TITLE PAGE

Activation of DREAM, a calcium-binding protein, reduces L-DOPA-induced dyskinesias in mice

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ABSTRACT

Background: Previous studies have implicated the cAMP/PKA pathway as well as FosB and dynorphin-B expression mediated by dopamine D1 receptor stimulation in the development of L-DOPA-induced dyskinesia (LID). The magnitude of these molecular changes correlates with the intensity of dyskinesias. The calcium-binding protein DREAM binds to DRE sites in the DNA and represses transcription of target genes such as c-fos, Fos-related antigen-2 (fra-2) and prodynorphin. This repression is released by Ca^{2+} and PKA activation. Dominant-active DREAM transgenic mice (daDREAM) and DREAM knockout mice ($\text{DREAM}^{-/-}$) were used to define the involvement of DREAM in dyskinesias.

Methods: Dyskinesias were evaluated twice a week in 6-OHDA-lesioned mice during chronic L-DOPA (25 mg/kg). The impact of DREAM on L-DOPA efficacy was evaluated using the rotarod and the cylinder test after the establishment of dyskinesia and the molecular changes by immunohistochemistry and Western blot.

Results: In daDREAM mice, LID was decreased during the entire treatment. In correlation with these behavioral results, daDREAM mice showed a decrease in FosB, P-Ach3, dynorphin-B and P-GluR1 expression. Conversely, genetic inactivation of DREAM potentiated the intensity of dyskinesia, and $\text{DREAM}^{-/-}$ mice exhibited an increase in expression of molecular markers associated with dyskinesias. Importantly, DREAM modifications did not affect the kinetic profile or antiparkinsonian efficacy of L-DOPA therapy.

Conclusion: DREAM decreases development of LID in mice and reduces L-DOPA-induced expression of FosB, P-Ach3 and dynorphin-B in the striatum. These data

suggest that therapeutic approaches that activate DREAM may be useful to alleviate L-DOPA-induced dyskinesia without interfering with its therapeutic motor effects.

INTRODUCTION

Despite extensive research focused on discovering therapeutic alternatives, the dopamine precursor molecule, 3,4-dihydroxyphenyl-L-alanine (L-DOPA), remains the most effective and widely used noninvasive therapy for Parkinson's disease (PD). Chronic administration of L-DOPA and disease progression cause the appearance of abnormal involuntary movements known as dyskinesias in the vast majority of patients. L-DOPA-induced dyskinesia (LID) is causally linked with hyperstimulation of dopamine D1 receptors (D1R) located on direct pathway medium spiny neurons (MSNs) of the severely denervated striatum (1-3). In the dopamine-depleted striatum, L-DOPA activates a cAMP-dependent signalling cascade via PKA activation (4,5), resulting in abnormally increased phosphorylation of DARPP-32 and activation of the Ras/ERK signalling pathway in the same neurons (1,6-9). These events induce transcriptional changes resulting in increased expression of FosB, P-Ach3 and dynorphin-B (10,11).

In addition, the increased activation of the cAMP/PKA/DARPP-32 pathway observed in dyskinesia induces persistent phosphorylation of the AMPA receptor subunit GluR1 at Ser845 (6,7) via protein phosphatase-1 inhibition, enhancing excitatory glutamatergic transmission in D1R-containing neurons and increasing NMDA receptor (NMDAR) activation (12-15). Enhanced synaptic transmission in LID has been associated with altered redistribution and trafficking of D1R (16) along with a

massive accumulation of PSD-95 in the synaptic membrane (17). Interestingly, PSD-95 has been shown to modulate LID in rats and monkeys by its direct interaction with D1R (18). In addition, D1R-mediated activation of adenylyl cyclase/PKA triggers calcium entry through voltage-dependent calcium channels (19) and treatment with the L-type Ca^{2+} channel antagonist isradipine diminishes dyskinesias (20), further supporting the idea that calcium entry into direct pathway striatal neurons after D1R stimulation contributes to LID.

Despite the progress made in recent years, the intracellular signalling mechanism downstream of D1R-PKA-dependent activation is not fully established. The downstream regulatory element antagonist modulator (DREAM), also known as calsenilin and KChIP3, is a Ca^{2+} -binding protein that mediates calcium- and cAMP-dependent transcriptional responses (21,22). In striatal neurons moderate levels of DREAM are present in the neuropil and cell soma (23-25). In basal conditions, DREAM binds to a regulatory element called DRE, located downstream from the transcription initiation site, repressing the transcription of target genes including prodynorphin, c-fos and Fos-related antigen-2 (fra-2) (26,27). DREAM-mediated repression is reversed by Ca^{2+} and by its PKA-dependent interaction with phospho-CREM, the cAMP responsive element modulator (26,28-30). Moreover, outside the nucleus, DREAM directly interacts with several proteins (31) including NR1 (32) and PSD-95 (33), thus inhibiting NMDAR function and its surface expression.

