UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE MEDICINA

Departamento de Anatomía, Histología y Neurociencia

Mecanismos moleculares que contribuyen al desarrollo de las discinesias en la enfermedad de Parkinson

TESIS DOCTORAL

Presentada por: **Oscar Solís Castrejón**

Director: **Dra. Rosario Moratalla**

Afiliación: **Instituto Cajal, CSIC**

Madrid, 2017

Rosario Moratalla Villalba, Profesor Titular del Instituto Cajal, CSIC, avenida Doctor Arce 37, Madrid.

HACEN CONSTAR: Que Don Oscar Solís Castrejón ha realizado en el departamento de Neurobiología Funcional y de Sistemas del Instituto Cajal, bajo mi dirección, los trabajos correspondientes a la Tesis Doctoral titulada "Mecanismos moleculares que contribuyen al desarrollo de las discinesias en la enfermedad de Parkinson".

Revisado el presente trabajo, considero que la presente memoria reúne todos los requisitos necesarios para ser sometida a juicio de la comisión correspondiente

Para que conste y surta los efectos oportunos, firmo la presente en Madrid a 14 de junio de 2017.

Fdo. Rosario Moratalla Villalba

AVDA. DOCTOR ARCE, 37
28002 MADRID ESPAÑA
TEL.: 91 585 47 50
FAX: 91 585 47 54

Agradezco al Consejo Nacional de Ciencia y Tecnología (CONACYT-México) por la beca que me otorgó para realizar los estudios de Doctorado en la Universidad Autónoma de Madrid (Beca 203775/309450).

También agradezco a la Secretaría de Ciencia, Tecnología e Innovación de la Ciudad de México, por el apoyo otorgado bajo el Convenio SECITI/069/2014.

PRESENTACIÓN DE LA TESIS DOCTORAL COMO COMPENDIO DE PUBLICACIONES

Esta tesis doctoral se presenta como compendio de publicaciones, según la normativa aprobada por la Comisión de Dirección de Doctorado de la Universidad Autónoma de Madrid.

Los artículos que se presentan, son la producción científica de una línea de investigación centrada en el estudio de los mecanismos moleculares asociados a las discinesias inducidas por L-DOPA:

1. **Solís O**, García-Sanz P, Herranz AS, Asensio MJ, Moratalla R (2016) L-DOPA Reverses the Increased Free Amino Acids Tissue Levels Induced by Dopamine Depletion and Rises GABA and Tyrosine in the Striatum. Neurotoxicity Research 30:67–75. © Número de licencia: 4130250578592

2. **Solís O**, García-Montes JR, Garcia-Sanz P, Herranz AS, Asensio MJ, Kang G, Hiroi N, Moratalla R (2017) Human COMT over-expression confers a heightened susceptibility to dyskinesia in mice. Neurobiology of Disease 102:133–139. © Número de licencia: 4130241479596

3. **Solís O**, Espadas I, Del-Bel EA, Moratalla R (2015) Nitric oxide synthase inhibition decreases l-DOPA-induced dyskinesia and the expression of striatal molecular markers in Pitx3(-/-) aphakia mice. Neurobiology of Disease 73:49–59. © Número de licencia: 4130250221234

4. **Solís O**, Garcia-Montes JR, González-Granillo A, Xu M, Moratalla R (2017) Dopamine D3 Receptor Modulates l-DOPA-Induced Dyskinesia by Targeting D1 Receptor-Mediated Striatal Signaling. Cerebral Cortex 27:435–446. © Número de licencia: 4130241131349

ÍNDICE

RESUMEN

La L-DOPA es el fármaco más eficaz para tratar la enfermedad de Parkinson. Sin embargo, su administración crónica y la progresión de la enfermedad, conducen a la aparición de efectos motores adversos como son las discinesias. Resultados de nuestro laboratorio junto con los de otros grupos, han establecido el papel crítico del receptor dopaminérgico D1 (D1R) y el de su vía de señalización intracelular en las discinesias, de manera que modificaciones pre o postsinápticas a esta vía modifican el desarrollo de las mismas. Sin embargo, debido a la complejidad de las interacciones del sistema dopaminérgico con otros sistemas, muchos de estos mecanismos aún siguen siendo desconocidos. En esta tesis, se han establecido algunos de estos mecanismos gracias al estudio de los siguientes aspectos: 1) la interacción de la dopamina con los principales sistemas de neurotransmisión (glutamato y GABA) mediante HPLC; 2) el metabolismo de la dopamina con animales que sobrexpresan la catecol-*O*-metil transferasa (COMT); 3) la actividad sináptica de las neuronas de proyección dopaminoceptivas mediante la manipulación del sistema nitrérgico, y 4) la posible modulación del receptor D3 (D3R) en los efectos mediados por el D1R utilizando ratones transgénicos knockout para el D3.

Nuestros resultados demuestran que los ratones discinéticos tienen una desregulación en los niveles estriatales de diferentes aminoácidos neuroactivos como GABA y tirosina. Además, destacamos la importancia de varias dianas moleculares que pueden regular las discinesias inducidas por L-DOPA. Mostramos que, la sobrepresión genética de la COMT incrementa las discinesias y sus marcadores moleculares, posiblemente debido al mayor metabolismo de la dopamina y al incremento de 3-MT en el estriado. Por otro lado, mostramos que el sistema nitrérgico está implicado en las discinesias, ya que la inhibición de la síntesis del óxido nítrico las alivia. Finalmente, demostramos que la L-DOPA induce una expresión aberrante del D3R en el estriado y que la inactivación de este receptor disminuye las discinesias y sus determinantes moleculares, al modular la vía de señalización del D1R. Nuestro trabajo contribuye a una mejor comprensión de los mecanismos que subyacen a las discinesias inducidas por L-DOPA, y además, sugieren dianas terapéuticas que pueden modular principalmente la señalización del receptor D1R. Así, la manipulación de varios sistemas de neurotransmisores podría reducir eficazmente las discinesias.

ABSTRACT

L-DOPA is the most effective treatment of Parkinson's disease available today; however, chronic L-DOPA administration and the progression of the disease lead to side motor effects, such as dyskinesia. Results from our laboratory and others have established the critical role of the dopamine D1 receptor and its intracellular signaling pathway in dyskinesia, so that pre- or post-synaptic modifications of this pathway could modulate dyskinesia. However, because of the complexity of the interactions of the dopaminergic system with other systems, many of these mechanisms remain unknown. In this thesis, we studied some of these mechanisms by studying the following aspects: 1) the interaction of dopamine with major neurotransmitter systems (glutamate and GABA) by HPLC; 2) the metabolism of dopamine in animals that over-express COMT; 3) the synaptic activity of dopaminoceptive projection neurons by manipulation of the nitrergic system; and 4) the possible modulation of the D3 receptor in the effects mediated by the D1 receptor by using D3 receptor knockout mice.

Our results demonstrate that dyskinetic mice have a striatal dysregulation of different neuroactive amino acids, such as GABA and tyrosine. In addition, we highlight the importance of several molecular targets that can regulate L-DOPA-induced dyskinesia. We show that the genetic overexpression of catechol-*O*-methyl transferase increases dyskinesia and L-DOPA-induced molecular markers, possibly due to the greater metabolism of dopamine and increased 3-MT in the striatum. On the other hand, we show that the nitrergic system is involved in dyskinesia, since the inhibition of nitric oxide synthesis relieves them. Finally, we demonstrate that L-DOPA induces aberrant D3 receptor expression in the dorsal striatum whereas the genetic inactivation of the D3 receptor decreases dyskinesia and L-DOPA-induced molecular markers by modulating D1 receptor signaling. Our work contributes to a better understanding of the mechanisms underlying L-DOPA-induced dyskinesia and also suggests therapeutic targets that can modulate D1 dopaminergic receptor signaling. Thus, manipulation of various neurotransmitter systems could effectively reduce dyskinesia.

INTRODUCCIÓN

Sistema dopaminérgico mesencefálico

La dopamina es un neurotransmisor perteneciente a las catecolaminas que está ampliamente distribuido en el sistema nervioso central. Los grupos dopaminérgicos del mesencéfalo, núcleo rojo (A8), sustancia nigra compacta (SNc, A9) y área tegmental ventral (ATV, A10), forman las vías dopaminérgicas nigroestriatal y mesocorticolímbica (Gerfen & Bolam, 2017) (Fig. 1). A través de estas vías, la dopamina participa en varias funciones fisiológicas como: el control del movimiento voluntario, emociones, aprendizaje, memoria y recompensa (Björklund & Dunnett, 2007; Beaulieu *et al.*, 2015). Además, las alteraciones en la transmisión dopaminérgica se han relacionado con diferentes trastornos neurológicos como son la enfermedad de Parkinson (EP), la enfermedad de Huntington, la esquizofrenia y la drogadicción (Rangel-Barajas *et al.*, 2015).

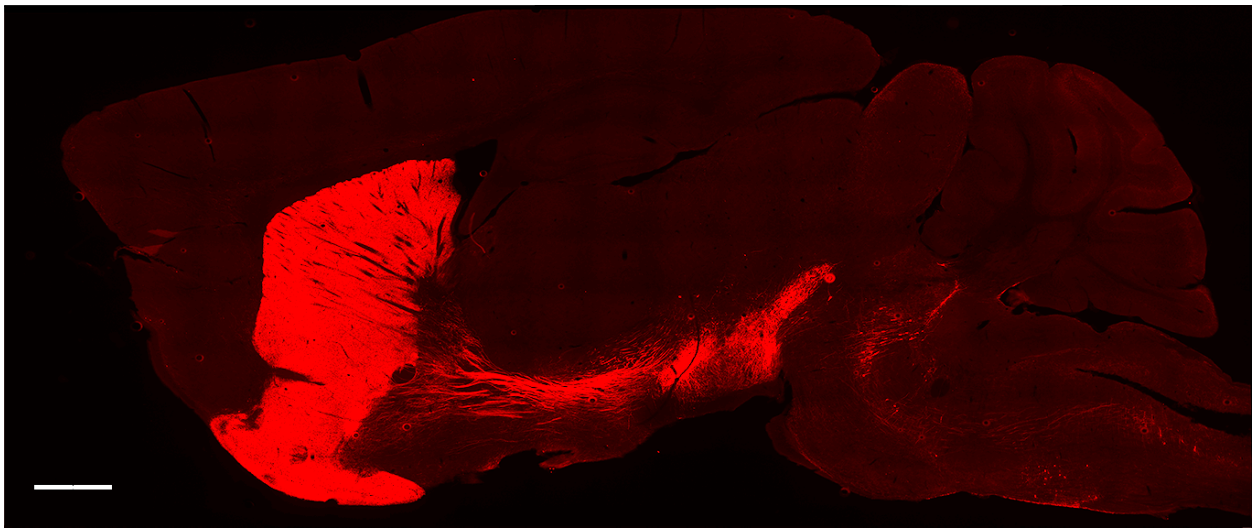


Figura 1. Vía nigroestriatal. Sección sagital de cerebro de ratón teñida para la tirosina hidroxilasa. Barra de calibración = 1000 μm.

En las neuronas dopaminérgicas, la dopamina se sintetiza a partir de la tirosina en una reacción enzimática de dos pasos. Primero, la tirosina se convierte en L-3,4-dihidroxifenilalanina (L-DOPA) por la tirosina hidroxilasa (TH) en el citosol. Segundo, la L-DOPA se metaboliza a dopamina por la DOPA descarboxilasa (AADC), y es transportada a los compartimentos vesiculares. Tras el impulso nervioso, la dopamina vesicular se libera al espacio sináptico para activar los receptores dopaminérgicos, y la dopamina restante es

recaptada por la terminal presináptica o por las células gliales con ayuda de los transportadores de dopamina. Una vez recaptada, la DA puede ser metabolizada o transportada a las vesículas para su almacenamiento y reutilización (Chinta & Andersen, 2005) (Fig. 2).

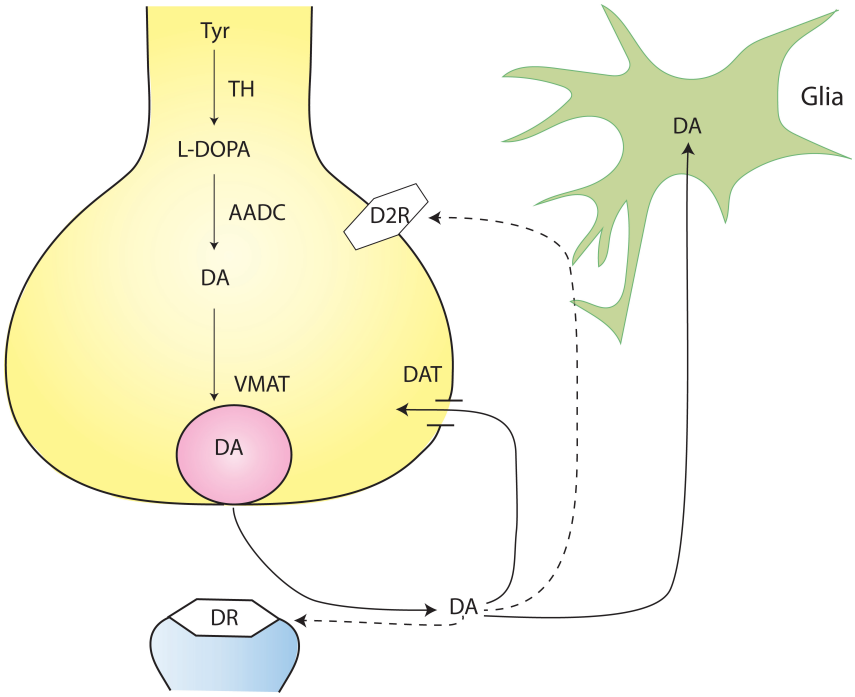


Figura 2. Terminal presináptica dopaminérgica. Esquema de la síntesis, almacenamiento, liberación y recaptación de la dopamina. AADC, dopa descarboxilasa; DA, dopamina; DAT, transportador de de dopamina; DR, receptor dopaminérgico; D2R, receptor dopaminérgico D2; Tyr, Tirosina; TH, tirosina hidroxilasa; VMAT, transportador de monoaminas vesicular;. Modificado de Gerfen & Bolam, *2017*.

La dopamina ejerce sus efectos a nivel presináptico y/o postsináptico, a través de la activación de los receptores dopaminérgicos. Estos receptores están clasificados en dos familias en base a su estructura, farmacología y sus cascadas de señalización. La familia D1 incluye los receptores D1 y D5, que se expresan en la membrana postsináptica y están acoplados a una proteína G_s que estimula la adenilato ciclasa y la producción de adenosín monofosfato cíclico (AMPc). La familia D2 incluye los receptores D2, D3 y D4, estos se encuentran tanto en la membrana presináptica como en la postsináptica, y están acoplados a una proteína G_i que provoca la inhibición de la adenilato ciclasa y la producción de AMPc (Rivera *et al.*, 2002, 2003; Beaulieu & Gainetdinov, 2011). Los receptores dopaminérgicos se expresan abundantemente en los ganglios basales y, en menor densidad, en otras áreas cerebrales relacionadas (Tabla 1).

	Familia D1		Familia D2		
	D1R	**D5R**	**D2R**	**D3R**	**D4R**
Localización	Estriado dorsal, estriado ventral, tubérculo olfatorio, tálamo, corteza frontal	Estriado dorsal, estriado ventral, hipocampo, corteza cerebral (baja expresión en todas las áreas)	Estriado dorsal, estriado ventral, tubérculo olfatorio, corteza cerebral (baja expresión)	Estriado ventral, tubérculo olfatorio, islas de Calleja, estriado dorsal (baja expresión)	Estriado dorsal, núcleo subtalámico, amígdala, hipocampo, hipotálamo (baja expresión en todas las áreas)
Respuesta	Incrementa adenilato ciclasa	Incrementa adenilato ciclasa	Disminuye adenilato ciclasa	Disminuye adenilato ciclasa	Disminuye adenilato ciclasa
Ki (nM) Dopamina	2340	228	1705	27	450

Tabla 1. Familias y subtipos de receptores dopaminérgicos. Elaborado a partir de Rivera *et al.*, 2002, 2003; Beaulieu & Gainetdinov, 2011.

