DOPA would be exerting its maximal effects. The brains were removed and immediately flash-frozen in isopentane at −45 °C, then stored at −80 °Cuntil processing.

[^{125}I]-RTI-121 binding autoradiography

The levels of striatal DAT binding were assessed by [^{125}I]-RTI-121 binding autoradiography in 20 µm cryostat-cut sections prepared from fresh-frozen tissue, using the method reported by Quik et al., (2003) with minor modifications. Briefly, thawed slides were placed in binding buffer (2 × 15 min, room temperature) containing 50 mM Tris, 120 mM NaCl and 5 mM KCl. Sections were then placed in the same buffer containing 50 pM [^{125}I]-RTI-121, (Perkin-Elmer, Waltham, MA, USA) specific activity 2200 Ci/µmol for 120 min at 25 °C to determine total binding. Non-specific binding was defined as that observed in the presence of 100 µM GBR 12909 (Tocris Bioscience, Ellisville, MO, USA). All slides were then washed (4 × 15 min) in ice-cold binding buffer, rinsed in ice-cold distilled water and air-dried. Together with [^{125}I]-microscale standards (Amersham, Piscataway, NJ, USA) slides were

then opposed to autoradiographic film (Kodak Biomax MR) and left for 2 days at 4 °C before developing.

Autoradiograms were analysed using MCID 6.0 Elite Image analysis system software (InterFocus Imaging Ltd, Linton, U.K.). Densiometric analysis of 3 striata from each animal was carried out whereby a reference curve of c.p.m. versus optical density was calculated from γ-emitting [^{125}I] microscale standards and used to quantify the intensity of signal as nCi/g. Background intensity was subtracted from each reading. Non-specific binding was calculated in the same way and subtracted from the total to give specific binding. Non-specific binding was found to account for <1% of total binding. All animals with <95% loss of striatal [^{125}I]-RTI-121 binding, as compared to the intact hemisphere were excluded from the study ($N = 14$ of 68 excluded).

In situ hybridisation

Antisense oligonucleotide probes (Sigma-Genosys, Oakville, ON, Canada) complementary to mRNA encoding PPE-A (sequence 5′–3′ CTT CAT GAA GCC TCC ATA CCG TTT CAT GAA CCC TCC ATA), PPE-B (sequence 5′–3′ GCT CCT CTT GGG GTA TTT GCG CAA AAA GCC GCC ATA GCG TTT GGC, the D_1 dopamine receptor, (sequence 5′–3′ AAT CGA TGC AGA ATG GCT GGG TCT CCT CAG AGC CAC AGA AGG GCA CCA) and D_2 dopamine receptor, (sequence 5′–3′ GCA AGA TCT TCA TGA AGG CCT TGC GGA ACT CGA TGT TGA AGG TGG TGT) were designed complementary to the sequences reported by (Bunzow et al., 1988; Horikawa et al., 1983; Howells et al., 1984; Zhou et al., 1990). In situ hybridization was carried out as previously described (Visanji et al., 2006c). 60 ng of each probe was 3′-end labelled with [^{35}S]dATP (Perkin-Elmer, Waltham, MA, USA) by incubation in a reaction mixture containing [^{35}S]dATP (approx 33 mCi), 30 U terminal deoxynucleotidyl transferase, 100 mM sodium cacodylate, 1 mM $CoCl_2$ (all Fermentas, Burlington, ON, Canada) 0.1 mM dithiothreitol (Sigma-Aldrich, St Louis, MO, USA) and sterile water at 37 °C for 1 h. The reaction was terminated by immersion in a 70 °C water bath for 10 min. Labelled probe was then purified by passing through a polyacrylamide gel column (Amersham, Piscataway, NJ, USA) and 5 × v/v 1 M dithiothreitol added to prevent probe oxidation. Labelling efficiency of the probe was established by scintillation counting (Beckman Coulter) and the appropriate dilution of probe calculated to give approx 3×10^6 c.p.m. ml^{-1} hybridization solution.

Briefly, at room temperature, sections were fixed for 10 min in 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA), incubated for a further 10 min in 0.25% acetic anhydride (Sigma-Aldrich, St Louis, MO, USA) and 0.1 M triethanolamine (Sigma-Aldrich, St Louis, MO, USA) (pH buffered to 8.0), dehydrated in increasing strengths of ethanol (70–100%); defatted in 100% chloroform then rehydrated into 95% ethanol. Sections were then hybridized overnight (minimum 18 h) at between 37 to 42 °C with [^{35}S]dATP-labelled probe (3×10^6 c.p.m. ml^{-1}) in a solution containing:— 10% w/v dextran sulphate, 50% deionised formamide, 4× standard sodium citrate solution (SSC; 1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH buffered to 7.0), 0.02 M DTT, 0.5 × Denhardt's solution, 0.2 mg ml^{-1} denatured salmon sperm, 0.1 mg ml^{-1} polyadenylic acid (all Sigma-Aldrich, St Louis, MO, USA). Following hybridization, sections were washed in 2× SSC for 2 min, 1× SSC for 30 min, 1× SSC for 30 min at 60 °C before dehydrating again in 70 and 95% ethanol and being allowed to air dry.

Sections, together with β-emitting ^{14}C microscale standards, range, 1–877 nCi/g (Amersham Biosciences, Piscataway, NJ, USA) were exposed to ^{35}S-sensitive film (Kodak Biomax MR) prior to developing. MCID 6.0 Elite Image analysis system software was used to quantify the intensity of signal expressed as nCi g^{-1} of tissue, using standard curves produced from the ^{14}C microscale standards. Analysis was focused on the dorsal striatum as previous studies have highlighted this region as the site of ectopic D_3 receptor expression following L-DOPA therapy (Bordet et al., 1997; Guigoni et al., 2005). The region measured was at the level of + 1.7 mm from Bregma (as defined by the atlas of Paxinos and Watson (1986) and included both lateral and medial parts of the striatum. Readings from three sections per animal were averaged and corrected for background.

