RESEARCH ARTICLE

Dopamine Agonist Cotreatment Alters Neuroplasticity and Pharmacology of Levodopa-Induced Dyskinesia

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ABSTRACT: Background: Current models of levodopa (L-dopa)-induced dyskinesia (LID) are obtained by treating dopamine-depleted animals with L-dopa. However, patients with LID receive combination therapies that often include dopamine agonists.

Objective: Using 6-hydroxydopamine-lesioned rats as a model, we aimed to establish whether an adjunct treatment with the D2/3 agonist ropinirole impacts on patterns of LID-related neuroplasticity and drug responses.

Methods: Different regimens of L-dopa monotreatment and L-dopa-ropinirole cotreatment were compared using measures of hypokinesia and dyskinesia. Striatal expression of ΔFosB and angiogenesis markers were studied immunohistochemically. Antidyskinetic effects of different drug categories were investigated in parallel groups of rats receiving either L-dopa monotreatment or L-dopa combined with ropinirole.

Results: We defined chronic regimens of L-dopa monotreatment and L-dopa-ropinirole cotreatment inducing overall similar abnormal involuntary movement scores. Compared with the monotreatment group, animals receiving the L-dopa-ropinirole combination exhibited an overall lower striatal expression of ΔFosB with a distinctive compartmental distribution. The expression of angiogenesis markers and blood–brain barrier hyperpermeability was markedly reduced after L-dopa-ropinirole cotreatment compared with L-dopa monotreatment. Moreover, significant group differences were detected upon examining the response to candidate antidyskinetic drugs. In particular, compounds modulating D1 receptor signaling had a stronger effect in the L-dopa-only group, whereas both amantadine and the selective NMDA antagonist MK801 produced a markedly larger antidyskinetic effect in L-dopa-ropinirole cotreated animals.

Conclusions: Cotreatment with ropinirole altered LID-related neuroplasticity and pharmacological response profiles. The impact of adjuvant dopamine agonist treatment should be taken into consideration when investigating LID mechanisms and candidate interventions in both clinical and experimental settings. © 2023 The Authors. *Movement Disorders* published by Wiley Periodicals LLC on behalf of International Parkinson and Movement Disorder Society.

Key Words: Parkinson's disease; animal models of L-dopa-induced dyskinesia; ropinirole; antidyskinetic treatments

L-Dopa is the most effective medication for the symptoms of Parkinson's disease (PD) but has a high propensity to induce motor complications, which unfortunately affect the vast majority of patients (reviewed in[1]). L-Dopa-induced dyskinesia (LID) has a negative impact on health-related quality of life, representing a significant

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clinical-therapeutic problem (reviewed in[1,2]). The development of LID is attributed to treatment-induced fluctuations in brain dopamine (DA) levels leading to an abnormal stimulation of DA receptors, which in turn engenders a multitude of maladaptive plastic changes in both neurons and non-neuronal cells in the brain.[3,4]

Animal models of LID are widely used to test antidyskinetic treatments.[5,6] The therapeutic development pipeline typically involves an evaluation of candidate treatments in rodent LID models, followed by studies in non-human primate (NHP) models, where parkinsonian and dyskinetic features are assessed with rating scales analogous to those used in PD patients.[6] If positive results are obtained in this preclinical setting, the treatment can be advanced to phase 1b/2 clinical trials.[6]

Currently, all rodent and NHP models of LID are produced using severely DA-denervated animals that receive L-dopa as a monotreatment to induce and maintain a robust dyskinetic phenotype.[7] Although widely validated across different laboratories, these models do not reflect the fact that PD patients affected by LID usually receive L-dopa in combination with adjunct dopaminergic agents to allow for L-dopa-dose reductions (reviewed in[1]). In particular, in patients with advanced PD and motor complications, it is quite common to combine L-dopa with substances that directly stimulate dopamine (DA) receptors, referred to as DA agonists (reviewed in[1]). Currently, ropinirole and pramipexole are the most commonly used DA agonists for oral treatment,[8] and both of them have selective affinity for D2-class DA receptors, in particular for the D2 and D3 subtypes.[9,10] The impact of DA agonist cotreatment on the pathophysiology and pharmacology of LID has thus far remained unknown.

Prompted by these considerations, we set out to compare profiles of behavioral and cellular effects induced by chronic treatment with L-dopa and ropinirole, alone or in combination, in 6-hydroxydopamine (6-OHDA)-lesioned rats. Using drug-naïve animals, we first evaluated different doses of ropinirole and L-dopa on both lesion-induced motor deficits and dyskinetic behaviors. Because ropinirole did not induce any overt dyskinesia when given alone (nor could it maintain a previously induced LID), we then compared the pharmacological properties of dyskinesias induced by equipotent regimens of L-dopa-monotreatment vs. L-dopa-ropinirole cotreatment. Our results reveal a previously unappreciated and remarkable impact of DA agonist cotreatment on LID-related neuroplasticity and pharmacological response profiles.

Study Design

The study was performed in rats with unilateral 6-OHDA lesions of the nigrostriatal DA pathway. A detailed description of all experimental and statistical procedures is provided in the Supplementary Methods.

In the first experiment (Fig. 1A,B), a total of 57 rats were treated daily with either L-dopa (3 or 6 mg/kg termed LD3, LD6) or ropinirole (0.5 or 1.5 mg/kg, termed R0.5, R1.5) for 3 weeks. Animals were then assigned to the second treatment phase, where the previous LD3, R0.5, and R1.5 groups received LD3 and R0.5 in combination. Animals in the initial LD6 group were either switched to R0.5 or continued treatment with LD6 (Fig. 1B). Dyskinesia rating sessions and tests of forelimb hypokinesia were carried out in both treatment periods. After the second treatment phase was completed, a group of animals from each treatment arm was killed for immunohistochemical examinations. Antidyskinetic effects of different compounds were sequentially evaluated in other groups of rats (Fig. 1A,B).

Quantitative Immunohistochemistry

Immunohistochemical analysis was performed using primary antibodies for tyrosine hydroxylase (TH), FosB/ΔFosB, μ opioid receptor (MOR), nestin, albumin, and rat endothelial antigen-1 (RECA-1). Perivascular hemosiderin deposits were visualized on Reca-1 immunostained sections using Prussian blue. A full description of sampling procedures and image analysis methods is provided in the Supplementary Methods.

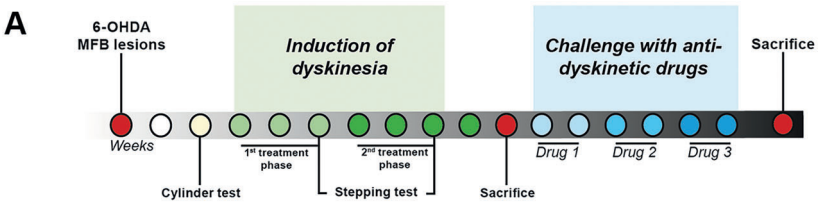
Statistical Analyses

Comparisons of treatment effects over time were performed with repeated measures two-way ANOVA. Overall antidyskinetic effects of drug challenges were examined on the area under the curve (AUC) values from the plot of AIM [abnormal involuntary movement] scores/monitoring period, comparing these values between drug and vehicle treatment. Analyses of treatment effects on single data sets were carried out using non-parametric tests or one-way ANOVA as appropriate. Statistical significance was set at $\alpha = 0.05$. For a detailed report of the statistical analyses and the corresponding data see Supplementary Methods and Supplemental Table S2, respectively.