Organización de los Ganglios Basales

Los ganglios basales son un conjunto de estructuras subcorticales formados principalmente por el núcleo estriado (núcleos caudado y putamen), núcleo accumbens, globo pálido externo (GPe) e interno (GPi), núcleo subtalámico (NST), sustancia nigra reticulada (SNr) y sustancia nigra compacta (SNc). Estas estructuras se interconectan entre sí para constituir una organización muy compleja, la cual desempeña un papel básico en el control de la conducta motora y en patologías como la enfermedad de EP (Albin *et al.*, 1989; Gerfen & Bolam, 2017) (Fig. 3).

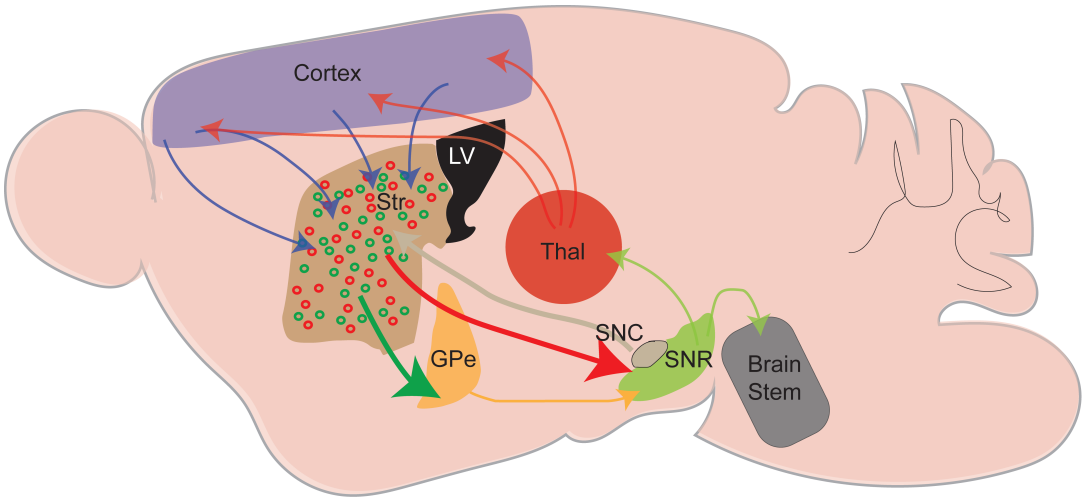


Figura 3. Esquema de los ganglios basales. Esquema de un corte sagital de ratón en donde se muestra el circuito de los ganglios basales. Str: estriado; GPe: globo pálido externo; SNc: Sustancia nigra compacta; SNr: sustancia nigra reticulada; Thal: tálamo. Modificado de Gerfen & Bolam, *2017*.

El principal núcleo de entrada de los ganglios basales es el estriado, que está compuesto principalmente por neuronas espinosas medianas de proyección (constituyendo el 95-97% de la población neuronal), mientras que el 3-5% restante de las neuronas estriatales está representado por interneuronas, que pueden ser clasificadas en interneuronas colinérgicas e interneuronas gabaérgicas. Las neuronas colinérgicas liberan acetil colina como neurotransmisor y son las únicas neuronas tónicamente activas del estriado. Las interneuronas gabaérgicas pueden ser divididas en: 1) parvalbumina positivas, 2) calretinina positivas, 3) somatostatina, neuropeptido Y y óxido nítrico sintasa positivas y 4) tirosina hidroxilasa positivas (Darmopil *et al.*, 2008; Tepper & Koós, 2017).

Las neuronas espinosas medianas de proyección utilizan al ácido γ-amino butírico (GABA) como neurotransmisor y pueden ser divididas en dos clases: las neuronas estriatonigrales que dan lugar a la vía directa (dSPN; del inglés direct striatal projection neurons), y las neuronas estriatopalidales que forman la vía indirecta (iSPN; del inglés indirect striatal projection neurons). Las primeras expresan el receptor dopaminérgico D1 (D1R), mientras que las segundas expresan el receptor dopaminérgico D2 (D2R) (Plenz & Wickens, 2017) (Fig. 4). Aunque el D1R se expresa mayoritariamente en las neuronas de la vía directa y el D2R en las de la vía indirecta, el 2% de las neuronas espinosas medianas de proyección expresan los dos tipos de receptores (Gagnon *et al.*, 2017).

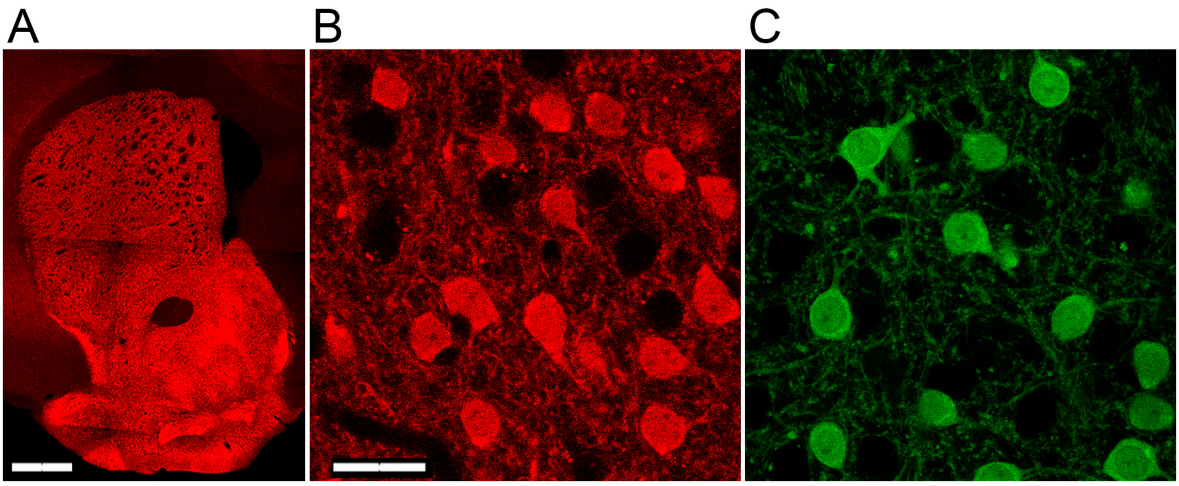


Figura 4. Neuronas espinosas medianas de proyección. Fotografías de un corte coronal de un ratón D1R-tomato, que muestra la expresión del D1R en las neuronas de la vía directa en el estriado (A y B). Fotografía de un corte coronal de un ratón D2R-GFP, que muestra la expresión del D2R en las neuronas de la vía indirecta en el estriado (C). Barras de calibración para las fotografías de bajo y alto aumento son de 500 μm y 25 μm respectivamente.

La vía dopaminérgica nigroestriatal regula la actividad de las vías directa e indirecta al modular la estimulación glutamatérgica proveniente principalmente de la corteza (Gerfen & Surmeier, 2011). Las vías directa e indirecta llevan información hacia los núcleos de salida de los ganglios basales mediante fibras inhibitorias GABAérgicas. Las neuronas de la vía directa envían sus fibras a la SNr y al GPi, mientras que las de la vía indirecta las mandan al GPe, que a su vez envía fibras inhibitorias al NST y éste conecta con fibras excitatorias glutamatérgicas con los núcleos de salida. Finalmente, el GPi/SNr envía proyecciones inhibitorias hacia el tálamo, que proyecta vías excitatorias a áreas corticales. El modelo clásico de los ganglios basales sugiere que la vía directa facilita el movimiento voluntario, mientras que la vía indirecta lo suprime (Albin *et al.*, 1989; Bateup *et al.*, 2010; Kravitz *et al.*, 2010) (Fig. 5), sin embargo, experimentos con optogenética indican que ambos tipos de neuronas deben excitarse para que se produzca el movimiento (Cui *et al.*, 2013; Jin *et al.*, 2014).

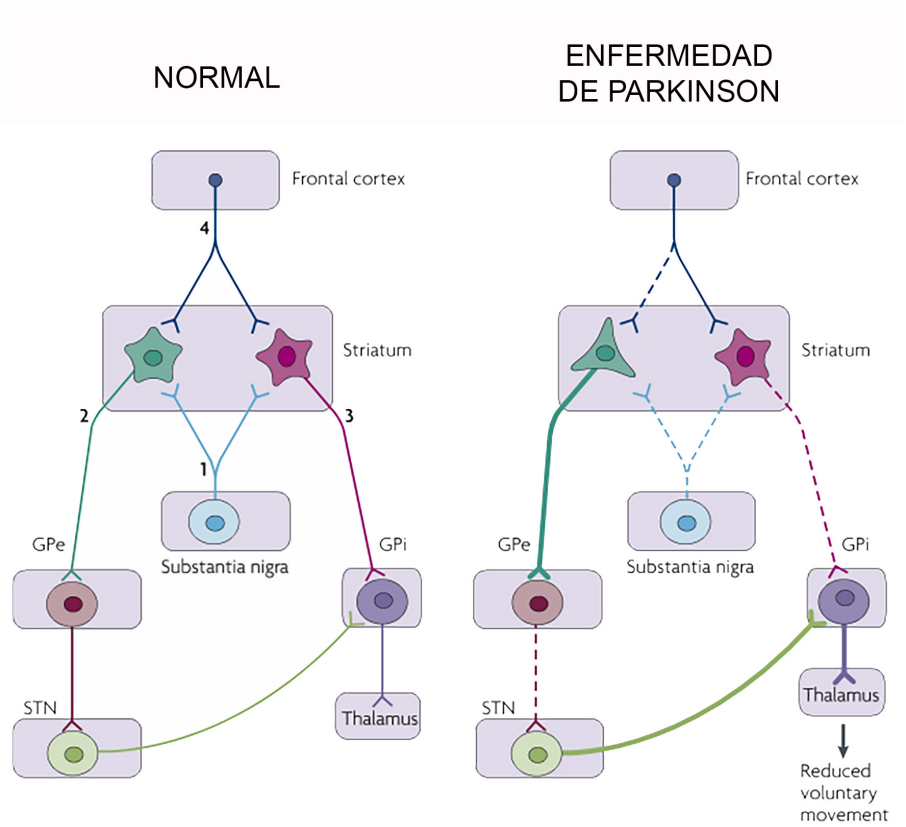


Figura 5. Esquema simplificado del módelo clásico de los ganglios basales en un individuo normal y un paciente con la EP. En el estriado se originan la vía directa e indirecta. El grosor de las proyecciones indica el grado de actividad de la proyección. Las proyecciones discontinuas indican que la proyección es hipoactiva. Modificado de *Jenner, 2008*.

Enfermedad de Parkinson

Hace ahora doscientos años, en 1817, James Parkinson realizó la primera descripción detallada de la enfermedad que lleva su nombre, en su monografía "Ensayo sobre la parálisis agitante" (Parkinson, 2002). La enfermedad de Parkinson (EP) es un trastorno neurodegenerativo, severo e incapacitante, siendo uno de los trastornos neurológicos de mayor incidencia a nivel mundial, ya que afecta al 1-2% de la población mayor de 60 años (Hirsch *et al.*, 2016). La prevalencia en Europa es de más de 1,2 millones de personas, mientras que en España se estima que existen al menos 160.000 personas que la padecen (Andlin-Sobocki *et al.*, 2005; Peñas *et al.*, 2015). Debido a que la edad es el principal factor de riesgo (Collier *et al.*, 2011), y a que la población de adultos mayores de 60 años y la esperanza de vida van en aumento, también aumentará el número de personas que sufren la EP (Hirsch *et al.*, 2016). En la actualidad, no existe cura para esta enfermedad y su progresión hace que la calidad de vida del paciente se vea deteriorada.

La EP se caracteriza por una pérdida progresiva de las neuronas dopaminérgicas de la SNc (Ehringer & Hornykiewicz, 1960; Andén *et al.*, 1966), y por la aparición de agregados proteicos que contienen α-sinucleina, entre otras muchas proteínas, llamados cuerpos de Lewy (Goedert *et al.*, 2013). La muerte de las neuronas de la SNc produce una disminución en los niveles de dopamina en el núcleo caudado y putamen (Ehringer & Hornykiewicz, 1960; Hornykiewicz, 1963). Esta disminución de dopamina en el núcleo caudado y putamen (estriado) está relacionada con las alteraciones motoras que ocurren en la EP. Los síntomas motores cardinales son: temblor en reposo, rigidez muscular, bradicinesia e inestabilidad postural, y se ha demostrado que estos síntomas motores aparecen cuando la muerte de las neuronas de la SNc sobrepasa el 60% (Fearnley & Lees, 1991; Kalia & Lang, 2015). Adicionalmente a los síntomas motores aparecen alteraciones en la olfacción, daños cognitivos, desordenes del sueño, disfunciones autonómicas, síntomas psiquiátricos, dolor y fatiga (Kalia & Lang, 2015).

Existen varios mecanismos que han sido implicados en la muerte de las neuronas dopaminérgicas, como son: el estrés oxidativo, la disfunción mitocondrial, agregación

proteica, autofagia, neuroinflamación y excitotoxicidad (Dauer & Przedborski, 2003). La falta de estas neuronas de la SNc produce alteraciones morfológicas y funcionales en las neuronas estriatales de proyección (Solis *et al.*, 2007; Suárez *et al.*, 2014; Suarez *et al.*, 2016; Moratalla *et al.*, 2017). La falta de dopamina produce un desequilibrio entre las dos vías, promoviendo la hiperactividad de la vía indirecta, lo que produce acinesia y depresión de la vía directa. Esto lleva a su vez a la pérdida del movimiento voluntario (Albin *et al.*, 1989, 1995; Grace, 2008) (Fig. 5). Por tanto, los efectos funcionales de este desequilibrio van en el mismo sentido, dificultando el movimiento.

Tratamiento de la enfermedad de Parkinson

En 1957, Carlsson y colaboradores demostraron que la L-DOPA era capaz de aliviar el parkinsonismo inducido por reserpina en modelos animales (Carlsson *et al.*, 1957). Birkmayer y Hornykiewicz reportaron que la inyección intravenosa de L-DOPA reducía de forma significativa la acinesia que caracteriza a la EP (Birkmayer & Hornykiewicz, 1961; Lees *et al.*, 2015). Posteriormente, Cotzias y colaboradores publicaron que la administración crónica de dosis altas de L-DOPA mejoraba significativamente los síntomas motores de la EP (Cotzias *et al.*, 1967). Actualmente, la L-DOPA continúa siendo el tratamiento de elección de esta enfermedad. Este fármaco se administra con inhibidores de la DOPA descarboxilasa, como son la carbidopa y la benserazida, que bloquean la conversión de L-DOPA a dopamina en la periferia. A diferencia de la dopamina, la L-DOPA atraviesa la barrera hematoencefálica y accede al cerebro, en donde puede ser descarboxilada y almacenada en las vesículas sinápticas de las neuronas dopaminérgicas supervivientes o de las neuronas serotonérgicas para su posterior liberación (Jenner, 2008; Prashanth *et al.*, 2011). Otra de las estrategias para paliar los síntomas motores de la enfermedad se basa en la administración de agonistas dopaminérgicos directos. Sin embargo, estos tienen un menor beneficio motor comparados con la L-DOPA. Los agonistas dopaminérgicos se usan frecuentemente como terapia inicial en pacientes jóvenes (<50 años), para así retrasar el tratamiento con L-DOPA (Obeso *et al.*, 2000; Rascol et al., 2001, 2006; Huot *et al.*, 2013;).