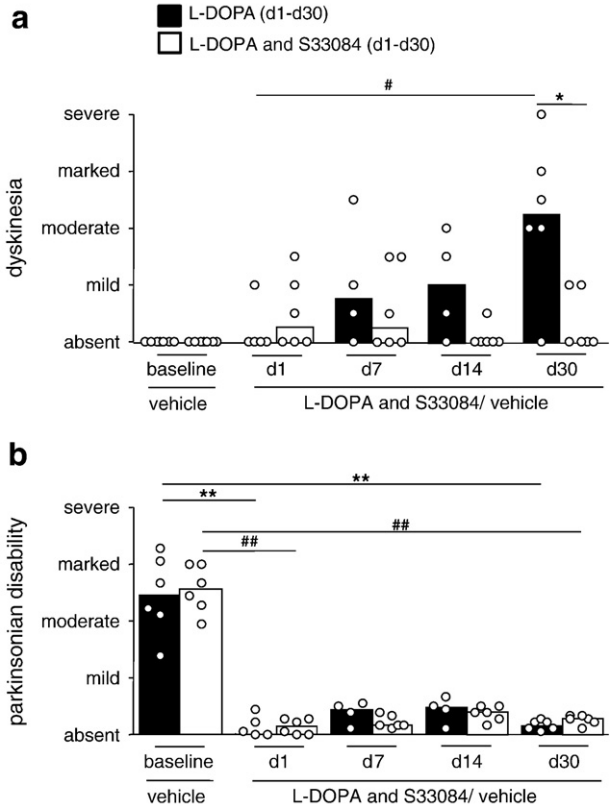

Fig. 1. Co-treatment with the dopamine D_3 antagonist S33084 prevents the development of L-DOPA-induced dyskinesia (LID), without reducing the anti-parkinsonian benefit of L-DOPA, in the MPTP-lesioned, non-human primate. MPTP-lesioned marmosets were treated twice daily for 30 days (d1-d30) with L-DOPA (15 mg/kg/benserazide 3.75 mg/kg) in combination with either vehicle p.o. (■) or S33084 (2.5 mg/kg) p.o. (□). a shows peak-dose dyskinesia (cumulative score 40–110 min post drug); where severe represents the greatest possible dyskinesia score = 8, marked = 6, moderate = 4, mild = 2, absent = 0 as defined in the methods. # represents $P<0.05$, ANOVA, followed by Dunn's multiple comparison test, L-DOPA/vehicle group day 1 (d1) compared to day 30 (d30), *$P<0.05$, Mann Whitney test, ($N = 6$/group), L-DOPA and vehicle on day 30 (d30) compared to L-DOPA and S33084 on day 30 (d30). b shows peak-dose parkinsonian disability (cumulative score 40–110 min post drug) where severe represents the greatest possible score = 72, marked = 54, moderate = 36, mild = 18, absent = 0 as defined in the methods; **$P<0.01$, ANOVA, followed by Dunn's multiple comparison test, L-DOPA/vehicle group at baseline compared to day 1 and day 30 ($N = 5/6$/group); ##$P<0.01$, ANOVA followed by Dunn's multiple comparison test, L-DOPA/S33084 group at baseline compared to day 1 and day 30 ($N = 6$/group). Data are individual animals (○) with the median indicated as a bar.

Western blotting

The dorsal striata of both the intact and lesioned hemispheres from each animal were dissected out at − 20 °C and homogenised in lysate buffer at 4 °C containing Tris at pH 7.4 (50 mM), NaCl (50 mM), 1 mM each of EDTA, EGTA, PMSF, sodium orthovanadate, sodium fluoride, 1% SDS and a protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada). Proteins were separated by gel electrophoresis and immunoblotting performed as described previously (Hallett et al., 2005). Briefly, intact and lesioned samples of one animal from each treatment group were loaded onto a single gel. 40 μg of protein was diluted (1:1) in Lammeli buffer (BioRad, Hercules, CA, USA), boiled for 2 min, loaded and separated in 10% SDS-PAGE gels and transferred to PVDF membranes (Amersham, Piscataway, NJ, USA). Total DARPP-32 was detected using a rabbit polyclonal antibody (PhosphoSolutions, Aurora, CO, USA) dilution 1:1000. Phospho[Thr34]DARPP-32 was

detected using a rabbit polyclonal antibody (PhosphoSolutions, Aurora, CO, USA) dilution 1:1000. Dopamine D_3 receptor protein was detected using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) dilution 1:500. β-actin was detected using a mouse monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA) dilution 1:1000. Labelled protein was revealed using anti-rabbit or anti-mouse HRP IgGs followed by ECL detection (all Amersham, Piscataway, NJ, USA). Protein levels were quantified by densitometric analysis (MCID 6.0 Elite Image analysis system). Data for each protein investigated was expressed as% of β-actin to control for variability in volume loaded. Levels of phospho[Thr34]DARPP-32 were expressed relative to total DARPP-32.

Statistical analysis

Marmoset behavioural data are expressed as median ± range cumulated peak dose score (40–110 min post L-DOPA) these data are non-parametric and were analyzed using a Kruskal Wallis followed by Dunn's test for multiple comparisons in the case of comparing data over multiple days, or a Mann–Whitney U test. All rodent data, expressed as mean ± s.e.m, conformed to normal distribution as assessed by a Kolmogorov–Smirnov Goodness-of-Fit Test and were thus analyzed using parametric statistics, either employing a one-way or two-way analysis of variance (ANOVA), as indicated in the text, followed by Bonferroni post-hoc analysis where appropriate. All analyses were carried out using GraphPad Prism 4.0 (San Diego, CA, USA). In all cases, $P<0.05$ was taken as the level of significance.

Results

Treatment with the dopamine D_3 antagonist, S33084, prevents the development of LID in the MPTP-lesioned primate model of PD

MPTP treatment of marmosets resulted in a parkinsonian syndrome, characterized by bradykinesia, reduced motor activity, reduced attention and a hunched posture. Animals were subsequently divided into two treatment groups; there was no significant difference in baseline total parkinsonian disability scores between the two groups (median parkinsonian disability score 44 (range 25–59) and 46 (range 35–54), respectively, (Mann Whitney, $P=1.00$, $N=6$/group, Fig. 1b). Similarly, in comparing each of the four individual components of the parkinsonian disability score, no statistically-significant differences were observed between the two groups at baseline (data not shown). No animal displayed any dyskinesia at baseline (Fig. 1a).