Based on these observations and on our previous data demonstrating a close association between LID and overexpression of FosB and dynorphin-B (1,10), we propose that DREAM, located downstream of D1R-dependent cAMP/PKA activation, plays an important role in the cascade of molecular events leading to LID. If this is so,

DREAM could provide a site for intervention to improve the efficacy of L-DOPA treatment while preventing the upregulation of target genes associated with dyskinesia. To investigate this possible role of DREAM in LID, we used DREAM knockout and dominant active DREAM transgenic mice in our mouse model of dyskinesias.

METHODS AND MATERIALS

Animals

This study was carried out in 3-6 months-old transgenic mice expressing a dominant active mutant DREAM (daDREAM) (34,35) or in DREAM-deficient mice (22,36). Both genetically modified animals and wild-type controls were maintained in a C57-BL/6 background. In the daDREAM transgenic mice, expression of the transgene, a bi-cistronic construct including the daDREAM mutant, an IRES sequence and the lacZ reporter, was driven by the CaMK-II alpha promoter (37). The transgenic line (JN26) used in this study has a telencephalic-specific expression with high levels of transgene expression in the striatum (Suppl. Fig S1). Homozygous daDREAM and DREAM^{-/-} mice were derived from mating the corresponding heterozygous mice. Genotyping was performed by PCR as described (35,36). Bacterial artificial chromosome (BAC)-transgenic D1R-tomato mice (38) were used to study DREAM striatal localization. All animal procedures followed guidelines from European Union Council Directive (86/609/European Economic Community) and experimental protocols were approved by the CSIC Ethics Committee.

6-OHDA lesion and L-DOPA treatment

Animals received unilateral stereotaxic injections (2 x 2 μ l) of 6-OHDA-HBr (20 mM, containing 0.02% ascorbic acid; Sigma-Aldrich) as described previously (39). After 3 weeks of recovery, mice received a daily intraperitoneal injection of 25 mg/kg of L-DOPA methyl ester (Sigma-Aldrich) 20 minutes after benserazide hydrochloride (10 mg/kg, Sigma-Aldrich), for a 3 week period.

Behavioral analysis

Locomotor activity and motor coordination. Horizontal and vertical activity as well as total distance traveled was recorded in mice as described previously (40). Motor coordination was measured in the rotarod (Ugo Basile, Italy) following an accelerating protocol, with increasing speed from 4 to 40 rpm over a 5 minute period as described (41). Mice were tested in 6 consecutive trials 20 minutes apart. Measurements were done before 6-OHDA (naïve), 3 weeks after lesion (parkinsonian) and during L-DOPA treatment (dyskinetic) on day 9, 24 hours after the last L-DOPA injection in order to avoid exhaustion and the peak dyskinesia.

Dyskinetic score. L-DOPA-induced dyskinesias were evaluated twice a week for 3 weeks, 40 minutes after L-DOPA using a 0-4 severity scale as described (1,38). The time course of L-DOPA response for each genotype was evaluated on day 20, for 1 minute every 20 minutes during 160 minutes post-L-DOPA injection.

Cylinder test was performed according to Espadas *et al.* (42) before the 6-OHDA lesion, 3 weeks after lesion and on day 17 of the chronic L-DOPA treatment, 140 minutes after L-DOPA injection to avoid dyskinetic symptoms. Spontaneous ipsilateral and contralateral forelimb touches to the cylinder were counted for 3 minutes to assess forelimb asymmetry.

Immunohistochemistry and image analysis

Mice were killed 1 hour after the last L-DOPA injection and immunohistochemistry studies were performed as described (43,44), using the following antibodies: tyrosine hydroxylase (TH 1:1000, Millipore, Temecula, CA, USA), FosB (1:7500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), dynorphin-B 1-29 leumorphin (1:10000, Serotec, Oxford, UK), phospho-(Ser10)-acetyl-(Lys14)-histone-3 (P-AcH3, 1:1500, Upstate, Cell Signaling Solutions, Lake Placid, NY, USA), DREAM (FL-214) (1:250, Santa Cruz Biotechnology) and HOECHST (1 μ g/ml, Sigma-Aldrich).

The extent of dopaminergic lesions was quantified using Neurolucida software (Microbrightfield, Colchester, VT, USA), depicting the border of striatal areas with complete loss of TH-immunoreactive fibers with a 4x lens using 7-9 serial rostrocaudal sections per animal. Quantification of FosB, P-AcH3 and dynorphin-B immunoreactivity was carried out as described (10,45).

