Pharmacological Modulation of Glutamate Transmission in a Rat Model of L-DOPA-Induced Dyskinesia: Effects on Motor Behavior and Striatal Nuclear Signaling^[S]

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ABSTRACT

L-DOPA-induced dyskinesia (LID) in Parkinson's disease has been linked to altered dopamine and glutamate transmission within the basal ganglia. In the present study, we compared compounds targeting specific subtypes of glutamate receptors or calcium channels for their ability to attenuate LID and the associated activation of striatal nuclear signaling and gene expression in the rat. Rats with 6-hydroxydopamine lesions were treated acutely or chronically with L-DOPA in combination with the following selective compounds: antagonists of group I metabotropic glutamate receptors (mGluR), (2-methyl-1,3-thiazol-4-yl) ethynylpyridine (MTEP) for mGluR5 and (3-ethyl-2-methyl-quinolin-6-yl)-(4-methoxy-cyclohexyl)-methanone methanesulfonate (EMQMCM) for mGluR1; an agonist of group II mGluR, 1*R*,4*R*,5*S*,6*R*-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268); *N*-methyl-D-aspartate (NMDA)-R2B subunit (NR2B)-selective NMDA receptor antagonists 1-[2-(4-hydroxyphenoxy)ethyl]-4-[(4-methylphenyl)methyl]-4-piperidinol hydrochloride (Ro631908) and (±)-(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidine propanol (Ro256981); and an L-type calcium channel antagonist, 4-(4-

benzofurazanyl)-1,-4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid methyl 1-methylethyl ester (isradipine). Dyskinesia and rotarod performance were monitored during chronic drug treatment. The striatal expression of phospho-extracellular signal-regulated kinase (ERK) 1/2 and mitogen- and stress-activated kinase (MSK)-1, or prodynorphin mRNA was examined after acute or chronic treatment, respectively. In the acute treatment studies, only MTEP and EMQMCM significantly attenuated L-DOPA-induced phospho-ERK1/2 and/or phospho-MSK-1 expression, with MTEP being the most effective (70–80% reduction). In the chronic experiment, only MTEP significantly attenuated dyskinesia without adverse motor effects, whereas EMQMCM and LY379268 inhibited the L-DOPA-induced improvement in rotarod performance. The NR2B antagonist had positive antiakinetic effects but did not reduce dyskinesia. Only MTEP blocked the up-regulation of prodynorphin mRNA induced by L-DOPA. Among the pharmacological treatments examined, MTEP was most effective in inhibiting LID and the associated molecular alterations. Antagonism of mGluR5 seems to be a promising strategy to reduce dyskinesia in Parkinson's disease.

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An overactive glutamate transmission in the basal ganglia has been suggested to play a key role in the pathophysiology of Parkinson's disease (PD) and L-DOPA-induced dyskinesia (LID) (for reviews, see Chase et al., 2000; Cenci, 2007). Parkinsonian motor symptoms are often attributed to an excessive glutamatergic activity in subthalamo-pallidal projections (Levy et al., 2002), whereas LID has been linked to abnormal glutamate transmission in the striatum (for review, see Chase and Oh, 2000). Indeed, animal models of LID exhibit altered corticostriatal synaptic plasticity (Picconi et

ABBREVIATIONS: PD, Parkinson's disease; LID, L-DOPA-induced dyskinesia; NMDA, *N*-methyl-D-aspartate; mGluR, metabotropic glutamate receptor; MSK, mitogen- and stress-activated kinase; 6-OHDA, 6-hydroxydopamine; NR, *N*-methyl-D-aspartate receptor; DA, dopamine; ERK, extracellular signal-regulated kinase; PDyn, prodynorphin; MTEP, (2-methyl-1,3-thiazol-4-yl) ethynylpyridine; EMQMCM, (3-ethyl-2-methyl-quinolin-6-yl)-(4-methoxy-cyclohexyl)-methanone methanesulfonate; LY379268, 1*R*,4*R*,5*S*,6*R*-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate; Ro256981, (±)-(R*,S*)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidine propanol; Ro631981, 1-[2-(4-hydroxyphenoxy)ethyl]-4-[(4-methylphenyl)methyl]-4-piperidinol hydrochloride; AIM, abnormal involuntary movement; ANOVA, analysis of variance; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; p-, phosphorylated.

