Dysregulation of CalDAG-GEFI and CalDAG-GEFII predicts the severity of motor side-effects induced by anti-parkinsonian therapy

Jill R. Crittenden[a], Ippolita Cantuti-Castelvetri[b], Esen Saka[c], Christine E. Keller-McGandy[a], Ledia F. Hernandez[a], Lauren R. Kett[b], Anne B. Young[b], David G. Standaert[d], and Ann M. Graybiel[a,1]

[a]Department of Brain and Cognitive Sciences and the McGovern Institute for Brain Research, Massachusetts Institute of Technology, 43 Vassar Street, Cambridge, MA 02139; [b]MassGeneral Institute for Neurodegenerative Diseases, Massachusetts General Hospital, Neurology Department, Charlestown, MA 02129; [c]Department of Neurology, Faculty of Medicine, Hacettepe University, 06100 Ankara, Turkey; and [d]Center for Neurodegeneration and Experimental Therapeutics, University of Alabama at Birmingham, Birmingham, AL 35294

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Voluntary movement difficulties in Parkinson's disease are initially relieved by L-DOPA therapy, but with disease progression, the repeated L-DOPA treatments can produce debilitating motor abnormalities known as L-DOPA-induced dyskinesias. We show here that 2 striatum-enriched regulators of the Ras/Rap/ERK MAP kinase signal transduction cascade, matrix-enriched CalDAG-GEFI and striosome-enriched CalDAG-GEFII (also known as RasGRP), are strongly and inversely dysregulated in proportion to the severity of abnormal movements induced by L-DOPA in a rat model of parkinsonism. In the dopamine-depleted striatum, the L-DOPA treatments produce down-regulation of CalDAG-GEFI and up-regulation of CalDAG-GEFII mRNAs and proteins, and quantification of the mRNA levels shows that these changes are closely correlated with the severity of the dyskinesias. As these CalDAG-GEFs control ERK cascades, which are implicated in L-DOPA-induced dyskinesias, and have differential compartmental expression patterns in the striatum, we suggest that they may be key molecules involved in the expression of the dyskinesias. They thus represent promising new therapeutic targets for limiting the motor complications induced by L-DOPA therapy.

dyskinesia | ERK | L-DOPA | Parkinson's disease | striatum

Parkinson's disease is characterized by progressive neurodegeneration that includes loss of neurons in the dopamine-containing pars compacta of the substantia nigra, the nucleus that provides dopaminergic input to the striatum, a central motor-control region in the basal ganglia. This loss of dopamine is often part of a more widespread pattern of cell dysfunction and loss that are thought to account for the diversity of symptoms experienced by Parkinson's disease patients (1), but it is primarily loss of nigrostriatal dopamine that leads to the motor deficits in this disorder. This loss first affects the caudal, mainly sensorimotor, part of the striatum (2), and in a primate model exhibiting this progression, the loss of dopamine-containing terminals also first affected the motor-related matrix compartment of the striatum rather than the limbic-related striosomal compartment (3). The dysfunction of striatal matrix neurons is especially severe for the indirect pathway neurons of the striatum, which in the sensorimotor striatum receive powerful inputs from the motor cortex (4–6). In different models of parkinsonian states, the gradients and compartmental expression of dopaminergic deficits vary (7–9), but it seems likely that dysfunctional dopaminergic modulation of corticostriatal motor pathways leading through the matrix compartment is a major contributor to the motor symptoms of Parkinson's disease, and that in a range of movement disorders, compartmentally selective striatal dysfunction is present (10–17).

Therapy with L-DOPA, a metabolic precursor of dopamine, offers relief from these pathway dysfunctions and allows patients with Parkinson's disease to regain volitional movement control. However, L-DOPA therapy often must be curtailed within a few years of its initiation because the treatment produces debilitating movements known as L-DOPA-induced dyskinesias. Learning how to curtail these would be a major advance in the treatment of Parkinson's disease. Rat models of L-DOPA-induced dyskinesia have shown preferential activation of immediate early genes in striosomes, relative to the surrounding matrix, in specific striatal regions (18, 19). Thus, models of parkinsonism and models of the motor difficulties produced by repeated L-DOPA treatment are both associated with distinct imbalances in striosome to matrix activity.

