S32504, a Novel Naphtoxazine Agonist at Dopamine D₃/D₂ Receptors: II. Actions in Rodent, Primate, and Cellular Models of Antiparkinsonian Activity in Comparison to Ropinirole

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Received November 5, 2003; accepted February 12, 2004

ABSTRACT

These studies evaluated the potential antiparkinsonian properties of the novel dopamine D₃/D₂ receptor agonist S32504 ([(+)-trans-3,4,4a,5,6,10b-hexahydro-9-carbamoyl-4-propyl-2H-naphth[1,2-b]-1,4-oxazine] in comparison with those of the clinically employed agonist ropinirole. In rats with a unilateral, 6-hydroxydopamine lesion of the substantia nigra, S32504 (0.0025–0.04 mg/kg, s.c.) more potently elicited contralateral rotation than S32601 ([–]-trans-3,4,4a,5,6,10b-hexahydro-9-carbamoyl-4-propyl-2H-naphth-[1,2-b]-1,4-oxazine (its less active enantiomer)), ropinirole, and L-3,4-dihydroxyphenylalanine (L-DOPA). Rotation elicited by S32504 was blocked by the D₃/D₂ receptor antagonists haloperidol and raclopride and the D₂ antagonist L741,626 but not S33084. Ropinirole was weakly neuroprotective in this model. In conclusion, S32504 displays potent and stereospecific activity in rodent, primate, and cellular models of antiparkinsonian properties. Although activation of D₂ receptors is crucial to the motor actions of S32504, engagement of D₃ receptors contributes to its neuroprotective properties.

Although there is increasing interest in the significance of perturbed noradrenergic and serotonergic transmission to the motor, cognitive, and emotional symptoms of Parkinson’s disease (PD), its cardinal features can principally be explained by the degeneration of dopaminergic pathways originating in the substantia nigra pars compacta (SNpc) (Gerlach and Riederer, 1996; Rascol et al., 2003). Correspondingly, PD is treated by administration of the dopaminergic precursor, L-DOPA, which is transformed in residual noradrenergic and serotonergic transmission to NA, noradrenaline; SNpc, substantia nigra pars compacta L-DOPA, L-3,4-dihydroxyphenylalanine; DA, dopamine; NA, noradrenaline; S32504, [(+)-trans-3,4,4a,5,6,10b-hexahydro-9-carbamoyl-4-propyl-2H-naphth[1,2-b]-1,4-oxazine; S32601, (3aR,9bS)-N-[4-(8-cyano-1,3a,4,5b-tetrahydro-3H-benzopyrano[3,4-c]pyrrole-2-yl)-butyl]-4-(phenyl)benzamide; S32304, (3aR,9bS)-N-[4-(8-cyano-1,3a,4,5b-tetrahydro-3H-benzopyrano[3,4-c]pyrrole-2-yl)-butyl]-4-(phenyl)benzamide; L741,626, 4-(4-chlorophenyl)phenylpyridinium, an action inhibited by ropinirole and S33084 but not L741,626. Ropinirole was weakly neuroprotective in this model. In conclusion, S32504 displays potent and stereospecific activity in rodent, primate, and cellular models of antiparkinsonian properties. Although activation of D₂ receptors is crucial to the motor actions of S32504, engagement of D₃ receptors contributes to its neuroprotective properties.

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ABBREVIATIONS: PD, Parkinson’s disease; SNpc, substantia nigra pars compacta L-DOPA, L-3,4-dihydroxyphenylalanine; DA, dopamine; NA, noradrenaline; S32504, (+)-trans-3,4,4a,5,6,10b-hexahydro-9-carbamoyl-4-propyl-2H-naphth[1,2-b]-1,4-oxazine; S32601, (3aR,9bS)-N-[4-(8-cyano-1,3a,4,5b-tetrahydro-3H-benzopyrano[3,4-c]pyrrole-2-yl)-butyl]-4-(phenyl)benzamide; L741,626, 4-(4-chlorophenyl)-1-(1H-indol-3-yl)piridin-4-ol; 6-OHDA, 6-hydroxydopamine; ANOVA, analysis of variance; ACh, acetylcholine; 5-HT, serotonin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP, 1-methyl-4-phenylpyridinium; MTT, 3-[4,5-dimethylthiazol-2,5-diphenyltetrazolium; S31411, trans-3,4,4a,5,6,10b-hexahydro-9-carbamoyl-4-propyl-2H-naphth[1,2-b]-1,4-oxazine HCl; S32601, (–)-trans-3,4,4a,5,6,10b-hexahydro-9-carbamoyl-4-propyl-2H-naphth[1,2-b]-1,4-oxazine.
dopaminergic (and other) neurons into dopamine (DA) (Bézard et al., 2001; Rascol et al., 2003). However, L-DOPA is suspected to exert neurotoxic properties that accelerate the loss of dopaminergic neurons (Blum et al., 2001; Jenner, 2003; Whone et al., 2003). Furthermore, the pharmacokinetic profile of L-DOPA is variable [leading to abrupt transitions between “on” (active) and “off” (inactive) phases], it is poorly effective against the amnesic and mood deficits of PD, it elicits marked dyskinesia, and its therapeutic efficacy gradually wanes over years of exposure (Bézard et al., 2001; Rascol et al., 2003).

There is, then, considerable interest in strategies to improve symptomatic control of motor and affective symptoms of PD, slow the loss of dopaminergic neurons, and delay the introduction of L-DOPA (Blum et al., 2001; Jenner, 2003; Rascol et al., 2003; Schapira and Olanow, 2003). Dopaminergic agonists retain their pertinence since, in addition to their utilization as adjuncts, their sustained efficacy as monotreatment permits postponement of treatment with L-DOPA (Brunet et al., 2002; Rascol et al., 2003). Moreover, upon long-term administration to Parkinson’s patients, dopaminergic agonists counter neurotoxic mechanisms underlying the degeneration of dopaminergic pathways (Carvey et al., 2001; Rascol et al., 2003; Whone et al., 2003). Finally, although dopaminergic agonists elicit their own spectrum of side effects, including sedation and sleep attacks, they elicit less dyskinesia than L-DOPA and possess superior pharmacokinetic profiles (Rascol et al., 2003).