al., 2003), along with an abnormal striatal expression, phosphorylation (for review, see Chase and Oh, 2000), and intracellular trafficking (Gardoni et al., 2006) of specific NMDA receptor subunits. Moreover, striatal extracellular glutamate levels are elevated in dyskinetic rats (Robelet et al., 2004), and excessive cortical activation has been detected in dyskinetic PD patients during the execution of simple movements (Rascol et al., 1998). The role of glutamate neurotransmission in the development and expression of dyskinesia is supported by the antidyskinetic activity of several glutamate-receptor antagonists in both animal models of PD and clinical studies (for review, see Fox et al., 2006). It is of interest that the only pharmacological agent so far designated as “efficacious” for the treatment of dyskinesia is amantadine, which exerts weak noncompetitive antagonism at the NMDA receptor channel (Kornhuber et al., 1994). We have recently reported that antagonism of metabotropic glutamate receptor (mGluR) type 5 (mGluR5) attenuates the gradual development of LID and the treatment-induced up-regulation of prodynorphin mRNA in rats with 6-OHDA lesions (Mela et al., 2007). Being highly enriched in the perisynaptic and postsynaptic membranes of striatal neurons (Lujan et al., 1997), mGluR5 is in a key position to modulate abnormal striatal plasticity in PD and LID. It has however remained unclear whether an antidyskinetic effect, similar or superior to that of mGluR5 blockade, can be achieved by drugs targeting other glutamate receptors or synaptic proteins. In addition to mGluR5, striatal neurons also express type 1 mGluR (mGluR1), which shares similar properties to mGluR5 (Testa et al., 1994; Pisani et al., 2001), but whose role in dyskinesia has not been examined. Moreover, mGluR5 has important functional interactions both with the NR2 subunits of NMDA receptors and with L-type calcium channels (Guo et al., 2004). Dysregulation of L-type calcium channels in striatal neurons is linked to atrophy of dendritic spines and loss of corticostriatal synapses (Day et al., 2006). The L-type calcium channel antagonist isradipine prevents these structural abnormalities and has partial prophylactic efficacy against LID (Schuster et al., 2008). It is, however, unknown whether isradipine can block the exuberant activation of striatal nuclear signaling and gene expression that is produced by L-DOPA in dyskinetic animals (Westin et al., 2007). The antidyskinetic potential of compounds targeting NR2-NMDA receptor subunits has been investigated in several studies with divergent results, which is probably due to differences between the animal models and compounds used by different laboratories (for reviews, see Fox et al., 2006; Cenci, 2007). In addition to the receptors and protein subunits mentioned above, group II mGluRs represent a drug target of potential interest for the treatment of dyskinesia. Picconi et al. (2002) have reported that selective agonists of group II mGluRs (i.e., mGluR2 and -3) potently decrease excitatory transmission at corticostriatal synapses via a pre-synaptic mechanism and that the magnitude of this effect is enhanced after dopamine (DA) denervation. In theory, agonists of group II mGluRs may thus attenuate dyskinesia by inhibiting glutamate transmission at presynaptic sites.

The aim of the present study was to try and relate antidyskinetic effects of selective compounds targeting mGluR5, mGluR1, mGluR2/3, NR2B, and L-type calcium channel to molecular changes believed to be implicated in the development of dyskinesia. The study was performed in a validated

model of LID in rats with 6-OHDA lesions. Behavioral effects were evaluated by repeated assessments of abnormal involuntary movements and rotarod performance during chronic drug treatment. As a marker of short-term molecular changes induced by L-DOPA, we examined the striatal phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and mitogen- and stress-activated kinase (MSK)-1, which is transiently induced by L-DOPA after each dose administration in both acute and chronic treatment regimens (Westin et al., 2007). As a marker of long-lasting molecular changes, we examined the up-regulation of prodynorphin (PDyn) mRNA in the striatum, which remains elevated during at least 16 days after discontinuation of chronic L-DOPA treatment (Andersson et al., 2003).

Materials and Methods

Subjects. Female Sprague-Dawley rats (225 g; Harlan, Venray, Holland) were housed under a 12-h light/dark cycle, with ad libitum access

to food and water. Animal care and experimental treatments were approved by the Malmö-Lund Ethical Committee on Animal Research.

Dopamine Denervating Lesions. In total, 7.5 and 6 μ g of free-base 6-OHDA (6-OHDA-HCl; Sigma-Aldrich Sweden AB, Stockholm, Sweden) were injected into the right ascending DA fiber bundle at two coordinates according to our standard procedure (Cenci et al., 1998; Westin et al., 2007). Two weeks after surgery, an amphetamine-induced rotation test (2.5 mg/kg D-amphetamine i.p.; 90-min recording) was applied to select rats with >90% striatal DA depletion (corresponding to more than five full turns per minute ipsilateral to the lesion) (Winkler et al., 2002).

Drug Treatments. Drug treatments were initiated 3 weeks after the amphetamine rotation test. L-DOPA methyl ester (Sigma-Aldrich Sweden AB) was administered intraperitoneally at the dose of 10 or 6 mg/kg/injection in the acute or chronic study, respectively, together with the peripheral DOPA-decarboxylase inhibitor, benserazide-hydrochloride (15 mg/kg/injection; Sigma-Aldrich Sweden AB). The following compounds were coadministered with L-DOPA (see Table 1; Supplemental Data): (2-methyl-1,3-thiazol-4-yl) ethynylpyridine (MTEP), a noncompetitive mGluR5 antagonist; (3-ethyl-2-methylquinolin-6-yl)-(4-methoxy-cyclohexyl)-methanone methane sulfonate (EMQMCM), a noncompetitive mGluR1 antagonist; LY379268, a group II mGluR (2/3) agonist; Ro256981 and Ro631981, NR2B-selective NMDA receptor antagonists; and 4-(4-benzofurazanyl)-1,4-dihydro-2,6-dimethyl-3,5-pyridinedicarboxylic acid methyl 1-methyl-ethyl ester (isradipine), an L-type calcium channel antagonist. Compounds were obtained from the following sources: MTEP and EMQMCM (Merz Pharmaceuticals GmbH, Frankfurt, Germany); LY379268 (Ascent Scientific, North Somerset, UK); Ro256981 (Sequoia Research Products Ltd., Pangbourne, UK; chronic experiment); Ro631981 (Tocris Bioscience, Bristol, UK; acute experiment); and isradipine (Sigma-Aldrich Sweden AB; acute experiment) or Boehringer Ingelheim Pharma, GmbH and Co. KG (München, Germany, chronic experiment). In general, doses of the substances (Table 1) were chosen based on indications of behavioral efficacy and in vivo receptor occupancy (where this information was available) in the literature. A detailed report on the choices of compounds and doses used is provided in Supplemental Data. If there were no dose-dependent effects in the acute study, we chose the most frequently reported (effective) dose of the substance for the chronic study. Substances were administered intraperitoneally simultaneously with L-DOPA (MTEP or EMQMCM) or 10 min before L-DOPA injection (LY379268, Ro256981, and Ro631981) with the exception of isradipine in the acute experiment, which was administered 30 min before L-DOPA (Supplemental Data). The ve-