RT-qPCR

Total RNA from isolated striata was extracted using the Illustra RNAspin kit (GE Healthcare-Europe, Barcelona, Spain). Quantitative PCR for DREAM and TH was performed with TaqMan Assay-on-Demand primers and the TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Madrid, Spain). PCR reactions were performed in triplicate in a 7900HT fast real-time PCR system (Applied Biosystems). Expression of target genes was normalized using the 18S expression and calculated using the double delta Ct method (46).

Western Blot

Animals were sacrificed by decapitation 30 minutes after the last L-DOPA injection and striatal tissue was dissected and homogenized. Western blot analysis was performed with primary antibodies against phospho-Ser845-GluR1 (1:7500, Millipore), TH (1:4000, Millipore) and β -actin (1:50000, Sigma-Aldrich) as a loading control. Blots were developed by chemiluminiscence (ECL-plus, GE Healthcare, UK) and quantified by densitometry.

Statistical Analysis

Behavioral data and quantifications of immunolabeling were analyzed by two-way ANOVA followed by Student-Newman-Keuls post hoc test. The extent of the dopaminergic lesion and DREAM mRNA expression were analyzed by one-way ANOVA. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Basal locomotor activity and motor coordination are normal in DREAM mutant mice

Although no morphological differences were evident between genotypes, we carried out spontaneous locomotor activity and rotarod tests prior to the 6-OHDA lesion to rule out any motor impairment in the mutant mice that could interfere with L-DOPA behavioral studies. In the absence of any external challenge, daDREAM, DREAM^{-/-} and WT mice showed similar horizontal and vertical activity, as indicated by the number of beam breaks measured in a multicage activity meter system (Figure 1A,B). Ambulatory activity measured as total distance traveled was also similar among the three genotypes (Figure 1C). Over time, as the mice habituated to the environment, the number of beam

breaks and the distance traveled diminished, with a similar profile in all three genotypes. Moreover, no statistically significant differences between genotypes were found in the rotarod test (Figure 1D). All animals achieved a significant increase ($p<0.001$) in the latency to fall from the rotating rod at the 6th and last trial compared to the first one, showing a similar capacity of motor learning in the rotarod. Taken together, these results indicated that alterations in DREAM expression do not modify spontaneous locomotor activity or motor coordination.

DREAM modulates L-DOPA-induced dyskinesias

To examine the role of DREAM in dyskinesias in hemiparkinsonian/6-OHDA-lesioned mice, we assessed axial dystonia and forelimb, orofacial and locomotive dyskinesia in daDREAM, DREAM^{-/-} and the corresponding wild-type littermates 40 minutes after L-DOPA administration. L-DOPA was given daily and assessments were done twice a week, as described (1,10).

In wild-type mice, LID symptoms were already apparent at the beginning of the treatment and progressively increased to reach a plateau in the second week of administration (Figure 2). Interestingly, daDREAM mice showed a significant reduction in dyskinetic movements, in particular for limb ($p<0.001$) and orofacial ($p<0.001$) symptoms as well as for axial dystonia ($p<0.001$) compared to their wild-type littermates. Meanwhile, DREAM^{-/-} mice showed a significant enhancement in limb ($p<0.05$) and orofacial ($p<0.01$) dyskinesia as well as axial dystonia ($p<0.05$). These changes were apparent from the first day of L-DOPA administration and lasted over the entire 3-week treatment. Notably, no difference in locomotive dyskinesia was observed

among genotypes, indicating that DREAM modification does not compromise the motor stimulant effect of L-DOPA.

We calculated the global dyskinetic score of daDREAM and DREAM^{-/-} mice and evaluated the time course of L-DOPA response in both genotypes. While dyskinesia was observed in both daDREAM and WT mice, the total score achieved by daDREAM mice was significantly lower ($p<0.001$) compared to WT mice for all time points evaluated (Figure 3A). On the other hand, the total dyskinetic score was higher in DREAM^{-/-} animals compared to their WT littermates. The difference was statistically significant ($p<0.05$), although it was smaller than that observed between the daDREAM and the WT mice.