Animals were treated twice daily for 30 days with L-DOPA in combination with either vehicle or S33084. MPTP-lesioned marmosets treated with L-DOPA alone developed a progressive increase in LID, in accordance with previous reports (Fox et al., 2002, 2006; Gomez-Ramirez et al., 2006; Pearce et al., 1995; Visanji et al., 2006a). Median dyskinesia score increased from 0 at baseline (range 0) and day 1 (range 0–2) to 4.5 (range 0–8) on day 30 ($P=0.0078$ vs. day 1, ANOVA, followed by Dunn's multiple comparison test; $N=4$–6, Fig. 1a). In contrast, animals treated with L-DOPA and S33084 did not develop significant LID over the 30 day treatment period. Thus, median dyskinesia score was 0 at baseline, 0.5 (range 0–3) on day 1 and 0 (range 0–2) on day 30 ($P=0.2247$, ANOVA, followed by Dunn's multiple comparison test, $N=4$–6). Furthermore, on day 30, LID was significantly greater in the L-DOPA/vehicle treated group compared to the L-DOPA/S33084 treated group ($P=0.0247$, Mann Whitney, $N=6$/group, Fig. 1a).

In a second phase of the study, days 31–60, all animals received twice daily treatment with L-DOPA alone. On days 33 and 44, i.e. following a 3 and 14 day period of S33084 washout respectively, the reduction of dyskinesia in animals previously treated with the combination of L-DOPA and S33084 was maintained. Thus, on day 33, the median LID score in animals previously treated with L-DOPA alone was 3 (range 0–8) compared to 0 (range 0–3) in the L-DOPA/S33084 prior treatment group ($P=0.0387$, Mann Whitney, $N=6$, Fig. 2a). On day 44 (i.e. after 14 days washout) the median LID score in animals previously treated with L-DOPA and vehicle was 3 (range 0–8) compared to 0 (range 0–0) in the L-DOPA/S33084 prior treatment group ($P=0.0165$, Mann-Whitney, $N=6$, Fig. 2a). On day 60, in the animals previously treated with L-DOPA/S33084 on days 1–30, the level of dyskinesia was 1 (range 0–5), which was not significantly different to that in animals previously treated with L-DOPA alone, 4 (range 2–6) in the prior L-DOPA/vehicle treated group ($P=0.1414$, Mann Whitney, $N=5/6$, Fig. 2a).

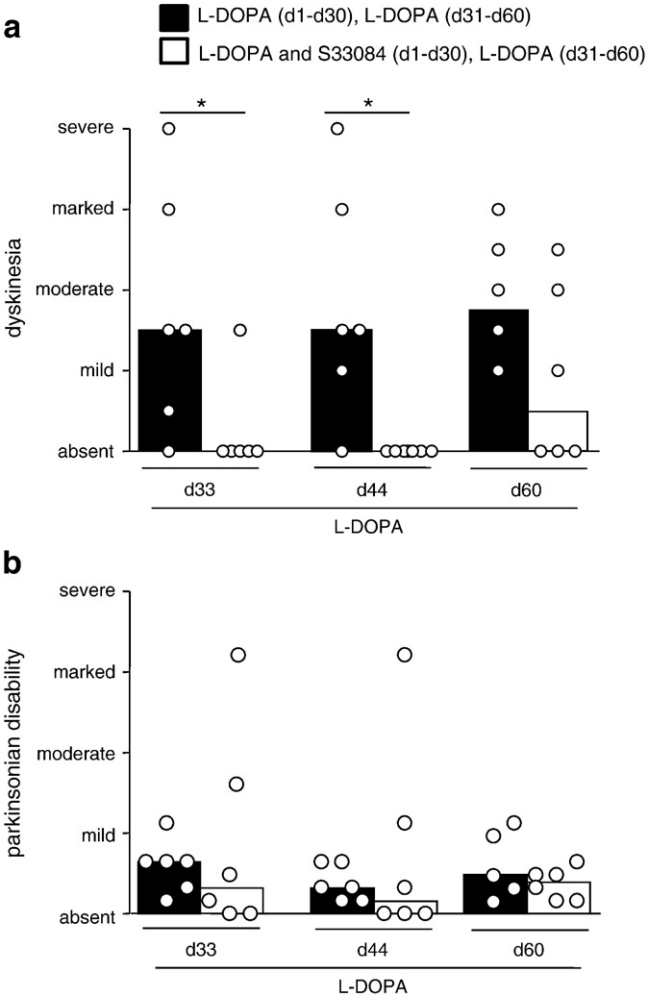

Fig. 2. The dopamine D_3 antagonist S33084 prevents the development, not the expression, of L-DOPA-induced dyskinesia (LID) in the MPTP-lesioned non-human primate. Following the 30 days priming period (d1–30) all animals received L-DOPA (15 mg/kg and benserazide 3.75 mg/kg) twice daily p.o. for a further 30 days (d31–60). a shows peak-dose dyskinesia (cumulative score 40–110 min post drug); where severe represents the greatest possible dyskinesia score = 8, marked = 6, moderate = 4, mild = 2, absent = 0 as defined in the methods; *$P<0.05$, Mann Whitney test, ($N=6$/group) prior L-DOPA/vehicle group (■) compared to prior L-DOPA and S33084 group (□) on day 33 and day 44. b shows peak-dose parkinsonian disability (cumulative score 40–110 min post drug); where severe represents the greatest possible score = 72, marked = 54, moderate = 36, mild = 18, absent = 0 as defined in the methods. Data are individual animals (○) with the median indicated as a bar.

D_3 receptor antagonism does not reduce the anti-parkinsonian effects of L-DOPA in the MPTP-lesioned primate

The reduction in the development of LID by day 30 with S33084 occurred without reducing the anti-parkinsonian action of L-DOPA (Fig. 1b). Thus, on day 30, there was no significant difference in total

parkinsonian disability, at time of peak L-DOPA action, between animals treated with L-DOPA alone and L-DOPA in combination with S33084 (total parkinsonian disability score 3 (range 1–5) and 5 (range 2–6), respectively ($P=0.0858$, Mann Whitney, $N=4$–6, Fig. 1b). Furthermore, there was no significant difference in the anti-parkinsonian effects of L-DOPA between animals treated with L-DOPA alone and L-DOPA in combination with S33084 during the S33084 washout period on days 33, 44 or 60 ($P=0.6279$, $P=0.6991$ and $P=0.4558$, respectively Mann Whitney, $N=5/6$, Fig. 2b). Similarly, in comparing each of the four individual components of the parkinsonian disability score, no statistically-significant differences were observed between the two groups on days 30, 33, 44 or 60 (data not shown).