TABLE 1
Experimental groups and drug treatments

Group	Target	Doses <i>mg/kg</i>	L-DOPA/Benserazide	Vehicle and i.p. Injection Volume	n/Group
Experimental design 1, acute drug treatment					
Vehicle			No	Physiological saline or 5% Tween 80/sterile water	6
L-DOPA only	All dopamineR	10.0	Yes	1 ml/kg in saline	6 to 8 × 3 ^a
MTEP	mGluR5	1.25 and 6.25	Yes	2 ml/kg in 5% Tween 80/sterile water	7 and 7
EMQMCM	mGluR1	1.25 and 5.0	Yes	2 ml/kg in 5% Tween 80/sterile water	7 and 7
LY379268	mGluR2/3	1.0 and 10.0	Yes	2.5 ml/kg in saline	4 and 6
Ro631908	NR2B	3.0 and 30.0	Yes	1 ml/kg in saline	3 and 7
Isradipine	L-type Ca ²⁺ channel	0.5 and 5.0	Yes	2 ml/kg in sterile water, sonicated	9 and 4
Experimental design 2, chronic drug treatment					
Vehicle			No	Physiological saline or 5% Tween 80/sterile water	4 × 5 ^a
L-DOPA only	All dopamineR	6.0	Yes	1 ml/kg in saline	8 to 9 × 5 ^a
MTEP	mGluR5	5.0	Yes	2 ml/kg in 5% Tween 80/sterile water	10
EMQMCM	mGluR1	5.0	Yes	2 ml/kg in 5% Tween 80/sterile water	11
LY379268	mGluR2/3	3.0	Yes	2.5 ml/kg in saline	10
Ro256981	NR2B	3.0	Yes	1 ml/kg in 37°C sterile water	8
Isradipine	L-type Ca ²⁺ channel	0.2	Yes	Subcutaneous continuous-release pellets	9

DopamineR, dopamine receptors.

^a In the acute drug treatment study, one separate "L-DOPA-only" group of six to eight rats was included in each experiment (i.e., one experiment testing MTEP, another experiment testing EMQMCM and isradipine, and a third experiment testing LY379268 and Ro631908). In the chronic drug treatment study, one separate L-DOPA-only group ($n = 8-9$) and one separate vehicle control group ($n = 4$) were included in each experiment (five separate experiments were performed to test the five compounds under investigation).

hicle solutions for each substance were chosen based on previous literature and/or on recommendations from the manufacturers (Table 1). Vehicle-treated control animals were administered with the same vehicle used to dissolve the drug of interest in each experiment (Table 1).

Experimental Design. The compounds under scrutiny were evaluated in two steps. In the acute experiment (Fig. 1), two doses of each compound were tested for their ability to block the striatal induction of phospho-ERK1/2 and phospho-MSK-1 by acute L-DOPA. Animals were killed 30 min after the L-DOPA challenge dose, a time point when kinase activation is maximal (Westin et al., 2007). In the chronic experiment (Fig. 1), one selected dose of each compound was administered daily for 3 weeks along with L-DOPA/benserazide for a behavioral evaluation of rotarod performance and L-DOPA-induced abnormal involuntary movements (AIMs).

Rats were killed at a long interval after L-DOPA injection (2 days) to evaluate long-lasting changes in striatal gene expression (as opposed to those induced by the last drug dose). We examined PDyn mRNA, whose striatal levels show a strong, positive correlation with the severity of LID in both rodents (Cenci et al., 1998) and nonhuman primate models of PD (Aubert et al., 2007).

Behavioral Tests. The dyskinetic effects of L-DOPA were evaluated using a validated rat AIMs scale, where axial, limb, and orolingual AIMs collectively represent the rodent equivalent of peak-dose dyskinesia in PD (Lundblad et al., 2002). In brief, rats were rated individually every 20th minute during 140 min after the injection of L-DOPA. Axial, limb, and orolingual AIMs were rated on a severity scale from 0 to 4 (1, occasional; 2, frequent; 3, continuous but interrupted by external stimuli; and 4, continuous and not interrupted by external stimuli). The AIMs rating method and defining criteria are extensively described in Cenci and Lundblad (2007). The theoretical maximal cumulative AIM score that an individual rat could reach in this study was 588 (maximum score per observation point, 12; number of observation points per session, 7; number of sessions, 7). The L-DOPA-only control groups in the different experiments had cumulative AIM scores of 163 ± 33 (experiment with MTEP; $n = 10$), 157 ± 44 (experiment with EMQMCM; $n = 11$), 172 ± 21 (experi-