Though dopamine D1 receptor agonists improve motor function in experimental models of PD, their long-term efficacy in man remains to be proven, and they do not possess neuroprotective properties (Rascol et al., 2003). Accordingly, most interest has been directed toward agonists of the D2 receptor family comprised of closely related D2, D3, and D4 receptors. Activation of D4 sites does not participate in the motor and neuroprotective actions of antiparkinsonian agents and may unfavorably influence mood and cognition (Millan et al., 1998). As regards D2 and D3 receptors, their respective significance in the treatment of PD remains under discussion as concerns both beneficial (motor and neuroprotective) actions and undesirable side effects (Joyce, 2001; Bézard et al., 2003; Van Kampen and Stoessl, 2003). One reason underlying this uncertainty is that all dopaminergic agonists therapeutically employed in the management of PD activate D2 and D3 receptors (Joyce, 2001; Millan et al., 2002; Newman-Tancredi et al., 2002). Indeed, even “preferential” agonists of D3 versus D2 receptors, such as ropinirole, engage D2 receptors in vivo (Eden et al., 1991; Coldwell et al., 1999; Joyce, 2001; Millan et al., 2004a,b).

Nevertheless, ropinirole shows pronounced functional selectivity for D2 and D3 receptors versus serotoninergic receptors and α-adrenoceptors, distinguishing it from many other antiparkinsonian drugs (Coldwell et al., 1999; Millan et al., 2002; Newman-Tancredi et al., 2002). This is important since actions of antiparkinsonian agents at nondopaminergic receptors greatly modify their functional profiles and complex interpretation of the significance of D2 versus D3 sites to their actions (Millan et al., 2002; Newman-Tancredi et al., 2002). In this light, the novel naphthoxazine derivative, S32504, is of interest since it is an even more selective D3/D2 receptor ligand than ropinirole. Furthermore, it behaves as an efficacious agonist at D3 and, at higher concentrations, D29 (short isoform) and D21 (long isoform) receptors (Millan et al., 2004b). Correspondingly, S32504 displays potent activity in vivo at both pre- and postsynaptic populations of D2 and D3 receptors (Millan et al., 2004a,b).

The studies described herein evaluated potential antiparkinsonian properties of S32504 in a broad range of cellular, neurochemical, and behavioral models reflecting both its influence upon motor function and its neuroprotective properties. In several procedures, parallel studies were undertaken with its less active enantiomer S32601 (Millan et al., 2004a,b). Furthermore, to evaluate the comparative significance of D3 versus D2 receptors in its effects, we employed the mixed D2/D3 receptor antagonists haloperidol and raclopride, the selective D3 receptor antagonist S33084, and the preferential D3 receptor antagonist L741,626 (Millan et al., 2000). In all studies, the actions of S32504 were compared with those of ropinirole, and, in key behavioral procedures, its effects were also compared with those of L-DOPA.

Materials and Methods

Animals. In rodent studies, animals were male Wistar rats weighing 220 to 250 g unless otherwise specified (Ifa Credo, L’Arbresle, France). They were maintained in sawdust-lined cages with unrestricted access to food and water. The laboratory temperature was held at 21 ± 1°C, and humidity was controlled at 60 ± 5%. There was a 12-h light/dark cycle, with lights on from 7:30 AM to 7:30 PM. Before experimentation, all animals were adapted for at least 1 week to laboratory conditions. All animal use procedures conformed to international European ethical standards (86/609-EEC) and the French National Committee (décret 87/848) for the care and use of laboratory animals. In primate studies, adult marmosets (Callithrix jacchus) of either sex were employed. They were kept in controlled housing conditions with the temperature held at 25°C to 27°C, 50% relative humidity, and a 12-h light/dark cycle (lights on from 8:00 AM to 8:00 PM). They had free access to food pellets, fresh fruit supplements, and water. All primate experiments were carried under Home Office License PPL 40/01487 in accordance with UK legal requirements.

Induction of Rotation by S32504 in Rats with a Unilateral Lesion of the Substantia Nigra Pars Compacta. The procedure employed was essentially as described in detail previously (Millan et al., 1998). Rats (300–330 g) received a unilateral, 6-hydroxydopamine (6-OHDA) (8 μg/4 μl)-induced lesion of the SNpc at the following coordinates: AP, +3.4; L, ±2.2; and H, +2.2. They were allowed 3 weeks after surgery for recovery before testing. Rotations were monitored automatically in rats coupled to a harness connected to a Rotacount 8 (Columbus Instruments, Columbus, OH) apparatus. Only animals that showed a pronounced response (>150 contralateral rotations/h) to apomorphine (0.04 mg/kg, s.c.; two sessions 1 week apart) and subsequently to quinpirole (0.02 mg/kg, s.c.; two further sessions, 1 week apart) were included in the behavioral and dialysis studies. After selection of animals for behavioral studies, sessions were performed once a week with an ABACADA design: “A” corresponds to a quinpirole control session, and “B to D” correspond to test sessions with variable drug treatment. In quinpirole-control sessions, rats were administered with vehicle 25 min before quinpirole (0.02 mg/kg, s.c.) and rotation measured for 1 h immediately after the quinpirole injection. In test sessions, vehicle was injected 25 min before S32504 (s.c.), ropinirole (s.c.), L-DOPA (i.p.), or vehicle (s.c. or i.p.) and rotation likewise measured for 1 h. The influence of S32504 and ropinirole was compared with vehicle by one-way analysis of variance (ANOVA) followed by Dunnett’s test. For antagonist studies, the procedure employed was exactly as described above except that, in the test sessions, haloperidol, raclopride, L741,626,
Rats were administered with saline or vehicle to rats 25 min before S25204 (0.01 mg/kg, s.c.). The influence of antagonists compared with vehicle upon the induction of rotation by S25204 was analyzed by one-way ANOVA followed by Dunnett’s test. ID₅₀ values were calculated for antagonists.