Results

Behavioral Characterization of Dyskinesias Induced by L-Dopa and Ropinirole

In the first experiment, we compared the motor effects of chronic treatment with ropinirole and L-dopa in drug-naïve animals. Ropinirole was given at either 0.5 or 1.5 mg/kg/day, and L-dopa was given at either a low dose (3 mg/kg) or a standard dose commonly used to induce dyskinesia in this animal model (6 mg/kg). Although all treatments had a motor stimulant effect and improved the animals´performance in a test of forelimb hypokinesia (Supplementary results and Fig. S1), only animals treated with LD6 developed axial, limb,



B

Experiment 1			
1st treatment phase	*2nd treatment phase*	*Drug Challenge*	*IHC analysis*
LD6	R0.5	none	ΔFosB (±MOR)
LD6	LD6	-D1R antagonist: SCH23390	Nestin
LD3	LD3+R0.5	-D2R antagonist: L741,626	Albumin
R0.5		-NMDAR antagonist: MK801	Reca-1+ PB
R1.5		-M4 PAM: VU0467154	TH

Experiment 2			
1st treatment phase	*2nd treatment phase*	*Drug Challenge*	*IHC analysis*
LD6	LD6	-Amantadine -mGluR5 antagonist: MTEP	TH
LD3+R0.5	LD3+R0.5	-5HT$_1$a+bR agonists: CP94253+8-OH-DPAT	TH

C

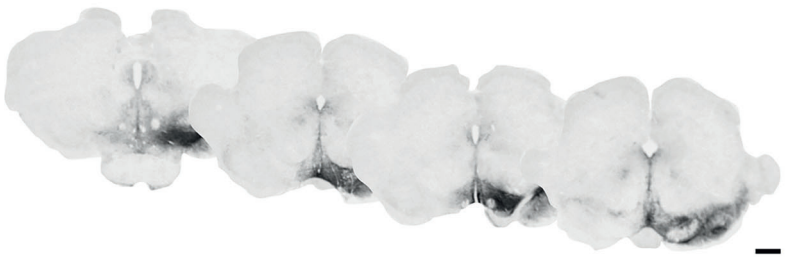


FIG. 1. Study design. (**A**) Timeline of Experiment 1. Rats received unilateral injections of 6-hydroxydopamine (6-OHDA) in the medial forebrain bundle (MFB), and animals with successful lesions were selected using a test of forelimb use asymmetry (cylinder test). Starting 4 weeks after the lesion, animals were randomized to four different pharmacological treatments (the first treatment phase). Three weeks later, animals were reallocated to three treatment groups (the second treatment phase). Ratings of dyskinesia were performed along the two treatment phases, whereas a test of forelimb adjusting steps was performed at the end of each phase. After completing the second treatment phase, a sample of rats from each group was killed for immunohistochemical analyses along with saline-injected control animals (n = 28 in total). Other animals from both LD6 (n = 9) and LD3 + R0.5 groups (n = 12, from the first treatment phase LD3 n = 6, R0.5 and R1.5 n = 6) received the same treatment with 2–4 drug administrations/week (over 8 weeks) to maintain stable abnormal involuntary movement (AIM) scores. During this period, rats were challenged with different compounds (see below). (**B**) Overview of the pharmacological treatments and immunohistochemical analyses carried out in Experiment 1 and Experiment 2. In Experiment 2, 22 rats with successful 6-OHDA lesions were randomized into two groups receiving daily injections of either LD6 or LD3 + R0.5 for a total of 6 weeks (same chronic treatment duration as in Experiment 1). This was followed by a regimen of 2–4 drug administrations/week to maintain stable AIM scores, during which drug challenge tests were carried out (details in the Supplemental Methods). Drug challenges, Experiment 1: the D1R antagonist SCH23390 (0.05 and 0.25 mg/kg i.p.); the D2R antagonist L741626 (1.0 and 3.0 mg/kg s.c.); the selective NMDA receptor antagonist MK801 (0.0175 and 0.035 mg/kg s.c.); the M4 positive allosteric modulator (PAM) VU0467154 (5 and 10 mg/kg s.c.). Drug challenges, Experiment 2: amantadine (20 and 40 mg/kg i.p.); the mGluR5 antagonist MTEP (2.5 and 5.0 mg/kg s.c.); two dose-combinations of the 5-HT1a and 5-HT1b receptor agonists CP94253 and 8-OH-DPAT (0.75 + 0.035 and 1.0 + 0.05 mg/kg, respectively s.c.). (**C**) Overview of midbrain sections immunostained for TH shows the typical MFB lesion-induced pattern of severe DA neuron loss in the substantia nigra on the side ipsilateral to the lesion. Scale bar: 1000 μm. *Abbreviations: LD, L-dopa; R, ropinirole; NMDAR, N-methyl-D-aspartate receptor; D1R, D1 receptor; D2R, D2 receptor; M4PAM, muscarinic M4 receptor positive allosteric modulator; mGluR5, metabotropic glutamate receptor type 5; 5-HT, serotonin; MOR, μ opioid receptor; Reca-1, rat endothelial cell antigen 1; PB, Perls' Prussian blue; TH, tyrosine hydroxylase.*

and orolingual AIM scores meeting the definition of moderate–severe LID (ie, basic severity score/monitoring period ≥2 on each AIM subtype[11,12]) (Fig. 2A,C). In contrast, treatment with LD3, R0.5, and R1.5 induced subthreshold levels of dyskinesia in all test sessions (Fig. 2A,C). Only 1/14 of animals treated with LD3 reached the criterion for moderate–severe LID, and the group as a whole did not differ from ropinirole-treated animals (Fig. 2A,C). Moreover, LD3-induced AIMs were dominated by orolingual components, whereas the AIM scores recorded from the other treatment groups had an overall similar representation of axial, limb, and orolingual components (Fig. 2E). Despite the low dyskinesiogenic effect, both ropinirole doses induced pronounced rotational locomotion (Fig. 2D), which is in agreement with previous observations.[13]

In the second treatment phase, animals from the previous LD3, R0.5, and R1.5 groups were given daily injections of LD3 + R0.5, whereas animals previously

treated with LD6 either continued on LD6 or were switched to R0.5 (Fig. 1A,B). Interestingly, LD3 + R0.5-cotreatment induced a gradual development of AIMs, which reached the same severity as LD6 treatment within 2 weeks independent of the initial treatment allocation (Fig. 2B,F). In addition, the subtype composition of AIM scores did not differ significantly between groups treated with LD3 + R0.5 vs. LD6 (Fig. 2H). Combined treatment with LD3 + R0.3 produced larger locomotive scores than did LD6 (Fig. 2G), but improved forelimb hypokinesia to a similar extent (Fig. S1). High locomotive scores were also measured in the animals switched to R0.5-monotreatment (Fig. 2G). However, switching from initial LD6 to R0.5 dramatically reduced the AIM scores already within 1 week (Fig. 2B), and none of the animals in this group met the criterion for moderate–severe LID by the end of the treatment period (Fig. 2F). The low levels of dyskinesia recorded from R0.5-treated rats predominantly consisted of axial AIMs (Fig. 2H).

Antidyskinetic Effects of D1 and D2 Receptor Antagonists

The results so far show that combining relatively low doses of L-dopa and ropinirole results in a robust model of dyskinesia that is phenotypically similar to that obtained with standard L-dopa-monotreatment. To assess whether the two models of dyskinesia differentially rely on D1 vs. D2 receptors, animals from each treatment group received challenge tests with antagonists of the two receptor classes.