Discinesias inducidas por L-DOPA

El tratamiento con L-DOPA resulta muy efectivo durante los primeros años y debido a esto, este periodo se le conoce como luna de miel. Sin embargo, después de 5-10 años, alrededor del 60% de los pacientes empiezan a desarrollar movimientos involuntarios coreicos y distónicos, conocidos como discinesias y fluctuaciones motoras (Evans *et al.*, 2011). Generalmente, las discinesias suelen aparecer cuando la concentración en cerebro de L-DOPA alcanza la máxima concentración (discinesias de pico de dosis). Las fluctuaciones motoras se describen como la alternancia entre periodos con respuesta adecuada a la medicación (on), y periodos con respuesta deficiente a la medicación (off), y el deterioro de fin de dosis (wearing-off) (Martínez-Lapiscina *et al.*, 2012). Se han propuesto varios factores de riesgo que contribuyen al desarrollo de las discinesias inducidas por L-DOPA, como son: la edad de inicio de la EP, la progresión de la enfermedad, y la administración pulsátil de la L-DOPA (Bastide *et al.*, 2015).

Mecanismos presinápticos relacionados con las discinesias

En condiciones basales, las neuronas dopaminérgicas son capaces de mantener los niveles extracelulares fisiológicos de dopamina. A pesar de que en los pacientes con la EP existe degeneración de estas neuronas, el tratamiento con L-DOPA produce un incremento en los niveles de dopamina, que resulta en un efecto terapéutico. Se ha demostrado que las neuronas serotonérgicas poseen la maquinaria enzimática capaz de convertir la L-DOPA exógena a dopamina. Sin embargo, las neuronas serotonérgicas no expresan proteínas capaces de recaptar la dopamina liberada por estas neuronas, dando como resultado un exceso en los niveles extracelulares de este neurotransmisor (Carta & Bezard, 2011; Mosharov *et al.*, 2015) (Fig. 6). Estos niveles elevados de dopamina estimulan los receptores dopaminérgicos y participan en el desarrollo de las discinesias. Estudios previos, han demostrado que el sistema serotonérgico está implicado en el desarrollo de las discinesias inducidas por L-DOPA, ya que la lesión de las neuronas serotonérgicas evita el desarrollo de este efecto secundario (Carta *et al.*, 2007; Bastide *et al.*, 2015; Beaudoin-Gobert *et al.*, 2015).

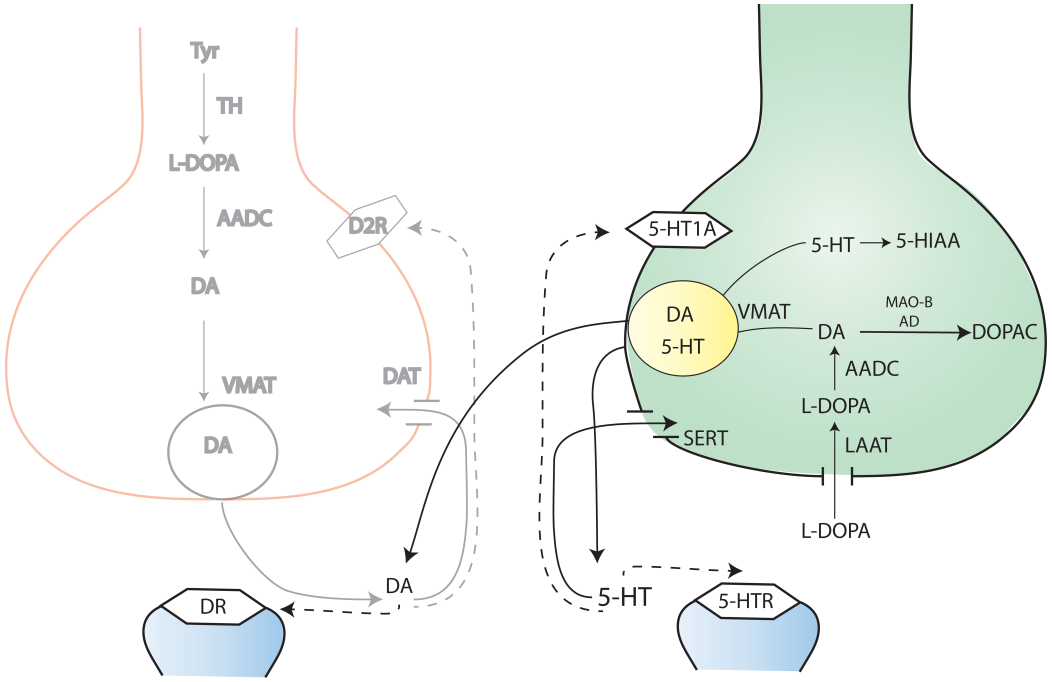


Figura 6. Terminales dopaminérgicas y serotonérgicas en la enfermedad de Parkinson. Las neuronas serotonérgicas captan a la L-DOPA a través del transportador de aminoácidos (LAAT), para su posterior conversión a dopamina y liberación junto a la serotonina (5-HT). Las neuronas no expresan transportadores de dopamina (DAT), lo que provoca un exceso en la dopamina extacelular, después del tratamiento con L-DOPA. 5-HTR, receptor serotonérgico; AADC, dopa descarboxilasa; MAO, monoamino oxidasa; SERT, transportador de serotonina; Modificado de Mosha*rov et al., 2015*.

Alteraciones postsinápticas en las neuronas estriatales

Se ha sugerido que, en el pico de las discinesias, los D1R y D2R están sobrestimulados por la dopamina generada por la L-DOPA, lo que potencia la activación de la vía directa e inhibe la vía indirecta. Esto provoca un decremento en la actividad de los núcleos de salida de los ganglios basales y, en consecuencia, un incremento en la actividad del tálamo, dando lugar a los movimientos involuntarios anormales (Pavón *et al.*, 2006; Jenner, 2008; Darmopil *et al.*, 2009; Alcacer *et al.*, 2017). Estos resultados también se han corroborado con estudios farmacológicos que muestran que tanto los agonistas dopaminérgicos D1 como los D2 inducen discinesias, aunque las generadas por los primeros, son más severas que las provocadas por los agonistas D2 (Rascol *et al.*, 2001, 2006).

Trabajos realizados en nuestro laboratorio han demostrado que el D1R, y no el D2R, es crítico para el desarrollo de las discinesias (Darmopil *et al.*, 2009), y que, el substra-

to anatómico de las discinesias son las neuronas estriatales completamente denervadas (Pavón *et al.*, 2006). Además, hemos demostrado que los cambios moleculares inducidos por la L-DOPA, pertenecen a la cascada de señalización del D1R y se expresan de manera aberrante en las neuronas denervadas de la vía directa tras el tratamiento de L-DOPA (Murer & Moratalla, 2011). La activación del D1R produce una sobreactivación de la proteína cinasa A (PKA, del inglés protein kinase A), que fosforila la fosfoproteína neuronal regulada por dopamina y AMPc (DARPP-32, del inglés dopamine and cAMP-regulated phosphoprotein of 32 kDa), y la proteína de unión al elemento de respuesta a AMPc (CREB), el cual induce la expresión de genes de expresión temprana como el FosB. Diferentes estudios han demostrado que la expresión aberrante de DARPP-32 fosforilado y FosB se correlacionan con la severidad de las discinesias (Andersson *et al.*, 1999; Pavón *et al.*, 2006; Santini *et al.*, 2007). Por otro lado, la estimulación del D1R también activa la vía de las cinasas activadas por una señal extracelular (ERK, del inglés extracellular signal-regulated kinases), que en condiciones de discinesias está incrementada, provocando un incremento en el ERK fosforilado (pERK), que a su vez fosforila a la histona 3 (pAcH3, del inglés phospho-acetylated-histone H3) (Pavón *et al.*, 2006; Darmopil *et al.*, 2009; Santini *et al.*, 2009). La pAcH3 en las neuronas de la vía directa está involucrada en los cambios en la expresión de genes relacionados con las discinesias, como es el aumento aberrante FosB (Ruiz-DeDiego *et al.*, 2015; Feyder *et al.*, 2016).

La activación anormal de la cascada de señalización del D1R en las neuronas de la vía directa del estriado se correlaciona con la aparición de las discinesias (Pavón *et al.*, 2006; Murer & Moratalla, 2011). Por este motivo, la disminución de los diferentes marcadores moleculares asociados a las discinesias (FosB, pAcH3 y pERK), disminuye el desarrollo de las discinesias inducidas por L-DOPA (Westin *et al.*, 2007; González-Aparicio & Moratalla, 2014; Engeln *et al.*, 2016).

Aminoácidos

Estudios recientes han demostrado que la falta de dopamina altera los niveles plasmáticos de los aminoácidos, y esta alteración puede contribuir a los síntomas motores

y no motores de la EP (Yuan *et al.*, 2013; Tong *et al.*, 2015; Zhang *et al.*, 2016). En el sistema nervioso central, el glutamato, GABA, glicina, taurina, aspartato y glutamina son aminoácidos neuroactivos que participan directamente en la transmisión sináptica. En el estriado, el glutamato es el responsable de la transmisión excitatoria y GABA de la inhibitoria. El estriado recibe inervación glutamatérgica proveniente principalmente de la corteza y del tálamo, y controla la actividad motora a través de las dSPN y las iSPN que son GABAérgicas (Gerfen & Surmeier, 2011). Además de glutamato y GABA, existen otros aminoácidos que regulan la neurotransmisión estriatal. Por ejemplo, la glicina actúa como un coagonista de los receptores glutamatérgicos de tipo NMDA (Sergeeva & Haas, 2001), y la taurina tiene un papel importante en el control de la inhibición GABAérgica en el estriado, y de la modulación de la actividad excitatoria glutamatérgica (Sergeeva *et al.*, 2007). Por otro lado, cuando se presentan niveles altos de aspartato en el estriado se induce una plasticidad sináptica aberrante (Errico *et al.*, 2008).

Los pacientes con EP presentan alteraciones en los niveles de varios aminoácidos tanto en muestras del fluido cerebroespinal como en suero (Engelborghs *et al.*, 2003). En un modelo animal de la EP se han encontrado alteraciones en los niveles de los aminoácidos neuroactivos en el estriado (Tanaka *et al.*, 1986), aunque estos cambios no se han observado en pacientes (Rinne *et al.*, 1988). No obstante, aún no se conoce si el tratamiento con L-DOPA afecta estos niveles, ni siquiera en el pico de las discinesias, cuando la concentración de L-DOPA en el estriado es máxima. La variación de estos niveles en discinesias nos ayudaría a entender la fisiopatología de las mismas.

Catecol-*O*-metil transferasa

Estudios previos sugieren que el metabolismo de la dopamina tiene un papel importante en el desarrollo de las discinesias (Espinoza *et al.*, 2012). La enzima catecol-*O*-metil transferasa (COMT) está involucrada en la degradación de los neurotransmisores catecolaminérgicos, entre los que se encuentra la dopamina. El gen que codifica la COMT se encuentra en el cromosoma 22, en la banda q11.2 (Grossman *et al.*, 1992), y se expresa principalmente en el cerebro, el hígado, y los riñones (Männistö & Kaakkola, 1999). En el

cerebro, se encuentra principalmente unida a la membrana de la glía y de las neuronas postsinápticas, respecto a las neuronas monoaminérgicas (Schendzielorz *et al.*, 2013). La COMT tiene un papel importante en la regulación de las acciones sinápticas de la dopamina y la L-DOPA, ya que cataliza su *O*-metilación. La dopamina se metaboliza intraneuronalmente a ácido 3,4-dihidroxifenilacético (DOPAC), por la monoamino oxidasa (MAO), y extraneuronalmente a 3-metoxitiramina (3-MT) por la COMT. Posteriormente el DOPAC es convertido a ácido homovanílico (HVA) por medio de la COMT, y la 3-MT a HVA por medio de la MAO (Männistö & Kaakkola, 1999; Espinoza *et al.*, 2012). Por otro lado, en la periferia, la COMT metaboliza la L-DOPA a 3-*O*-metildopa para su excreción (Grossman *et al.*, 1992) (Fig. 7).

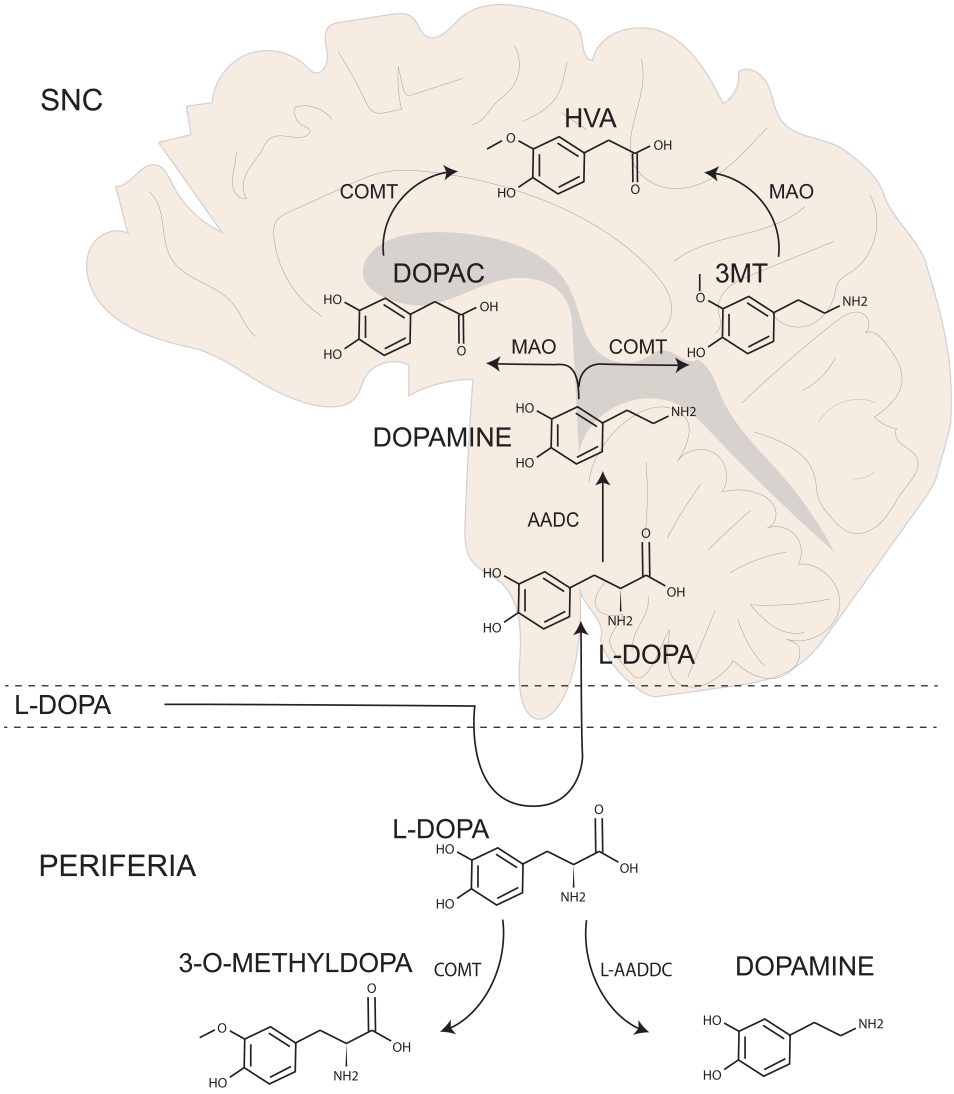


Figura 7. Principales mecanismos neuroquímicos que involucran a la COMT en el metabolismo de la L-DOPA y dopamina, tanto en la periferia como en el sistema nervioso central (SNC). Modificado de Espinoza et al., 2012).

En condiciones fisiológicas, el papel de la COMT en el metabolismo de la dopamina en el estriado es menos importante que en otras áreas como la corteza frontal y el hipotálamo (Gogos *et al.*, 1998). Sin embargo, estudios previos han demostrado que el tratamiento crónico de L-DOPA incrementa la expresión y la actividad de COMT en el estriado (Zhao *et al.*, 2001). Además, los inhibidores de la COMT podrían reducir las discinesias inducidas por L-DOPA, ya que incrementarían el acceso de la L-DOPA al cerebro, y también prologarían la actividad dopaminérgica en el estriado (Müller, 2015). Debido a que actualmente no existe un inhibidor específico de la COMT que pueda determinar el papel de esta enzima en el desarrollo de las discinesias, la utilización de ratones transgénicos de COMT es fundamental para abordar específicamente su estudio (Suzuki *et al.*, 2009).