To look for differences in the kinetic profile of L-DOPA responses among genotypes, we determined the duration of the dyskinetic response and the peak severity of the symptoms by evaluating the time course of L-DOPA response in daDREAM and DREAM^{-/-} mice on day 20 of L-DOPA administration (Figure 3B). Dyskinetic symptoms were scored for 1 minute every 20 minutes during the 160 minutes following L-DOPA administration. Dyskinesia was evident at 20 minutes, peaked between 40 and 60 minutes, and declined significantly at 100 minutes after L-DOPA before disappearing at 140 minutes for both genotypes. Although the time course of L-DOPA response was similar in both groups of mice, daDREAM mice exhibited significantly lower dyskinesia compared with WT across the entire duration of the L-DOPA response. Finally, there were also no statistically significant differences between DREAM^{-/-} and WT mice in the duration of the L-DOPA response: As in daDREAM mice, the response was evident 20 minutes after L-DOPA and disappeared after 140 minutes. However, a difference was again observed in the intensity of the dyskinetic

symptoms, which were stronger in DREAM^{-/-} mice until 120 minutes, when the dyskinetic scores were similar for the duration of the effect. These time course results show that changes in DREAM do not alter the kinetic profile of L-DOPA as demonstrated by the duration of the L-DOPA response.

To exclude that the extent of the dopaminergic lesion in the three genotypes was different, which could influence the results, we assessed the percentage of striatal volume with a complete loss of TH-immunoreactive (-ir) fibers for each group of animals (Figure 3C). We found no statistically significant difference between groups (daDREAM, $44.2 \pm 2\%$; DREAM^{-/-}, $48.7 \pm 2\%$; and WT, $46.2 \pm 2\%$).

The antiparkinsonian effect of L-DOPA is maintained in DREAM mutant mice

To rule out that the different dyskinetic score induced by L-DOPA in DREAM mutant mice is not due to an altered motor response to L-DOPA in these mice, we carried out the cylinder and rotarod tests to measure the antiparkinsonian effect of L-DOPA (42,47). In the cylinder test, naïve mice used both forelimbs similarly, while lesioned mice showed a significant ($p < 0.001$) forelimb use asymmetry (Figure 4A). As expected, L-DOPA significantly ($p < 0.001$) recovered the contralateral paw use. Importantly, no significant differences were observed among the three genotypes, indicating that the genetic manipulation of DREAM does not affect the motor impairment induced by the lesion or the therapeutic effect of L-DOPA. Similar results were observed with the rotarod that evaluates motor coordination (Figure 4B), strengthening the data from the cylinder test and further confirming that modifications in DREAM do not alter the antiparkinsonian efficacy of the L-DOPA.

DREAM is expressed in direct and indirect striatal projection neurons

Previous studies demonstrated that DREAM is expressed in the striatum and substantia nigra (SN) (23-25). Using naïve BAC-transgenic D1R-tomato mice to differentiate direct from indirect pathway neurons, we observed moderate DREAM signal in both, D1R-positive (red fluorescence) and D1R-negative (no fluorescence) MSNs (Figure 5A). This immunolabeling was preferentially nuclear, but was also evident in the cytoplasm and in the neuropil. Quantification experiments of DREAM carried out by RT-qPCR demonstrated that L-DOPA treatment to sham or lesioned animals did not modify DREAM mRNA expression in the striatum or in the SN (Figure 5B). TH mRNA in the SN was also measured in the same animals by RT-qPCR to confirm the efficacy of 6-OHDA lesion (Figure 5C).

DREAM regulates key molecular determinants of LID

Several studies have correlated the increased levels of FosB, P-Ach3 and dynorphin-B in the dorsolateral lesioned striatum with the appearance of LID (1,11,48). We evaluated the effect of the genetic modification of DREAM on the striatal levels of these markers after chronic L-DOPA administration in hemiparkinsonian mice. Notably, the number of FosB-positive neurons was 3-fold lower in the lesioned striatum of daDREAM transgenic mice ($p<0.001$) and just slightly increased in DREAM^{-/-} mice ($p<0.01$) compared to WT mice (Figure 6A,B). Furthermore, the presence of histone H3 phosphorylated on Ser10 and acetylated on Lys14 was significantly modified in the striatum ipsilateral to the lesion with a 2.5-fold decrease in the number of P-Ach3-

positive nuclei in daDREAM mice ($p<0.001$) and a 1.5-fold increase in DREAM^{-/-} mice compared to WT mice ($p<0.001$) (Figure 6C,D). Finally, scanning of the dynorphin-B-positive area, including cytoplasm and neuropil, revealed a 39% reduction ($p<0.01$) and a 65% increase ($p<0.001$) relative to the total area scanned in daDREAM and DREAM^{-/-} mice, respectively (Figure 6E,F). No statistically significant differences were found in any of these molecular markers in the unlesioned striatum of daDREAM, DREAM^{-/-} and WT mice, which served as a negative control for these experiments. Furthermore, as an additional control for the specificity of these changes, we verified that L-DOPA-induced changes were strictly restricted to the completely denervated striatal area (Suppl. Fig S2), as we have shown before (2,10,38). These results indicate that genetic manipulation of DREAM does not modify the expression pattern of the molecular markers of dyskinesia in mice. These findings correlate well with our behavioral results and implicate DREAM in the regulation of FosB, P-AcH3 and dynorphin-B levels.