The selective dopamine D_3 antagonist, S33084, reduces behavioural sensitization to repeated L-DOPA treatment in the 6-OHDA-lesioned rat model of PD

In accordance with previous reports (Papa et al., 1994), the rotational response to L-DOPA increased significantly with repeated administration ($F_{2,19}=9.4$, $P<0.0014$, 1-way ANOVA), from 196 ± 1 mean total contraversive rotations elicited over the 3 h period following L-DOPA administration on the first day of treatment (day 1) compared to 1368 ± 263 following 13 days treatment ($P<0.01$, Bonferroni, $N=9$) and 1514 ± 8 after 21 days of L-DOPA ($P<0.01$, Bonferroni, $N=9$ (Fig. 3). There was a significant effect of treatment on the development of this rotational response to repeated L-DOPA ($F_{\text{treatment } 5,213}=30.72$, $P<0.001$, 2-way ANOVA). Raclopride reduced the number of contraversive rotations elicited by L-DOPA, by ~61% on day 1,~62% on day 5, ~68% on day 9 ($P<0.05$, Bonferroni, $N=6$), ~83% on day 13 ($P<0.001$, Bonferroni, $N=8$), ~79% on day 17 ($P<0.001$, Bonferroni, $N=7$) and ~83% on day 21 ($P<0.001$, Bonferroni, $N=7$) (Fig. 3). In contrast, the D_3 antagonist S33084 had no significant effect on L-DOPA-induced rotations on days1 to 13($P>0.05$, $N=8$–9, Fig. 3). However, on days 17 and 21, the number of contraversive rotations elicited by L-DOPA was reduced by ~53% and ~48% (both $P<0.05$, Bonferroni, $N=7$–8) in animals co-administered with L-DOPA and S33084 as compared to those co-administered L-DOPA and vehicle (Fig. 3).

Vehicle treated animals showed a small ipsiversive bias on day 1, 6.2 ± 0.04 total ipsiversive rotations elicited over the 3 h period, this did not sensitize over time, being 7.6 ± 0.05 after 21 days of treatment ($P>0.05$, Bonferroni, $N=9$, Fig. 3).

Following a 24 h washout, on day 22, animals previously treated with L-DOPA combined with S33084 or raclopride, exhibited

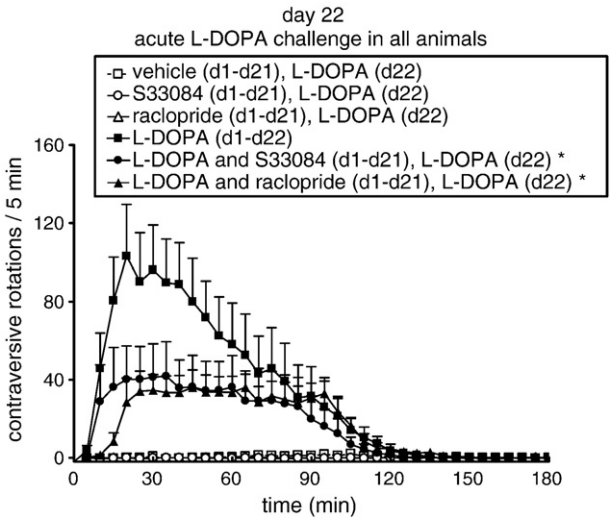

Fig. 4. The dopamine D_3 antagonist, S33084 attenuates the development, but not the expression, of L-DOPA-induced contraversive rotations in the 6-OHDA-lesioned rat. Animals were divided into 6 treatment groups and treated twice daily for 21 days; vehicle + vehicle (□), vehicle + raclopride 0.16 mg/kg (△) vehicle + S33084 0.63 mg/kg (○), L-DOPA 15 mg/kg + vehicle (■), L-DOPA 15 mg/kg + raclopride 0.16 mg/kg (▲) or L-DOPA 15 mg/kg + S33084 0.63 mg/kg (●). On day 22, following a 24 h drug washout, all animals received an L-DOPA (15 mg/kg) challenge. Data are mean (+s.e.m.) contraversive rotations/5 min time frame for duration of 3 h, $N=8$–10. * indicates significant difference of cumulative rotations elicited 3 h post-drug treatment to L-DOPA + vehicle group, (*$P<0.05$, 1-way ANOVA with Bonferroni post hoc analysis).

significantly less rotations in response to a challenge with L-DOPA alone than those previously treated with L-DOPA alone ($F_{3,22}=3.975$, $P=0.021$, 1 way ANOVA, both $P<0.05$ Bonferroni, $N=8$, Fig. 4).

The ability of S33084 to attenuate L-DOPA-induced behavioural sensitization in the 6-OHDA-lesioned rat is not associated with changes in D_1, D_2 or D_3 receptor levels

In keeping with previous reports, the striatal levels of D_1 receptor mRNA in the lesioned hemisphere were significantly enhanced by repeated L-DOPA treatment (Bordet et al., 1997; Gerfen et al., 1990) ($F_{\text{treatment } 5,49}=36.44$, $P<0.0001$, 2 way ANOVA). This elevation was apparent in all L-DOPA treated animals, irrespective of co-administration of vehicle, raclopride or S33084 (all $P<0.001$,