The molecular events leading to the abnormal movements induced by L-DOPA therapy are complex (20), but striatal signaling through the extracellular-regulated kinases (ERK1/2) has been shown to be directly correlated with, and necessary for, the development of motor complications in rodent models of L-DOPA-induced dyskinesias (21–24). It is therefore of special interest that 2 regulators of ERK signaling have striatum-enriched expression (25) and have opposite preferential distributions in projection neurons of the striosome and matrix compartments of the striatum (25–27). These molecules are the matrix-enriched CalDAG-GEFI and the striosome-enriched CalDAG-GEFII, homologous exchange factors for the Ras superfamily (25). By binding calcium and diacylglycerol (DAG), these proteins can target either Rap1/2 (CalDAG-GEFI) or Ras (CalDAG-GEFII) through their guanine nucleotide exchange factor (GEF) domains, and they thus can have either cooperative or antagonistic effects on ERK signaling (25, 28–31), depending on the cell-type of expression.

Given the hypothesis that differential signaling efficacy in the striosomes and matrix could be related to the expression of L-DOPA-induced dyskinesias, as has been suggested for repetitive movements induced by repeated dopamine-receptor agonist treatments (15, 18, 19, 32), we tested in a rodent model of parkinsonism whether the CalDAG-GEFs were differentially affected in relation to the induction of abnormal movements by L-DOPA. Our findings were unequivocal in demonstrating that the matrix-enriched CalDAG-GEFI is down-regulated and the striosome-enriched CalDAG-GEFII is up-regulated in relation to the severity of the L-DOPA-induced movement abnormalities expressed by the animals. We suggest that the differential regulation of Rap/Ras/ERK signaling by these striatum-enriched CalDAG-GEFs could be critical in producing the abnormal

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[1]To whom correspondence should be addressed. E-mail: graybiel@mit.edu.

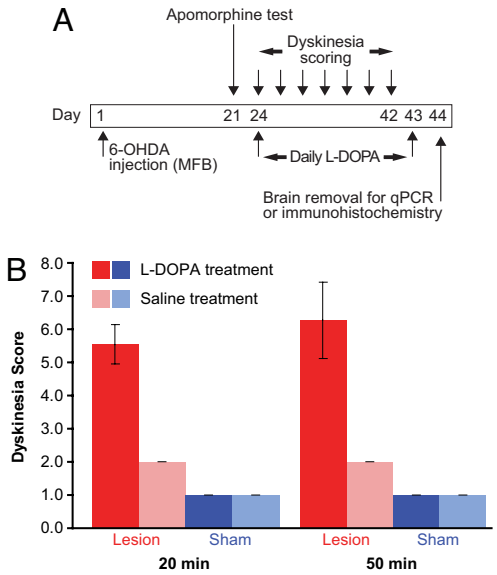

Fig. 1. Generation of hemi-parkinsonian rats with L-DOPA-induced dyskinesia. (*A*) Timeline of treatments and observations. (*B*) Dyskinesia scores for rats in 4 different treatment groups, as indicated. Abnormal movements developed in dopamine-depleted rats treated with L-DOPA, but not in control rats that received the dopamine-depleting 6-OHDA lesion alone or L-DOPA treatment alone. Scores shown are averages of the last 3 sets of behavioral observations made 20 min and 50 min after L-DOPA injection. Error bars represent SD from the mean. MFB, medial forebrain bundle.

movements induced by L-DOPA therapy. Controlling the differential activity of these CalDAG-GEF exchange factors could be beneficial in the treatment of L-DOPA induced dyskinesias in Parkinson's disease.