The selective D2 receptor antagonist L741,626 produced a modest, dose-dependent AIMs reduction in both LD6- and LD3 + R0.5-treated animals, and the effect was mainly apparent in the end phase of the dyskinesia time curve (Fig. 3A,B). A comparison of the AIMs AUC (expressed as a percentage of baseline in each group) revealed a similar effect size in the two groups, consisting of ∼25%–30% reduction in AUC values by the higher dose of L741626 (Fig. 3C).

Investigating the effects of the D1 receptor antagonist SCH23390, we found that LD6-induced AIMs were markedly reduced throughout the test session by both antagonist doses (Fig. 3D). In animals treated with LD6 + R0.5, SCH23390 had a significant effect between 80 and 140 minutes after drug administration for the higher dose tested, and only at 100–120 minutes for the lower dose (Fig. 3E). The analysis of AUC values revealed a marked dose-dependent reduction in overall dyskinesia expression by SCH23390 in LD6-treated animals, whereas only the higher dose of SCH23390 had a significant effect in the LD3-R0.5 group (Fig. 3F). In addition, the effect size in the latter was moderate compared with LD6 animals (−25% vs −50% AUC, $P < 0.05$; Fig. 3F).

Taken together, these results suggest that LD6-induced dyskinesia relies more on D1 than D2 receptor stimulation, whereas dyskinesias induced by combined treatment with LD3 + R0.5 are less dependent on D1 stimulation, relying to a similar extent on the two receptor classes.

Expression of LID-Related Neuroplasticity Markers

The development of LID is associated with a long-lasting upregulation of transcription factor ΔFosB in striatal neurons, a response mediated by D1 receptors.[14,15] We therefore compared the striatal expression of ΔFosB among groups of animals completing the second treatment phase in Experiment 1. Automated counts of ΔFosB-positive neurons revealed markedly increased cell numbers in LD6-treated animals ($P < 0.05$ vs all other groups; Fig. 3G,G'). An increased number of ΔFosB-positive cells were also detected in animals treated with LD3 + R0.5 (Fig. 3G,G"), although with ∼50% lower cell counts relative to LD6-treated animals ($P < 0.05$). In contrast, animals receiving R0.5-monotreatment (and previously primed with LD6) did not exhibit any ΔFosB upregulation above saline-treated controls (Fig. 3G).

Because the relative stimulation of D1 vs D2 receptors affects the compartmental patterning of Fos protein expression,[16-18] we counted ΔFosB-positive cells within areas marked as striosomes or matrix using MOR-immunolabeling. This analysis revealed a distinctively high striosome/matrix expression ratio in LD3-R0.5-treated animals (Fig. 3H,H"; $P < 0.05$ vs all other groups), with values ∼2.5-fold larger than those measured in LD6-treated animals (Fig. 3H', H"). The different distribution of ΔFosB in LD3-R0.5 vs LD6-treated animals was not attributable to possible differences in DA denervation patterns, as all animals exhibited severe TH loss in the striatum (Fig. S2).

One important facet of LID-related plasticity consists in neurovascular changes, including angiogenesis and altered blood–brain barrier (BBB) permeability,[4] which correlate with dyskinesia severity[19,20] and depend on D1 receptor stimulation.[21] We therefore examined markers of angiogenesis and BBB hyperpermeability in the dorsolateral striatum and the substantia nigra pars reticulata (SNr), two regions exhibiting prominent neurovascular plasticity upon L-dopa treatment.[11,21,22] In both these regions, LD6-treated animals exhibited a significant increase in nestin-immunoreactive microvessels, a marker of ongoing angiogenesis (Fig. 4A, A', B, B'; $P < 0.05$ for LD6 vs. all other groups). The upregulation of microvessel nestin expression did not reach statistical significance in the LD3 + R0.5 group (Fig. 4A,A",B,B"), despite that these animals had exhibited levels of dyskinesia similar to those of the LD6 group (Fig. 2B,F). Animals receiving R0.5-monotreatment did not exhibit any sign of