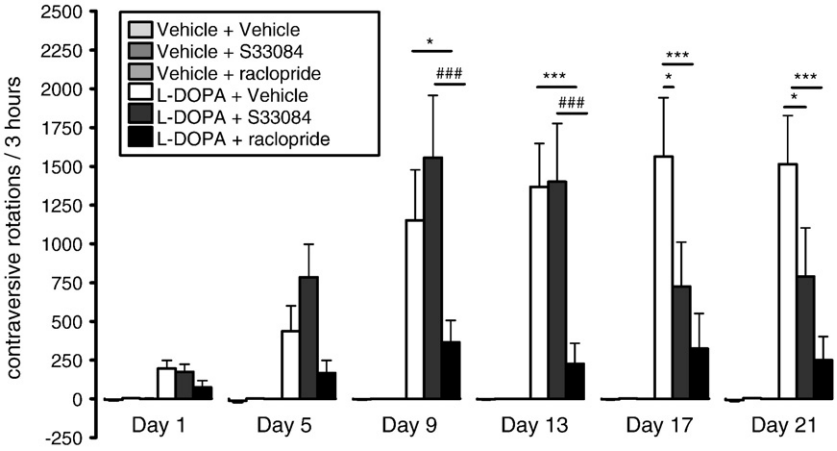

Fig. 3. Co-administration of L-DOPA with S33084 reduces sensitization of L-DOPA-induced contraversive rotations in the 6-OHDA-lesioned rat. Animals were divided into 6 treatment groups and treated twice daily for 21 days; vehicle + vehicle, vehicle + raclopride 0.16 mg/kg, vehicle + S33084 0.63 mg/kg, L-DOPA 15 mg/kg + vehicle, L-DOPA 15 mg/kg + raclopride 0.16 mg/kg or L-DOPA 15 mg/kg + S33084 0.63 mg/kg. This figure shows the total number of contraversive rotations elicited over 3 h pot-treatment on days 1, 5, 9, 1, 17 and 21. Data are mean (+s.e.m) contraversive rotations/3 h, $N=8$–10. * indicates significant difference to L-DOPA + vehicle group and # indicates significant difference L-DOPA + S33084 treated group, (*$P<0.05$; ***$P<0.001$, ###$P<0.001$, 2-way ANOVA with Bonferroni post hoc analysis).

Bonferroni, $N=8$–10, Fig. 5a) and not significantly different between L-DOPA treatment groups. In addition, and in accordance with previous reports (Creese et al., 1977; Gerfen et al., 1990; Lee et al., 1978), the level of D_2 receptor mRNA was significantly enhanced in the lesioned hemisphere of all animals bearing a 6-OHDA lesion, irrespective of treatment with either L-DOPA or S33084 or raclopride ($F_{\text{treatment } 5,\ 47}=22.1$, $P<0.0001$, 2 way ANOVA, all $P<0.01$, Bonferroni, $N=8$–10, Fig. 5b). Furthermore, there were no significant differences between the lesioned striata in any of the L-DOPA treated groups. The most prominent change found in relation to dopamine receptors in the dorsal striatum was in the levels of D_3 receptor protein (Fig. 5c) ($F_{\text{treatment } 5,\ 34}=1.0$, $P<0.0001$, 2 way ANOVA, Fig. 5c). Thus, in animals treated with L-DOPA, striatal D_3 receptor expression in the lesioned hemisphere was significantly increased by ~132, 66, and 91% compared to the intact hemisphere in animals co-treated with vehicle, raclopride and S33084 respectively (all $P<0.05$, Bonferroni, $N=8$–10). However, within these groups there were no significant differences in D_3 protein levels.