Results

To model the generation of abnormal movements in Parkinson's disease by repeated L-DOPA therapy, we employed a well established hemi-parkinsonian rat model in which rats were first rendered hemi-parkinsonian by unilateral injection of the neurotoxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle and, following nearly a month of recovery and a behavioral test for the adequacy of the lesions, were then given L-DOPA therapy each morning and afternoon for 20 days following standardized protocols (Fig. 1*A*). We analyzed the levels of the CalDAG-GEF proteins and their mRNAs in the striatum on the side of the 6-OHDA injection and on the noninjected control side, and we also performed saline control injections both for the initial 6-OHDA injections and for the L-DOPA treatments. We thus produced 4 treatment groups, receiving (1) 6-OHDA lesions and L-DOPA, (2) saline sham lesions and L-DOPA, (3) 6-OHDA lesions and saline sham treatment, or (4) saline sham lesions and saline sham treatment.

All of the dopamine-depleted rats treated with L-DOPA developed abnormal motor responses (Table 1 and Fig. 1*B*). These included repetitive movements and dystonic postures of the limbs contralateral to the 6-OHDA lesion, evaluated according to a rating scale modified from Cenci *et al.* (19) (Table 1). None of the control animals exhibited such behaviors (Fig. 1*B*).

Fig. 2 *A–D* illustrates the striatal expression of the CalDAG-GEF proteins following the unilateral dopamine depletion. There was a striking reduction in CalDAG-GEFI immunostaining in the dopamine-depleted striatum of rats treated with L-DOPA (Fig. 2*A*). On the control side, the striatal matrix was heavily immunostained relative to the striosomes, which appeared as regions of pale staining. On the side of the 6-OHDA

Table 1. Scale for rating L-DOPA-induced dyskinesias in rats

Observed behavior	Severity	Score
Contralateral turning behavior	<6/min	1
	>6/min	2
Contralateral involuntary repetitive forelimb movements	Mild	1
	Moderate	2
	Severe	3
Hindlimb dystonia	Absent	0
	Present	1
Oral stereotypy	Absent	0
	Present	1
Twisted posture of head and/or body to contralateral side	Mild	1
	Moderate	2
	Severe	3

lesion, it was still possible to distinguish the 2 striatal compartments, but the matrix compartment had lost much of its immunostaining. We found an equally striking increase in CalDAG-GEFII immunostaining in the dopamine-depleted striatum of