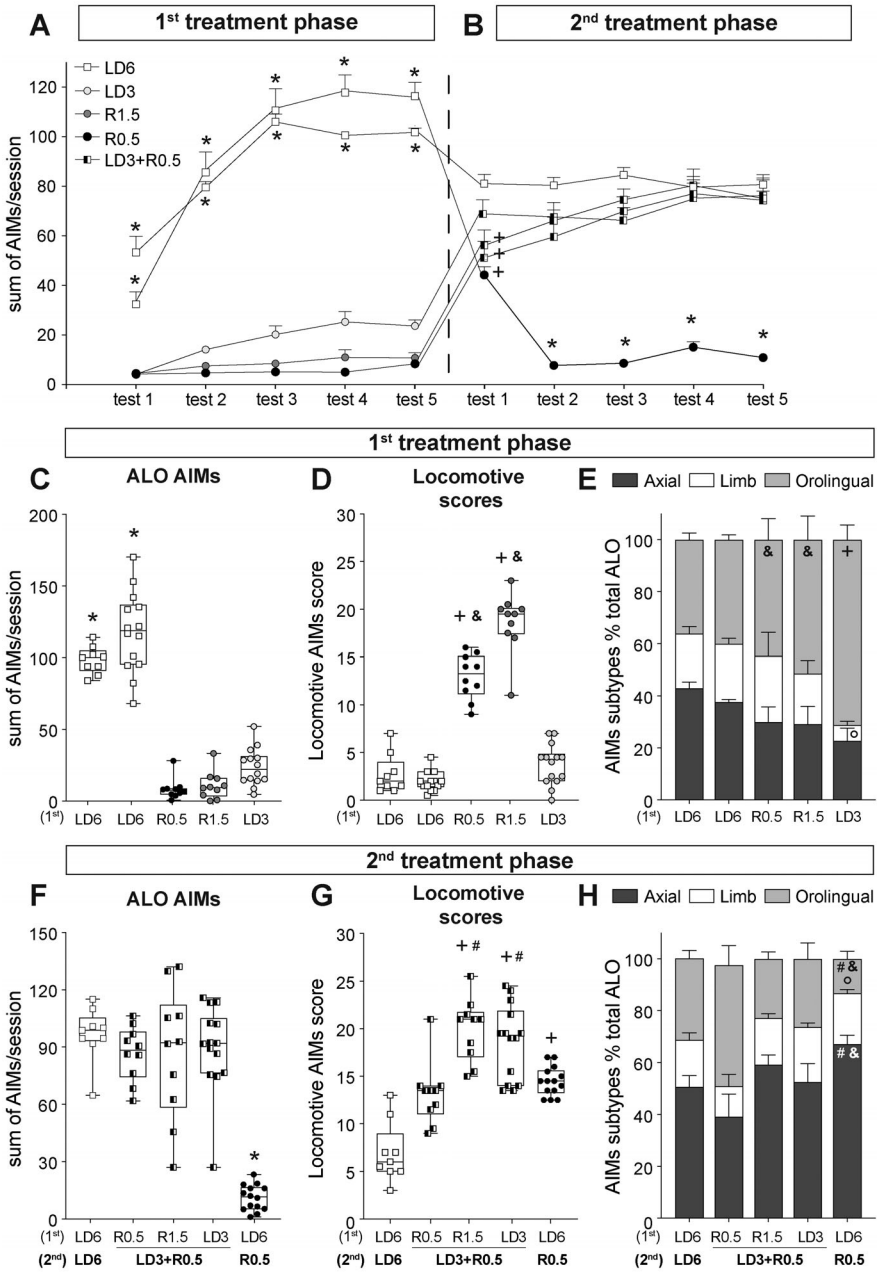


FIG. 2. Development of abnormal involuntary movements (AIMs) during the chronic treatments evaluated in Experiment 1. In the first treatment phase, L-dopa was tested at the doses of 3 (LD3; n = 14) and 6 mg/kg s.c. (LD6; n = 23 in total), and ropinirole at the doses of 0.5 (R0.5; n = 10) and 1.5 mg/kg s.c. (R1.5; n = 10). (**A**) Time course of axial, limb, and orolingual (ALO) AIM scores during the first treatment phase, Bonferroni's post hoc: *$P < 0.05$ vs. all other groups. (**B**) Time course of ALO AIM scores during the second treatment phase, Bonferroni's post hoc: *$P < 0.05$ vs. all other groups, +$P < 0.05$ vs. LD6. (**C**) ALO AIMs (total AIM score on the last test session) at the end of the first treatment phase, Dunn's post hoc: *$P < 0.05$ vs. all other groups. (**D**) Locomotive scores at the end of the first treatment phase, Dunn's post hoc: +$P < 0.05$ vs. LD6, &$P < 0.05$ vs. LD3. (**E**) Representation of ALO AIM scores as a percentage of the total AIMs at the end of the first treatment phase, Tukey's post hoc: +$P < 0.05$ vs. LD6; °$P < 0.05$ vs. R1.5; &$P < 0.05$ vs. LD3. (**F**) ALO AIMs (total AIM score on the last test session) at the end of the second treatment phase, Dunn's post hoc: *$P < 0.05$ vs. all other groups. (**G**) Locomotive scores at the end of the second treatment phase, Dunn's post hoc: +$P < 0.05$ vs. LD6, #$P < 0.05$ vs. (R0.5)/LD3 + R0.5. (**H**) Representation of ALO AIM scores as a percentage of the total AIMs at the end of the second treatment phase, Tukey's post hoc: #$P < 0.05$ vs. (R0.5)/LD3 + R0.5, °$P < 0.05$ vs. (R1.5)/LD3 + R0.5, &$P < 0.05$ vs. (LD3)/LD3 + R0.5. The treatment indicated in brackets is the one given to the same rats during the first treatment phase. See Table S2 for statistical analysis. *Abbreviations: AIMs, abnormal involuntary movements; ALO, axial, limb, orolingual; (1st), first treatment phase; (2nd), second treatment phase.*

nestin upregulation (Fig. 4A,B), despite their previous treatment with LD6.

As LID-related-angiogenesis concurs with focal increases in BBB permeability,[19,22] we measured parenchymal albumin immunoreactivity through the dorsolateral striatum and the SNr to estimate the overall degree of BBB leakage. In the striatum, LD6-treated animals showed a marked increase in albumin

immunoreactivity (Fig. 4C,C'; $P < 0.05$ vs. all other groups), whereas the LD3 + R0.5 group did not differ significantly from saline-treated controls (Fig. 4C,C"). In the SNr, LD6 was the only treatment inducing a significant upregulation of parenchymal albumin immunostaining, which was however overall modest in this region (Fig. 4D,D'). To better appreciate BBB dysregulation in the SNr, we counted the number of perivascular hemosiderin deposits (marker of extravasated erythrocytes) using a PB staining method.[23] Animals treated with LD6 exhibited a large number of PB-positive perivascular deposits (Fig. 3E,E', $P < 0.05$ vs. all other groups). Although the number of PB deposits tended to increase also in LD3-R.05-treated

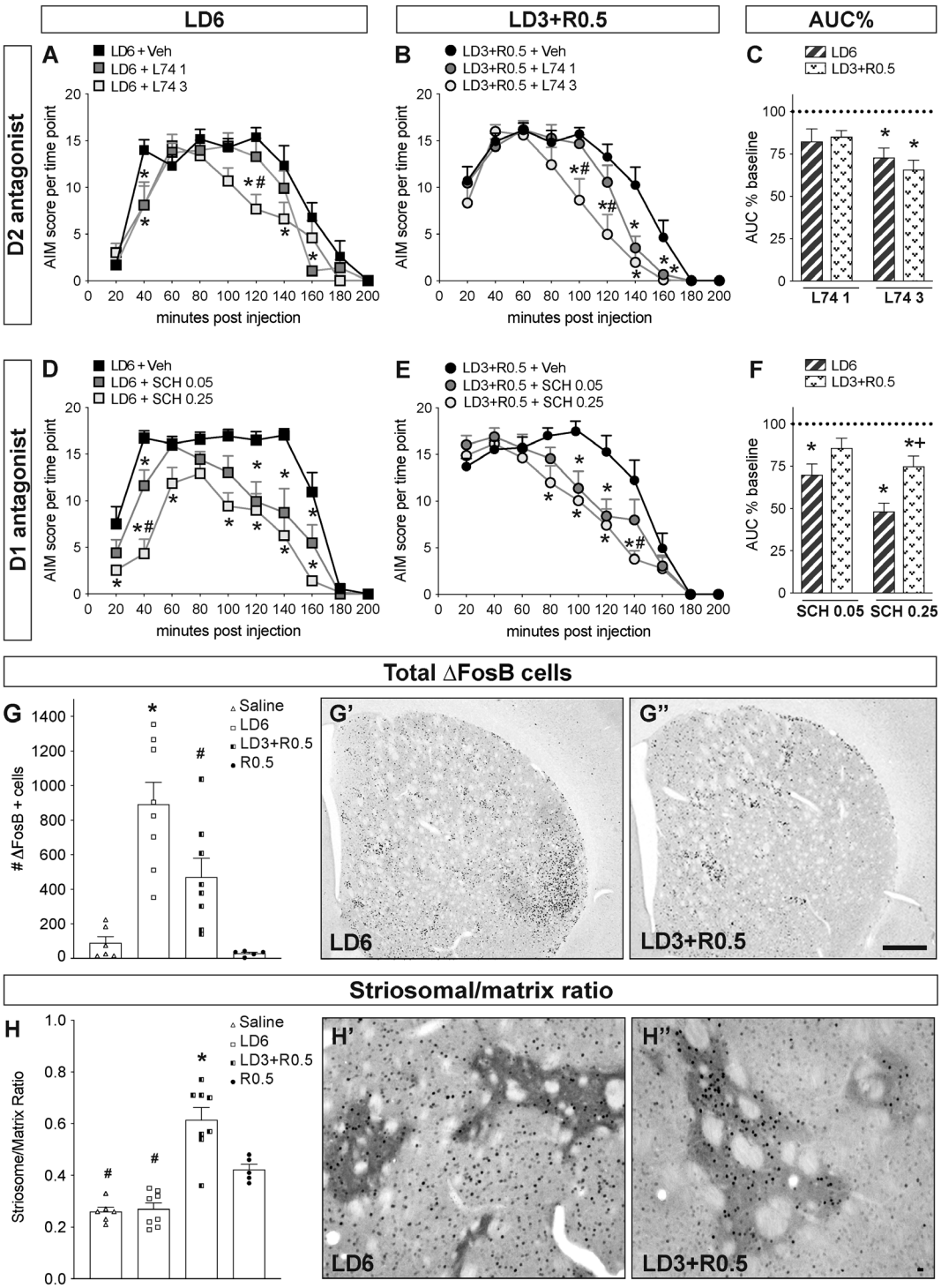


FIG. 3. Legend on next page.

animals (Fig. 4E,E"), the difference from saline-treated controls did not reach significance.

Despite their previous treatment with LD6, animals receiving R0.5-monotreatment did not differ from saline-injected controls on any of the above BBB permeability markers (Fig. 4C–E).