LID-associated phosphorylation of GluR1 is attenuated in daDREAM mice

It is well documented that in the striatum of 6-OHDA lesioned mice (6) and MPTP-lesioned monkeys (49), chronic L-DOPA administration results in PKA-dependent hyperphosphorylation of the GluR1 subunit of the glutamate AMPA receptor at serine 845. Therefore, we examined whether the genetic modification of DREAM modifies the phosphorylation state of GluR1 induced by L-DOPA. Analysis of striatal extracts showed that L-DOPA treatment induces an increase in P-GluR1 levels in WT lesioned mice, as expected. This increase was evident also in the DREAM^{-/-} mice. In contrast, DREAM overexpression in daDREAM mice inhibited the hyperphosphorylation of GluR1 induced by L-DOPA (Figure 7A). In parallel,

measurement of levels of TH protein confirmed a similar efficacy of the 6-OHDA lesion in all samples (Figure 7B).

DISCUSSION

These studies provide evidence that the DREAM protein plays a modulatory role in LID in mice and in the underlying molecular changes within the denervated striatum. Overexpression of a dominant active version of DREAM in transgenic mice dramatically reduced LID, whereas genetic inactivation of DREAM increased dyskinetic symptoms. In strict correspondence with the behavioral observations, molecular markers of dyskinesia induced by chronic L-DOPA treatment, including FosB, dynorphin-B, P-Ach3 and P-GluR1, were decreased in daDREAM mice but increased in DREAM knockout mice. Importantly, modifications in DREAM did not alter the kinetic profile or the antiparkinsonian efficacy of L-DOPA, suggesting that modulation of DREAM function could serve as an intervention target in therapies designed to alleviate LID without interfering with the beneficial effects of L-DOPA treatment.

In agreement with previous observations in naïve DREAM^{-/-} (36,50) and daDREAM (51) adult mutant mice, spontaneous locomotor activity and motor coordination were indistinguishable from WT littermates, suggesting that the differences observed in LID are not due to any motor impairment, but rather to the modifications related to DREAM levels. Moreover, dyskinetic differences among genotypes after L-DOPA treatment are not due to alterations of the kinetic profile of L-DOPA by DREAM, as all animals showed a similar duration of the L-DOPA response. Interestingly, mutant mice show similar motor performance skills in the cylinder and

rotarod tests, demonstrating that the antiparkinsonian effect of L-DOPA is preserved in mutant animals despite DREAM modifications.

DREAM is a multi-functional calcium binding protein (52) that acts as a transcriptional repressor to regulate activity-dependent gene expression through direct interaction with regulatory sites in the DNA (26). Our data show that DREAM is present in the nucleus of striatal projection neurons. In these neurons, increased expression of FosB and dynorphin-B after L-DOPA treatment correlates with dyskinesias in PD patients (53,54), MPTP-treated monkeys (55,56), and in 6-OHDA-treated rats (48) and mice (1,10,38). Thus, transcriptional repression of FosB and prodynorphin genes in daDREAM mice could be directly related to the observed reduction in LID development.

Correlating with the behavioral response to L-DOPA, our results show that overexpression of daDREAM diminishes FosB and dynorphin-B expression induced by L-DOPA in denervated striatal neurons, while genetic inactivation of DREAM induces the opposite effect. The small increase of FosB and dynorphin-B expression observed in the striatum of DREAM-deficient mice after L-DOPA is in agreement with similar increases observed previously in the spinal cord of DREAM^{-/-} mice (36). DREAM represses the transcription of the immediate early genes Fos-related antigen-2 (fra-2) and c-fos as well as prodynorphin by binding to DRE sites located in the 5'-untranslated sequence downstream from the TATA box (26,27). Although not previously studied, our data indicate that DREAM may also repress FosB, consistent with the presence of two DRE sites in this gene (57). Dynorphin-B expression is also regulated by D1R activation (58) and by binding of Fos/Jun heterodimers to an AP-1 site in the prodynorphin gene (59). Moreover, overexpression of FosB after L-DOPA treatment

induces a concomitant increase of dynorphin-B expression in the 6-OHDA lesioned striatum (48). Therefore, our data support the notion that DREAM exerts a synergistic double effect: directly, blocking prodynorphin transcription by anchoring to its DRE site, and indirectly, diminishing FosB expression in turn resulting in dynorphin-B down-regulation via AP-1.