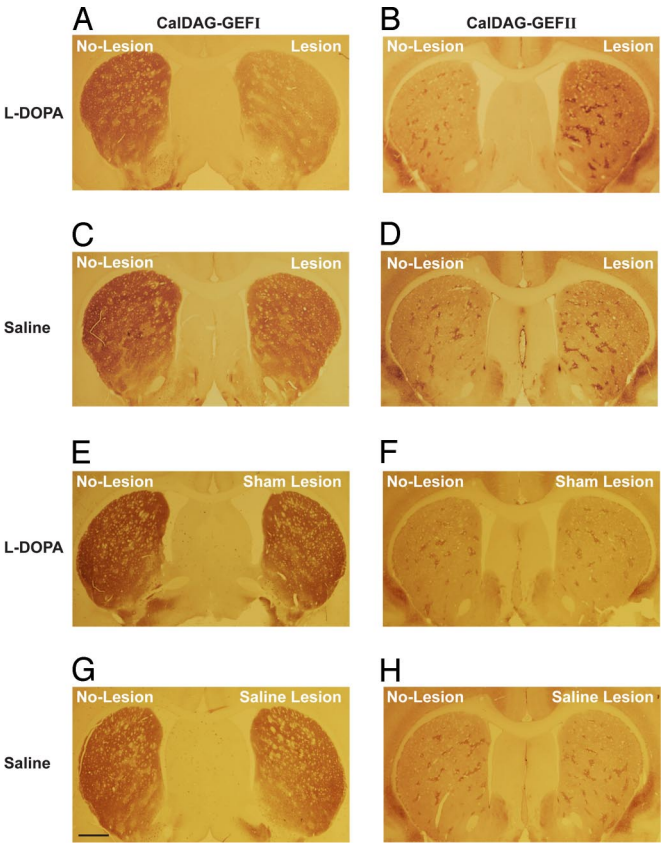

Fig. 2. CalDAG-GEFI and CalDAG-GEFII proteins are severely dysregulated in the dopamine-depleted striatum of rats exhibiting L-DOPA-induced dyskinesias. (*A*) CalDAG-GEFI immunostaining is diminished in the dopamine-depleted (Lesion) striatum of rats that exhibited L-DOPA-induced dyskinesias, relative to that in the contralateral (No-Lesion) striatum. (*B*) CalDAG-GEFII immunostaining is up-regulated by the same 6-OHDA lesion plus L-DOPA treatment schedule. (*C* and *D*) Dopamine depletion combined with daily saline treatment, which did not induce dyskinesias, does not produce strong dysregulation of either CalDAG-GEFI (*C*) or CalDAG-GEFII (*D*). Treatment with daily L-DOPA alone did not appear to change immunoreactivity for CalDAG-GEFI (*E*) or CalDAG-GEFII (*F*) following 6-OHDA injections that failed to produce decreased striatal tyrosine hydroxylase immunostaining or turning asymmetry on apomorphine testing relative to a saline-treated control (*G* and *H*). Scale bar, 1 mm.

the L-DOPA treated rats (Fig. 2*B*). In the control striatum contralateral to the dopamine depletion, striosomes exhibited stronger CalDAG-GEFII staining than the surrounding matrix. In the dopamine depleted striatum, the levels of CalDAG-GEFII immunostaining were higher in both striosomes and matrix, and the striosomes were vividly demarcated by dense immunostaining (Fig. 2*B*). The adjoining claustrum was also strongly stained and appeared to have elevated CalDAG-GEFII levels relative to those on the control side.

For both CalDAG-GEFI and CalDAG-GEFII, the combined dopamine depletion and L-DOPA treatment thus appeared to affect protein expression in both compartments. Given that the striosome-enriched CalDAG-GEFII immunostaining was increased, and the matrix-enriched CalDAG-GEFI immunostaining was diminished; this inverse regulation in the expression of the 2 CalDAG-GEFs could lead to an exaggerated divergence in the compartmental expression of the 2 gene products.

In the animals given unilateral 6-OHDA injections but then treated only with saline, there were also perceptible decreases in CalDAG-GEFI immunostaining and slight increases in CalDAG-GEFII immunostaining (Figs. 2 *C* and *D*), but these were small by comparison with the changes induced by combined dopamine depletion and L-DOPA treatment. These results suggest that the dopamine depletion alone in this parkinsonian model leads to a small down-regulation of CalDAG-GEFI and concomitant small up-regulation of CalDAG-GEFII. We found no apparent differences between the CalDAG-GEF immunostaining in control rats treated with L-DOPA after receiving 6-OHDA lesions that were ineffective as determined by tyrosine hydroxylase immunohistochemistry and apomorphine testing (Figs. 2 *E* and *F*), and the CalDAG-GEF immunostaining in rats that received saline treatment after sham saline lesions (Figs. 2 *G* and *H*). Thus, the L-DOPA treatment did not by itself alter CalDAG-GEF expression at levels detectable by immunohistochemistry.

To obtain quantitative estimates of the degree of regulation of the CalDAG-GEFs, and in particular to assess their levels of dysregulation in relation to the degree of dyskinesia evidenced by individual animals, we conducted experiments in which we performed the same set of 6-OHDA or saline injections and subsequent L-DOPA or saline treatments in a second set of rats and then used quantitative PCR (qPCR) to measure the levels of *CalDAG-GEFI* and *CalDAG-GEFII* mRNAs. In these animals, we measured the behavioral effects of the L-DOPA and sham saline therapeutic treatments by making behavioral observations at 20 and 50 min time-points after the morning L-DOPA injection (Table 1 and Fig. 1*B*). This protocol allowed us to compare behavioral changes to changes in mRNA levels that were associated with (*i*) the 6-OHDA dopamine-depleting treatment alone, (*ii*) the L-DOPA dopamine-enhancing treatment alone, (*iii*) the combined 6-OHDA and L-DOPA treatment that led to abnormal involuntary movements, and (*iv*) successive saline treatments as controls.