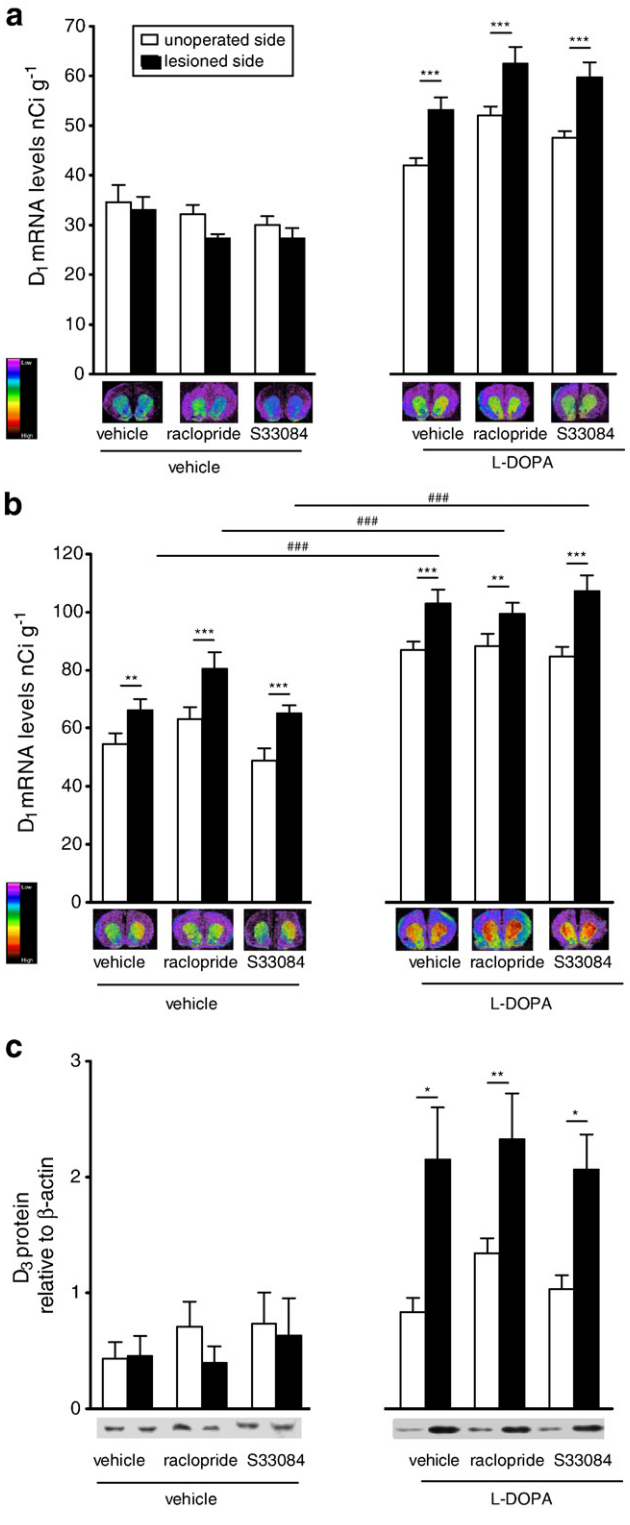

Fig. 5. The effect of co-administration of L-DOPA with S33084 on reducing L-DOPA-induced sensitization in the 6-OHDA-lesioned rat is not mediated by changes in dopamine D_1, D_2 or D_3 receptor expression. Animals were divided into 6 treatment groups as noted and treated twice daily for 21 days. On day 23, 1 h post drug administration, animals were killed, the brains removed and frozen in isopentane. a, D_1 and b, D_2 receptor mRNA in the dorsal striatum was quantified by *in situ* hybridization in 3 consecutive 20 μM sections. c, D_3 receptor protein was quantified by western blot analysis of striatal homogenates, representative images illustrating D_3 receptors expression are show below each bar. Data are mean (±s.e.m) $N=8/10$. * indicates significant difference between L-DOPA treated lesioned hemisphere and L-DOPA-treated intact hemisphere and # indicates significant difference between L-DOPA treated lesioned hemisphere and respective vehicle treated lesioned hemisphere (*$P<0.05$; **$P<0.01$, ***$P<0.001$, ###$P<0.001$ 2-way repeated measures ANOVA with Bonferroni post hoc analysis). Representative psudocolour images of the level of striatal D_1 and D_2 receptor mRNA expression and representative images of the level of striatal D_3 receptor protein are shown below each histogram.

The ability of S33084 to attenuate L-DOPA-induced behavioural sensitization in the 6-OHDA-lesioned rats is associated with reduced striatal levels of the opioid precursor PPE-A

The level of striatal PPE-A mRNA in the lesioned hemisphere of L-DOPA treated animals was ~94% greater than in the lesioned hemisphere of respective vehicle treated animals ($F_{11,78}=10.97$, $P<0.0001$, 1 way ANOVA, $P<0.01$ Bonferroni, $N=6$–9, Fig. 6a). This L-DOPA enhanced increase in PPE-A mRNA was not apparent in animals treated with L-DOPA in combination with either raclopride or S33084. Thus, in animals treated with L-DOPA and either raclopride or S33084, there was no significant difference in PPE-A in the lesioned hemisphere when compared to the level of PPE-A mRNA in the lesioned hemisphere of vehicle treated animals (both $P>0.05$, Bonferroni, $N=6$–9, Fig. 6a).

A significant rise in striatal mRNA levels of PPE-B was apparent in the lesioned hemisphere of all animals receiving L-DOPA, irrespective of co-administration of vehicle, raclopride or S33084 ($F_{11,76}=19.15$, $P<0.0001$, 1 way ANOVA, all $P<0.001$ Bonferroni, $N=6$–9, Fig. 6b). Furthermore, the level of striatal PPE-B mRNA in the lesioned hemisphere of animals treated with L-DOPA in conjunction with either vehicle, raclopride or S33084 was significantly higher than the level of PPE-B mRNA in the lesioned hemisphere of 6-OHDA-lesioned animals treated with vehicle (all $P<0.001$, Bonferroni, $N=6$–9). There was no significant difference in the level of striatal PPE-B mRNA in the intact or lesioned hemisphere of 6-OHDA-lesioned animals treated with vehicle irrespective of co-administration with vehicle, raclopride or S33084 (all $P<0.05$, Bonferroni, $N=6$–9, Fig. 6b).

There was no significant difference in the level of phospho[Thr^{34}] Dopamine- and cyclic AMP-regulated phosphoprotein with molecular weight 32 kDa (phospho[Thr34]DARPP-32), relative to total DARPP-32, in the lesioned or intact hemispheres of animals treated with vehicle in conjunction with either vehicle, raclopride or S33084 ($F_{\text{lesion } 2,\ 14}=1.005$, $P>0.05$, 2 way ANOVA, $N=5$–7, Fig. 7). However, in L-DOPA-treated, 6-OHDA-lesioned rats, the level of phospho[Thr^{34}] DARPP-32 expression, relative to total DARPP-32, in the lesioned hemisphere was significantly increased, by ~122%, as compared to the intact hemisphere ($F_{\text{lesion } 2,23}=17.67$, $P=0.0003$, 2 way ANOVA, $P<0.05$, Bonferroni, $N=9$, Fig. 7). This elevation in phospho[Thr34] DARPP-32 was also apparent in animals treated with L-DOPA in conjunction with S33084 ($P<0.05$, Bonferroni, $N=8$). There was no