Modulation of Extracellular Levels of ACh by S25204 Compared with Ropinirole in the Striatum of Freely Moving, 6-OHDA-Lesioned and Intact Rats. Dialysis studies were undertaken either in rats with a unilateral lesion of the SNpc or in intact animals. In both cases, they were performed in freely moving animals. As previously described (Gobert et al., 2003), rats were anesthetized with pentobarbital (60 mg/kg, i.p.), placed in a Kopf stereotaxic frame, and guide cannulae implanted in the striatum with coordinates as follows: AP, +0.5 from bregma; L, −2.8; and H, −3.0 from dura. Lesioned animals were behaviorally validated with apomorphine as described above, then, 3 months after lesioning, two guide cannulae were implanted. One was located on the side of the lesion and the other in the contralateral striatum. For intact animals, only one cannula was implanted in the right or left striatum. Rats were single housed and allowed to recover for 5 days before dialysis. On the day of dialysis, a cuprophan CMA/11 probe (4 mm in length, 0.24 mm o.d.) was slowly lowered into position. It was perfused at 1 µl/min with a phosphate-buffered solution of NaCl (147.2 mM), KCl (4 mM), and CaCl₂ (2.3 mM) (pH 7.3). No inhibitor of acetylcholinesterase was added. Two hours after implantation, 20-µl dialysate samples were collected every 20 min. In lesioned animals, the first two basal samples were taken for quantification of monoamines. The following three basal samples were retained for quantification of ACh. Thereafter, S25204 (0.16 mg/kg, s.c.) was injected and ACh levels measured for 3 h. In intact animals, the same procedure was used, but monoamines were quantified in basal samples, and S25204, ropinirole, or vehicle were administered and ACh levels measured over 3 h. In the antagonist studies of ACh levels in intact animals, haloperidol, raclopride, L741,626, S33084, or vehicle were injected, 20 min later by S25204 (0.63 mg/kg, s.c.) or vehicle, and dialysis was undertaken for a further 2 h.

Electrochemical Quantification of Extracellular Levels of ACh and Monoamines. ACh was quantified, as indicated above, in the absence of acetylcholinesterase inhibitors, as described by Gobert et al. (2003). In brief, 20-µl dialysate samples were collected on 10 µl of acetic acid (0.01%), and 20-µl aliquots were then analyzed by high-performance liquid chromatography. The mobile phase was composed of NaH₂PO₄ (50 mM) and Proclin (0.5%), adjusted to pH 8.2 with H₃PO₄. The stationary phase was comprised of a cation ion exchanger (Sephadex, 530 × 1.0 mm, 10-μm particle size) BAS, (BAS, Congleton, UK), a precolumn (preimmobilized enzyme reactor, 55 × 1 mm) of choline oxidase/catalase (BAS), and a postcolumn (postimmobilized enzyme reactor, 50 × 1 mm) of choline oxidase/acetylcholinesterase (BAS) maintained at 35°C. An amperometric detector set at +100 mV versus Ag/AgCl was used for quantification. The glassy carbon electrode (MF2098, BAS) was coated with a peroxidase-redox polymer. The mobile phase was delivered at a flow rate of 0.14 ml/min. The sensitivity of the assay for ACh was 0.1 pg (injected in a volume of 20 µl). Monoamines were quantified as extensively described previously (Millan et al., 1998, 2000) by high-performance liquid chromatography and coulometric detection. The detection limits for DA, NA, and 5-HT were 0.01 to 0.2 pg/sample in each case. Basal levels of ACh, DA, NA, and 5-HT in the striatum unilateral compared with contralateral to the lesion were compared by use of a paired Student’s t test. The influence of drugs upon ACh levels were analyzed by ANOVA followed by Bonferroni’s test with sampling time as the repeated within-subject factor.

Reversal of Reserpine-Induced Hypolocomotion in Rats by S25204 Compared with Ropinirole. Rats were administered with reserpine (2.5 mg/kg, i.p.) or saline (i.p.) 18 h before injection of S25204, ropinirole, or vehicle. Immediately after administration of the latter drugs, animals were individually placed in transparent polycarbonates cages (45 × 30 × 20 cm) located in activity chambers (Labline System; Coulbourn Instruments, Allentown, PA). Locomotion was monitored for 60 min. One movement corresponded to the consecutive interruption of two infrared beams situated 24 cm apart and 4 cm above the cage floor. The effects of reserpine (i.p.), S25204 (10.0 mg/kg, s.c.), or ropinirole (10.0 mg/kg, s.c.) alone upon locomotion were analyzed by Student’s two-tailed t test, and the effects of S25204 and ropinirole upon the hypolocomotion induced by reserpine were analyzed by ANOVA followed by Dunnett’s test.

Restoration of Motor Function by S25204 in Unprimed, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-Treated Marmosets. The procedure employed was essentially as described previously (Smith et al., 1996). Marmosets were rendered parkinsonian by administration of MPTP hydrochloride (2 mg/kg, s.c. for 5 consecutive days). The parkinsonian state stabilized within 8 to 10 weeks following administration, after which time studies were performed. On the experimental day, MPTP-treated parkinsonian marmosets were placed individually for 45- to 60-min habituation in activity cages fitted with eight infrared photocells and a clear plastic front for observation. Following habituation, they received either S25204 or vehicle. Thereafter, locomotor activity was monitored for 2 consecutive h. Locomotor counts were measured as the number of light beam interruptions as the animals moved about and were cumulated over 10-min periods. The disability of the animals was scored as follows: alertness (0, normal to 2, sleepy), reaction (0, normal to 3, absent); checking movements (0, present to 2, absent); attention and eye movements (0, normal to 4, grossly abnormal); balance/coordination (0, normal to 3, spontaneous falls); and vocalization (0, normal to 2, absent). The maximal disability score was 18, where an animal showed pronounced parkinsonian deficits. Disability scores were attributed during a 10-min observation period immediately after drug or vehicle administration for every 10 min for the following 2 h, starting at 10 min after drug or vehicle administration. Locomotor activity and disability scores were analyzed with two-way ANOVA with repeated measures of time, followed by Dunnett’s test.