Óxido nítrico

Trabajos anteriores han mostrado que en la EP, existe un desbalance de diferentes sistemas de transmisores diferentes a la dopamina, entre ellos se ha sugerido al sistema del óxido nítrico (nitrérgico) (Jenner, 2008). El óxido nítrico (ON) es un gas que actúa como mensajero celular y se sintetiza a partir de la L-arginina por medio de la óxido nítrico sintasa (NOS, del inglés nitric oxide synthase). La NOS cuenta con tres tipos de enzima: la neuronal (nNOS), la endotelial (eNOS) y la inducible (iNOS). La nNOS y la eNOS sintetizan ON de manera constitutiva, cuando las células que contienen esta enzima reciben un influjo de Ca^{2+}, mientras que la iNOS lo hace como una respuesta inmunológica (Calabrese *et al.*, 2007). El ON se une a la guanilato ciclasa, e induce la activación de guanosín monofosfato cíclico (GMPc), que es un segundo mensajero que activa diferentes cinasas para producir una respuesta celular (Guix *et al.*, 2005). En los ganglios basales, el ON se sintetiza en las interneuronas nNOS-positivas y participa en la regulación de la transmisión sináptica corticoestriatal. Además, se ha demostrado que el ON modula la conducta motora al interferir con diferentes sistemas de neurotransmisión en el estriado, como son: el dopaminérgico, el glutamatérgico, el colinérgico y el serotonérgico (Del Bel *et al.*, 2005; Park & West, 2009; Sagi *et al.*, 2014).

Cuando el ON se produce en cantidades excesivas, cambia de neuromodulador fi-

siológico a factor neurotóxico (Guix *et al.*, 2005). El sistema del ON podría contribuir a las discinesias ya que el tratamiento con L-DOPA incrementa la producción de ON en modelos animales (Itokawa *et al.*, 2006; Del-Bel *et al.*, 2015), y en pacientes con la EP se ha encontrado un aumento en los niveles séricos de GMPc (Chalimoniuk & Stepień, 2004). Además, estudios realizados en nuestro laboratorio mostraron que la L-DOPA, en ratones lesionados con 6-OHDA, induce la expresión aberrante de FosB en las dSPN y las interneuronas nNOS positivas (Pavón *et al.*, 2006). Por otro lado, estudios realizados en ratas lesionadas con 6-OHDA muestran que la inhibición de la nNOS atenúa las discinesias inducidas por L-DOPA, aunque los mecanismos por los cuales se produce este decremento siguen siendo desconocidos (Padovan-Neto *et al.*, 2009; Takuma *et al.*, 2012).

Receptor dopaminérgico D3

Estudios en modelos animales han implicado al receptor dopaminérgico D3 (D3R) en varios mecanismos que pueden regular a las discinesias inducidas por L-DOPA (Bezard *et al.*, 2003). El D3R pertenece a la familia de los receptores D2. En el sistema nervioso central, se expresa principalmente en las neuronas del estriado ventral, las islas de calleja y el tubérculo olfatorio (Sokoloff *et al.*, 1990; Xu *et al.*, 1997) (Fig 8). No obstante, el patrón de expresión del D3R cambia cuando hay modificaciones en el contenido de dopamina en el estriado. Por ejemplo, tanto en animales lesionados con 6-OHDA como en pacientes con la EP que no han sido tratados con L-DOPA, existe una disminución en los niveles del D3R (Lévesque *et al.*, 1995; Boileau *et al.*, 2009). Los animales lesionados tratados con L-DOPA desarrollan sensibilización conductual y un aumento del D3R en las dSPN del estriado dorsal (Bordet *et al.*, 1997, 2000). Cabe destacar que el aumento del D3R en el estriado dorsal normal mediante infección con partículas adenovirales provoca la aparición de síntomas discinéticos (Cote *et al.*, 2014).

Recientemente, se ha descrito la formación de heterómeros D1R-D3R en ratones discinéticos (Farré *et al.*, 2015). A pesar de que el D3R está acoplado a una proteína G_i, la presencia de estos heterómeros produce una sobreactivación de la vía de señalización dependiente del D1R en las dSPN mediante la amplificación de las vías de señalización

PKA y ERK (Fiorentini *et al.*, 2008; Marcellino *et al.*, 2008; Guitart *et al.*, 2014). Además, la actividad anormal de las dSPN también puede estar relacionada con alteraciones en la internalización del D1R provocado por la interacción con el D3R (Berthet *et al.*, 2009). Se ha descrito que el tratamiento con L-DOPA promueve la formación de heterómeros D1R-D3R, incrementando la señalización dependiente del D1R y contribuyendo al desarrollo de las discinesias (Farré *et al.*, 2015; Fiorentini *et al.*, 2015). Además, estudios previos han mostrado que el tratamiento con antagonistas del D3R atenúan el desarrollo de las discinesias (Bézard *et al.*, 2003; Kumar *et al.*, 2009), sin embargo, otros no encontraron ningún efecto (Mela *et al.*, 2010). Es posible que estos resultados controvertidos sean debidos a que los antagonistas del D3R que se utilizan actualmente no son totalmente selectivos, ya que también se unen al D2R.

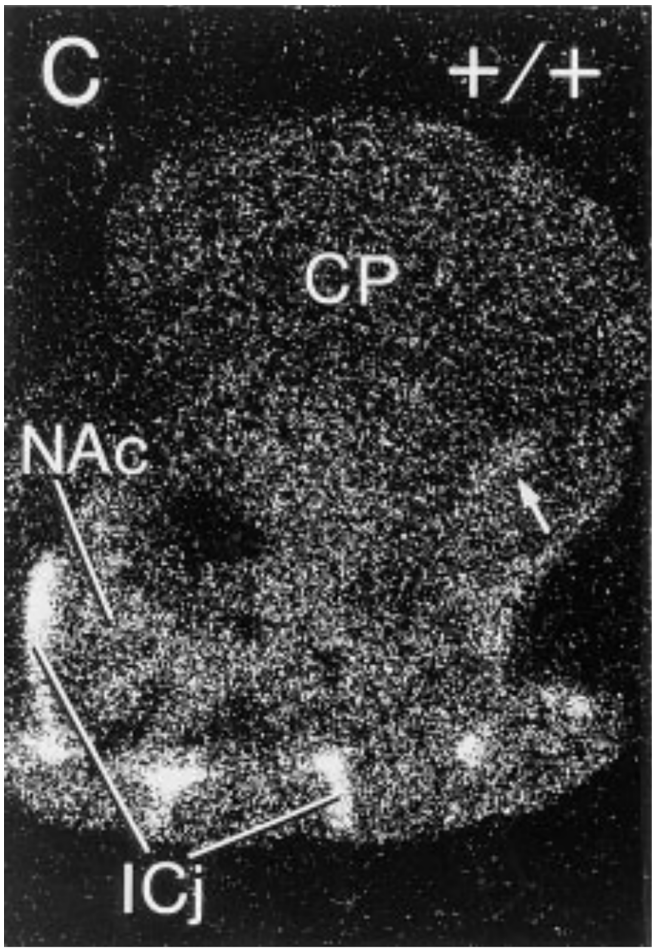


Figura 8. Expresión del receptor dopaminérgico D3 en el estriado. El D3R se expresa principalmente en el núcleo accumbens (NAc) y en las islas de Calleja (ICj), y en menor medida en el estriado dorsal (CP). Modificado de *Xu et al., 1997.*

Modelos animales de las discinesias inducidas por L-DOPA

Debido a que la L-DOPA es muy efectiva para tratar los síntomas motores de la enfermedad de Parkinson, existe la necesidad de encontrar estrategias farmacológicas que eviten la aparición de las discinesias (Iderberg *et al.*, 2012). Para este fin, es indispensable el uso de modelos animales que las desarrollen. Actualmente, los modelos más estudiados y aceptados se basan en la lesión con MPTP en primates (ya que en roedor no se desarrollan las discinesias) o con 6-hidroxidopamina en roedor y en el único modelo genético de la EP (el ratón aphakia) que las desarrolla (Iderberg *et al.*, 2012; Morin *et al.*, 2014).

Modelo de lesión con 6-hidroxidopamina

El modelo de la EP basado en la lesión unilateral con 6-hidroxidopamina (6-OHDA) en roedores fue desarrollado a finales de la década de 1960 (Ungerstedt, 1968). La 6-OHDA no atraviesa la barrera hematoencefálica, por lo tanto, la administración de esta toxina se realiza directamente en el estriado, en el haz prosencefálico medial o en la SNc, lo que provoca la muerte de las neuronas dopaminérgicas y la disminución en los niveles estriatales de dopamina (Herrera-Marschitz *et al.*, 2010). El mecanismo de acción de esta toxina está relacionado principalmente con la formación de H_2O_2 y radicales libres. El modelo de lesión unilateral con 6-OHDA está resultando muy útil en la investigación de nuevos compuestos para tratar la EP (Duty & Jenner, 2011), y para desentrañar los mecanismos moleculares de las discinesias.

El tratamiento crónico con L-DOPA en los roedores lesionados provoca la aparición de discinesias inducidas por L-DOPA (Cenci *et al.*, 1998; Pavón *et al.*, 2006). Los síntomas discinéticos reconocidos en los roedores son: las discinesias axiales, de las extremidades y las orolinguales. Utilizando este modelo se han descrito los mecanismos principales que subyacen a las discinesias, y se han probado diferentes fármacos con potencial efecto antidiscinético (Andersson *et al.*, 1999; Pavón *et al.*, 2006; Santini *et al.*, 2007, 2009; Darmopil *et al.*, 2009; Espadas *et al.*, 2012; González-Aparicio & Moratalla, 2014; Ruiz-DeDiego *et al.*, 2015a, 2015b).

Modelo genético aphakia

Los ratones modificados genéticamente permiten estudiar específicamente mecanismos fisiopatológicos que subyacen a determinadas enfermedades. El ratón aphakia tiene una deficiencia en el factor de transcripción Pitx3 ($Pitx3^{-/-}$) que es crítica para el nacimiento y desarrollo de las neuronas dopaminérgicas de la SNc, dando como resultado una denervación dopaminérgica bilateral en el estriado dorsal, mientras que la parte ventral permanece sin cambios (Hwang, 2003; Nunes *et al.*, 2003; van den Munckhof *et al.*, 2003; Espadas *et al.*, 2012).

El ratón $Pitx3^{-/-}$ desarrolla discinesias bilaterales después del tratamiento con L-DOPA o con agonistas dopaminérgicos, que son atenuadas con agentes antidiscinéticos como la amantadina. Cabe destacar que el ratón Pitx3 es el único modelo genético de la EP que desarrolla las discinesias inducidas por L-DOPA (Duty & Jenner, 2011; Iderberg *et al.*, 2012; Morin *et al.*, 2014). Además, las discinesias en el ratón $Pitx3^{-/-}$ están acompañadas con la aparición de los marcadores moleculares asociados a este efecto secundario motor (Ding *et al.*, 2007, 2011).

HIPÓTESIS Y OBJETIVOS

Hipótesis

1. **El tratamiento crónico con L-DOPA produce un desequilibrio en los niveles estriatales de aminoácidos neuroactivos en ratones lesionados con 6-OHDA**. Estudios previos en modelos animales y en pacientes con la EP, indican que existe una interacción estriatal entre la dopamina y los diferentes sistemas de neurotransmisión, incluyendo los de los aminoácidos GABA y glutamato entre otros. Así, cambios en los niveles de dopamina afectan a estos aminoácidos que participan en la fisiopatología de la EP.

2. **La sobrexpresión genética de COMT aumenta las discinesias inducidas por L-DOPA.** El tratamiento crónico con L-DOPA incrementa la expresión y la actividad de COMT en el estriado disminuyendo la vida media de la dopamina y por tanto aumentando las discinesias. Por otro lado, estudios clínicos con inhibidores selectivos de COMT mejoran los síntomas motores, aunque no arrojan datos concluyentes sobre las discinesias.

3. **La inhibición de la síntesis del óxido nítrico disminuye las discinesias inducidas por L-DOPA en un modelo genético de la enfermedad de Parkinson.** El óxido nítrico regula la transmisión sináptica de las neuronas estriatales de proyección y estudios previos de nuestro laboratorio, demuestran que el tratamiento crónico con L-DOPA aumenta la actividad de las interneuronas NOS positivas en el estriado lesionado y la producción de óxido nítrico en los ganglios basales.

4. **El receptor dopaminérgico D3 regula las discinesias inducidas por L-DOPA a través de la señalización dependiente del receptor dopaminérgico D1**. La L-DOPA aumenta la expresión del receptor D3 en animales discinéticos y la formación de heterodímeros con el receptor D1 (D1R-D3R) potenciando la señalización intracelular dependiente de AMPc y aumentando la expresión de los determinantes moleculares de las discinesias en las neuronas D1-positivas.

Objetivos

1. Determinar las alteraciones de los niveles estriatales de los aminoácidos neuroactivos y de los que no participan directamente en la transmisión sináptica en animales parkinsonianos y discinéticos.

2. Evaluar el efecto de la sobrexpresión genética de la COMT en el desarrollo de las discinesias en el modelo de 6-OHDA en ratón.

3. Evaluar el papel del sistema nitrérgico en las discinesias inducidas por L-DOPA en el modelo genético Pitx3$^{-/-}$ de la enfermedad de Parkinson.

4. Estudiar la contribución específica del receptor dopaminérgico D3 en el desarrollo de las discinesias inducidas por L-DOPA y su mecanismo de acción.

RESUMEN DE RESULTADOS

Artículo I.

En este artículo hemos abordado el objetivo 1 mediante el estudio de la interacción entre la dopamina y aminoácidos estriatales y explorando su posible implicación en los síntomas motores del Parkinson y de las discinesias. Los niveles estriatales de los aminoácidos se estudiaron por cromatografía líquida de alta eficacia (HPLC, del inglés *high performance liquid chromatography*) en animales hemilesionados con 6-OHDA, tratados o no con L-DOPA, para producir animales parkinsonianos y discinéticos. Encontramos que, los niveles de glutamato y aspartato no cambian ni en el parkinsonismo ni en las discinesias. Sin embargo, la falta de dopamina aumenta los niveles de glutamina, glicina y taurina, mientras que la L-DOPA los restablece, indicando una interacción inversa entre la dopamina y estos aminoácidos. Además, la L-DOPA incrementa significativamente los niveles de GABA y de tirosina en animales discinéticos, lo que concuerda con la actividad aberrante de las neuronas GABAérgicas de la vía directa en discinesias, y con la aparición de neurons TH-positivas en el estriado.

Artículo II

En este artículo hemos abordado el objetivo 2 para estudiar el papel de la COMT en el desarrollo de las discinesias, utilizando ratones que sobrexpresan a esta enzima (Tg-COMT). Observamos que los ratones Tg-COMT tienen síntomas discinéticos más severos que los WT a lo largo de todo el tratamiento, así como niveles más altos de los marcadores moleculares FosB y pAcH3. También encontramos que los animales Tg-COMT tienen menos dopamina y más 3-metoxitiramina (3-MT) que los WT, lo que indicaría un mayor metabolismo de la dopamina. Además, la L-DOPA produce un incremento de 3-MT significativamente mayor en los animales Tg-COMT que en los WT, como cabía esperar. El aumento de discinesias en los ratones Tg-COMT podría deberse a que la 3MT incrementa la vía de señalización del receptor D1 mediante la activación de los receptores asociados a aminas traza (TAAR1) y/o a la disminución de la vida media de la dopamina.