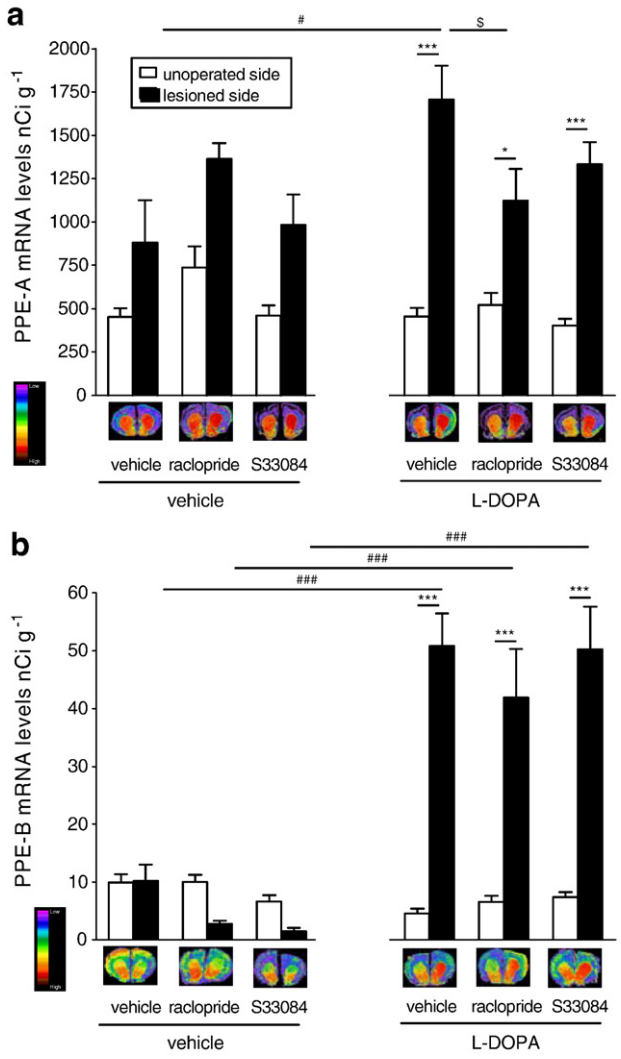

Fig. 6. The effect of the dopamine D_3 antagonist, S33084, on reducing L-DOPA-induced sensitization in the 6-OHDA-lesioned rat may involve reduced striatal pre-proenkephalin A (PPE-A) but not pre-proenkephalin B (PPE-B). Animals were divided into 6 treatment groups as noted and treated twice daily for 21 days. On day 23, 1 h post drug administration, animals were killed, the brains removed and frozen in isopentane. a, PPE-A and b, PPE-B mRNA in the dorsal striatum was quantified by *in situ* hybridization in 3 consecutive 20 μM sections. Data are mean (±s.e.m) $N=8/10$. * indicates significant difference between L-DOPA treated lesioned hemisphere and L-DOPA-treated intact hemisphere; # indicates significant difference between L-DOPA treated lesioned hemisphere and respective vehicle treated lesioned hemisphere and $ indicates significant difference between L-DOPA treated lesioned hemisphere and L-DOPA/raclopride treated lesioned hemisphere (*$P<0.05$; **$P<0.01$, ***$P<0.001$, #$P<0.05$, ###$P<0.001$; $$P<0.05$, 2-way repeated measures ANOVA with Bonferroni post hoc analysis). Representative psudocolour images of the level of striatal PPE-A and PPE-B mRNA expression are shown below each histogram.

significant increase in striatal phospho[Thr34]DARPP-32 expression in the lesioned hemisphere of animals treated with L-DOPA and raclopride ($P>0.05$, Bonferroni, $N=9$).

Discussion

We demonstrate that combined treatment with a selective D_3 receptor antagonist, S33084, prevents the development of LID over a 30 day treatment period in MPTP-lesioned marmosets. This action was without detriment to the anti-parkinsonian actions of L-DOPA. Following a 14 day washout period, when challenged with L-DOPA in the absence of S33084 these animals continue to exhibit a reduced level of LID, suggesting that this effect of D_3 antagonism is truly one of reducing sensitization to repeated L-DOPA treatment, rather than merely reducing expression of the behaviour. However, following a 30 day washout period, when challenged with L-DOPA, in the absence of S33084, these animals began to exhibit a low level of LID suggesting that D_3 antagonists do not permanently protect against the development of dyskinesia but that combination therapy must be maintained to avoid LID development. Interestingly, there was a trend for there to be a less rapid development of LID, following switching, from L-DOPA/S33084 to L-DOPA alone on day 31, compared to initiating L-DOPA therapy *de novo*. Further studies may define whether treatment with a D_3 antagonist during the initial priming stage might slow subsequent development of dyskinesia.

The mechanisms underlying the ability of D_3 receptor antagonism to attenuate the development of LID require further evaluation. On the basis of our rodent data, we propose there are at least two phases of L-DOPA-induced sensitization. The first phase, represented in the rodent model at day 13, develops in a D_3-independent manner and is thus unaffected by the D_3 antagonist S33084. Indeed, the elevation of D_3 receptors following L-DOPA therapy has previously been suggested as being dependent upon D_1/D_5 stimulation and elevation of BDNF levels (Guillin et al., 2001, 2003). A second phase of sensitization, represented after day 13, is blocked by S33084 and thus D_3-dependent. The delayed effect of S33084 is unsurprising given that in drug naive parkinsonian animals, striatal D_3 binding in the dorsal striatum is minimal (Levesque et al., 1992; Sokoloff et al., 1990). However, repeated L-DOPA treatment in parkinsonian animals induces ectopic striatal D_3 expression in the dorsal striatum (Bordet et al., 1997; Guillin et al., 2001). We propose that repeated L-DOPA induces the expression of dorsal striatal D_3 receptors, by a D_3 receptor-independent mechanism, and this precedes a D_3-dependent mechanism responsible for the further development of sensitization to L-DOPA. It is of interest that an involvement of abnormal D_3 signalling in the development of dyskinesia is also suggested by reports of a role for D_3 receptors in a related disorder, tardive dyskinesia (Bakker et al., 2006).

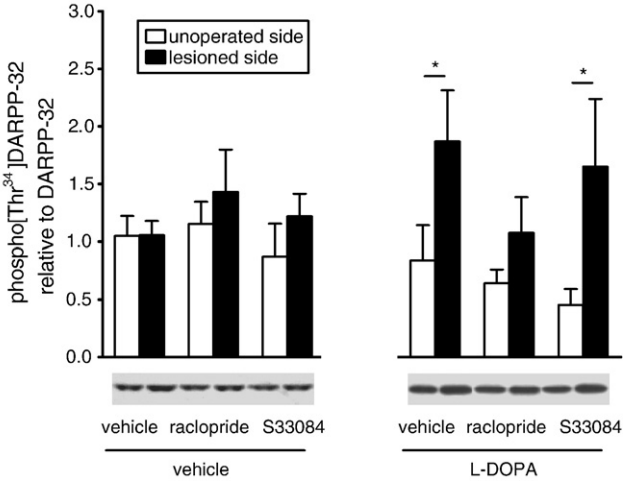

Fig. 7. The effect of the dopamine D_3 antagonist, S33084, on reducing L-DOPA-induced sensitization in the 6-OHDA-lesioned rat does not involve alteration in striatal phospho [Thr34]DARPP-32. Animals were divided into 6 treatment groups as noted and treated twice daily for 21 days. On day 23, 1 h post drug administration, animals were killed, the brains removed and frozen in isopentane. Phospho[Thr34]DARPP-32 protein was quantified by Western blot analysis of striatal homogenates, representative images illustrating phospho[Thr^{34}]DARPP-32 expression are show below each bar. Data are presented relative to total DARPP-32 levels as mean (±s.e.m) $N=8/10$. * indicates significant difference between L-DOPA treated lesioned hemisphere and L-DOPA-treated intact hemisphere; (*$P<0.05$; **$P<0.01$, ***$P<0.001$, #$P<0.05$, ###$P<0.001$; $$P<0.05$, 2-way repeated measures ANOVA with Bonferroni post hoc analysis). Representative images of the level of striatal phospho[Thr34]DARPP-32 protein are shown below each histogram.