Restoration of Motor Function by S25204 Compared with Ropinirole and L-DOPA in Primed, MPTP-Treated Marmosets. The procedure followed was essentially that described in detail previously (Fox et al., 2002). Following stabilization of the Parkinsonian state induced by MPTP (2 mg/kg s.c., for 5 consecutive days) administration, the animals were treated with 12.5 mg/kg l-DOPA plus 3.125 mg/kg benserazide twice daily for 3 weeks during which time they developed dyskinesia. Then, on the experimental day, the animals were administered drug or vehicle and their behavior recorded on video for 4 h. Parkinsonian disability and dyskinesia were assessed immediately after drug or vehicle administration by an observer blinded to the treatment. A global parkinsonian disability score (0, none; 36, severe) was calculated (Fox et al., 2002) from range of movement, bradykinesia, and posture scores. Parkinsonian disability scores were attributed as follows: (18 + (range of movement × 2) + (bradykinesia × 5) + (posture × 9)); range of movement (0, no movement to 9, running, jumping, wide range of motion); bradykinesia (0, normal speed and initiation of movement to 3, akinetic); and postural abnormality (0, normal, upright; 1, abnormal, crouched). A dyskinesia score was also attributed employing a non-parametric scale (0, absent to 4, severe, continuous). For individual parameters, statistical analyses were performed for the first 90 min of observations since S25204, ropinirole, and L-DOPA all expressed their maximal actions over this time period. Data were analyzed with Friedman’s, nonparametric one-way repeated measure ANOVA followed by Dun’s multiple comparison tests.

Neuroprotective Properties of S32504 in Transformed Human, SH-5YSY Neuroblastoma Cells. The procedures employed were essentially those described in detail elsewhere (Joyce et al., 2003a,b). In brief, SH-SY5Y cells (ATCC, Manassas, VA) were grown to confluence, then subcultured for differentiation in 48-well culture plates (Corning Glassworks, Corning, NY) by addition of 10 µM retinoic acid media for 3 days. The media was removed and replaced with fresh media containing 80 nM 12-O-tetradecanoyl-phorbol-13-
acetate for a further 3 days. After this 6-day differentiation protocol, cells were pretreated with media containing S32504, S32601, or ropinirole for 3 days, then treated with 1-methyl-4-phenylpyridinium (MPP+) (100 μM)-containing media for 3 days. The media was then replaced with MPP+ (100 μM)-containing media without drugs and cell viability assayed 3 days later. In antagonist studies, cells were similarly subcultured and differentiated for 6 days. They were then pretreated with media containing S32504 (7 μM, see Results) and raclopride, L741,626, or S33084 for 3 days before MPP+ (100 μM) plus the same drug combinations for a further 2 days. Thereafter, the media was replaced with MPP+ (100 μM)-containing media alone and cell viability assayed 3 days later. Control experiments were also run in which antagonists were evaluated in the same manner without S32504. The cytotoxic actions of MPP+ were assayed using a MTT salt assay (Sigma-Aldrich, St. Louis, MO). MTT stock solution (5 mg/ml) was added to each well at one-tenth the total media volume and incubated at 37°C for 4 h. The end point was measured at 570 nM. Cytotoxicity was also evaluated in 48-well culture plates by detaching cells and staining with trypan blue (final concentration 0.023% w/v) for 3 min. Viable, noncolored compared with dead, blue cells were counted from an aliquot (1 ml) of cell suspension using a hemocytometer under 400× magnification. Data presented are means ± S.E.M. (generally too small to be seen on the graphs). The influence of MPP+ was compared with the vehicle (control) condition of no MPP+ treatment (defined as 100%) by a Student's t test. The influence of S32504, ropinirole, and S32601 upon the cytotoxic actions of MPP+ were compared with vehicle treatment. The influence of antagonists upon the neuroprotective actions of S32504 were compared with vehicle treatment. For the MTT procedure, these differences between groups were analyzed by ANOVA followed by Dunnett's t test and, for the trypan blue procedure, they were analyzed by χ² tests.

**Drugs.** In general, full dose (concentration)-response curves were generated for S32504, ropinirole, and l-DOPA. As concerns the antagonists employed to explore the role of D2 or D3 receptors in the actions of S32504, their essential properties may be briefly summarized as follows. The substituted benzamide, raclopride, presents high and balanced affinity for dopamine D2 and D3 receptors and behaves as a potent antagonist of cerebral populations of these sites in vivo; it is virtually devoid of other receptor interactions (Millan et al., 1998, 2004c). The butyrophenone derivative, haloperidol, shares the potent and equilibrated antagonist properties of raclopride at dopamine D2 and D3 receptors in vitro and in vivo, although it also possesses high affinity for dopamine D3 receptors (Joyce, 2001; Newman-Tancredi et al., 2001; Millan et al., 2004c). Finally, the benzopyridine, S33084, is a potent and highly selective antagonist at dopamine D3 sites, whereas L741,626 behaves, in an opposite fashion, as a preferential antagonist at dopamine D2 versus D3 receptors (Millan et al., 2000a,b, 2004c; Silverdale et al., 2002). The dose ranges employed herein were based upon our previous characterization of selective actions at their respective targets (see preceding citations). With the exception of reserpine, drugs were dissolved in sterile water. If necessary, a few drops of lactic acid were added, and the pH was adjusted to as close to neutrality as possible (pH > 5.0). They were injected s.c. in a volume of 1 ml/kg body weight unless indicated. Reserpine (0.25 mg/ml) was dissolved in warm 1N acetic acid (3.1%, pH = 3.5) and was injected i.p. in a volume of 10 ml/kg body weight. l-DOPA (methyl ester) and benserazide were injected in a ratio of 1:4. All drug doses (milligrams per kilogram) refer to the base. Drugs sources, salts, and structures were as follows: reserpine base, haloperidol base, raclopride base, l-DOPA (methyl ester), and benserazide (HI) (Sigma, St. Quentin-Fallavier, France); L741,626 (Tocris Cookson Inc., Bristol, UK); ropinirole, racemic (±) trans-3,4,4a,5,6,10b-hexahydro-9-carbamoyl-4-propyl-2H-naphthalene-1,2-bis-(1,4-oxazine HCl (S31411), (+)-S32504, and its entantiomer (−)-S32601 were synthetized by J.-L. Pégion (Institut de Recherches Servier, Paris, France); and S33084 was synthesized by G. Lavielle (Institut de Recherches Servier).