Challenge Tests with Antidyskinetic Treatment Principles

Lastly, we compared the responsiveness of LD6- vs LD3 + R0.5-induced dyskinesias to pharmacological principles that are currently being used or considered for the treatment of LID. Compounds were evaluated at two doses each, selected for their reported antidyskinetic activity in rodent models of LID (see Table S1).

Modulators of *N*-methyl-D-Aspartate Receptors

Amantadine is the only drug currently used for the management of LID in PD.[6] Its antidyskinetic action is partly attributed to non-competitive antagonism of glutamate *N*-methyl-D-aspartate (NMDA) receptors.[1]

Amantadine dose-dependently improved LD6-induced AIMs, being effective at the beginning and the peak of the dyskinesia time curve, though not in the end phase (Fig. 5A). A stronger effect was found in the LD3 + R0.5 group, where both doses of amantadine largely reduced the AIM scores in all phases of the dyskinesia curve (Fig. 5B), with a complete suppression of dyskinesia by the higher dose tested. A comparison of AIMs AUC confirmed that the response to amantadine was significantly larger in the LD3 + R0.5 vs LD6 group for both tested doses (Fig. 5C, cf. –92% vs –60% AUC for Ama-40 in LD3 + R0.5 vs LD6, $P < 0.05$).

A similar pattern of group differences was found using MK801 (dizocilpine), a selective uncompetitive antagonist of NMDA receptors in their open-channel (active) conformation.[24] Whereas LD6-induced AIMs were significantly reduced only by the higher MK801 dose (Fig. 5D), LD3 + R0.5-treated rats showed a pronounced antidyskinetic response to both compound doses (Fig. 5E), and a nearly complete AIMs suppression with the higher dose (Fig. 5F, − 84% vs –39% AUC for MK-0.035 in LD3 + R0.5 vs LD6 group, $P < 0.05$).

Altogether, these data indicate that AIMs induced by LD3 + R0.5 cotreatment rely on NMDA receptor activity to a larger extent than those induced by standard L-dopa-monotreatment (LD6). This may be related to a stronger relative dependence of LD3 + R0.5-induced dyskinesia on D2 vs. D1 receptors, as we found that MK801 did not improve but rather aggravated dyskinesias induced by a selective D1 receptor agonist (Fig. S3B,B′).

Non-dopaminergic Modulators of D1-Dependent Signaling

Next, we compared the two dyskinesia models using drugs proven to modulate D1 receptor-mediated striatal signaling in LID.[1]

To inhibit metabotropic glutamate receptor type 5 (mGluR5), we used the selective allosteric antagonist 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP), which has a strong antidyskinetic action against D1 agonist-induced AIMs[25] (Fig. S3C,C′). In LD6-treated animals, 5 mg/kg MTEP produced a marked reduction in peak dyskinesia severity (40–80 minutes) without affecting the decline phase of the AIMs (Fig. 5G), which is consistent with previous reports.[25,26] In contrast, LD3 + R0.5-treated animals showed a significant, non-dose-dependent response to MTEP specifically at 100 minutes post injection (Fig. 5H). When the AIMs AUC values were examined, the strongest response to MTEP was found in LD6-treated animals challenged with the higher dose (−32% AUC), with modest trends in the other conditions tested (Fig. 5G).

Acetylcholine muscarinic receptor M4 inhibits abnormal D1 receptor-dependent synaptic plasticity,

FIG. 3. Pharmacological and molecular indicators of DA receptor-type engagement. (**A–F**) Results of challenge tests with D2- vs. D1 selective antagonists in dyskinetic animals treated either with L-dopa alone (6 mg/kg, LD6, n = 9) or with the combination of 3 mg/kg L-dopa and 0.5 mg/kg ropinirole (LD3 + R0.5, n = 12). The D2 receptor antagonist L-741626 (L74) was tested at the doses of 1 and 3 mg/kg s.c. and the D1 antagonist SCH23390-HCL (SCH) at the doses of 0.05 and 0.25 mg/kg i.p. (**A, B**) Effects of the D2 antagonist on the time course of ALO abnormal involuntary movement (AIM) scores induced by LD6: ([A] Bonferroni's post hoc: *$P < 0.05$ vs. LD6 + veh, #$P < 0.05$ vs. LD6 + L74 1 mg/kg; or LD3 + R0.5; [B] Bonferroni's post hoc: *$P < 0.05$ vs. R0.5 + LD3 + veh, #$P < 0.05$ vs. R0.5 + LD3 + L74 1 mg/kg). (**C**) Area under the curve (AUC) of AIMs/monitoring period through the test session (180 minutes), expressed as a percentage of baseline (dashed line), Tukey's post hoc: *$P < 0.05$ vs. the corresponding baseline values. (**D, E**) Effects of the D1 antagonist on the time course of ALO AIM scores induced by LD6: ([D] Bonferroni's post hoc: *$P < 0.05$ vs. LD6 + veh, #$P < 0.05$ vs. LD6 + SCH 0.05 mg/kg; or LD3 + R0.5; [E] Bonferroni's post hoc: *$P < 0.05$ vs. R0.5 + LD3 + veh, #$P < 0.05$ vs. LD3 + R0.5 + SCH 0.05 mg/kg). (**F**) AUC as a percentage of baseline (dashed line), Tukey's post hoc: *$P < 0.05$ vs. respective baseline values, +$P < 0.05$ vs. LD6 + SCH 0.25 mg/kg. (**G**) Automated cell counts of ΔFosB immunoreactive cells across the striatum, Tukey's post hoc: *$P < 0.05$ vs. all other groups, #$P < 0.05$ vs. R0.5; saline n = 6, LD6 n = 8, LD3 + R0.5 n = 8, R0.5 n = 5. (**G', G"**) Low-magnification photomicrographs showing the distribution pattern of ΔFosB-positive cells in LD6 (G') and LD3 + R0.5 (G"). (**H**) Striosomal/matrix expression ratio of ΔFosB-positive cells (as counted at high magnification in striosomal (MOR positive) and matrix (MOR negative) areas, Tukey's post hoc: *$P < 0.05$ vs. all other groups, #$P < 0.05$ vs. R0.5; saline n = 6, LD6 n = 8, LD3 + R0.5 n = 8, R0.5 n = 5. (**H', H"**) Photomicrographs of double ΔFosB-MOR immunostained sections from a LD6-treated animal (H') and an LD3 + R0.5-treated case (H") (scale bar: 50 μm). See Table S2 for statistical analysis. *Abbreviations: AIM, abnormal involuntary movements, MOR, μ opioid receptor.*