Fig. 1. Experimental design of the acute and chronic experiments. Experiment 1: acute dose-response effect on p-ERK1/2 and p-MSK-1. Rats with 6-OHDA lesions ($n = 87$) were randomly divided into 12 groups to receive a single injection of vehicle, L-DOPA only, or L-DOPA in combination with the testing compound (see Table 1). Experiment 2: chronic effects on behavior and PDyn gene expression. In five separate experiments (one for each drug), 112 rats in total were allocated to different treatment groups (see Table 1). For every experiment 1, L-DOPA only was included in addition to the drug tested. During 21 days, rats were injected once daily (intraperitoneally) and tested on drug-induced behavior. In total, seven AIM tests and three rotarod tests were performed during the treatment period (between 9:00 AM and 5:00 PM).

ment with LY379268; $n = 10$), 150 ± 27 (experiment with Ro256981; $n = 7$), and 222 ± 20 (experiment with isradipine; $n = 6$) (values indicate group means $\pm$ S.E.M.). This score corresponded to AIM severity grade ≥ 2 on at least two of the three AIM subtypes in the majority of the animals.

The antiakinetetic effects of different treatments were evaluated on the rotarod test, which reveals a motor impairment in 6-OHDA-lesioned rats that is significantly improved by L-DOPA (Lundblad et al., 2003). The test was applied according to procedures described previously (Lundblad et al., 2003). In brief, 1 to 2 weeks before drug treatment, rats were pretrained on the rotarod on accelerating speed (from four to 44 turns/min over 90 s) until they reached a stable baseline performance. During the chronic drug treatment, rats were tested on the rotarod 40 and 60 min after injecting L-DOPA using the same acceleration mode as in the training phase. The data (time on the rod) from these two time points were then averaged. To compensate for interindividual variation in absolute levels of performance, the time on the rod after drug treatment was expressed as a percentage of the baseline values in each animal.

Tissue Preparation. In experiment 1, rats were deeply anesthetized with sodium pentobarbital (240 mg/kg i.p.; Apoteksbolaget AB, Stockholm, Sweden) 30 min after the acute L-DOPA challenge and then transcardially perfused with ice-cold 4% paraformaldehyde (VWR, West Chester, PA) in 0.1 M phosphate buffer, pH 7.4. Brains were postfixed in the same solution for 2 h before being transferred to 20% buffered sucrose overnight. They were coronally sectioned on a microtome at 40 μ m and stored in cryoprotective solution at -20°C until processed.

In experiment 2, rats were deeply anesthetized with sodium pentobarbital and decapitated. Brains were rapidly extracted, frozen on crushed dry ice, and stored at -80°C . Coronal sections were cut on cryostat at 16 μ m in thickness and thaw-mounted on adhesive glass slides (Superfrost Plus; Electron Microscopy Sciences, Hatfield, PA). The slides were air-dried and stored at -20°C .

In Situ Hybridization Histochemistry. PDyn mRNA was measured using quantitative in situ hybridization histochemistry according to well established procedures (Cenci et al., 1998). In brief, the 48-mer oligonucleotide probe was labeled at the 3' end with [α - ^{35}S]deoxy-ATP using 15 U of terminal deoxynucleotidyltransferase (PerkinElmer Life and Analytical Sciences, Boston, MA) for 2 h at 37°C . The labeled probe (specific activity, $>10^6$ cpm/ μ g) was purified with a Chroma Spin column (Clontech, Mountain View, CA) and mixed in a hybridization cocktail. The slide-mounted sections were pretreated with 0.2 M HCl and 25% acetic anhydride in 0.1 M triethanolamine. They were dehydrated and air-dried for approximately 10 min before adding hybridization cocktail (40 μ l/section). After coverslipping, sections were incubated at 42°C overnight. The slide-mounted sections were washed four times in $1\times$ sodium citrate buffer at 55°C , dehydrated, and apposed to autoradiographic films (BioMax MR-1; Eastman Kodak, Rochester, NY) together with radioactivity standards (autoradiographic microscale; GE Healthcare, Freiburg, Germany). Film exposure was carried out at 4°C for 3 weeks. Films were developed in Kodak D19 and fixed in High Speed Fixer (Stena, Stockholm, Sweden).

Bright-Field Immunohistochemistry. Immunohistochemistry was performed as detailed in Westin et al. (2007) using a peroxidase-based detection method and 3–3'-diaminobenzidine (Sigma-Aldrich Sweden AB) as the chromogen. The following primary antisera were used: anti-phospho-(Thr202/Tyr204) p44/42 mitogen-activated protein kinase (1:200 overnight; Cell Signaling Technology Inc., Danvers, MA) and rabbit monoclonal anti-phospho-(Ser376)-MSK-1 (1:200 for 48 h; Epitomics Inc., Burlingame, CA).

Image Analysis and Cell Counts. Cells immunoreactive for phospho-ERK1/2 and phospho-MSK-1 were counted by a blinded investigator using the ImageJ software (Westin et al., 2007). Two striatal sections were considered for this analysis, corresponding to the rostrocaudal levels 1.00 and 0.50 mm rostral to bregma in the atlas of Paxinos and Watson (1998). Six sample areas (0.82×0.66 mm) per side were visualized under a $10\times$ objective in an Eclipse 80i microscope (Nikon, Tokyo, Japan) and digitized through a Nikon DMX 1200F videocamera.

The expression of prodynorphin mRNA was measured on film autoradiographs and digitized with the Nikon DM1200F camera.