**Results**

**Induction of Rotation in Unilateral, SNpc-Lesioned Rats by S32504 Compared with Ropinirole and l-DOPA (Fig. 1).** In rats sustaining a unilateral 6-OHDA lesion of the SNpc, the reference D2/D3 agonist quinpirole dose-dependently (0.0025–0.02 mg/kg, s.c.) elicited contralateral (contraversive) rotation at a daily dose of 12 ± 9 turns; 0.0025 mg/kg, 205 ± 62; 0.005, 317 ± 75; 0.01, 417 ± 76 and 0.02, 625 ± 129, F(4,35) = 18.2, P < 0.001. Likewise, racemic (±)-S31411 dose-dependently induced contralateral rotation. This action was potently mimicked by its (+)-enantiomer S32504, which attained a maximal effect at a dose of 0.01 mg/kg, s.c., whereas its less active (−)-enantiomer S32601, which shows low affinity for dopamine D2 and D3 receptors (Millan et al., 2004b), was inactive, even at higher doses. Ropinirole likewise elicited contralateral rotation in this paradigm, although it was less potent than S32504. In comparison with S32504, only high doses of l-DOPA elicited contralateral rotation.

**Influence of Antagonists at D2 and/or D3 Receptors upon the Induction of Contralateral Rotation by S32504 (Fig. 1).** The dopamine D2/D3 receptor antagonists haloperidol and raclopride both dose-dependently and completely blocked induction of contralateral rotation by S32504 (0.01 mg/kg, s.c.) with ID₅₀s of 0.03 mg/kg in each case. Their actions were mimicked by the preferential dopamine D2 receptor antagonist L741,626 (ID₅₀ 1.7 mg/kg), whereas the selective D3 receptor antagonist S33084 (0.04–0.63 mg/kg) was inactive. None of the antagonists elicited contralateral (or ipsilateral) rotation alone (not shown).

**Influence of S32504 upon Extracellular Levels of ACh in Unilateral, SNpc-Lesioned Rats (Fig. 2; Table 1).** In rats sustaining a unilateral lesion of the SNpc, as determined in freely moving animals, there was a substantial reduction in extracellular levels of DA in the ipsilateral compared with contralateral striatum: levels of NA and 5-HT were unaffected in the same samples, indicating the selectivity of lesions. In contrast to DA, there was a modest but significant elevation in dialysis levels of ACh. Administration of S32504 (0.16 mg/kg, s.c.) provoked a pronounced, rapid, and sustained reduction in levels of ACh in both the ipsilateral and contralateral striatum such that the levels of ACh in the lesioned striatum reached a level similar to that of basal levels in the nonlesioned site. Although the maximal percentage reduction in levels of ACh induced by S32504 did not differ between lesioned and nonlesioned striatum (59.8 ± 2.7% versus 58.4 ± 7.5%, respectively), “area under the curve” analysis revealed that the overall effect of S32504 was significantly (P < 0.01) greater in the striatum ipsilateral compared with contralateral to the SNpc lesion: 12.7 ± 1.2 versus 6.7 ± 1.8, respectively. Correspondingly, ANOVA revealed an interaction between the influence of S32504 and that of the lesion: F(1,11) = 3.7, P < 0.01. Nevertheless, full dose-response curves would be desirable to consolidate this notion of increased sensitivity to S32504 in ipsilateral versus contralateral striatum, as well as studies at time-points closer to the lesion.
Modulation of Extracellular Levels of ACh in Nonlesioned Rats by S32504 Compared with Ropinirole: Influence of Antagonists (Figs. 3 and 4). In the striatum of freely moving (nonlesioned) rats, S32504 elicited a pronounced, sustained, and dose-dependent diminution in dialysis levels of ACh (Fig. 3). Ropinirole also exerted a robust, potent, and dose-dependent diminution in striatal levels of ACh (Fig. 3). In the presence of haloperidol or raclopride, which did not themselves modify extracellular levels of ACh, the reduction of ACh levels elicited by S32504 (0.63 mg/kg, s.c.) was abolished (Fig. 4). Furthermore, the preferential dopamine D2 receptor antagonist L741,626 dose-dependently (and almost entirely) suppressed the inhibitory influence of S32504 upon ACh without itself modifying their levels (Fig. 4). In contrast, the selective dopamine D3 receptor antagonist S33084 did not significantly modify the action of S32504 (Fig. 4). It was inactive alone.

Reversal of Reserpine-Induced Hypolocomotion in Rats by S32504 Compared with Ropinirole and L-DOPA (Fig. 5). Eighteen hours after administration of reserpine (2.5 mg/kg, i.p.), rats displayed a marked reduction in locomotor activity. S32504 dose-dependently and fully reversed this effect of reserpine. At a dose of 10.0 mg/kg, s.c., S32504 completely restored locomotor activity to a level equivalent to that of control, vehicle-treated animals without itself enhancing locomotor activity. In contrast to S32504, ropinirole only partially and less potently reversed the reduction in locomotor activity elicited by reserpine.

Restoration of Locomotor Activity in Unprimed, MPTP-Treated Marmosets by S32504 (Fig. 6). Subcutaneous administration of S32504 over a dose range (0.01–0.04 mg/kg) comparable with that which evoked rotation in rats elicited a pronounced increase in the locomotor activity of drug-naive, MPTP-treated, akinetic marmosets. The onset of action was within 10 min, a peak effect was obtained within 20 to 30 min (at doses of 0.02 and 0.04 mg/kg), and it was active for 70 to 80 min. Subcutaneous administration of S32504 also resulted in a rapid and robust reduction in disability scores that was similarly rapid in onset (within 10 min) and lasted for ca. 70 to 80 min. Administered by the oral route, S32504 (0.04–1.25 mg/kg) similarly produced a marked, dose-dependent, and sustained increase in locomotor activity and a reduction in disability scores. The peak effect was seen within 10 to 20 min, and its effects lasted for at least 2 h at the highest doses (0.64 and 1.25 mg/kg).
Influence of S32504 upon extracellular levels of acetylcholine in the ipsilateral compared with contralateral striatum of freely moving, unilateral substantia nigra-lesioned rats. Data are means ± S.E.M. n ≥ 5 per value. The difference between basal levels of acetylcholine in contralateral versus ipsilateral striatum was significant (P < 0.02) in a paired Student’s t test (see Table 1). ANOVA for influence of S32504 as follows: lesioned, ipsilateral, F(5,11) = 21.6, P < 0.01; contralateral, intact, F(5,11) = 3.6, P < 0.01. Asterisks indicate significance of difference of drug versus basal, preinjection values. *, P < 0.005.