The relevance of the D_3-indpendent phase to dyskinesia is less clear as in the primate the development of dyskinesia was essentially prevented by S33084, i.e. there was no apparent D_3-independent phase. The nature of the D_3-indepenent mechanism remains to be resolved but many systems, including D_1 receptors and modulators of dopaminergic signalling such as RGS9-2, which are not functionally linked to D_3 receptors, have been suggested as being involved in the expression of dyskinesia once established, though it is not clear if they contribute to the development of dyskinesia (Aubert et al., 2005; Gold et al., 2007).

The mechanisms underlying the ability of S33084 to attenuate sensitization to L-DOPA appear not to be related to alterations in striatal dopamine receptor levels as the effects of both raclopride and S33084 in reducing L-DOPA-induced behavioural sensitization in the 6-OHDA-lesioned rat are not accompanied by a change in expression of either dopamine D_1 or D_2 mRNA or D_3 receptors. However, though the elevation of dopamine receptors by repeated L-DOPA treatment does not appear sufficient to provoke development of sensitization, it may be permissive. Indeed, the severity of LID in MPTP-lesioned non-human primates correlates with changes in D_1 receptor linked GTPγS signalling rather than number of D_1 receptors *per se* (Aubert et al., 2005). While D_3 receptor antagonism reduced the development of dyskinesia and sensitization, it did not normalize the elevated levels of dorsal striatal D_3 receptors themselves. This suggests that D_3 receptor levels are not regulated by D_3 stimulation and that the mechanisms responsible for the elevation of D_3 receptors in dyskinesia are not D_3-dependent. This is consistent with the hypothesis, stated above, that there is an initial D_3-indepenent mechanism during which D_3 receptors become elevated.

It has previously been suggested the elevated opioid transmission might contribute to the process of sensitization to L-DOPA in LID. Indeed, increased striatal expression of preproenkephalin A (PPE-A) (Gerfen et al., 1990; Nisenbaum et al., 1994; Voorn et al., 1987) and B (PPE-B) mRNA (Aubert et al., 2007; Duty and Brotchie, 1997; Gerfen and Young, 1988; Henry et al., 2003; Westin et al., 2001) is well-documented in animal models of LID. Our data point to an L-DOPA-induced elevation of PPE-A as a component of the mechanisms responsible for the development of sensitization. Thus, striatal PPE-A expression was only elevated above levels seen in vehicle-treated-lesioned animals (i.e. equivalent to untreated Parkinsonism), in animals exhibiting behavioural sensitisation. On the other hand, a significant rise in striatal mRNA levels of PPE-B was apparent in all animals receiving L-DOPA, irrespective of treatment given or the presence of behavioural sensitisation. The suppression of an elevation in PPE-A is a potential mechanism by which D_3 antagonists may reduce the development of LID. Indeed, Schneider et al. have suggested that elevated PPE-A might be necessary for the development of LID in the MPTP-lesioned primate (Schneider et al., 1999, 2003). Furthermore, PD patients without LID do not have elevated striatal PPE-A following L-DOPA treatment, whereas those with LID do (Calon et al., 2002). However, it is unclear whether changes in PPE-A mRNA represent a mechanism underlying sensitization to L-DOPA or are a response to it.

Phosphorylation of the threonine 34 residue of DARPP-32 is enhanced in 6-OHDA-lesioned rats treated with repeated L-DOPA by a D_1 receptor-dependent mechanism (Picconi et al., 2005). We demonstrate that the L-DOPA-induced increase in striatal phospho [Thr34]DARPP-32 is also apparent following co-treatment with S33084. However, in animals treated with raclopride, no L-DOPA-induced increase in striatal phospho[Thr34]DARPP-32 was apparent. These data suggest that elevation of phospho[Thr34]DARPP-32 may be related to the first, D_3-independent, phase of sensitization.

The lack of effect of S33084 on the anti-parkinsonian actions of L-DOPA in MPTP-lesioned marmosets suggests that D_3 receptor activation does not make a major contribution to the anti-parkinsonian actions of L-DOPA. These data thus complement our previous finding that anti-parkinsonian effects of the mixed D_3/D_2 receptor agonist S32504 result from activation of D_2 rather than D_3 receptors (Hill et al., 2006) and suggest that the D_3 activity of clinically-available dopamine agonists such as pramipexole and ropinirole may not be important for their symptomatic efficacy. However, we cannot discount that long term benefit of D_3 agonism with these agents may accrue from D_3 involvement in neurogenesis which might suspport re-modelling of damaged neuronal architecture (Van Kampen and Eckman, 2006; Van Kampen and Robertson, 2005). The maintenance of anti-parkinsonian benefit, suggests that the reduction in LID by S33084 is not simply due to a non-specific reduction in motor activity, perhaps due to sedation or an alteration in L-DOPA metabolism, and also suggests a lack of any D_2 antagonist properties of S33084 at the dose used. Furthermore, the demonstration of good anti-parkinsonian benefit with less dyskinesia highlights the potential clinical utility of a D_3 antagonist/L-DOPA approach to treating Parkinson's disease.

The above discussion on the role of D_3 receptors in anti-parkinsonian actions of drugs highlights that D_3 receptor stimulation has been implicated in several different processes related to Parkinsonism. Each of these may have different neural mechanisms. Here we show that D_3 receptor stimulation prevents the development of dyskinesia *de novo*, i.e. priming. This is distinct from the mechanisms that might underlie the expression of dyskinesia once the system has been primed. Indeed the data here, and those we previously published, suggest that D_3 stimulation is not involved in expression of established dyskinesia. On the other hand, D_3 stimulation may be involved in the expression of other complications of L-DOPA therapy, i.e. wearing-off.

In conclusion, these observations suggest that initial, and continued, treatment of PD patients with L-DOPA combined with a selective dopamine D_3 antagonist may provide effective anti-parkinsonian benefit whilst avoiding the development of LID. However, we find no evidence to support the case that D_3 antagonists may suppress dyskinesia once it has been established.

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