Optical density was calibrated against a standard scale provided by the software and analyzed using the ImageJ software. An average of four sections per animal was analyzed. The entire cross-sectional area of the caudate-putamen was included in the analysis (see Fig. 6) on both the DA-denervated and the intact side.

Statistical Analysis. All comparisons between treatments were carried out using one-factor analysis of variance (ANOVA) and post hoc Student-Newman-Keuls test. The expression of phospho-ERK1/2, phospho-MSK-1, and PDyn mRNA was compared among treatments on the side ipsilateral to the lesion after having ascertained that no differences occurred on the intact side. Comparisons of dyskinesia severity were carried out using the cumulative AIM scores of the rats (i.e., the sum of all scores collected from each rat in seven testing sessions), and the results were verified with nonparametric Kruskal-Wallis test and post hoc Mann-Whitney test. For comparisons of rotarod performance, the time spent on the rod on three tests was averaged. The α level of statistical significance was set at $p < 0.05$. To enable comparisons of data collected in different experiments, the results obtained with each compound were expressed as a percentage of the values measured in a simultaneously processed L-DOPA control group (see legend to Table 1).

Results

Antagonism of Group I mGluRs Reduces the Activation of ERK1/2 and MSK-1 by L-DOPA. In the present study, phospho-ERK1/2 and phospho-MSK-1 were analyzed in the striatum of rats killed 30 min after an acute injection of L-DOPA, which was given either alone or together with the compounds under investigation (Table 1). In agreement with a previous report (Westin et al., 2007), immunoreactivity for phospho-ERK1/2 and phospho-MSK-1 was induced by L-DOPA only on the side of the striatum ipsilateral to the lesion (Fig. 2A; $p < 0.001$ for treatment effect in one-factor ANOVA; compare A and B in Figs. 3 and 4), and cell counts from this side provide the basis for the comparisons reported here. Both doses of MTEP reduced the expression of phospho-ERK1/2 by approximately 70% compared with the values in L-DOPA-only animals (Fig. 2A; $p < 0.05$ for MTEP versus L-DOPA-only, LY379268, Ro631908, and isradipine; Fig. 3, C–C'). Cotreatment with EMQMCM reduced the number of phospho-ERK1/2-immunoreactive cells, too (Fig. 3, D–D'), although the difference from L-DOPA-only treatment did not reach statistical significance. Supporting the trend toward an effect of EMQMCM, a comparison only involving three groups (L-DOPA-only, EMQMCM, and vehicle) revealed a significant reduction in phospho-ERK1/2 expression by the mGluR1 antagonist ($p < 0.001$ for treatment effect in one-way ANOVA, $p < 0.05$ for L-DOPA-only versus EMQMCM). The groups cotreated with either LY379268, Ro631908, or isradipine did not differ significantly from the L-DOPA-only controls (Figs. 2A and 3, E–G').

The histone kinase MSK-1 is a nuclear substrate of ERK1/2 (Deak et al., 1998), and phospho-MSK-1 immunoreactivity was examined to verify that the activation of ERK1/2 by L-DOPA had affected downstream nuclear signaling events. The levels of phospho-MSK-1 immunoreactivity after a challenge injection of L-DOPA alone or combined with the compounds of interest are shown in Fig. 2B. When given alone, L-DOPA induced an extensive activation of phospho-MSK-1 in the dopamine-denervated striatum (Fig. 2B; $p < 0.001$ for treatment effect in one-factor ANOVA; compare A and B in Fig. 4). This induction was greatly reduced (70–80%) by both doses of MTEP (Fig. 2B; $p < 0.05$ versus

Fig. 2. Acute dose-response effect on p-ERK1/2 and p-MSK-1. A, phospho-ERK1/2 is induced on lesion side by acutely L-DOPA challenge ($p < 0.001$ for treatment effect). MTEP cotreatment attenuates this induction at both doses, whereas the other cotreatments have no significant effect. B, phospho-MSK-1 is induced by acutely L-DOPA challenge. Both MTEP and EMQMCM cotreatment attenuates the induction ($p < 0.001$ for treatment effect). See Table 1 for respective doses. $p < 0.05$ versus *, L-DOPA only; #, vehicle; ○, MTEP (high and low dose); +, EMQMCM (high and low dose); §, EMQMCM low dose. Values represent group mean $\pm$ S.E.M. (bars).

Fig. 3. Photomicrographs of striatal phospho-ERK1/2-immunoreactive cells in the acute dose-response experiment. A, vehicle. B, L-DOPA-only control group. C, L-DOPA + MTEP low dose. C', L-DOPA + MTEP high dose. D, L-DOPA + EMQMCM low dose. D', L-DOPA + EMQMCM high dose. E, L-DOPA + LY379268 low dose. E', L-DOPA + LY379268 high dose. F, L-DOPA + Ro631908 low dose. F', L-DOPA + Ro631908 high dose. G, L-DOPA + isradipine low dose. G', L-DOPA + isradipine high dose. For respective doses, see Table 1. Photos were taken on the side of the striatum ipsilateral to the 6-OHDA lesions. Scale bar, 100 μ m.