Artículo III

En este artículo hemos abordado el objetivo 3, que consiste en el estudio del papel del sistema nitrérgico que potencia la transmisión sináptica de las neuronas estriatales en las discinesias, y para ello empleamos un modelo genético (ratones Pitx3$^{-/-}$) de la EP. Encontramos que la inhibición de la síntesis de ON mediante el uso de 7-nitroindazole (7-NI), un inhibidor específico de la nNOS, reduce tanto el desarrollo, como las discinesias que ya han sido establecidas previamente. Cabe destacar que el 7-NI no afecta el efecto terapéutico de la L-DOPA. Asimismo, el 7-NI atenúa la expresión de FosB y pAcH3 en los ratones tratados con L-DOPA. Por otro lado, observamos que al aumentar la señalización del óxido nítrico mediante la administración de zaprinast (un inhibidor de la fosfodiesterasa) y molsidomina (un donador de NO), también se reducen las discinesias. Sin embargo, estos efectos interfieren con el efecto terapéutico de la L-DOPA.

Artículo IV

En este artículo hemos abordado el objetivo 4 para estudiar el papel específico del receptor D3R en las discinesias (Solís et al., 2017b), y hemos utilizado ratones knockout del D3R (D3$^{-/-}$) lesionados con 6-OHDA. Observamos que la inactivación genética del D3R atenúa el desarrollo de las discinesias, sin afectar el efecto antiparkinsoniano de la L-DOPA, disminuyendo al mismo tiempo la expresión de los marcadores moleculares asociados a las discinesias. Para descartar que los resultados observados en los ratones D3$^{-/-}$ no se deban a mecanismos de compensación durante el desarrollo, administramos un antagonista del D3R a ratones WT, y encontramos resultados similares a los observados en los ratones D3$^{-/-}$. Por otra parte, mediante el uso de ratones transgénicos que expresan la proteína fluorescente rojo tomate bajo el promotor del D1R (D1R-tomato) o la proteína fluorescente verde bajo el promotor del D2R (D2R-GFP), analizamos la expresión del D3R en el estriado dorsal. Encontramos que en ratones intactos y en parkinsonianos, el D3R se expresa en menos del 2% de las neuronas de proyección, mientras que la L-DOPA aumenta la expresión del D3R al 60% de las neuronas de la vía directa y al 20% en las de la vía indirecta. Finalmente, para ver si el modulador de las discinesias

del D3R se debe a una interacción D1R y D3R, utilizamos ratones heterocigotos para el D1R ($D1^{+/-}$), que muestran menos discinesias que sus WT. Curiosamente, el bloqueo farmacológico del D3R en estos ratones disminuye aún más las discinesias, así como sus marcadores moleculares asociados, sugiriendo que efectivamente hay una interacción directa entre el D1R y D3R.

TRABAJOS PUBLICADOS

L-DOPA Reverses the Increased Free Amino Acids Tissue Levels Induced by Dopamine Depletion and Rises GABA and Tyrosine in the Striatum.

Solís O, García-Sanz P, Herranz AS, Asensio MJ, Moratalla R.

Neurotox Res. 2016; 30:67-75. doi: 10.1007/s12640-016-9612-x.

Neurotox Res (2016) 30:67–75
DOI 10.1007/s12640-016-9612-x

ORIGINAL ARTICLE

L-DOPA Reverses the Increased Free Amino Acids Tissue Levels Induced by Dopamine Depletion and Rises GABA and Tyrosine in the Striatum

Oscar Solís[1,2] · Patricia García-Sanz[1,2] · Antonio S. Herranz[3] · María-José Asensio[3] · Rosario Moratalla[1,2]

Received: 12 November 2015 / Revised: 23 February 2016 / Accepted: 25 February 2016 / Published online: 10 March 2016

Abstract Perturbations in the cerebral levels of various amino acids are associated with neurological disorders, and previous studies have suggested that such alterations have a role in the motor and non-motor symptoms of Parkinson's disease. However, the direct effects of chronic L-DOPA treatment, that produces dyskinesia, on neural tissue amino acid concentrations have not been explored in detail. To evaluate whether striatal amino acid concentrations are altered in peak dose dyskinesia, 6-hydroxydopamine (6-OHDA)-lesioned hemiparkinsonian mice were treated chronically with L-DOPA and tissue amino acid concentrations were assessed by HPLC analysis. These experiments revealed that neither 6-OHDA nor L-DOPA treatment are able to alter glutamate in the striatum. However, glutamine increases after 6-OHDA and returns back to normal levels with L-DOPA treatment, suggesting increased striatal glutamatergic transmission with lack of dopamine. In addition, glycine and taurine levels are increased following dopamine denervation and restored to normal levels by L-DOPA. Interestingly, dyskinetic animals showed increased levels of GABA and tyrosine, while aspartate striatal tissue levels are not altered. Overall, our results indicate that chronic L-DOPA treatment, besides normalizing the altered levels of some amino acids after 6-OHDA, robustly increases striatal GABA and tyrosine levels which may in turn contribute to the development of L-DOPA-induced dyskinesia.

✉ Rosario Moratalla
moratalla@cajal.csic.es

[1] Instituto Cajal, CSIC, Av. Dr. Arce 37, 28002 Madrid, Spain

[2] CIBERNED, Instituto de Salud Carlos III, Madrid, Spain

[3] Servicio Neurobiología, Hospital Universitario Ramón y Cajal, IRYCIS, 28034 Madrid, Spain

Keywords Tyrosine · GABA · Glutamine · Parkinson's disease · Abnormal involuntary movements · Glutamate · Taurine · Glycine

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive motor dysfunction with bradykinesia and akinesia, cognitive decline, and psychiatric symptoms such as depression and mood disorders. PD is caused by the death of dopaminergic neurons in the substantia nigra pars compacta, leading to a dramatic reduction of dopamine (DA) levels in the striatum (Dexter and Jenner 2013; Granado et al. 2013) causing bradykinesia. DA replacement by its precursor L-3,4-dihydroxyphenylalanine (L-DOPA) remains the primary treatment for PD (LeWitt 2015), however, chronic L-DOPA exposure leads to severe motor side effects, including L-DOPA-induced dyskinesia (LID). The mechanisms underlying the development of LID remain largely obscure (Murer and Moratalla 2011), although it has been associated with dysfunction of several neurotransmitter systems, including dopaminergic, glutamatergic, serotonergic, cholinergic, GABAergic, and endocannabinoid signaling (Carta et al. 2007; Darmopil et al. 2009; Mela et al. 2012; González-Aparicio and Moratalla 2014; Lim et al. 2015; Solís et al. 2015b).

A growing body of evidence suggests that altered plasma levels of amino acids may contribute to the motor and non-motor symptoms of PD (Yuan et al. 2013; Tong et al. 2014). In addition to glutamate and GABA, respectively, the major excitatory and inhibitory neurotransmitters, several other amino acids regulate striatal neurotransmission (Bido et al. 2011; Rangel-Barajas et al.

2011; Mela et al. 2012). For example, glycine, besides activating glycine receptors, acts as a co-agonist at NMDA-type glutamate receptors (Sergeeva and Haas 2001). Taurine plays a key role in the control of GABAergic inhibition in the striatum and is involved in the regulation of movement (Sergeeva et al. 2007). Taurine also modulates the excitatory activity of glutamate through regulation of intracellular calcium concentrations (El Idrissi and Trenkner 1999) and causes long-lasting enhancement of synaptic transmission in corticostriatal slices (Chepkova et al. 2002). In addition, the extracellular concentration of the excitatory amino acid aspartate increases in the striatum following electrical or chemical stimulation of the cortex (Parrot et al. 2003), and abnormally high levels of aspartate induce aberrant striatal synaptic plasticity (Errico et al. 2008).

In PD patients, dopamine depletion alters the levels of several amino acids in both serum (Yuan et al. 2013) and cerebrospinal fluid (Engelborghs et al. 2003). Yet, other studies showed no changes in the caudate nucleus or in the temporal cortex of PD patients (Rinne et al. 1988), despite the close relationship between the levels of amino acids in plasma and brain tissue (Bongiovanni et al. 2010). In 6-OHDA-lesioned rats, striatal tissue levels of several amino acids are altered due to dopamine depletion in line with the changers showed in the cerebrospinal samples in PD patients (Tanaka et al. 1986; Lindefors and Ungerstedt 1990). However, it is not known how L-DOPA treatment affects striatal amino acids content in peak dose dyskinesia, when high levels of L-DOPA triggered dopamine turnover and abnormal involuntary movements (Zetterström et al. 1986; Herrera-Marschitz et al. 2010; Del-Bel et al. 2014). The present study measured striatal tissue levels of neuroactive amino acids including glutamate, glycine, aspartate, GABA, and taurine, as well as amino acids that do not directly participate in synaptic transmission (e.g., glutamine, alanine, lysine, and tyrosine) in a mouse model of dyskinesia (Pavón et al. 2006) induced by chronic L-DOPA administration. Changes in levels of these amino acids may have an important role in the aberrant synaptic plasticity that underlies L-DOPA-induced dyskinesia, and to understand the pathophysiology of the disorder.

Materials and Methods

Animals

This study was performed in C57BL/6J mice that were 5–7 months old. The animals were housed with a 12-h light/dark cycle with food and water available ad libitum. All studies were conducted in accordance with the European Union Council Directive (86/609/European Economic Community) and approved by the Consejo Superior de Investigaciones Científicas Ethics Committee.

6-Hydroxydopamine Lesion and L-DOPA Treatment

Surgical procedures used in this study were as previously reported (Suárez et al. 2014; Ruiz-DeDiego et al. 2015a). Mice were placed in a stereotaxic surgery apparatus (Kopf instruments, CA, USA) and anesthetized by isoflurane inhalation (5 % for induction and 2 % for maintenance). Unilateral infusions were made into the dorsal striatum with 2 × 2 µl of 6-OHDA HBr (20 mmol/l, containing 0.02 % ascorbic acid; Sigma-Aldrich, Spain; $n = 24$) at the following coordinates from bregma and dura: anteroposterior (+0.65 mm), lateral (−2.0 mm), and dorsoventral (−4.0 and −3.5 mm). Thirty minutes before the intrastriatal injection of 6-OHDA, mice were injected with desipramine (20 mg/kg, i.p.; Sigma-Aldrich, Spain) to avoid the destruction of noradrenergic neurons. Three weeks after surgery, to inhibit de L-DOPA decarboxylation, mice received daily injections of benserazide (10 mg/kg, i.p.; Sigma-Aldrich, Spain), followed by either L-DOPA (20 mg/kg, i.p.; Sigma-Aldrich, Spain) or saline for 2 weeks. SHAM-lesioned animals were subjected to the stereotaxic surgery but received saline infusion instead of 6-OHDA.

Behavioral Testing

Two weeks after the lesion, mice were assessed for forelimb asymmetry using the cylinder test, as previously described (Espadas et al. 2012). Briefly, SHAM-lesioned and 6-OHDA-lesioned mice were individually placed in a transparent cylinder and videotaped for 3 min. We scored the number of supporting wall contacts made by the mice with the ipsilateral and the contralateral forepaw, relative to the lesion. Data are expressed as percentage of contralateral paw touches to the wall. Motor coordination and balance were evaluated in the rotarod (UgoBasile, Rome, Italy), as previously described (Granado et al. 2008; Solís et al. 2015a). All mice underwent training for one trial on the rotarod (Ugo Basile) at a constant speed (10 rpm) for 10 min. If the mouse fell from the rotarod during this period, it was placed back on. On the test day, animals were evaluated following a uniformly accelerating protocol from 4 rpm to a maximum of 40 rpm over a 5 min period and latency to fall off the rod was measured.

To quantify LID, lesioned mice were randomized to one of the following groups: (i) parkinsonian treated with saline (saline, $n = 16$) or (ii) treated with L-DOPA (dyskinetic; $n = 8$). Each animal was individually videotaped 3 times per week in a transparent cylinder, and their behavior was

analyzed during a 4-min time period at 40 min after the injection of L-DOPA or saline. The videotapes were analyzed for axial (A), limb (L), and orolingual (O) dyskinesia subtypes by a trained observer blind to the treatment of each animal. The rating for dyskinesia was based on a scale ranging from 0 (not present) to 4 (severe) (Suárez et al. 2014; Solís et al. 2015b). The total score represents the sum of the scores for the three dyskinetic subtypes (Sum of ALO score). On day 15, mice were evaluated every 20 min over a period of 180 min after L-DOPA injection. The investigator performing behavioral experiments was blind to lesion condition and treatment.

Amino Acid Quantification

Free tissue levels of amino acids and dopamine were assessed by high-performance liquid chromatography (HPLC), indicating that our results represent the total content, including the extracellular and the intracellular compartments. Mice were sacrificed by decapitation 1 h after the last L-DOPA or saline administration. The brain was quickly removed, and the striatum was dissected on ice. Tissue samples were taken separately from the left and right striatum and frozen at −80 °C until analysis. The amino acids were analyzed using HPLC, as previously described (Perucho et al. 2015). Briefly, striatal tissue was homogenized in 0.4 N perchloric acid for deproteinization. Pellets were used for protein quantification (BCA assay). Supernatants were precolumn derivatized with ortho-phthal-dialdehyde (OPA). The reagent was a mixture of 32 mg OPA in borate buffer 0.4 M pH 9.5 (7140 µl) containing 60 µl of 3-mercaptopropionic acid. The fluorescent derivatized amino acids were separated by a "Ultrasphere ODS Beckman" (150 × 4.6 mm, particle size 5 µm) using gradient elution. Gradients were performed with two degassed mixture solvents. Solvent A was 0.05 M sodium acetate pH 5.88: methanol (90:10), and solvent B was methanol: H2O (70:30). (Gradient profile: time = 0 min % B 2, time = 0.1 min % B 15, time = 1 % B 47, time = 6 % B 100, time = 9 % B 2); at time = 13 the column is ready for a new sample injection. The solvent flow rate was adjusted to 1 ml/min and the injection volume was 10 µl. Fluorescence detection was accomplished with Jasco detector (model FP-2020) at 240 and 450 nm for excitation and emission wavelengths, respectively. Amino acids were identified by their retention times, and their concentrations were calculated by comparison to calibrated amino acid external standard solutions (1.5 µM).

Dopamine Determination

The levels of DA were measured by HPLC with an ESA Coulochem III detector, according to Mena et al. (1984) with minor modifications. Briefly, samples from the same brain region indicated above were sonicated in 8 volumes (w/v) of 0.4 N perchloric acid (PCA) with 0.5 mM $Na_2S_2O_5$ and 2 % EDTA and then centrifuged for 10 min. Tissue dopamine levels were determined from 20 µl of the supernatant. The chromatographic conditions were as follows: a column ACE 5 C18, 150 × 4.6 mm (UK); the mobile phase, a 109.3 mM citrate buffer/1.1 mM acetate buffer, pH 3.55 with 10 % methanol, 1 mM EDTA and 5 mM sodium 1-heptanesulfonate, flow rate 1 ml/min. Dopamine was identified by their retention time, and its amount calculated against a calibrated external standard solution (0.6 µM).

Statistical Analysis

Motor performance and forelimb asymmetry data were analyzed using an unpaired *t* test. Repeated measures ANOVA followed by the Bonferroni post hoc comparison was used to determine the statistical significance of dyskinesia scores over time. The amino acid levels were normalized such that the mean amino acid value of SHAM-lesioned mice was set to 100 %. The amino acid levels of parkinsonian and dyskinetic mice were expressed as percentage of SHAM-lesioned mice. DA and amino acid data were analyzed by a one-way ANOVA followed by Bonferroni post hoc tests. Statistical analysis was performed with GraphPad Prism 5 (La Jolla, CA, USA). Data are expressed as the mean ± standard error of the mean (SEM). The significance level was set at $p < 0.05$.