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<tr>
<th>Intact</th>
<th>6-OHDA-Lesioned</th>
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<tr>
<td>DA</td>
<td>5.96 ± 1.08</td>
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<tr>
<td>NA</td>
<td>0.10 ± 0.02</td>
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<tr>
<td>5-HT</td>
<td>1.07 ± 0.24</td>
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<td>ACh</td>
<td>1.34 ± 0.35</td>
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*Significance of differences between contralateral (intact) and ipsilateral (lesioned) striatum in paired Student’s t test. P < 0.05.

Restoration of Motor Function Compared with Induction of Dyskinesias in 1-DOPA-Primed, MPTP-Treated Marmosets by S32504 Compared with Ropinirole and 1-DOPA (Figs. 7–9). In an independent series of studies, oral administration of S32504 (0.16–2.5 mg/kg) produced a dose-dependent (as evaluated over 4 h) and pronounced alleviation of motor symptoms in primed, MPTP-treated marmosets (Fig. 7) as evaluated by parkinsonian disability. Range of movement was increased, whereas bradykinesia and postural abnormalities were both abolished. At the highest dose evaluated (2.5 mg/kg, s.c.), the influence of S32504 was maintained throughout the 4-h period of observation. S32504 also dose-dependently elicited dyskinesia with a maximal intensity of “marked” on the nonparametric scale employed (Fig. 7). Ropinirole (0.16–2.5 mg/kg, p.o.) likewise elicited marked antiparkinsonian properties that were maintained over 3 to 4 h of observation (Fig. 8). It also elicited dyskinesia, which, like S32504, attained a maximal intensity of “marked” on the semiquantitative scale employed (Fig. 8). Administration of 1-DOPA (4.0–15.0 mg/kg, p.o.) was associated with marked antiparkinsonian effects (Fig. 9). Furthermore, 1-DOPA elicited dyskinesia, which, in contrast to S32504 (and ropinirole), reached an intensity of “severe” (Fig. 9).

Neuroprotective Effects of S32504 Compared with Ropinirole in MPP⁺-Treated SH-SY5Y Cells (Fig. 10). Treatment of transformed SH-SY5Y cells possessing a dopaminergic phenotype with MPP⁺ (100 μM) resulted in severe neurotoxic effects as quantified by compromise of mitochondrial function (inhibition of MTT) and cell loss (measured by trypan blue staining). S32504 concentration dependently (0.1–10 μM) and markedly attenuated these effects of MPP⁺. Higher concentrations of S32504 were not evaluated since its effects were already maximal for both parameters at a concentration of 10 μM. The actions of S32504 were exerted stereospecifically inasmuch as its less active enantiomer, (−)-S32601, did not show significant activity in the trypan blue assay and was (only weakly) active in the MTT procedure even at concentrations of 10 and 50 μM. In comparison with S32504, ropinirole showed weak neuroprotective properties that failed to attain statistical significance in the trypan blue procedure and that were significant only at concentrations of 10 and 50 μM in the MTT assay.

Influence of Antagonists at Dopamine D₂ and/or D₃ Receptors upon the Neuroprotective Actions of S32504 (Fig. 11). Administered at a fixed concentration (7 μM),
Fig. 4. Attenuation of the influence of S32504 upon dialysis levels of acetylcholine in the striatum by the D2/D3 receptor antagonists haloperidol and raclopride and by the preferential D2 receptor antagonist L741,626, but not by the selective D3 receptor antagonist S33084. Panel A, blockade by haloperidol; panel B, blockade by raclopride; panel C, lack of influence of S33084; panels D to F, dose-dependent blockade by L741,626. Data are means ± S.E.M. n = 5 per value. ANOVAs as follows: panel A, influence of haloperidol, F(1,11) = 0.3, P > 0.05; influence of S32504, F(1,12) = 71.7, P < 0.01; interaction, F(1,9) = 38.5, P < 0.01; panel B, influence of raclopride, F(1,11) = 0.3, P > 0.05; influence of S32504, F(1,12) = 56.2, P < 0.01; interaction, F(1,9) = 13.1, P < 0.01; panel C, influence of S33084, F(1,12) = 2.4, P > 0.05; influence of S32504, F(1,12) = 45.0, P < 0.01; interaction, F(1,9) = 0.8, P > 0.05; panel D, influence of L741,626 (0.16), F(1,11) = 0.7, P > 0.05; influence of S32504, F(1,12) = 48.7, P < 0.01; interaction, F(1,9) = 0.2, P > 0.05; panel E, influence of L741,626 (2.5), F(1,11) = 1.1, P > 0.05; influence of S32504, F(1,12) = 63.3, P < 0.01; interaction, F(1,9) = 52.2, P < 0.01; panel F, influence of L741,626 (40.0), F(1,11) = 0.1, P > 0.05; influence of S32504, F(1,12) = 59.9, P < 0.01; interaction, F(1,9) = 33.4, P < 0.01. Asterisks indicate significance of antagonist + S32504 versus vehicle + S32504 values.

Fig. 5. Influence of S32504 compared with ropinirole upon reserpine-induced hypolocomotion in rats. Panel A, influence of S32504 upon the reduction of locomotor activity by reserpine in rats; panel B, influence of ropinirole. Data are means ± S.E.M. n = 5 per value. ANOVA as follows: S32504, F(4,25) = 17.5, P < 0.01; ropinirole, F(4,25) = 7.3, P < 0.01. Asterisks indicate significance of difference of drug + reserpine versus vehicle + reserpine values in Dunnett’s test following ANOVA. ∗, significance of difference of vehicle + reserpine versus vehicle + vehicle values in Student’s two-tailed t test. ∗, P < 0.05.
S32504 displayed robust protective properties against MPP\(^+\). In both the MTT and tryptan blue assays, cotreatment with raclopride concentration dependently antagonized the neuroprotective properties of S32504, an action reproduced by S33084, whereas L741,626 was ineffective. None of the antagonists, upon administration alone across equivalent concentration ranges, modified the influence of MPP\(^+\) (not shown).