L-DOPA-only, LY379268, Ro631908, and isradipine; Fig. 4, C–C'). L-DOPA-induced phospho-MSK-1 immunoreactivity also was reduced (55–65%) by cotreatment with EMQMCM

Fig. 4. Photomicrographs of striatal phospho-MSK-1-immunoreactive cells in the acute dose response experiment. A, vehicle. B, L-DOPA-only control group. C, L-DOPA + MTEP low dose. C', L-DOPA + MTEP high dose. D, L-DOPA + EMQMCM low dose. D', L-DOPA + EMQMCM high dose. E, L-DOPA + LY379268 low dose. E', L-DOPA + LY379268 high dose. F, L-DOPA + Ro631908 low dose. F', L-DOPA + Ro631908 high dose. G, L-DOPA + isradipine low dose. G', L-DOPA + isradipine high dose. For respective doses, see Table 1. Photos were taken on the side of the striatum ipsilateral to the 6-OHDA lesions. Scale bar, 100 μ m.

at both doses tested (Fig. 2B; $p < 0.05$ versus L-DOPA-only; Fig. 4, D–D'). In contrast, cotreatment with LY379268, Ro631908, or isradipine did not attenuate the induction of phospho-MSK-1 (Fig. 4, E–G').

Pharmacological Modulation of the Motor Response to L-DOPA. For a behavioral analysis, the compounds under scrutiny were coadministered with L-DOPA in a chronic treatment regimen (see Fig. 1, experimental design 2), during which ratings of AIMs and rotarod performance were assessed on several occasions. The severity of L-DOPA-induced AIMs differed significantly among treatments ($p < 0.001$ for treatment effect in one-factor ANOVA; Fig. 5A). Coadministration of MTEP reduced the cumulative axial, limb, and orolingual AIM scores by approximately 70% compared with L-DOPA-only treatment (hatched bars in Fig. 5A; $p < 0.05$ versus L-DOPA-only, LY379268, Ro256981, and isradipine). A clear trend toward lower AIM scores was seen in the EMQMCM cotreatment group (gray bars in Fig. 5A; $p < 0.05$ for EMQMCM versus Ro256981 and isradipine). Although the difference from L-DOPA-only animals did not reach statistical significance, a comparison only involving three groups (L-DOPA-only, EMQMCM, and vehicle) revealed a significant reduction in AIM scores by the cotreatment group ($p < 0.001$ for treatment effect in one-way ANOVA; $p < 0.05$ L-DOPA-only versus EMQMCM). Cotreatment with LY379268, Ro256981, or isradipine had no alleviating effect on dyskinesia (Fig. 5A).

To evaluate treatment effects on general motor skill and coordination, rats were tested on the rotarod 40 and 60 min post-L-DOPA injection. Performance on the rotarod differed significantly among treatments ($p < 0.001$ for treatment effect in one-factor ANOVA). As expected, the time spent on the rod was significantly improved by L-DOPA (Fig. 5B; $p < 0.05$ for L-DOPA only versus vehicle). Coadministration of MTEP produced an additional improvement (hatched bar in Fig. 5B; $p < 0.05$ versus L-DOPA only). In contrast, EMC-MCM and LY379268 blocked the antiakinetic action of L-DOPA, because both of these treatment groups showed levels of performance similar to vehicle-injected animals (grey and

Fig. 5. Chronic effects on behavior (experiment 2). L-DOPA cotreatment in comparison with L-DOPA-only. A, AIM scores as percentage of L-DOPA-only group. Only cotreatment with mGluR5 antagonist (L-DOPA + MTEP) attenuated the development of dyskinesia significantly ($p < 0.001$ for treatment effect). B, time on the rotarod performance (seconds) expressed as percentage of baseline performance. L-DOPA-only group performed significantly better on the rotarod compared with vehicle control. Cotreatment with EMQMCM and LY379268 blocked the antiakinetik effect of L-DOPA ($p < 0.001$ for treatment effect). MTEP, Ro256981, and isradipine had no such effects. C, striatal PDyn mRNA levels as percentage of L-DOPA only. Chronic L-DOPA treatment up-regulates the PDyn mRNA levels compared with vehicle controls ($p < 0.001$ for treatment effect). MTEP cotreatment blocked L-DOPA-induced up-regulation. For respective doses, see Table 1. $p < 0.05$ versus *, L-DOPA only; #, vehicle; O, MTEP; +, EMQMCM; @, Ro256981. Values represent group mean $\pm$ S.E.M. (bars).

striped bars in Fig. 5B; $p < 0.05$ for EMQMCM and LY379268 versus L-DOPA only). Similar to MTEP, coadministration of Ro256981 further improved the rotarod performance compared with L-DOPA (black striped bar in Fig. 5B; $p < 0.05$ versus L-DOPA only). Cotreatment with isradipine did not significantly modify the time spent on the rod compared with L-DOPA alone, although it improved the performance compared with vehicle (dotted bar in Fig. 5B; $p < 0.05$ versus vehicle and EMQMCM).