The qPCR results (Fig. 3) demonstrated that *CalDAG-GEFI* mRNA was down-regulated in the dopamine-depleted striatum in the L-DOPA treatment group relative to its levels in all other conditions (Fig. 3*A*), and that *CalDAG-GEFII* mRNA was up-regulated in the dopamine-depleted striatum of the L-DOPA treatment group (Fig. 3*B*). As we found for the CalDAG-GEF proteins, dopamine depletion alone produced at most small changes in the mRNAs, especially for *CalDAG-GEFII*, but these changes were not significant in formal (ANOVA) tests. There was not an observable increase or decrease in the mRNA levels following L-DOPA treatment alone, in the absence of prior dopamine depletion, for either *CalDAG-GEFI* or *CalDAG-GEFII* (Fig. 3 *A* and *B*). These findings at the mRNA level were thus consistent with our observations at the protein level in suggesting that only the combination of dopamine depletion and L-DOPA treatment was sufficient to induce major changes in the striatal expression of the 2 *CalDAG-GEFs*.

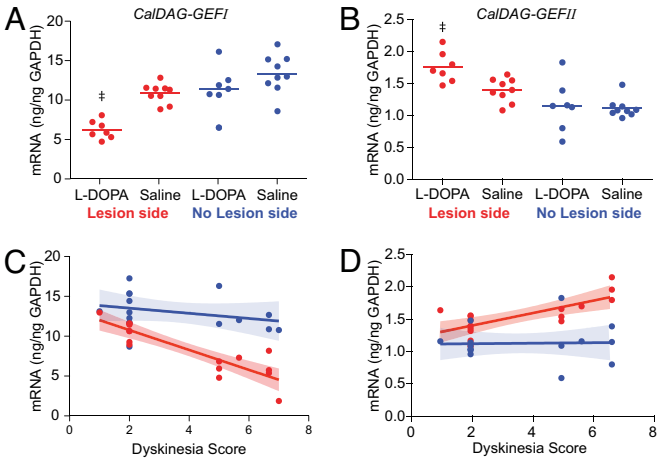

Fig. 3. *CalDAG-GEFI* and *CalDAG-GEFII* mRNAs are oppositely dysregulated in parkinsonian rats with L-DOPA-induced dyskinesias, and the degree of that dysregulation is correlated with L-DOPA-induced dyskinesias severity. (*A* and *B*) As measured by qPCR, *CalDAG-GEFI* transcript is reduced (*A*) whereas *CalDAG-GEFII* transcript is up-regulated (*B*) in the dopamine-depleted striatum of rats that exhibited contralateral L-DOPA-induced dyskinesias (L-DOPA, red), relative to the nondepleted side of the same rats (L-DOPA, blue), and the depleted and nondepleted sides of rats given repeated saline (Saline red and blue, respectively; $P < 0.05$, indicated by ‡). (*C* and *D*) Correlations between dyskinesia scores (averages of last 3 observations at 20 min) and the down-regulation of *CalDAG-GEFI* (*C*, red; $r = 0.88$, $P < 0.0001$) and up-regulation of *CalDAG-GEFII* (*D*, red; $r = 0.74$, $P < 0.0011$) in the dopamine-depleted striatum. Samples for *CalDAG-GEFII* from 1 rat were lost. No significant correlations were found for the contralateral striatum for either *CalDAG-GEFI* (*C*, blue; $r = 0.30$, $P = 0.274$) or *CalDAG-GEFII* (*D*, blue; $r = 0.03$, $P = 0.91$). Shading shows 95% confidence limits. For *A*–*D*, the *y* axes represent nanogram equivalents of mRNA, normalized to GAPDH.