Results

Cylinder and Rotarod Tests

Motor impairments in the cylinder and rotarod tests were used to behaviorally evaluate the extent of dopamine depletion (Heuer et al. 2012; Espadas et al. 2012; Solís et al. 2015b). In the cylinder test (Fig. 1a), SHAM-lesioned animals did not present any significant asymmetry of forepaw use, as cylinder touches with the contralateral paw were ~50 % of total forepaw touches. In contrast, the dopamine-depleted mice displayed severe forelimb use asymmetry (ca. 20 %; $p < 0.001$). Similarly, lesioned mice exhibited greatly impaired performance in the rotarod task, as their latency to fall (Fig. 1b) was significantly lower than that of SHAM-lesioned mice ($p < 0.001$).

L-DOPA-Induced Dyskinesia

Three weeks after 6-OHDA injection, lesioned mice were chronically injected with saline or L-DOPA.

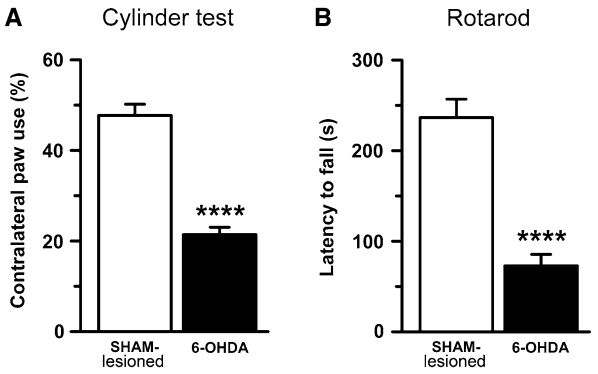


Fig. 1 Mice unilaterally lesioned with 6-OHDA exhibit motor impairment. Hemiparkinsonian mice displayed forelimb asymmetry measured by the cylinder test (**a**). Motor coordination and balance assessed in the rotarod was impaired in the 6-OHDA-injected mice (**b**). $^{****}p < 0.0001$ versus SHAM-lesioned animals (unpaired t test). Data are expressed as the mean ± SEM. $n = 5$–23 for each group

Administration of L-DOPA resulted in a progressive increase in the development of dyskinetic symptoms during the first week of treatment, and reached a plateau in the second week of treatment, we found significant main effects of time and treatment, as well as a significant time and treatment interaction ($p < 0.0001$; $F_{5,70} = 51.22$). Saline-treated hemiparkinsonian mice did not develop dyskinesia (Fig. 2a). The time course analysis showed that L-DOPA-treated mice displayed the highest intensity of dyskinesia between 40 and 60 min post-injection. LID was present for at least 160 min after administering L-DOPA (Fig. 2b). This behavioral pharmacological profile of LID is in register with those published earlier by our group (Darmopil et al. 2009; Suárez et al. 2014; Ruiz-DeDiego et al. 2015a; Solís et al. 2015a, b) and those of others (Mela et al. 2012; Del-Bel et al. 2014).

DA Content in the Striatum

After the completion of all behavioral experiments, brains were analyzed to assess the degree of DA denervation in the lesioned mice (Fig. 3). We measured the total (intracellular and extracellular) DA tissue content in the ipsilateral and contralateral striatum relative to the lesion using HPLC analysis. SHAM-lesioned animals showed no difference in DA levels between hemispheres. However, in accordance with the behavioral results in lesioned animals, we found that 6-OHDA injection induced a severe decrease (91 %) in DA concentration in the ipsilateral striatum compared with the contralateral, or compared with samples from SHAM-lesioned animals. Dyskinetic mice also showed a marked reduction (93 %) of DA tissue content in the ipsilateral striatum. Somewhat surprisingly, the tissue levels of DA in either the contralateral or ipsilateral striatum did not differ between parkinsonian and dyskinetic mice in agreement with Del-Bel et al. (2014). Finally, DA tissue content in the contralateral striatum of parkinsonian and dyskinetic animals was similar to that of the SHAM-lesioned animals.

Amino Acid Levels in the Striatum

As expected, the tissue levels of the amino acids tested in the ipsilateral and contralateral striatum of SHAM-lesioned animals were very similar. Therefore, to reduce the number of animals to the minimum required for valid statistical analysis, the data of the amino acid tissue content in the ipsilateral and contralateral striatum of SHAM-lesioned mice ($n = 6$) were analyzed as a single collective group for statistical analysis to reach $n = 12$. Similarly, because the total (intracellular and extracellular) amino acid tissue content in the contralateral striatum of lesioned animals is

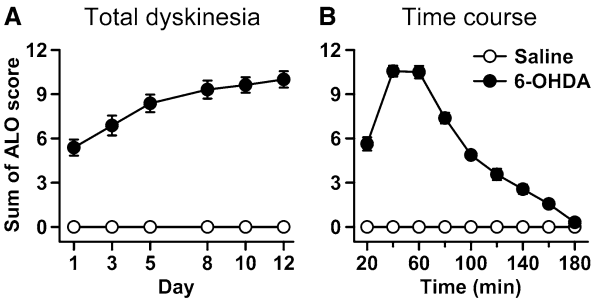


Fig. 2 L-DOPA induces severe dyskinesia in hemiparkinsonian mice. Cumulative axial, limb, and orolingual (ALO) scores were measured 40 min after L-DOPA injection on the indicated days (**a**). On day 15 of L-DOPA treatment, dyskinetic scores were measured every 20 min during 180 min (**b**). Data are expressed as the mean ± SEM. $n = 8$–16 for each group

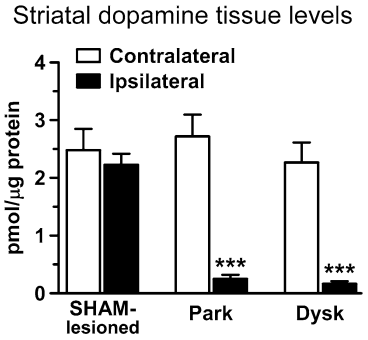


Fig. 3 Dopamine tissue content in the striatum of hemiparkinsonian mice. Both parkinsonian and dyskinetic mice exhibit decreases in dopamine content in the ipsilateral striatum. $^{***}p < 0.001$ versus contralateral striatum (two-way ANOVA followed by a Bonferroni test). Data are expressed as the mean ± SEM. $n = 6$–16 for each group

Neurotox Res (2016) 30:67–75

very similar to that in SHAM-lesioned animals, we only represent the data of the ipsilateral striatum.

Total tissue levels of the excitatory amino acid glutamate in the striatum did not differ significantly in the parkinsonian and dyskinetic groups compared to the control group (Fig. 4a). Then, we also studied glutamine which is involved in the metabolism of glutamate and GABA in neurons and astroglia (Bak et al. 2006). Tissue levels of glutamine significantly increased after dopamine depletion indicating an over-activation of the glutamatergic system by the lack of dopamine ($p < 0.05$). Interestingly, glutamine tissue levels returned to control values with chronic L-DOPA administration ($p < 0.01$) (Fig. 4a). Aspartate levels were not altered either by 6-OHDA or by subsequence treatment with L-DOPA (Fig. 4a), although we observed a slight increase in dyskinetic animals. In term of the inhibitory amino acids, analysis of GABA levels showed no difference between SHAM-lesioned and parkinsonian mice, but showed a significant increase in dyskinetic animals treated with L-DOPA ($p < 0.05$) (Fig. 4b). In addition, glycine and taurine striatal tissue concentration showed a significant increase in parkinsonian compared with SHAM-lesioned mice, and, interestingly, L-DOPA treatment in dyskinetic mice returned these elevated glycine and taurine levels to control values (Fig. 4b).

Like glycine and taurine, lysine increased after dopamine depletion and returned to control values with chronic L-DOPA administration ($p < 0.05$) (Fig. 5). Interestingly, alanine levels, though unchanged after the lesion, were significantly lower in dyskinetic mice compared with SHAM-lesioned animals ($p < 0.05$) (Fig. 5). In contrast, tyrosine striatal tissue concentrations were not changed after dopamine depletion in parkinsonian animals, but were significantly and robustly increased in dyskinetic mice compared with both the SHAM-lesioned and the parkinsonian groups ($p < 0.001$) (Fig. 5).

Discussion

Our results reveal an overall loss of homeostasis in total levels of free neuroactive amino acids in the DA-depleted striatum, in mouse models of PD and LID. Although dopamine denervation with 6-OHDA does not alter glutamate in the striatum, it increases glutamine, indicating an enhanced glutamatergic neurotransmission, while dopaminergic activation with L-DOPA returns glutamine levels to control values, restoring glutamatergic transmission. Accordingly, the inhibitory amino acids glycine and taurine increase following the lesion and decrease after L-DOPA treatment possibly to counteract glutamatergic activity. In addition, we found that GABA tissue levels are increased in dyskinetic animals in agreement with the hyperactivation of direct pathway striatal neurons in this condition. For the non-neuroactive amino acids, alanine is decreased in dyskinetic animals while tyrosine is robustly increased in line with the increase in TH-positive neurons in the striatum after L-DOPA (Espadas et al. 2012). These

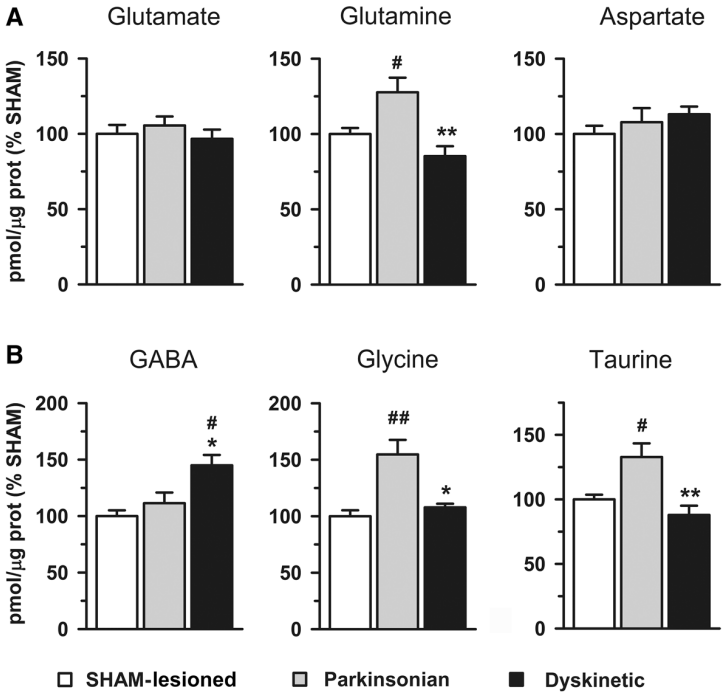


Fig. 4 Effect of chronic L-DOPA treatment on the tissue levels of neuroactive amino acids in the striatum of 6-OHDA-injected mice. Striatal content of glutamate, glutamine, and aspartate (**a**) at peak LID. Striatal content of GABA, glycine, and taurine (**b**) at peak LID. $^{\#\#}p < 0.01$ versus SHAM-lesioned; $^{*}p < 0.05$ versus parkinsonian mice (one-way ANOVA followed by a Bonferroni test). Data are expressed as the mean ± SEM. Striatal content of glutamate (406 pmol/µg protein), glutamine (293 pmol/µg protein), aspartate (90 pmol/µg protein), GABA (106 pmol/µg protein), glycine (35 pmol/µg protein), and taurine (591 pmol/µg protein) in SHAM-lesioned animals. $n = 8$–16 for each group

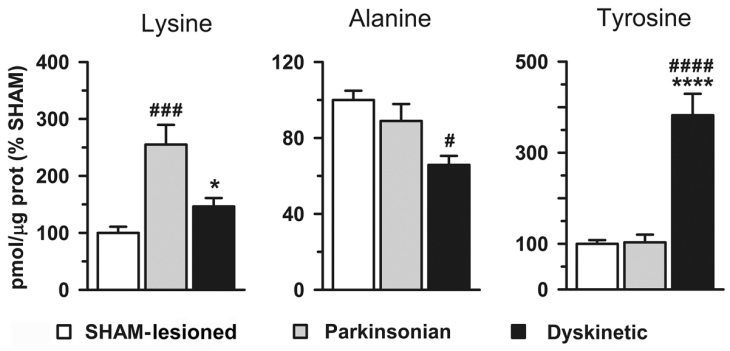


Fig. 5 Effect of chronic L-DOPA treatment on the tissue levels of non-neuroactive amino acids in the striatum of 6-OHDA-injected mice. Striatal content of lysine, alanine, and tyrosine at peak LID. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, and $^{\#\#\#\#}p < 0.0001$ versus SHAM-lesioned; $^{*}p < 0.05$ and $^{****}p < 0.0001$ versus parkinsonian mice (one-way ANOVA followed by a Bonferroni test). Data are expressed as the mean ± SEM. Striatal content of lysine (66 pmol/µg protein), alanine (25 pmol/µg protein), and tyrosine (1.96 pmol/µg protein) in SHAM-lesioned animals. $n = 8–16$

results provide further evidence of the modulation of glutamatergic neurotransmission by dopamine and suggest that dysregulation of amino acid levels of glutamate, glutamine, GABA, glycine, and taurine contributes to the motor deficiencies in PD and LID.

Previous reports have shown that striatal 6-OHDA lesion in mice causes impaired motor performance, indexed as forelimb asymmetry and deficits in the rotarod task. These behaviors are related with dopamine denervation in the striatum and are capable of differentiating a near-complete lesion from a SHAM-lesioned animal (Herrera-Marschitz and Ungerstedt 1984; Heuer et al. 2012; Espadas et al. 2012; Ruiz-DeDiego et al. 2015a). The degree of denervation can predict the development of LID after L-DOPA administration (Darmopil et al. 2009; Smith et al. 2012). The LID model used in this study has been well validated and recapitulates the major clinical symptoms and molecular markers of L-DOPA-induced dyskinesia in PD patients (Pavón et al. 2006). We and others have previously used this model to show that repeated L-DOPA exposure in parkinsonian animals causes long-lasting changes in the striatonigral neurons that are critical for the development of dyskinesia and is associated with the expression of FosB, activation of histone 3, and externally regulated kinase ERK phosphorylation (Pavón et al. 2006; Darmopil et al. 2009; Ruiz-DeDiego et al. 2015a). The role of the striatopallidal pathway in dyskinesia remains uncertain, we demonstrated that the dopamine D2 receptor is not critical for LID (Darmopil et al. 2009). However, L-DOPA selectively restores the number of the dendritic spines in the D2R-striatopallidal neurons (Suárez et al. 2014) that decreases following 6-OHDA lesion (Solis et al. 2007). In addition to alterations in dopaminergic signaling, it has shown disruptions in the glutamatergic, and GABAergic circuitry (Rangel-Barajas et al. 2011; Mela et al. 2012; Engeln et al. 2015). However, only a few previous studies have investigated the amino acid levels.

To study neurotransmission in the striatum, we performed "ex vivo" experiments in striatal homogenates by HPLC (Mena et al. 1984; Perucho et al. 2015). This methodology allows us to determine the total content (extracellular and intracellular) of neurotransmitters, but cannot differentiate between intra or extracellular compartments.

We found no differences in the levels of striatal glutamate in either parkinsonian or dyskinetic mice in agreement with a former study in rats sacrificed 1 month after 6-OHDA-surgery (Tanaka et al. 1986). However, glutamine levels were significantly increased in parkinsonian mice, indicating an increased activation of the glutamatergic neurotransmission suggested by the increased transformation of glutamate into glutamine in dopamine-depleted conditions. In the glutamate/glutamine cycle, glutamate released by neurons is rapidly taken up by astroglia cells that convert it into glutamine by glutamine synthetase, thus, glutamatergic neurotransmission activity can be measured by the metabolism of glutamate into glutamine (Shen 2013). Interestingly, chronic L-DOPA restored the increased levels of glutamine to control values, while glutamate levels were still unchanged, suggesting the restoration of the glutamatergic neurotransmission. These results are in line with a previous work from our lab, using nuclear magnetic resonance spectroscopy in the reserpine mouse model. We showed that dopamine depletion elevates the concentration of cerebral glutamine and L-DOPA treatment reverted the glutamine levels to normal (Rodrigues et al. 2007) without changing glutamate levels. Thus, in dopamine-depleted conditions after 6-OHDA, there is an increase in striatal glutamatergic activity, that it is restored back to normal by L-DOPA. Our results further

confirm a strong inverse interaction between the dopaminergic and the glutamatergic neurotransmission systems in the striatum, possibly via D1 receptors stimulation as suggested by Rodrigues et al. (2007).