The non-transcriptional effects of DREAM have been shown to regulate cell function and synaptic activity, mostly through a variety of specific protein-protein interactions (31). The list of target proteins includes cationic channels (60), membrane receptors (32,61) and membrane docking proteins (33). Since a wide variety of mechanisms underlie synaptic changes associated with LID, including abnormal dendritic spine increases in denervated D2R-containing striatal neurons (38) involved in corticostriatal synapses (62), several additional mechanisms could be related to DREAM-mediated changes in LID based on its presence in the cell body as discussed below.

In addition to D1R stimulation, NMDAR activation plays an important role in dyskinesia, as treatment with NMDAR antagonists such as MK-801 or amantadine completely abolishes LID (63,64). Interestingly, DREAM has been shown to diminish NMDAR activation (32,33). DREAM knockdown with siRNAs significantly enhanced NMDAR-mediated currents in cultured neurons, while overexpression of DREAM reduced NMDAR activation and its presence in the plasma membrane, indicating that DREAM modulates NMDAR function. Taken together, these data correlate well with our dyskinetic behavioral results. Inactivation of DREAM, which potentiates NMDAR-mediated currents, potentiates dyskinesias, while DREAM overexpression, which inhibits NMDAR function, decreases dyskinesia. Thus, it is possible that the inhibitory

role of DREAM in LID reported here could be partially related to the negative regulation exerted by DREAM on NMDAR by directly interacting with the NR1 subunit preventing full activation of NMDAR complex as shown in cultured hippocampal neurons (32). On the other hand, the documented capacity of DREAM to bind PSD-95 (33), impairing the recruitment of PSD-95 by D1 receptors (18) could contribute to the observed reduction of LID. Disruption of the NMDAR-PSD-95 complex increases LID (14), probably by making PSD-95 available for interaction with D1R in the synaptic membrane. Thus, displacement of the normal PSD-95 interactome from D1R due to increased DREAM levels could explain the decrease in dyskinesia in daDREAM mice.

Acetylated histone H3 is associated with active chromatin and gene transcription (65), and this effect is synergistically potentiated by phosphorylation (66). Since phosphoacetylation of histone H3 correlates with the intensity of dyskinesias (6,10,11,67), the presence of increased phosphoacetylated histone H3 in DREAM-deficient animals is consistent with increased dynorphin-B and FosB expression and with increased LID. On the contrary, in the presence of the daDREAM repressor, we observed decreased P-AcH3, dynorphin-B, FosB and LID, as expected. In line with these observations, DREAM has been shown to inhibit transcription by blocking the CREB-CBP histone acetylase complex (29). On the other hand, histone H3 phosphorylation depends on the stimulation of the D1R-pathway (10). Although it is possible that this occurs through the D1R/cAMP/PKA/DARPP-32 signaling pathway, it could also be mediated by the reported NMDAR-DREAM interaction through the NR1 receptor subunit (32).

Although a direct DREAM and AMPA receptor interaction has not been described, it is possible that an indirect interaction occurs, since overexpression of DREAM significantly blocked L-DOPA-induced phosphorylation of the PKA substrate AMPA receptor subunit GluR1 at Ser845. GluR1-phosphorylation after L-DOPA is in line with recent work from our laboratory (38) showing dendritic spine regrowth in denervated striatal areas. Indeed, spine regrowth leads to a recruitment of AMPA receptors to the post-synaptic density (68-70) in an activity-dependent manner, i.e. L-DOPA activation, since in basal conditions this recruitment is inactive. This is in line with our western blot experiments, which revealed similar levels of GluR1-phosphorylation in basal conditions in all mutant mice, consistent with previous work (33).

Finally, DREAM overexpression could control LID development through the regulation of voltage-dependent calcium channels. It has been shown that blockade of L-type Ca^{2+} channels using the antagonist isradipine decreases dyskinetic symptoms (20), while DREAM overexpression reduces the expression of the Cav1.2 gene, encoding the L-type Ca^{2+} channel (52) and decreases calcium permeability by direct protein-protein interaction with the L- and the T-type channel complexes (31,71,72).

In summary, this study demonstrates the inhibitory role of DREAM in LID evidenced by reduction of dyskinetic symptoms in daDREAM mice and a potentiation in DREAM^{-/-} mice. In addition, we demonstrate that this action occurs at least in part by transcriptional repression of target genes, although non-transcriptional mechanisms could also be involved. Thus, DREAM regulates dynorphin-B and possibly FosB expression, repressing their transcription by anchoring to DRE sites in the DNA, and reducing LID. In addition, outside the nucleus DREAM could directly interact with the

NR1 subunit and PSD-95, regulating activation of NMDAR and D1R (Figure 8). The findings we describe here validate DREAM as a novel therapeutic target against LID, and we propose that specific modulators of DREAM could be useful in alleviating L-DOPA-induced dyskinesias without interfering with the antiparkinsonian effect of L-DOPA. However, other transcriptional and non-transcriptional targets of DREAM, as yet uncharacterized, could also be important, directly or indirectly, in the regulation of LID. Future genome-wide analysis during LID establishment in daDREAM and DREAM^{-/-} mice might expand our understanding of the role of DREAM in LID.