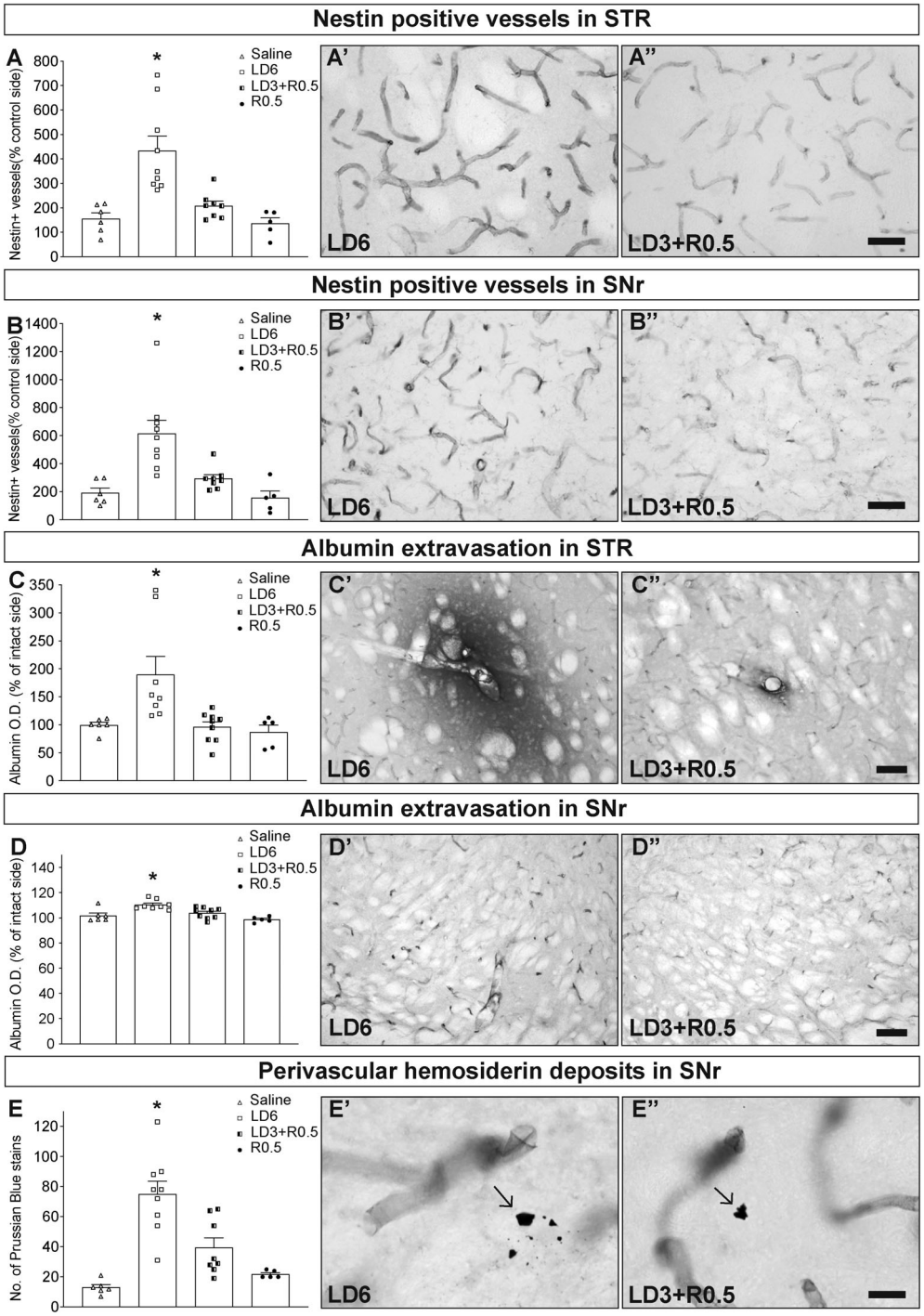


FIG. 4. Markers of angiogenesis and BBB hyperpermeability. Treatments and animals: same as in Figure 3G,H. (**A, B**) Nestin-immunopositive vessels were measured using an image segmentation method in the dorsolateral striatum (STR) (A) and substantia nigra pars reticulata (SNr) (B). Data from the DA-denervated side are expressed as a percentage of values measured on the contralateral intact side ([A] Tukey's post hoc: $^*P < 0.05$ vs. all other groups; [B] Tukey's post hoc: $^*P < 0.05$ vs. all other groups; saline n = 6, LD6 n = 9, LD3 + R0.5 n = 8, R0.5 n = 5). (**A', A"**) Nestin-positive microvessels in STR of 6-OHDA-lesioned rats treated with LD6 (A') vs. LD3 + R0.5 (A") (scale bar: 50 μm). (**B'-B"**) Nestin-positive microvessels in SNr of 6-OHDA-lesioned rats treated with LD6 (B') vs. LD3 + R0.5 (B") (scale bar: 50 μm). (**C, D**) Albumin extravasation was quantified with optical density (O.D.) measurements in STR (C) and SNr (D). Data from the DA-denervated side are expressed as a percentage of those measured on the intact side ([C] Tukey's post hoc: $^*P < 0.05$ vs. all other groups; [D]: Tukey's post hoc: $^*P < 0.05$ vs. all other groups; saline n = 6, LD6 n = 8, LD3 + R0.5 n = 9, R0.5 n = 5). (**C', C"**) Albumin immunostaining adjoining blood vessels in STR of 6-OHDA-lesioned rats treated with LD6 (C') vs. LD3 + R0.5 (C") (scale bar: 50 μm). (**D', D"**) Albumin immunostaining adjoining blood vessels in SNr of 6-OHDA-lesioned rats treated with LD6 (D') vs. LD3 + R0.5 (D") (scale bar: 50 μm). (**E**) Perivascular hemosiderin deposits visualized with Prussian blue dye (PB) on Reca-1-immunostained sections; data show the number of PB-positive dots in the SNr (Tukey's post hoc: $^*P < 0.05$ vs. all other groups). (**E', E"**) Photomicrographs show Reca-1 positive blood vessels and the presence of PB positive deposits (black dots) in the adjacent space in the SNr of 6-OHDA rats treated with LD6 (E') vs LD3 + R0.5 (E") (scale bar: 10 μm). See Table S2 for statistical analysis. *Abbreviation: Reca-1, rat endothelial cell antigen.*