Pharmacological Modulation of L-DOPA-Induced Prodynorphin Gene Expression. At the end of the behav-

ioral studies, the striata of the rats were used for an analysis of PDyn mRNA by in situ hybridization. The expression of this transcript differed significantly among the groups on the side ipsilateral to the lesion ($p < 0.001$ for treatment effect in one-factor ANOVA; Figs. 5C and 6). As expected, chronic L-DOPA treatment causes a significant up-regulation of PDyn mRNA levels compared with vehicle (Fig. 5C; $p < 0.05$

Fig. 6. Chronic effects on prodynorphin gene expression (experiment 2). Photomicrographs of prodynorphin mRNA in striatum, lesion side. L-DOPA-only or with cotreatment of LY379268, Ro256981, and isradipine up-regulated PDyn compared with vehicle group ($p < 0.001$ for treatment effect). MTEP cotreatment attenuated the up-regulation. Scale bar, 1 mm.

versus vehicle; Fig. 6, B and C). This up-regulation was blocked by MTEP cotreatment (hatched bar in Fig. 5C; $p < 0.05$ versus L-DOPA only; Fig. 6D). Also EMQMCM cotreatment lowered the expression of PDyn mRNA to similar levels as in vehicle controls (gray bars in Fig. 5C; $p < 0.05$ versus LY379268, Ro256981, and isradipine; Fig. 6E), although the difference from L-DOPA-only animals did not reach significance (a comparison only involving L-DOPA-only, EMQMCM, and vehicle treatment revealed a significant reduction of PDyn mRNA levels by the mGluR1 antagonist; $p < 0.001$ for treatment effect in one-way ANOVA; $p < 0.05$ EMQMCM versus L-DOPA only). All other cotreatments (LY379268, Ro256981, and isradipine) failed to attenuate the L-DOPA-induced up-regulation of prodynorphin mRNA expression (Figs. 5C and 6, F–H).

Discussion

Glutamate receptor antagonists have been proposed as a strategy to alleviate or prevent LID. Multiple types of glutamate receptors and/or subunits exist whose activity is modulated by several proteins in the postsynaptic density. Hence, there are potentially many targets for an “antiglutamatergic” therapy of LID, but the specific functional properties of different targets are poorly understood.

The present study provides the first systematic comparison of behavioral and molecular effects produced by different pharmacological modulators of glutamate transmission in a well characterized animal model of LID. Our results point to antagonism of group I mGluR as being an effective strategy to reduce the alterations in striatal nuclear signaling associated with LID. However, not all group I mGluR antagonists are equally effective. Indeed, the mGluR5 antagonist MTEP was more potent than the mGluR1 antagonist EMQMCM in reducing L-DOPA-induced AIM scores, as well as the activation of ERK1/2 signaling and the up-regulation of striatal PDyn mRNA. It is most important to note that MTEP achieved these effects at a dose that enhanced the positive action of L-DOPA on the rotarod performance of the rats. By contrast, the mGluR1 antagonist EMQMCM produced some improvement of dyskinesia at a dose that had a motor depressant effect on the rotarod. Among the other substances tested, the mGluR2/3 agonist LY379268, the NR2B antagonist Ro256981, and the L-type calcium channel antagonist isradipine had no significant effect on LID and the associated molecular alterations. However, the NR2B antagonist potentiated the positive effect of L-DOPA on the rotarod test. In contrast, the mGluR2/3 agonist tended to negatively affect the rotarod performance of the rats.

Different types of mGluRs can be targeted to achieve either a presynaptic inhibition of glutamate release or a blockade of postsynaptic responses. Group II mGluRs are expressed in presynaptic axon terminals in the striatum as well as striatal output pathways (for review, see Gubellini et al., 2004). These receptors couple to G_i/G_o and cause inhibition of adenylyl cyclase (Pin and Duvoisin, 1995). Group I mGluR have been located to postsynaptic and perisynaptic membranes in striatal neurons (Lujan et al., 1997) where they couple primarily to G_q and stimulate phosphoinositide hydrolysis, playing a key role in the regulation of calcium release from intracellular stores (Pin and Duvoisin, 1995). Agonists of group I mGluR exert a strong modulatory effect on NMDA

receptor-mediated responses, being implicated in activity-dependent synaptic plasticity (for review, see Gubellini et al., 2004). In addition, group I mGluRs regulate striatal gene expression via the ERK1/2 pathway (Choe and Wang, 2001).

In this study, the mGluR5 antagonist MTEP markedly attenuated the phosphorylation of ERK1/2 and MSK-1 induced by L-DOPA in the DA-denervated striatum. The mGluR1 antagonist EMQMCM was also able to reduce phospho-ERK1/2 and phospho-MSK-1 immunoreactivity, although to a lesser extent. We have proposed that the activation of ERK1/2 and MSK-1 is instrumental to the development of dyskinesia and the associated long-term molecular plasticity during chronic drug treatment (Westin et al., 2007). In support of this proposal, of all the compounds tested in this study, only MTEP and EMQMCM, which inhibited phospho-ERK1/2 and/or phospho-MSK-1, reduced the AIM scores and the up-regulation of PDyn mRNA upon chronic L-DOPA treatment. These results indicate that antagonists of both mGluR5 and mGluR1 can potentially inhibit the exuberant striatal activation of nuclear signaling pathways and gene expression that is produced by L-DOPA. Antagonism of mGluR5 is however a more effective approach. Compared with EMQMCM, MTEP inhibited dyskinesia without interfering with the antiakinetic action of L-DOPA. In contrast, EMQMCM produced a marginal attenuation of the AIMs at a dose that interfered with the antiakinetic effect of L-DOPA on the rotarod test. The untoward effect of EMQMCM on the rotarod may be related to the high cerebellar expression of mGluR1 and to the importance of cerebellar glutamate transmission to the control of motor coordination (Nakao et al., 2007). Taken together, these data indicate that antagonism of mGluR1 is not a good strategy for the treatment of dyskinesia in PD. This conclusion is further supported by the lack of acute antidyskinetic efficacy of EMQMCM when given to animals already primed for LID (Dekundy et al., 2006).