**Discussion**

**Induction of Rotation by S32504 in Unilateral, SNpc-Lesioned Rats.** Unilateral elimination of striatal dopaminergic input leads to a motor disequilibrium revealed in the presence of dopaminergic receptor agonists (Gerlach and Riederer, 1996). Accordingly, S32504 dose-dependently, stereospecifically, and potently elicited contralateral rotation, an action mimicked (less potently) by ropinirole and L-DOPA (Eden et al., 1991). Although structures other than the striatum are implicated in the motor drive underlying rotation, it principally reflects a supersensitivity (enhanced density) of striatal D\(_2\) receptors (Koshikawa et al., 1996; Araki et al., 2000; Newman-Tancredi et al., 2001). Correspondingly, extending studies with a further D\(_2\)/D\(_3\) agonist, quinergoline (Newman-Tancredi et al., 2001), S32504-induced rotation was blocked by the preferential D\(_2\) receptor antagonist L741,626 but not by the selective D\(_3\) receptor antagonist S33084 (Millan et al., 2000). In contrast to D\(_2\) sites, either no change or a decrease in striatal D\(_3\) receptor density has been reported following destruction of dopaminergic input in rodents and in primates (Morissette et al., 1998; Quik et al., 2000; Bézard et al., 2003). Nevertheless, upon long-term exposure to L-DOPA, striatal D\(_3\) sites may be up-regulated, so a possible role of D\(_3\) receptors in the motor actions of S32504 under such conditions would be of interest to evaluate (Morissette et al., 1998; Joyce, 2001; Bézard et al., 2003; Van Kampen and Stoesjll, 2003). Although inhibition of DA reuptake can elicit rotation, S32504 exhibits negligible affinity for DA transporters and reduces extracellular levels of...
DA, so this mechanism cannot be involved in its actions (Millan et al., 2004b).

**Modulation of Striatal Cholinergic Transmission by S32504.** Cholinergic interneurons in the basal ganglia control motor behavior (Di Chiara et al., 1994; Raz et al., 1996) and an alteration in their activity contributes to motor dysfunction in PD (Di Chiara et al., 1994; Araki et al., 2000). Dopaminergic terminals target striatal cholinergic interneurons, providing a substrate for reports that D2/D3 receptor agonists inhibit ACh release (Di Chiara et al., 1994; De Boer...
and Abercrombie, 1996; Alcantara et al., 2003). Such studies were, however, undertaken employing acetylcholinesterase inhibitors to boost basal levels of ACh. Since their use modifies drug actions (De Boer and Abercrombie, 1996; Ichikawa et al., 2002; Gobert et al., 2003), it is of significance that S32504 and ropinirole suppressed extracellular ACh in the striatum of freely moving rats in the absence of acetylcholinesterase inhibitors. Furthermore, the actions of S32504 were blocked by raclopride, haloperidol, and L741,626 but not S33084, indicating their mediation by D2 receptors.

**Fig. 8.** Influence of oral administration of ropinirole upon the motor behavior of primed, parkinsonian marmosets treated with the dopaminergic neurotoxin MPTP. Values in panels B to E are the medians for n = 7. For a detailed description of behaviors corresponding to these panels, see text. As evaluated for 0 to 90 min, over which period maximal effects were obtained, Friedman analysis followed by Dunn’s test showed that the following doses exerted statistically significant (P < 0.05) effects upon specific behaviors. Parkinson disability, 0.64, 1.25, and 2.5; dyskinesia, 0.64, 1.25, and 2.5; range of movement, 0.64, 1.25, and 2.5; bradykinesia, 0.16, 0.64, and 2.5; postural abnormalities, 0.16, 0.64, and 2.5. In F, the dose-response relationship for the influence of S32504 upon overall Parkinson disability is shown. Open asterisks indicate significance of differences (Friedman analysis) to vehicle values. +, P < 0.05.
finding coincides with reports that cholinergic interneurones express D_2 sites (Joyce, 2001; Alcantara et al., 2003). However, D_3 sites may be inhibitory to ACh release in nucleus accumbens (Yamada et al., 1999). Thus, further study of a possible contribution of D_3 sites to modulation of striatal ACh release is justified.

Inasmuch as lesions of striatal dopaminergic pathways up-regulate striatal levels of D_2 but not D_3 receptors (see
above), the enhanced inhibitory influence of S32504 upon striatal ACh levels in lesioned versus contralateral striatum is consistent with a role for D2 receptors. Sato et al. (1994) also reported a reinforced inhibitory influence of apomorphine upon striatal ACh release following 6-OHDA lesions. Interestingly, basal dialysis levels of ACh were elevated in the lesioned versus contralateral striatum in the present study, likewise mimicking the findings of Sato et al. (1994) and clinical reports of alterations in cholinergic markers in parkinsonian patients (Joyce, 1993). Indeed, the responsivity of striatal cholinergic interneurones is modified upon loss of striatal dopaminergic input (Marti et al., 2003), whereas
changes in their firing pattern contribute to tremor in PD (Raz et al., 1996). In line with the effects of 6-OHDA lesions, studies performed in the presence of acetylcholinesterase inhibitors found that haloperidol elevates resting dialysis levels of ACh in the striatum (De Boer and Abercrombie, 1996). However, haloperidol, raclopride and L741,626 were inactive herein, underpinning a recent study of Ichikawa et al. (2002) also undertaken in the absence of acetylcholinesterase inhibitors. Thus, acute blockade of D2 receptors may not influence striatal release of ACh, questioning the significance of disinhibition of cholinergic pathways to short-term extrapyramidal properties of neuroleptics (Karasawa et al., 2003).

Reversal of Reserpine-Induced Hypokinesia. Reserpine depletes central pools of DA (and other monoamines), leading to a marked suppression of locomotor activity (Gerlach and Riederer, 1996; Skalas et al., 2002). Reflecting engagement of postsynaptic D2/D3 receptors in striatal and limbic structures, comparatively high doses of dopaminergic agonists abrogate reserpine-induced hypokinesia (Zarrindast and Minaian, 1991). Accordingly, S32504 restored locomotor activity in reserpine-treated rats, more efficaciously and more potently than ropinirole, and at doses that did not enhance spontaneous locomotion. However, the significance of D3 versus D2 receptors to this action remains to be elucidated.