We next compared the qPCR data from individual rats to their abnormal movement scores (Figs. 3 *C* and *D*). There was a strong correlation between the degree of dysregulation of each of the *CalDAG-GEFs* and the severity of abnormal involuntary movements exhibited by the rats. The greater the severity of motor abnormalities exhibited, the more pronounced was the down-regulation of *CalDAG-GEFI* in the dopamine-depleted striatum, and the more pronounced was the up-regulation of *CalDAG-GEFII*. We found no such correlation between the abnormal movement scores and mRNA levels in the contralateral, control striatum of animals with L-DOPA-induced motor abnormalities (Figs. 3 *C* and *D*).

Discussion

Our findings demonstrate that the striatum-enriched genes, *CalDAG-GEFI* and *CalDAG-GEFII*, are strongly and inversely dysregulated in an animal model of the involuntary movement disorder that is produced by prolonged L-DOPA treatment of Parkinson's disease patients. Our findings further demonstrate that the dysregulation of these genes is highly correlated with the severity of the abnormal motor responses evoked by the L-DOPA treatment. The contrasting signaling and expression profiles of these genes suggest that their dysregulation could be important correlates of L-DOPA induced dyskinesias, and raise the possibility that CalDAG-GEF dysregulation contributes to these motor difficulties.

CalDAG-GEFII strongly activates ERK both in vitro and in vivo, whereas CalDAG-GEFI has a cell-type dependent effect (25, 28–31). If these signaling patterns hold for the striatum, the 2 CalDAG-GEF proteins could, through their respective target-

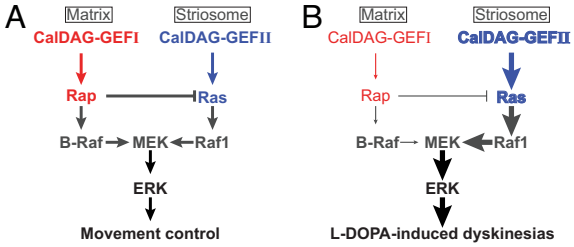

Fig. 4. Hypothetical scheme of matrix-enriched CalDAG-GEFI and striosome-enriched CalDAG-GEFII signaling to ERK, implicated in relation to the expression of L-DOPA-induced dyskinesia (20, 22–24, 36, 38, 42). (*A*) Normal striatum. (*B*) Dopamine-depleted striatum with L-DOPA treatment.

ing of Rap and Ras, regulate the key ERK signaling pathways implicated in the genesis of L-DOPA-induced dyskinesias. In situ hybridization studies suggest that the 2 *CalDAG-GEF* mRNAs coexist in individual striatal projection neurons (26), despite their differential striosome-matrix distributions. This evidence further suggests that their inverse regulation by L-DOPA under conditions of dopamine depletion could affect ERK signaling cascades in the same neurons.

We found that L-DOPA treatment affected the expression of the CalDAG-GEFs in both striatal compartments, but the opposite predominant expression patterns of the 2 CalDAG-GEFs yielded a pattern in which CalDAG-GEFII expression was yet further enhanced in the striosomal compartment relative to CalDAG-GEFI, whereas CalDAG-GEFI, normally predominant in the matrix compartment, had weakened expression there. Thus overall, the compartment-enriched expression patterns of the CalDAG-GEFs in the dopamine-depleted striatum after L-DOPA treatment suggests that CalDAG-GEFII, as an exchange factor for Ras, could dominantly drive ERK activation in striosomes (Fig. 4).