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FIGURE LEGENDS

Figure 1. Genetic modification of DREAM protein levels does not alter basal locomotor activity. (A) Horizontal and (B) vertical locomotor activity (beam breaks) and (C) total distance traveled (cm) during 60 min were measured in a multicage activity meter system. (D) Latency (s) to fall in the rotarod test at constant acceleration. Transgenic (daDREAM) and knockout (DREAM^{-/-}) mice behave similarly to wild-type mice and show similar improvement in motor coordination. The data expressed as mean $\pm$ SEM were analyzed by two-way ANOVA with repeated measures followed by Student-Newman-Keuls test. Significant differences for trial were found [$F(2,64) = 22.38$; $p < 0.001$]. # $p < 0.01$ vs. trial 1, $n = 10-12$.

Figure 2. DREAM overexpression in daDREAM mice decreases L-DOPA-induced dyskinesia in hemiparkinsonian mice while genetic inactivation of DREAM potentiates it. Time course of appearance of dyskinetic symptoms: limb dyskinesia (Limb), orofacial dyskinesia (ORF), axial dystonia (Axial) and locomotive dyskinesia (Loc). Movements were evaluated 40 min after L-DOPA (25 mg/kg) administration at the indicated days. daDREAM mice show a lower dyskinetic score for limb and orofacial dyskinesia and for axial dystonia compared to wild-type mice, while inactivation of DREAM in DREAM^{-/-} mice significantly increased these dyskinetic symptoms. No statistically significant differences were found for locomotive dyskinesia. Data are expressed as mean $\pm$ SEM. Two-way ANOVA with repeated

measures followed by Student-Newman-Keuls test showed significant differences for genotype [$F(2,190) = 25.52$; $p < 0.001$ for Limb; $F(2,190) = 34.47$; $p < 0.001$ for ORF; $F(2,190) = 28.43$; $p < 0.001$ for Axial] and for time [$F(5,190) = 14.00$; $p < 0.001$ for Limb; $F(5,190) = 14.23$; $p < 0.001$ for ORF; $F(5,190) = 8.92$; $p < 0.001$ for Axial]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs WT. n=8-15.

Figure 3. DREAM content modulates the global dyskinetic score. (A) Time course of global dyskinetic score (Global dysk) in daDREAM and in DREAM^{-/-} mice compared to their wild-type littermates, evaluated 40 min after L-DOPA (25 mg/kg) administration at the indicated days. DREAM overexpression decreased LID score for the entire treatment period, while inactivation of DREAM increased LID score. Two-way ANOVA with repeated measures followed by Student-Newman-Keuls test showed significant differences for genotype [$F(2,190) = 34.01$, $p < 0.001$], time [$F(5,190) = 19.34$; $p < 0.001$] and genotype x time [$F(10,190) = 2.12$, $p < 0.05$]. **(B)** Kinetic profile of dyskinetic symptoms evaluated one every 20 min during 160 min on day 20 of L-DOPA treatment. DREAM overexpression reduced LID score during the entire half-life of L-DOPA, while genetic inactivation of DREAM increased dyskinesia. Two-way ANOVA with repeated measures followed by Student-Newman-Keuls test showed significant differences for genotype [$F(2,196) = 13.51$, $p < 0.001$], time [$F(7,196) = 99.56$; $p < 0.001$] and genotype x time [$F(14,196) = 5.44$, $p < 0.001$]. **(C)** Scatter diagram of the extent of striatal lesions assessed by percentage of striatal volume completely denervated. No statistically significant differences were found among genotypes by one-way ANOVA analysis. Global LID scores expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs WT. n=8-15.

Figure 4. Genetic manipulation of DREAM does not modify the therapeutic effect of L-DOPA. Forelimb use asymmetry was assessed with the cylinder test **(A)** and motor coordination with the rotarod **(B)** before (naïve), 3 weeks after 6-OHDA lesion (parkinsonian, Park) and during chronic L-DOPA (dyskinetic, Dysk) on day 17, 140 min post-L-DOPA **(A)** or on day 9, 24 hours after the administration of L-DOPA **(B)**. Data from the cylinder test are expressed as % of wall contacts performed with the contralateral limb and in sec for the rotarod. Mean $\pm$ SEM were analyzed by two-way ANOVA followed by Student-Newman-Keuls test showing significant differences for treatment: **(A)** [$F(2,61) = 48.23; p < 0.001$], **(B)** [$F(2,83) = 27.70; p < 0.001$]. # $p < 0.001$ vs. naïve; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Park group. No statistically significant differences among genotypes were found in any of the three conditions tested, naïve, parkinsonian or dyskinetic. $n=6-9$.