Group II mGluRs have a presynaptic localization on excitatory corticostriatal (or thalamostriatal) terminals in the forebrain (Testa et al., 1994). Selective agonists of group II mGluRs potently decrease excitatory transmission at corticostriatal synapses via a presynaptic mechanism, and the magnitude of this effect is enhanced after DA denervation (Picconi et al., 2002). This is the first study in which a selective mGluR2/3 agonist was tested in an animal model of LID. Contrary to our initial hypothesis, cotreatment with LY379268 had no significant effect on L-DOPA-induced molecular changes and did not reduce dyskinesia. However, LY379268 tended to reduce rotarod performance. Studies in MPTP monkeys have shown that the expression of mGluR2/3 in the basal ganglia does not change with L-DOPA treatment and dyskinesia development (Samadi et al., 2008a). In line with the lack of effect of LY379268 on the rodent AIMs, these data would support the view that group II mGluRs do not play a significant role in LID. In keeping with our rotarod data, experiments in reserpine-treated rats have failed to detect antiakinetic effects by LY379268 (Murray et al., 2002). Taken together, all of these findings discourage a further pursuit of group II mGluR agonists as an antiparkinsonian therapy.

The NR2B subunit of NMDA receptors is particularly abundant in the striatum (for review, see Gubellini et al., 2004). Its functional properties and intracellular trafficking

are heavily dependent on its phosphorylation state, which is in turn modulated by group I mGluRs (Guo et al., 2004). Tyrosine phosphorylation of NR2B is increased in the striatum after chronic L-DOPA treatment (Dunah et al., 2004), and the trafficking of NR2B subunits between synaptic and extrasynaptic membranes is altered in dyskinetic rats (Gardoni et al., 2006). Because of these considerations, we had hypothesized that a NR2B-selective NMDA receptor antagonist would decrease LID and the associated molecular plasticity with an efficacy equal or superior to that of MTEP. Although cotreatment with the NR2B-selective antagonist Ro256981 enhanced the antiakinet effect of L-DOPA on the rotarod, it failed to reduce LID and the concomitant molecular changes (phospho-ERK1/2, phospho-MSK-1, and PDyn mRNA). Our results are apparently at variance with some reports from MPTP-treated monkeys (Hadj Tahar et al., 2004) or PD patients (Nutt et al., 2008) showing that NR2B-selective antagonists have some antidyskinetic properties. However, there also have been reports of increased dyskinesia severity after treatment with NR2B antagonists (Nash et al., 2004). The lack of inhibition of phospho-ERK1/2 and phospho-MSK-1 by Ro631908 is in line with the finding that NMDA receptor antagonists cannot block phospho-ERK1/2 (Gerfen et al., 2002) or immediate-early gene induction by DA agonists in the DA-denervated striatum (Ganguly and Keefe, 2000).

L-type calcium channels are key modulators of membrane excitability, synaptic plasticity, and gene expression in striatal medium spiny neurons (Chan et al., 2007) and mediate spine pruning and synaptic loss after DA depletion (Day et al., 2006). Medium-sized striatal neurons express the L-type channel $\text{Ca}_v1.3$ $\alpha 1$ subunit that interacts with Shank (Olson et al., 2005), which is the anchoring protein of mGluR1 and -5 in the postsynaptic density (Tu et al., 1999). Based on these considerations, we set out to examine the effects of isradipine, a dihydropyridine antagonist of $\text{Ca}_v1.2$ – 1.3 L-type calcium channels. Because of its neuroprotective effects in rodent models, isradipine is being considered for clinical application in PD (Surmeier, 2007). It has recently been reported that chronic isradipine treatment to 6-OHDA-lesioned rats attenuates the development of certain types of AIMs only if started simultaneously with the 6-OHDA lesion but not when given after the lesion has already produced spine pruning and loss of corticostriatal synapses (Schuster et al., 2008). Here, we show that, when given to animals with severe and stable DA-denervating lesions, isradipine cannot block the abnormal nuclear signaling responses induced by L-DOPA in striatal neurons. Accordingly, isradipine does not alleviate LID and the associated up-regulation of PDyn mRNA. Our results suggest that treatment with isradipine would not have antidyskinetic effects in patients with advanced PD.

Concluding Remarks. This study represents the first comparison of molecular and behavioral effects exerted by a range of compounds with postulated antilutamatergic actions in an animal model of LID. Of the approaches examined, specific antagonism of mGluR5 was the most effective in inhibiting nuclear signaling pathway activation and up-regulation of late-response genes by L-DOPA. Supporting the close link between these molecular changes and dyskinesia, antagonism of mGluR5 also was the most effective approach in reducing the AIMs and did, in addition, potentiate the

improvement in rotarod performance produced by L-DOPA. Genetic ablation of mGluR5 has produced adverse effects in hippocampal-dependent memory tasks in rodents (Lu et al., 1997). However, pharmacological blockade of mGluR5 improves spatial memory deficits in rodent models of PD (De Leonibus et al., 2009). Thus, although blockade of mGluR5 in a normal brain may negatively affect cognition, the same treatment would ameliorate cognitive deficits in situations where this receptor is overstimulated, which is the case in LID (Samadi et al., 2008b). We conclude that pharmacological approaches targeting mGluR5 seem promising for therapeutic development in PD.

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