An imbalance in activity in the 2 compartments favoring striosomes over matrix has been suggested as a correlate of exaggerated and repetitive movement syndromes induced by repeated exposure to drug treatments that activate dopamine receptors in the striatum (14, 15, 18, 32–34). Viewed in this context, our findings raise the possibility that in the parkinsonian striatum, repeated L-DOPA treatment enhances striosome-predominant activity ratios through coordinated, but opposite, regulation of the matrix-enriched CalDAG-GEFI (down-regulated) and the striosome-enriched CalDAG-GEFII (up-regulated). The fact that the CalDAG-GEF proteins have calcium binding domains suggests that excitatory inputs from the neocortex or other sources could be affected as part of this activity dysregulation, thus potentially implicating abnormal CalDAG-GEF-associated corticostriatal transmission as part of the dysregulation related to the expression of dyskinesias following L-DOPA treatment. In addition, multiple neuromodulatory systems follow striosome-matrix organization, such as serotonergic and cholinergic systems, and might also be involved as a result of the CalDAG-GEF regulation demonstrated here (35). Because our immunohistochemical findings depend on antibodies that likely detect only one or a subset of the possible CalDAG-GEF isoforms, our findings do not address the possible compartmental distributions of all proteins derived from the *CalDAG-GEF* genes. Nonetheless, our evidence does suggest that the differential anatomical distribution of these gene products may be critical to their functions in addition to the differential Rap/Ras/ERK targeting of CalDAG-GEFI and CalDAG-GEFII.

Striatal ERK is strongly activated by L-DOPA treatment in rodent models of Parkinson's disease (21–24). In rodent models of L-DOPA-induced motor abnormalities, treatment with the MEK/ERK inhibitor, SL327, or with the Ras/ERK inhibitor, lovastatin, inhibits both the development and the expression of these motor problems (23, 36). Thus, the Ras/MEK/ERK cascade has been implicated in the genesis of L-DOPA-induced dyskinesias, but the Ras activators that initiate this cascade in the striatum remain unknown. Our findings suggest that coordinated dysregulation of the Ras/Rap exchange factors, CalDAG-GEFI and CalDAG-GEFII, might drive the Ras/MEK/ERK cascade that contributes to L-DOPA-induced motor dyskinesias (Fig. 4). Striatal ERK, once phosphorylated, is maintained in the active state by DARPP-32, a phosphatase inhibitor that is up-regulated in rodents with L-DOPA-induced motor abnormalities (23, 37). However, it has been suggested that the requirement for DARPP-32 in maintaining ERK phosphorylation is restricted to the ventral striatum (38). By contrast, in the dorsal striatum, we found that up-regulation of the Ras/MEK/ERK-activator CalDAG-GEFII was highly correlated with L-DOPA-induced dyskinesias and that the CalDAG-GEFII isoform we detected was particularly intensely immunostained in striosomes.

In a parallel microarray study aimed at discovering striatal genes differentially regulated by L-DOPA following dopamine depletion, we have observed elevated *CalDAG-GEFII* mRNA, in accord with our present qPCR and immunohistochemistry; *CalDAG-GEFI* was not represented on the microarray chip that we used. Moreover, in preliminary qPCR analysis of laser-captured samples of striosomes and matrix in these experiments, we found that the *CalDAG-GEFII* up-regulation was even greater in the matrix compartment than in striosomes. If confirmed, these findings would suggest, as we found here, an overall dominance of striatal signaling mediated by CalDAG-GEFII following L-DOPA therapy for dopamine depletion. The striosomes, highly CalDAG-GEFII-enriched, could be dominant relative to the severely CalDAG-GEFI-depleted matrix in signal processing depending on a balance between the actions of these Rap/Ras exchange factors. Until signaling studies have been done in vivo, this hypothesis of CalDAG-GEFII predominance in signaling cannot be adequately tested, but the correlations that we found between increased abnormal movement and the coordinate; opposite changes in expression of the 2 CalDAG-GEFs raises the possibility that the signaling dysregulation resulting from these expression changes could be an important correlate or even mediator of the L-DOPA induced movement abnormalities (Fig. 4).

Despite evidence for the involvement of Ras/MEK/ERK signaling in the genesis of dyskinesias induced by repeated L-DOPA treatment, the wide-ranging brain distributions and functions of the Ras/MEK/ERK proteins make them poor targets for therapeutic intervention in Parkinson's disease. However, the identification of striatum-enriched members of this signaling cascade might provide possible targets for treating the dyskinesias. Our findings suggest that the striatum-enriched Ras/MEK/ERK regulators, CalDAG-GEFI and CalDAG-GEFII, represent such new potential therapeutic targets for the prevention and alleviation of L-DOPA-induced dyskinesias.