Figure 5. DREAM expression in striatum and substantia nigra. **(A)** Representative high-resolution striatal confocal images of naïve BAC-transgenic D1R-tomato mice illustrating DREAM (green) expression in D1R-positive (red) and in D1R-negative MSN. HOECHST (blue) stain identifies the nuclei. Arrows indicate examples of dual DREAM- and D1R-positive neurons while arrowheads point to DREAM-positive/D1R-negative neuron. Scale bar = 25 μ m. Histograms show DREAM **(B)** and TH **(C)** mRNAs levels analyzed by RT-qPCR and normalized to 18S mRNA in WT mice. Note that 6-OHDA or/and L-DOPA treatment do not modify DREAM transcripts in striatum or SN. **(C)** The TH levels in SN were quantified to confirm the efficacy of 6-OHDA lesion. Data (mean $\pm$ SEM) are expressed as normalized values and analyzed by one-way ANOVA followed by Student-Newman-Keuls test. [$F(2,17) = 10.54; p < 0.001$]. * $p < 0.01$ vs. WT naïve animals. $n=5-7$.

Figure 6. DREAM overexpression reduces, while its inactivation potentiates FosB, P-Ach3 and dynorphin-B protein expression induced by L-DOPA. (A, C, E) High power photomicrophotographs of unlesioned and lesioned striatum of WT, daDREAM and DREAM^{-/-} mice sacrificed 1 hour after the last L-DOPA injection, immunostained for FosB, P-Ach3 and dynorphin-B. Scale bar = 50 μ m. (B, D, F) Histograms represent the quantification of FosB and P-Ach3 immunoreactive positive nuclei and dynorphin-B immunoreactive area in the unlesioned and lesioned striatum of hemiparkinsonian WT, daDREAM and DREAM^{-/-} mice. Note the higher expression in DREAM^{-/-} compared to WT and the decreased expression in daDREAM mice. Data (mean $\pm$ SEM) representing positive nuclei (B and D) and proportional stained area (F) were analyzed by two-way ANOVA followed by Student-Newman-Keuls test. Significant differences were found for genotype x treatment [$F(2,47) = 30.55$; $p < 0.001$ for FosB; $F(2,41) = 21.67$; $p < 0.001$ for P-Ach3; $F(2,24) = 12.13$; $p < 0.001$ for DynB] * $p < 0.01$, ** $p < 0.001$ vs lesioned WT.

Figure 7. DREAM overexpression inhibits L-DOPA-induced phosphorylation of GluR1 at Ser845. Upper panels show representative autoradiograms of western blots of striatal extracts from sham-operated and hemiparkinsonian, unlesioned (U) and lesioned (L) striatum, WT, daDREAM and DREAM^{-/-} mice chronically treated with 25 mg/kg L-DOPA, incubated with antibodies against P-GluR1 (A) and TH (B). Lower panels show the quantification of western blots in triplicates. (A) Chronic L-DOPA increases GluR1-phosphorylation at Ser 845 in DREAM^{-/-} and WT mice, whereas DREAM overexpression blocks L-DOPA-induced GluR1-phosphorylation. (B) The TH levels were quantified in order to confirm the efficacy of 6-OHDA lesion and no statistically

significant differences were found among genotypes. Relative intensities versus loading control actin (normalized to WT Sham) were analyzed by two-way ANOVA followed by Student-Newman-Keuls test. Significant differences for genotype [$F(2,55) = 3.23$; $p < 0.05$] and for treatment [$F(2,55) = 12.77$; $p < 0.001$] were found. # $p < 0.05$ vs WT lesioned + L-DOPA, * $p < 0.01$, ** $p < 0.001$ vs WT sham-operated mice.

Figure 8. Schematic diagram illustrating the regulatory role of DREAM in the transcriptional and non-transcriptional mechanisms involved in LID. In the nucleus, the DREAM protein regulates dynorphin-B and possibly FosB expression (and subsequent LID) by anchoring to DRE sites located downstream from the transcription initiation site of the DNA. This repression is released under conditions of Ca^{2+} and PKA-dependent interaction with phospho-CREM. In addition, outside the nucleus, DREAM could directly interact with NR1 subunit, inhibiting activation of NMDA receptors, and with PSD-95, impairing its recruitment by D1R and decreasing the cAMP/PKA signaling pathway.

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