We found that striatal GABA was unaltered in the parkinsonian group but significantly increased in dyskinetic mice. It is possible that this higher GABA concentration in the striatum of dyskinetic mice may be due to an increased activation of GABAergic striatal neurons via D1 receptors by repeated exposure to L-DOPA (Darmopil et al. 2009; Ruiz-DeDiego et al. 2015b). In fact, these neurons are hypersensitive and respond to L-DOPA with an aberrant FosB induction (Solis et al. 2015b). Because glutamate levels are not changed, the GABA increase could be achieved at the expense of glutamine through glutamate metabolism. This would be in line with the decrease in glutamine we observed after L-DOPA treatment and with the increased activity of glutamic acid decarboxylase (enzyme that catalyzes the decarboxylation of glutamate to GABA) observed in the hemiparkinsonian rat (Segovia et al. 1991).

Taurine and glycine levels were elevated in the striatum of parkinsonian mice and restored to SHAM-lesioned levels by L-DOPA treatment. To our knowledge, this is the first examination of taurine and glycine levels at peak LID in the striatum. These two amino acids are inhibitory and bind glycine receptors present in the striatum with different affinities (Han et al. 2004). Glycine can also modulate striatal GABAergic transmission (Chepkova et al. 2002) affecting synaptic plasticity in the striatum. Therefore, it is possible that their increase after dopamine depletion responds to a homeostatic mechanism to counteract the increased glutamatergic activity after the dopamine lesion. The decrease after L-DOPA could also be part of this counteracting mechanism since glutamatergic activity returns to normal in dyskinetic animals. In fact, we and others have recently demonstrated that L-DOPA re-establishes the functional corticostriatal connectivity that is lost in PD (Suárez et al. 2014; Fieblinger et al. 2014). Therefore, it is possible that taurine and glycine return to control levels as a consequence of this adaptation.

Another important modulatory amino acid in the striatum is aspartate, which activates the NMDA receptor and facilitates NMDA receptor-dependent synaptic plasticity (Errico et al. 2008). Interestingly, a mouse model with increased levels of aspartate exhibited loss of corticostriatal synaptic depotentiation and a facilitated onset of LID (Errico et al. 2011). Interestingly, Pettersson et al. (1996) showed that D1R stimulation, in the dopamine-depleted striatum, increases the presence of aspartate-immunoreactive interneurons. These results could be related to the slight increase in the content of aspartate we found in dyskinetic mice, although it did not reach statistical significance. It is possible that the levels of aspartate we observed in dyskinetic mice may be a compensatory mechanism to avoid further alterations in glutamatergic transmission.

We also studied the concentration of amino acids that do not directly participate in synaptic transmission such as lysine, alanine, and tyrosine. We found that lysine tissue levels are increased in the parkinsonian mice, but are restored after L-DOPA in dyskinetic mice, however, its role remains elusive. Tyrosine is a precursor of the catecholamine neurotransmitters dopamine and norepinephrine. We found a several-fold increase in the concentration of tyrosine in the striatum of dyskinetic animals compared to the parkinsonian and the SHAM-lesioned mice. This increase is in line with an increase in tyrosine hydroxylase, as demonstrated by the appearance of tyrosine hydroxylase-positive neurons in the striatum (Darmopil et al. 2008; Espadas et al. 2012). Although the role of increased tyrosine levels in LID is currently unknown, previous studies demonstrated that tyrosine depletion influences the release of DA (Le Masurier et al. 2013). It is possible that under dyskinetic conditions, the remaining catecholaminergic neurons increase tyrosine concentration to provide more DA. Alternatively, it may be possible as well that the increase in tyrosine reflects a decrease turnover induced by the chronic treatment with L-DOPA. Nevertheless, the elevated levels of tyrosine in LID suggest that this amino acid could be involved in LID pathophysiology.

In summary, our results show that L-DOPA restores back to normal the increased amino acid levels in the striatum of 6-OHDA-lesioned mice, in line with the synaptic plasticity. Moreover, L-DOPA produces a strong increase in GABA and tyrosine levels that correlate with the hyperactivation of D1R-containing striatal neurons and with the appearance of striatal TH-positive neurons, respectively. These findings advance our understanding of the striatal mechanisms underlying development of dyskinesia and behavioral abnormalities. Further studies are needed to determine the role of these amino acid level changes in LID, and how it affects dopaminergic, serotonergic, and cholinergic systems.

Acknowledgments This work was supported by Grants from the Spanish Ministerios de Economía y Competitividad (SAF2013-48532-R) and of Sanidad Política Social e Igualdad (ISCIII, CIBERNED CB06/05/0055) and Comunidad de Madrid ref. S2011/BMD-2336 to RM. OS has a CONACYT-Mexico doctoral scholarship. We thank Beatriz Pro and Emilia Rubio for technical assistance.

Compliance with Ethical Standards

Conflict of Interest The authors declare no competing financial interests.

References

Bak LK, Schousboe A, Waagepetersen HS (2006) The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. J Neurochem 98:641–653. doi:10.1111/j.1471-4159.2006.03913.x

Bido S, Marti M, Morari M (2011) Amantadine attenuates levodopa induced dyskinesia in mice and rats preventing the accompanying rise in nigral GABA levels. J Neurochem 118(6):1043–1055. doi:10.1111/j.1471-4159.2011.07376.x

Bongiovanni R, Kirkbride B, Newbould E, et al (2010) Relationships between large neutral amino acidlevels in plasma, cerebrospinal fluid, brain microdialysate and brain tissue in the rat. Brain Res 1334:45–57. doi:10.1016/j.brainres.2010.03.111

Carta M, Carlsson T, Kirik D, Björklund A (2007) Dopamine released from 5-HT terminals is the cause of L-DOPA-induced dyskinesia in parkinsonian rats. Brain 130:1819–1833. doi:10.1093/brain/awm082

Chepkova AN, Doreulee N, Yanovsky Y et al (2002) Long-lasting enhancement of corticostriatal neurotransmission by taurine. Eur J Neurosci 16:1523–1530

Darmopil S, Muñetón-Gómez VC, de Ceballos ML, Bernson M, Moratalla R (2008) Tyrosine hydroxylase cells appearing in the mouse striatum after dopamine denervation are likely to be projection neurones regulated by L-DOPA. Eur J Neurosci 27:580–592. doi:10.1111/j.1460-9568.2008.06040.x

Darmopil S, Martín AB, De Diego IR et al (2009) Genetic inactivation of dopamine D1 but not D2 receptors inhibits L-DOPA–induced dyskinesia and histone activation. Biol Psychiatry 66:603–613. doi:10.1016/j.biopsych.2009.04.025

Del-Bel E, Padovan-Neto FE, Szawka RE et al (2014) Counteraction by nitric oxide synthase inhibitor of neurochemical alterations of dopaminergic system in 6-OHDA-lesioned rats under L-DOPA treatment. Neurotox Res 25:33–44. doi:10.1007/s12640-013-9406-3

Dexter DT, Jenner P (2013) Parkinson disease: from pathology to molecular disease mechanisms. Free Radic Biol Med 62:132–144. doi:10.1016/j.freeradbiomed.2013.01.018

El Idrissi A, Trenkner E (1999) Growth factors and taurine protect against excitotoxicity by stabilizing calcium homeostasis and energy metabolism. J Neurosci 19:9459–9468

Engelborghs S, Marescau B, De Deyn PP (2003) Amino acids and biogenic amines in cerebrospinal fluid of patients with Parkinson's disease. Neurochem Res 28:1145–1150

Engeln M, De Deurwaerdère P, Li Q et al (2015) Widespread monoaminergic dysregulation of both motor and non-motor circuits in Parkinsonism and Dyskinesia. Cereb Cortex 25:2783–2792. doi:10.1093/cercor/bhu076

Errico F, Rossi S, Napolitano F et al (2008) d-Aspartate prevents corticostriatal long-term depression and attenuates schizophrenia-like symptoms induced by amphetamine and MK-801. J Neurosci 28:10404–10414. doi:10.1523/JNEUROSCI.1618-08.2008

Errico F, Bonito-Oliva A, Bagetta V et al (2011) Higher free D-aspartate and N-methyl-D-aspartate levels prevent striatal depotentiation and anticipate L-DOPA-induced dyskinesia. Exp Neurol 232:240–250. doi:10.1016/j.expneurol.2011.09.013

Espadas I, Darmopil S, Vergaño-Vera E et al (2012) L-DOPA-induced increase in TH-immunoreactive striatal neurons in parkinsonian mice: insights into regulation and function. Neurobiol Dis 48:271–281. doi:10.1016/j.nbd.2012.07.012

Fieblinger T, Graves SM, Sebel LE et al (2014) Cell type-specific plasticity of striatal projection neurons in parkinsonism and L-DOPA-induced dyskinesia. Nat Commun 5:5316. doi:10.1038/ncomms6316

González-Aparicio R, Moratalla R (2014) Oleoylethanolamide reduces L-DOPA-induced dyskinesia via TRPV1 receptor in a mouse model of Parkinsońs disease. Neurobiol Dis 62:416–425. doi:10.1016/j.nbd.2013.10.008

Granado N, Ortiz O, Suárez LM et al (2008) D1 but not D5 dopamine receptors are critical for LTP, spatial learning, and LTP-Induced arc and zif268 expression in the hippocampus. Cereb Cortex 18:1–12. doi:10.1093/cercor/bhm026

Granado N, Ares-Santos S, Moratalla R (2013) Methamphetamine and Parkinson's disease. Parkinsons Dis 2013:308052. doi:10.1155/2013/308052

Han NL, Clements JD, Lynch JW (2004) Comparison of taurine- and glycine-induced conformational changes in the M2-M3 domain of the glycine receptor. J Biol Chem 279:19559–19565. doi:10.1074/jbc.M400548200

Herrera-Marschitz M, Ungerstedt U (1984) Evidence that apomorphine and pergolide induce rotation in rats by different actions on D1 and D2 receptor sites. Eur J Pharmacol 98:165–176

Herrera-Marschitz M, Arbuthnott G, Ungerstedt U (2010) The rotational model and microdialysis: significance for dopamine signalling, clinical studies, and beyond. Prog Neurobiol 90:176–189. doi:10.1016/j.pneurobio.2009.01.005

Heuer A, Smith GA, Lelos MJ et al (2012) Unilateral nigrostriatal 6-hydroxydopamine lesions in mice I: Motor impairments identify extent of dopamine depletion at three different lesion sites. Behav Brain Res 228:30–43. doi:10.1016/j.bbr.2011.11.027

Le Masurier M, Zetterström T, Cowen P, Sharp T (2013) Tyrosine-free amino acid mixtures reduce physiologically-evoked release of dopamine in a selective and activity-dependent manner. J Psychopharmacol 28:561–569. doi:10.1177/0269881113507642

LeWitt PA (2015) Levodopa therapy for Parkinson's disease: pharmacokinetics and pharmacodynamics. Mov Disord 30:64–72. doi:10.1002/mds.26082

Lim SA, Xia R, Ding Y et al (2015) Enhanced histamine H2 excitation of striatal cholinergic interneurons in L-DOPA-induced dyskinesia. Neurobiol Dis 76:67–76. doi:10.1016/j.nbd.2015.01.003

Lindefors N, Ungerstedt U (1990) Bilateral regulation of glutamate tissue and extracellular levels in caudate-putamen by midbrain dopamine neurons. Neurosci Lett 115:248–252

Mela F, Marti M, Bido S et al (2012) In vivo evidence for a differential contribution of striatal and nigral D1 and D2 receptors to L-DOPA induced dyskinesia and the accompanying surge of nigral amino acid levels. Neurobiol Dis 45:573–582. doi:10.1016/j.nbd.2011.09.015

Mena MA, Aguado EG, de Yebenes JG (1984) Monoamine metabolites in human cerebrospinal fluid. HPLC/ED method. Acta Neurol Scand 69:218–225

Murer MG, Moratalla R (2011) Striatal signaling in L-DOPA-induced dyskinesia: common mechanisms with drug abuse and long term memory involving D1 dopamine receptor stimulation. Front Neuroanat 5:51. doi:10.3389/fnana.2011.00051

Parrot S, Bert L, Renaud B, Denoroy L (2003) Glutamate and aspartate do not exhibit the same changes in their extracellular concentrations in the rat striatum after N-methyl-D-aspartate local administration. J Neurosci Res 71:445–454. doi:10.1002/jnr.10489

Pavón N, Martín AB, Mendialdua A, Moratalla R (2006) ERK phosphorylation and FosB expression are associated with L-DOPA-induced dyskinesia in hemiparkinsonian mice. Biol Psychiatry 59:64–74. doi:10.1016/j.biopsych.2005.05.044

Perucho J, Gonzalo-Gobernado R, Bazan E et al (2015) Optimal excitation and emission wavelengths to analyze amino acids and optimize neurotransmitters quantification using precolumn OPA-derivatization by HPLC. Amino Acids 47:963–973. doi:10.1007/s00726-015-1925-1

Pettersson E, Herrera-Marschitz M, Rodriguez-Puertas R, et al (1996) Evidence for aspartate-immunoreactive neurons in the neostriatum of the rat: modulation by the mesencephalic dopamine pathway via D1-subtype of receptor. Neuroscience 74:51–66. doi:10.1016/0306-4522(96)00124-8

Rangel-Barajas C, Silva I, Lopéz-Santiago LM et al (2011) L-DOPA-induced dyskinesia in hemiparkinsonian rats is associated with up-regulation of adenylyl cyclase type V/VI and increased GABA release in the substantia nigra reticulata. Neurobiol Dis 41:51–61. doi:10.1016/j.nbd.2010.08.018

Rinne JO, Halonen T, Riekkinen PJ, Rinne UK (1988) Free amino acids in the brain of patients with Parkinson's disease. Neurosci Lett 94:182–186

Rodrigues TB, Granado N, Ortiz O et al (2007) Metabolic interactions between glutamatergic and dopaminergic neurotransmitter systems are mediated through D(1) dopamine receptors. J Neurosci Res 85:3284–3293. doi:10.1002/jnr.21302

Ruiz-DeDiego I, Mellstrom B, Vallejo M et al (2015a) Activation of DREAM (downstream regulatory element antagonistic modulator), a calcium-binding protein, reduces L-DOPA-induced dyskinesias in mice. Biol Psychiatry 77:95–105. doi:10.1016/j.biopsych.2014.03.023

Ruiz-DeDiego I, Naranjo JR, Hervé D, Moratalla R (2015b) Dopaminergic regulation of olfactory type G-protein α subunit expression in the striatum. Mov Disord 30:1039–1049. doi:10.1002/mds.26197

Segovia J, Armstrong DM, Benzing WC, Hornby PJ (1991) Striatal glutamic acid decarboxylase immunoreactivity is increased after dopaminergic deafferentation: densitometric analysis. Neurosci Lett 122:252–256

Sergeeva OA, Haas HL (2001) Expression and function of glycine receptors in striatal cholinergic interneurons from rat and mouse. Neuroscience 104:1043–1055. doi:10.1016/S0306-4522(01)00130-0

Sergeeva OA, Fleischer W, Chepkova AN et al (2007) GABAA-receptor modification in taurine transporter knockout mice causes striatal disinhibition. J Physiol (Lond) 585:539–548. doi:10.1113/jphysiol.2007.141432

Shen J (2013) Modeling the glutamate-glutamine neurotransmitter cycle. Front Neuroenergetics 5:1. doi:10.3389/fnene.2013.00001