Antiparkinsonian Properties of S32504 in Primates. In line with previous studies, the dopaminergic neurotoxin MPTP severely compromised motor function in “drug-naive” marmosets (Gerlach and Riederer, 1996; Smith et al., 1996; Fox et al., 2002), and its effects were dose-dependently reversed by S32504 upon both s.c. and oral administration. These observations resemble those acquired with ropinirole under equivalent conditions (Smith et al., 1996; Maratos et al., 2001), although the rapidity of action of S32504 deserves emphasis. Notably, S32504 did not elicit dyskinesia, in line with studies employing acute administration of ropinirole and other antiparkinsonian drugs to unprimed primates (Gerlach and Riederer, 1996; Pearce et al., 1998; Bézard et al., 2001). Thus, like ropinirole, S32504 is of potential utility in the monotherapy of PD (Rascol et al., 2000).

In marmosets previously treated (“primed”) with L-DOPA, S32504 produced a robust, dose-dependent, and sustained reduction in motor disability comparable in magnitude to the effects of L-DOPA. In contrast to rodent studies, ropinirole was more potent than S32504 upon oral application in this primate model. This difference probably reflects catabolism of ropinirole into highly active metabolites possessing pronounced affinity for D2/D3 receptors (Coldwell et al., 1999). Once dyskinasias have been provoked by repeated L-DOPA administration, the threshold for their induction by D2/D3 agonists falls (Pearce et al., 1998; Bézard et al., 2001), explaining the induction of dyskinasias by S32504 and ropinirole in primed animals. Like ropinirole (Pearce et al., 1998), however, S32504 provoked less intense dyskinesia than L-DOPA. Moreover, the low prodyskinetic potential of S32504 was exemplified upon chronic (4 weeks) administration to naive primates in which dyskinesia did not develop despite a maintained therapeutic response (M. Hill, A. Crossman, and M. J. Millan, manuscript submitted for publication).

In MPTP-treated, primed primates, L741,626 suppresses the restitution of motor function by ropinirole (Silverdale et al., 2002), indicating a role for D3 receptors’ antiparkinsonian properties. Interestingly, D3 receptor blockade with S33084 enhances its antiparkinsonian actions (Silverdale et al., 2002), suggesting, in line with certain rodent studies (see above), that activation of D3 receptors may oppose the motor effects of D2 receptor engagement. However, the respective significance of D3 and D2 receptors to the influence of S32504 upon motor function in primates must be directly investigated in view of controversy concerning their roles in mediating the therapeutic and dyskinetic actions of dopaminergic agonists and l-DOPA (Blanchet et al., 1997; Morissette et al., 1998; Joyce, 2001; Bézard et al., 2003).

Neuroprotective Properties in Vitro. By analogy to other D2/D3 agonists, such as pramipexole (Carvey et al., 2001), the neurotoxic effects of MPP+ in transformed SH-SY5Y cells were concentration-dependently and stereospecifically abrogated by S32504 compared with S32601. Ropinirole protects dopaminergic neurones from 6-OHDA in mice in vivo (Iida et al., 1999) and delays dopaminergic neuron degeneration in Parkinson patients (Whone et al., 2003). However, in the present model, it was only weakly active, confirming our earlier work (Joyce et al., 2003b) and an independent study of a mesencephalic cell line (Carvey et al., 2001). Furthermore, in a parallel in vivo study, we recently showed (Joyce et al., 2003b) that S32504 is more potent and effective than ropinirole in mice in protecting nigrostriatal dopaminergic neurones from the neurotoxic effects of MPTP.

Though transformed SH-SY5Y cells bear both D2 and D3 receptors (Joyce et al., 2003b), blockade of the neuroprotective actions of S32504 by raclopride and S33084, but not L741,626, suggests a principle role of the former. D3 sites are similarly implicated in the neuroprotective effects of pramipexole both in this (J. Joyce and M. J. Millan, unpublished observations) and other (Carvey et al., 2001) cell lines. Furthermore, in vivo studies with mice either lacking D2 receptors or treated with selective D2 antagonists also demonstrated a role for D3 receptors in the neuroprotective actions of pramipexole (Ramirez et al., 2003). A major role for D3 receptors under the present conditions may partly explain the poor efficacy of ropinirole since it is a less efficacious agonist at these sites than S32504 (Millan et al., 2004b). However, by analogy to other agonists, the striking neuroprotective activity of S32504 may also reflect actions mediated independently of D3 receptors (Bennett et al., 2001; Blum et al., 2001; Carvey et al., 2001). That is, D3 receptor stimulation by S32504 may permit the expression of neuroprotective properties via complementary mechanisms such as free radical scavenging (Le et al., 2000; Carvey et al., 2001; Jenner, 2003; Joyce et al., 2003a). This issue is currently under investigation, together with the paradoxical resistance of the neuroprotective actions of S32504 and pramipexole to certain dopaminergic antagonists (such as haloperidol) in cellular models (Le et al., 2000; Bennett et al., 2001; Carvey et al., 2001; J. Joyce and M. J. Millan, unpublished observations). Furthermore, a possible role of D3 receptors in the neuroprotective properties of S32504 should not be excluded (Joyce, 2001; Nair et al., 2003).

Conclusions. In conclusion, the novel naphtoxazine dopamine D2/D3 agonist S32504 exerts potent and robust actions in rodent and primate models of antiparkinsonian activity.
Activation of D₂ receptors is primarily responsible for the motor actions of S32504 and its inhibitory influence upon striatal cholinergic transmission. However, a potential contribution of D₃ receptors sites justifies additional study. Furthermore, D₂ receptors are implicated in the neuroprotective properties of S32504 in vitro, observations requiring extension to other models of the loss of dopaminergic neurons in Parkinson's disease.

Acknowledgments
We thank M. Sobeyram for secretarial assistance and S.P. Pres- graves, H. Gressier, S. Veiga, L. Cistarelli, and R. Billiras for tech-

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