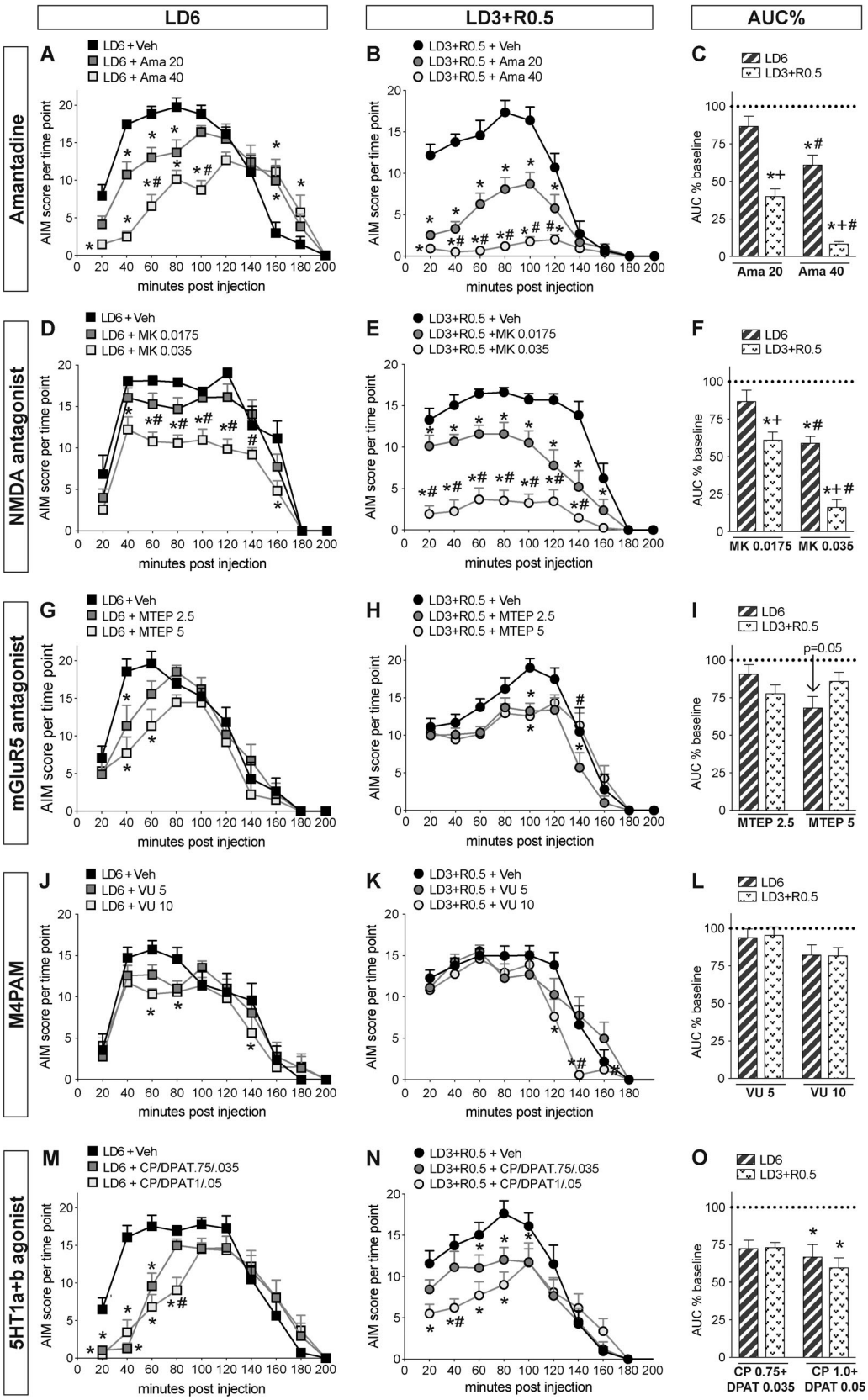


FIG. 5. Legend on next page.

and the pharmacological stimulation of M4 receptors has been proposed as a treatment for LID.[27] We therefore tested the M4 positive allosteric modulator (PAM) VU0467154[28] at doses having pronounced efficacy against D1 agonist-induced dyskinesia (Fig. S3D,D1). A dose-dependent reduction in dyskinesia severity was detected in LD6-treated animals, with an evident blunting of AIM scores at 60–80 minutes post injection by 10 mg/kg VU0467154 (Fig. 5J; $P < 0.05$ vs vehicle), though with little or no effect at other time points. In LD3 + R0.5-treated animals, the same dose of VU0467154 showed some efficacy in the AIMs decline phase (Fig. 5K; $P < 0.05$ for vs vehicle at 120–140 minutes). An analysis of AIMs AUC values, however, revealed that VU0467154 had a marginal antidyskinetic effect in both the LD6 and LD3 + R0.5 groups (Fig. 5L).

Modulators of Serotonin Receptors 5-HT1a/b

Low-dose combinations of serotonin 5HT1a and 5HT1b receptor agonists blunt peaks of striatal and nigral DA release that accompany the expression of LID.[29] We evaluated combinations of the 5HT1a and 5HT1b agonists, CP94253 and 8-OH-DPAT, at previously characterised doses.[29] When administered to LD6-treated rats, CP94253 and 8-OH-DPAT dramatically reduced the dyskinesia rising and peak phases at both dose combinations (Fig. 5M, $P < 0.05$ vs vehicle at 20–80 minutes). A strong antidyskinetic effect was apparent also in LD3 + R0.5-treated animals using the higher dose combination (Fig. 5N, $P < 0.05$ vs. vehicle at 20–80 minutes). The analysis of AIMs AUC values revealed that the antidyskinetic effect of CP94253/8-OH-DPAT was overall similar in the two dyskinesia models (Fig. 5O).

Discussion

Animal models of LID have shaped current pathophysiological notions and guided clinical proof-of-concepts trials across multiple therapeutic targets.[6,7] In many cases, however, failures have been encountered when attempting to translate promising antidyskinetic principles from the lab to the clinic. These failures indicate that additional efforts are needed to improve both clinical trial methodology and preclinical models in this translational area.[1,6] In particular, several authors have expressed a concern that the use of relatively high bolus doses of L-dopa in the experimental setting does not reflect DOPA-sparing strategies currently used in the clinic.[12,30] Indeed, advanced stages of PD are rarely managed using L-dopa as a monotherapy, as other dopaminergic agents are added to achieve longer-lasting therapeutic effects and reduce the daily L-dopa dose (reviewed in[1]). Some of these agents (eg, DA breakdown inhibitors) are unlikely to modify neuronal signaling events elicited by the primary treatment. Other agents, however, have a mechanism of action profoundly different from that of L-dopa, having a potential impact also on the mechanisms of LID, thus on the responsiveness to antidyskinetic drugs.

Non-ergoline class DA agonists (ropinirole, pramipexole, pergolide, and rotigotine) have a predominant or exclusive activity on dopaminergic receptors of D2-D3 type (reviewed in[31]). They can be used as monotherapy in the early stages of PD[32] and as an adjuvant to L-dopa in more advanced disease stages (reviewed in[1]). All these compounds have a longer duration of action than L-dopa, although they have inferior clinical efficacy,[33,34] possibly because they do not achieve the same profile of receptor stimulation in the brain.[35]

As an example of this class of compounds, we have chosen ropinirole, a potent agonist of D3/D2

FIG. 5. Effects of drug challenges on abnormal involuntary movements (AIM) induced by L-dopa alone vs. L-dopa-ropinirole combination. Results of the challenge tests: (**A–O**) amantadine (Ama) was tested in doses of 20 and 40 mg/kg (A–C), the NMDA receptor antagonist MK801 (MK) in doses of 0.0175 and 0.035 mg/kg (D–F), the mGluR5 antagonist MTEP in doses of 2.5 and 5.0 mg/kg (G–I), the M4PAM VU0467154 (VU) in doses of 5 and 10 mg/kg (J–L), and 5HT1a and b receptors agonists CP94253 (CP) and 8-OH-DPAT (DPAT) in dose combinations of 0.75 + 0.035 and 1.0 + 0.05 mg/kg, respectively (M–O). (**A, B**) Effects of amantadine on the time course of global AIM scores induced by LD6: ([A] Bonferroni's post hoc: *$P < 0.05$ vs. LD6 + veh, #$P < 0.05$ vs. LD6 + amantadine 20 mg/kg, n = 10; or LD3 + R0.5; [B] Bonferroni's post hoc: *$P < 0.05$ vs. R0.5 + LD3 + veh, #$P < 0.05$ vs. LD3 + R0.5 + amantadine 20 mg/kg, n = 12). (**C**) Area under the curve (AUC) as a percentage of baseline (dashed line) Tukey's post hoc: *$P < 0.05$ vs. respective baseline values, +$P < 0.05$ vs. LD6 + amantadine same dose, # $P < 0.05$ vs. low dose of the same treatment regimen. (**D, E**) Effects of MK801 on the time course of global AIM scores induced by LD6: ([D] Bonferroni's post hoc: *$P < 0.05$ vs. LD6 + veh, #$P < 0.05$ vs. LD6 + MK801 0.0175 mg/kg, n = 9; or LD3 + R0.5; [E] Bonferroni's post hoc: *$P < 0.05$ vs. R0.5 + LD3 + veh, #$P < 0.05$ vs. R0.5 + LD3 + MK801 0.0175 mg/kg, n = 12). (**F**) AUC as a percentage of baseline (dashed line) Tukey's post hoc: *$P < 0.05$ vs. respective baseline values, +$P < 0.05$ vs. LD6 + MK same dose, # $P < 0.05$ vs. low dose of the same treatment regimen. (**G, H**) Effects of MTEP on the time course of global AIM scores induced by LD6: ([G] Bonferroni's post hoc: *$P < 0.05$ vs. LD6 + veh, n = 10; or LD3 + R0.5; [H] Bonferroni's post hoc: *$P < 0.05$ vs. R0.5 + LD3 + veh, #$P < 0.05$ vs. LD3 + R0.5 + MTEP 2.5 mg/kg, n = 10). (**I**) AUC as a percentage of baseline (dashed line), Tukey's post hoc: $P = 0.05$ vs. respective baseline values. (**J, K**) Effects of VU0467154 on the time course of global AIM scores induced by LD6: ([J] Bonferroni's post hoc: *$P < 0.05$ vs. LD6 + veh, n = 9; or LD3 + R0.5; [K] Bonferroni's post hoc: *$P < 0.05$ vs. R0.5 + LD3 + veh, #$P < 0.05$ vs. R0.5 + LD3 + M4PAM 5 mg/kg, n = 12). (**L**) AUC as a percentage of baseline (dashed line) Tukey's post hoc: $P = 0.9813$. (**M, N**) Effects of the combination of CP94253 and 8-OH-DPAT on the time course of global AIM scores induced by LD6: ([M] Bonferroni's post hoc: *$P < 0.05$ vs. LD6 + veh, n = 10, #$P < 0.05$ vs. LD6 + CP + DPAT 0.75 + 0.035; or LD3 + R0.5; [N] Bonferroni's post hoc: *$P < 0.05$ vs. LD3 + R0.5 + veh, n = 12). (**O**) AUC as a percentage of baseline (dashed line) Tukey's post hoc: *$P < 0.05$ vs. respective baseline values. See Table S2 for statistical analysis. *Abbreviations: NMDA, N-methyl-D-aspartate; M4PAM, muscarinic M4 receptor positive allosteric modulator; mGluR5, metabotropic glutamate receptor 5; 5-HT, serotonin.*