Materials and Methods

Surgery. Male Sprague–Dawley rats (250–350 g, $n = 26$), treated in accordance with the policies of the Massachusetts Institute of Technology Committee on Animal Care, were housed under standard conditions with a 12 h light/dark cycle (lights on at 7 a.m.), and were given free access to food and water. Rats deeply anesthetized with ketamine hydrochloride (75 mg kg^{-1}) and xylazine (10 mg kg^{-1}) were given 6-OHDA (10 μg in 0.1% ascorbic acid in saline) or vehicle (0.1% ascorbic acid in saline) injections into the right medial forebrain bundle under stereotaxic guidance (AP = −4.0 mm, ML = −1.3 mm, DV = −8.4 mm) (39).

Behavioral Evaluation. Three weeks after surgery, the rats were injected with apomorphine (0.5 mg kg^{-1}, s.c.) to test for contraversive circling. Rats exhibiting more than 6 contraversive turns per minute at 10 min postinjection were chosen for further study, and 3 days later were treated at 9 a.m. and 5 p.m.

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with either L-DOPA (25 mg kg^{-1}, i.p.) and benserazide (6.25 mg kg^{-1}, i.p.) or saline for 20 days. Vehicle-injected rats were also treated with either L-DOPA/benserazide alone or saline alone. Abnormal motor behaviors were scored for 1 min observation periods on every third day at 20 and 50 min after the morning L-DOPA injection according to a standardized rating scale (Table 1).

Real-Time qPCR. Twelve hours after the last L-DOPA or saline injection, rats were decapitated and the striatum of each hemisphere was rapidly removed and individually frozen on dry ice. RNA was extracted as previously described (40). Primers for qPCR were designed with Primer3 software. *CalDAG-GEFI* transcript was detected with 5′-cttggaccagaaccaggatg-3′ forward and 5′-gtggcagttcacaccacaag-3′ reverse primers. *CalDAG-GEFII* transcript was detected with 5′-ggacctaccaagaactggaac-3′ forward and 5′-gatcccagtaaaccccgtctg-3′ reverse primers. Quantitative PCR was performed with an iCycler (BioRad) with SYBR Green PCR Master Mix (Applied Biosystems) with 6 min at 95 °C (first cycle only), 30 s at 95 °C, 30 s at 55–60 °C, elongation at 72 °C for 45 cycles, followed by eighty 0.5 °C increases in temperature (starting at 55 °C) to collect melting curve data. The data were quantified with the ΔΔCt method and normalized to GAPDH standards. One-way analysis of variance (ANOVA) with Bonferroni correction was used to evaluate statistical significance of differences among samples.

Immunohistochemistry. Rats terminally anesthetized with sodium pentobarbital (Nembutal; >25 mg kg^{-1}) 12 h after the final L-DOPA or saline injection were perfused transcardially with 4% paraformaldehyde in 0.1 M $NaKPO_4$, pH 7.4. Brains were soaked successively in 4% paraformaldehyde in 0.1 M $NaKPO_4$ and then 25% glycerol solution in 0.1 M $NaKPO_4$, pH 7.4, were frozen, and were cut transversely at 30 μm on a sliding Microtome. Sections were processed by conventional immunohistochemical methods with anti-RasGRP mouse monoclonal antibody m199 (Santa Cruz) or polyclonal anti-CalDAG-GEFI antiserum (41) (approximately 12 h at 4 °C) with ABC amplification (Vector Labs) and DAB detection with nickel enhancement.

Statistical Analysis. mRNA levels in the striatal samples were compared across groups by one-way ANOVA. Dyskinesia scores were evaluated with Brunner's variation of Friedman's two-way ANOVA by ranks for correlated samples followed by Dunn's multiple comparison test. Correlations between abnormal movement scores and mRNA levels were calculated by Spearman correlations for ranked data.

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