receptors[10] that has been frequently used to model both impulse-control disorders and motor effects of PD therapies in animal models. Our results show that, in the absence of L-dopa, de novo treatment with ropinirole produces very low AIMs but high rotational locomotion, which is consistent with previous reports using larger doses of ropinirole.[13,36,37] Moreover, we reproduce the observation that ropinirole can induce dyskinesia if administered to L-dopa-primed animals.[38] However, previously established AIM scores dramatically declined already within 1 week of switching treatment from LD6 to ropinirole. Interestingly, this decline was accompanied by a normalization of striatal ΔFosB, a very stable transcription factor protein implicated in the dyskinesia priming process (reviewed in[39]). Markers of LID-related neurovascular plasticity were also suppressed by ropinirole treatment. These interesting and novel results suggest that D2/D3 agonists may exert a "de-priming effect" on the dyskinesia-prone brain if chronically administered without L-dopa.

The central contribution of the present study is, however, the demonstration that an animal model with robust and stable dyskinesias can be obtained by combining a low dose of L-dopa[39] with ropinirole (here also used at a relatively low dose for rats). This combination treatment was necessary to maintain moderate–severe and reproducible AIM scores over time, a prerequisite for using this model in preclinical pharmacological studies. We could not reproduce results by other groups showing that low-dose L-dopa (2–3 mg/kg/day) leads to a gradual development of moderate–severe dyskinesia.[12]

An important question is whether dyskinesias induced with L-dopa-ropinirole cotreatment or standard L-dopa-monotreatment share the same cellular mechanisms. Our histomolecular analyses indicate that partially different mechanisms are at play. Thus, although both treatments induced striatal upregulation of ΔFosB, we found both lower expression levels and a different distribution of the ΔFosB-positive cells after treatment with L-dopa-ropinirole compared with L-dopa alone. Specifically, the combined treatment group exhibited an increased striosomal/matrix ΔFosB expression ratio, which is likely to reflect a stronger stimulation of D2-class receptors than that achieved by standard L-dopa monotreatment. Indeed, although D2 receptor agonists do not induce Fos family proteins in the striatum, they alter the compartmental pattern of D1 agonist-induced Fos expression, with a concomitant striosomal augmentation and matrix suppression of Fos induction.[16-18] These findings indicate that the relative balance between D1 and D2 receptor stimulation determines the patterning of striatal neuronal activity. In addition, the rats cotreated with L-dopa and ropinirole exhibited a very low expression of microvascular nestin upregulation and BBB hyperpermeability, neurovascular plasticity markers previously shown to depend on D1 receptor stimulation.[14,15,21] Taken together, these data indicate that dyskinesias induced by L-dopa-ropinirole cotreatment are less reliant on D1 receptors while engaging D2 receptors to a larger degree. Confirming this notion, the selective D1-class antagonist SCH23390 had a significantly weaker antidyskinetic effect in L-dopa-ropinirole-cotreated animals compared with those treated with full-dose L-dopa. Interestingly, the D2 receptor-selective antagonist L741626 was effective in the decline phase but not at the peak of the dyskinesia-time curve. This interesting finding calls for additional investigations addressing the relative engagement of D1 vs D2 receptors in different phases of the L-dopa dosing cycle (with a potential bearing on the understanding of diphasic dyskinesias).

In the last part of the study, we compared the responsiveness of the two dyskinesia models to compounds modulating different neurotransmitter receptor systems that represent possible targets for the development of antidyskinetic therapies (for a review see[1]). Compared with the standard LID model, dyskinesias induced by the L-dopa-ropinirole regimen showed a markedly larger response to both amantadine and MK801 (a selective NMDA antagonist), and a somewhat lower response to the selective mGluR5 antagonist MTEP. The latter is in line with previous observations linking mGluR5 to aberrant D1-dependent signaling in LID.[25,40] We also examined the effect of the M4PAM VU0467154, which has been proposed to reduce dyskinesia by alleviating synaptic abnormalities in direct pathway-D1 positive striatal neurons.[27] This compound slightly reduced peak-dose AIMs only in the standard L-dopa model, though having a modest effect overall. To probe the relative contribution of the serotonin system, we used low-dose combinations of serotonin 5HT1a and 5HT1b receptor agonists.[26] The latter treatment had a similarly robust effect in both models, suggesting that its antidyskinetic action is exerted upstream of D1- vs. D2-receptor-specific signaling mechanisms.

In conclusion, this study presents a new pharmacological model of LID dependent on L-dopa-DA agonist coadministration, a treatment regimen that is better aligned with the current clinical practice. We show that this "combination therapy" produces dyskinesias that are phenotypically similar to the classical LID model, although they depend on a different D1-D2 stimulation balance and they are associated with distinctive patterns of neuroplasticity and drug responsiveness. Our results highlight the importance of considering the regimen of DA replacement therapy when evaluating the efficacy of candidate antidyskinetic treatments in both clinical and preclinical settings. In animal studies, using pharmacologically distinct models of LID may increase the chances of identifying robust candidate

interventions for clinical translation. Finally, we here provide novel and important indications that an adjunct treatment with DA agonists may exert a protective effect on LID-related maladaptive plastic changes known to depend on D1 receptor stimulation.

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Data Availability Statement

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Data

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

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(1) Research project: A. Conception, B. Organization, C. Execution.
(2) Statistical analysis: A. Design, B. Execution, C. Review and critique.
(3) Manuscript preparation: A. Writing of the first draft, B. Review and critique.
E.E.: 1B, 1C, 2A, 2B, 2C, 3A, 3B
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