Smith GA, Heuer A, Dunnett SB, Lane EL (2012) Unilateral nigrostriatal 6-hydroxydopamine lesions in mice II: Predicting l-DOPA-induced dyskinesia. Behav Brain Res 226:281–292. doi:10.1016/j.bbr.2011.09.025

Solis O, Limón DI, Flores-Hernández J, Flores G (2007) Alterations in dendritic morphology of the prefrontal cortical and striatum neurons in the unilateral 6-OHDA-rat model of Parkinson's disease. Synapse 61:450–458. doi:10.1002/syn.20381

Solís O, Espadas I, Del-Bel EA, Moratalla R (2015a) Nitric oxide synthase inhibition decreases l-DOPA-induced dyskinesia and the expression of striatal molecular markers in Pitx3(−/−) aphakia mice. Neurobiol Dis 73:49–59. doi:10.1016/j.nbd.2014.09.010

Solís O, Garcia-Montes JR, González-Granillo A et al (2015b) Dopamine D3 receptor modulates l-DOPA-induced dyskinesia by targeting D1 receptor-mediated striatal signaling. Cereb Cortex. doi:10.1093/cercor/bhv231

Suárez LM, Solís O, Caramés JM et al (2014) L-DOPA treatment selectively restores spine density in dopamine receptor D2-expressing projection neurons in dyskinetic mice. Biol Psychiatry 75:711–722. doi:10.1016/j.biopsych.2013.05.006

Tanaka Y, Niijima K, Mizuno Y, Yoshida M (1986) Changes in gamma-aminobutyrate, glutamate, aspartate, glycine, and taurine contents in the striatum after unilateral nigrostriatal lesions in rats. Exp Neurol 91:259–268

Tong Q, Xu Q, Xia Q et al (2014) Correlations between plasma levels of amino acids and nonmotor symptoms in Parkinson's disease. J Neural Transm. doi:10.1007/s00702-014-1280-5

Yuan YS, Zhou XJ, Tong Q et al (2013) Change in plasma levels of amino acid neurotransmitters and its correlation with clinical heterogeneity in early Parkinson's disease patients. CNS Neurosci Ther 19:889–896. doi:10.1111/cns.12165

Zetterström T, Herrera-Marschitz M, Ungerstedt U (1986) Simultaneous measurement of dopamine release and rotational behaviour in 6-hydroxydopamine denervated rats using intracerebral dialysis. Brain Res 376:1–7. doi:10.1016/0006-8993(86)90893-0

Human COMT over-expression confers a heightened susceptibility to dyskinesia in mice.

Solís O, García-Montes JR, Garcia-Sanz P, Herranz AS, Asensio MJ, Kang G, Hiroi N, Moratalla R.

Neurobiol Dis. 2017; 102:133-139. doi: 10.1016/j.nbd.2017.03.006.

Neurobiology of Disease 102 (2017) 133–139

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Human COMT over-expression confers a heightened susceptibility to dyskinesia in mice

Oscar Solís [a,b], Jose-Rubén García-Montes [a,b], Patricia Garcia-Sanz [a,b], Antonio S. Herranz [c], Maria-José Asensio [c], Gina Kang [d], Noboru Hiroi [d], Rosario Moratalla [a,b,*]

[a] Instituto Cajal, CSIC, Madrid 28002, Spain
[b] CIBERNED, Instituto de Salud Carlos III, Madrid, Spain
[c] Servicio de Neurobiología, Hospital Universitario Ramón y Cajal (IRYCIS), Madrid 28034, Spain
[d] Department of Psychiatry and Behavioral Sciences, Dominick P. Purpura Department of Neuroscience, and Department of Genetics, Albert Einstein College of Medicine, Bronx, NY 10461, USA

ARTICLE INFO

Article history:
Received 1 December 2016
Revised 8 March 2017
Accepted 14 March 2017
Available online 16 March 2017

Keywords:
L-DOPA
LID
Abnormal involuntary movements
TXNRD2
COMT
ARVCF
22q11.2
Dopamine
Striatum

ABSTRACT

Catechol-*O*-methyltransferase (COMT) degrades dopamine and its precursor L-DOPA and plays a critical role in regulating synaptic dopamine actions. We investigated the effects of heightened levels of COMT on dopamine-regulated motor behaviors and molecular alterations in a mouse model of dyskinesia. Transgenic mice overexpressing human COMT (TG) and their wildtype (WT) littermates received unilateral 6-OHDA lesions in the dorsal striatum and were treated chronically with L-DOPA for two weeks. L-DOPA-induced dyskinesia was exacerbated in TG mice without altering L-DOPA motor efficacy as determined by contralateral rotations or motor coordination. Inductions of FosB and phospho-acetylated histone 3 (molecular correlates of dyskinesia) were potentiated in the lesioned striatum of TG mice compared with their WT littermates. The TG mice had lower basal levels of dopamine in the striatum. In mice with lesions, L-DOPA induces a greater increase in the dopamine metabolite 3-methoxytyramine in the lesioned striatum of dyskinetic TG mice than in WT mice. The levels of serotonin and its metabolite were similar in TG and WT mice. Our results demonstrate that human COMT overexpression confers a heightened susceptibility to L-DOPA-induced dyskinesia and alters molecular and neurochemical responses in the lesioned striatum of mice.

1. Introduction

Chronic use of the dopamine (DA) precursor L-3,4-dihydroxyphenylalanine (L-DOPA) for effective noninvasive treatment of Parkinson's disease (LeWitt, 2015) induces abnormal involuntary motor movements known as L-DOPA-induced dyskinesia (LID). In mice, these abnormal involuntary movements appear in the limbs as hyperkinetic and jerky stepping movements of the forelimbs or small circular movements of the forelimb to and from the snout. There are also axial (lateral flexion of the neck or torsional movements) and orolingual (bursts of masticatory movements from facial, jaw, and tongue muscles) areas (Pavón et al., 2006; Cenci and Lundblad, 2007). LID has been associated with pulsatile stimulation of dopamine receptors (Bastide et al., 2015)—mainly the sensitized dopamine D1 receptors (Darmopil et al., 2009; Hernández et al., 2017), which increase cell-surface expression and traffic (Porras et al., 2012; Berthet et al., 2009). This activation leads to aberrant expression of L-DOPA-induced molecular markers such as FosB and phospho-acetylated histone3 (pAcH3) (Ruiz-DeDiego et al., 2015; Solís et al., 2015).

The enzymes that regulate DA concentrations may play a role in the development of LID (Marin and Obeso, 2010; Müller, 2015). Catechol-*O*-methyltransferase (COMT) is encoded within the 22q11.2 chromosomal segment. It has been linked to LID. COMT catalyzes the *O*-methylation of catecholamines including DA and plays an important role in regulating the synaptic actions of DA and its precursor L-DOPA (Müller, 2015). The role of COMT in dopamine (DA) metabolism in the striatum is less important than in other areas such as the frontal cortex and the hypothalamus (Gogos et al., 1998). However, previous studies have demonstrated that L-DOPA treatment increases the expression and activity of COMT in the striatum (Zhao et al., 2001). Clinical observations suggest that the impact of COMT in regulating LID is

Abbreviations: BAC, bacterial artificial chromosome; COMT, catechol-*O*-methyltransferase; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; D1R, dopamine receptor 1; H3, histone 3; HPLC, high performance liquid chromatography; L-DOPA, L-3,4-dihydroxyphenylalanine; LID, L-DOPA-induced dyskinesia; pAcH3, phospho-acetyl-histone 3; TG, transgenic mice that carry a BAC comprising a 190-kb region of human chromosome 22q11.2 (BAC467.8); TH, tyrosine hydroxylase; WT, wild type; 3-MT, 3-methoxytyramine; 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; 6-OHDA, 6-hydroxydopamine hydrobromide.

* Corresponding author at: Cajal Institute, CSIC, Av. Dr. Arce 37, Madrid 28002, Spain.
E-mail address: moratalla@cajal.csic.es (R. Moratalla).

Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2017.03.006
0969-9961/

underscored by the fact that COMT inhibitors can reduce LID by enhancing the L-DOPA delivery to the brain and prolonging DA action in the striatum (Müller, 2015). However, these studies were not conclusive (Stocchi et al., 2010; Nyholm et al., 2011; Muhlack et al., 2014), possibly because of the lower efficiency of COMT inhibitors in the brain than in the periphery.

To establish the impact of COMT in LID, we used bacterial artificial chromosome (BAC)-transgenic mice (TG) that overexpress a 190-kb human chromosomal segment of 22q11.2 including the genes *TXNRD2*, *COMT*, and *ARVCF*. In this BAC-transgenic mouse, COMT enzymatic activity in the striatum, prefrontal cortex, and hippocampus is 2-fold greater compared with their wild-type littermates (Suzuki et al., 2009). We used a LID model as a reliable and robust method to induce abnormal limb movements (Pavón et al., 2006; Solís et al., 2017) and measured markers of cellular responses of striatal neurons FosB and histone 3 (H3) activation (Hiroi and Graybiel, 1996; Hiroi et al., 2002; Ruiz-DeDiego et al., 2015; Solís et al., 2015) and levels of DA, serotonin (5-HT), and their metabolites.

2. Materials and methods

2.1. Animals

This study was carried out using congenic transgenic mice that carry a BAC comprising a 190-kb region of human chromosome 22q11.2 (BAC467.8) containing *TXNRD2*, *COMT*, and *ARVCF*. These mice were backcrossed into the C57BL/6J line for more than ten generations (Suzuki et al., 2009). We used the offspring of BAC-transgenic (TG) and wild-type (WT) breeder pairs. Genotypes were determined by polymerase chain reactions of tail-tip DNA. Mice aged 5–7 months were used for all experiments. The animals were housed and maintained in accordance with the guidelines of the European Union Council Directive (86/609/European Economic Community). The protocol was approved by the Consejo Superior de Investigaciones Científicas ethics committee.

2.2. 6-OHDA lesions and treatment

Mice were anesthetized with isoflurane and placed in a stereotaxic apparatus (Kopf Instruments, CA, USA). As previously described (Solís et al., 2017), the animals received unilateral injections of either 2 × 2 μL of 6-hydroxydopamine hydrobromide (6-OHDA) (20 mmol/L containing 0.02% ascorbic acid; Sigma-Aldrich, Spain) or saline solution (0.9% NaCl) into the dorsal striatum at the following coordinates: (mm from bregma/dura) anteroposterior, +0.65; lateral, −2.0; and dorsoventral, −4.0 and −3.5. Desipramine (20 mg/kg, i.p.; Sigma-Aldrich, Spain) was injected 30 min before the intrastriatal injections of 6-OHDA to avoid destroying noradrenergic neurons. Two to three weeks after the lesion procedures, mice were treated daily with benserazide (10 mg/kg, i.p.; Sigma-Aldrich, Spain) and L-DOPA (20 mg/kg, i.p.; Sigma-Aldrich, Spain) for 2 weeks. Benserazide and L-DOPA were freshly prepared before use and injected in a volume of 10 mL/kg.

2.3. Behavioral assessment

To evaluate LID, animals were placed in clear-glass cylinders and were rated by a trained observer. Dyskinesia was evaluated for 4 min 2–3 times per week 40 min after L-DOPA was injected. Previous studies demonstrated that the incidence and intensity of abnormal involuntary movements are maximal 30 and 60 min following L-DOPA administration (Pavón et al., 2006; Solís et al., 2015). Three subtypes (axial, limb, and orolingual) of dyskinetic symptoms were give scores ranging from 0 (not present) to 4 (severe) (Suárez et al., 2014; Ruiz-DeDiego et al., 2015). The total score represents the sum of these three dyskinetic subtypes (ALO score). To evaluate the time course of LID, on day 16 of treatment, we evaluated LID for 1 min, every 30 min for a period of 180 min immediately after L-DOPA was injected.

Motor coordination was assessed using the rotarod test following an accelerating protocol with increasing speed from 4 to 40 rpm over a 5-min period as previously described (Ruiz-DeDiego et al., 2015; Solís et al., 2017). The latency to fall off the rod was measured before 6-OHDA lesions (prelesion), before treatment with L-DOPA (pre-L-DOPA; day 0), and during the chronic L-DOPA treatment on day 14 (post-L-DOPA). This latter measure was carried out 90 min after the L-DOPA, to avoid the peak of dyskinetic symptoms.

Rotational behaviors were measured to evaluate behavioral sensitization. All animals were videotaped with a vertically mounted video camera for 15 min starting 5 min after L-DOPA injection on days 3, 6, 9, 12, and 14. Contralateral turns were analyzed in Viewer2 (Biobserve GmbH, Bonn, Germany). All experiments were performed by investigators blinded to the treatment conditions and mouse genotypes.

2.4. Immunohistochemistry

The mice were deeply anesthetized with pentobarbital and perfused transcardially with cold saline followed by a solution of 4% formaldehyde in phosphate-buffered saline 1 h after the last L-DOPA injection. Immunostaining was performed on free-floating coronal brain sections as described previously (Solís et al., 2015) with the following rabbit antibodies: tyrosine hydroxylase (TH, 1:1000; Chemicon, Temecula, California), FosB (1:7500; Santa Cruz Biotechnology, Dallas, Texas), and phospho-(Ser10)-acetyl (Lys14)-histone 3 (pAcH3; 1:1500; Upstate Biotechnology, Inc., Lake Placid, New York).

The extent of the dopaminergic lesions was quantified using Stereo Investigator (MBF Bioscience, Williston, Vermont) by depicting the borders of the dorsal striatal areas with complete loss of TH-immunoreactive fibers under 4× objective in 5–7 serial rostrocaudal sections per animal. The lesioned areas were quantified as the percentage volume of the dorsal striatum without TH immunostaining (Supplementary Fig. 1). Quantification of FosB and pAcH3-positive cells was carried out using ImageJ. The numbers of immunolabeled cells were determined for all animals in each group using two serial rostrocaudal sections per animal from the lesioned dorsolateral striatum for a total of six images per animal. The digital images were obtained under a Leica microscope using a 40× objective. The data are presented as the number of stained nuclei per mm^2 in the lesioned striatum.

2.5. Neurochemical procedures

A separate cohort of mice was sacrificed by decapitation 60 min after the last injection of L-DOPA (dyskinetic group) or saline (sham-operated and Parkinsonian groups). The brains were removed rapidly, and the dorsal striata were dissected.

Separate tissue samples were taken from the left and right dorsal striata and were frozen at −80 °C for analyses of neurotransmitter contents. The levels of monoamine neurotransmitters and metabolites were determined by high performance liquid chromatography (HPLC) using electrochemical detection (Solís et al., 2016). Briefly, levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) were measured with an ESA Coulochem III detector according to Mena et al. (1984) with minor modifications. Samples were sonicated in 8 volumes (w/v) of 0.4 N perchloric acid with 0.5 mM $Na_2S_2O_5$ and 2% EDTA, and then centrifuged for 10 min. DA levels were determined from 20 μL of the supernatant. The chromatographic conditions were as follows: column ACE 5 C18, 150 × 4.6 mm (UK); mobile phase, 109.3 mM citrate buffer/1.1 mM acetate buffer (pH 3.55) with 10% methanol, 1 mM EDTA, and 5 mM sodium 1-heptanesulfonate at a flow rate of 1 mL/min. Peaks of DA, 5-HT, and their metabolites were identified by their retention time, and their amounts were calculated against a calibrated

external standard solution (0.6 μM). The investigator performing HPLC measurements was blinded to the sample identities.

2.6. Statistical analysis

Data were analyzed by repeated measures analyses of variance (ANOVAs) with levels of significance adjusted by Bonferroni corrections or by unpaired *t*-tests. Statistical analyses were performed with SPSS 23.0 for Windows. The data are expressed as the means ± standard errors (SEM). The minimal significance level was set at 5%.

3. Results

3.1. Overexpression of COMT increases LID

To assess the role of COMT overexpression in the development of LID, WT and TG mice were depleted of DA in the striatum in one hemisphere and treated daily with L-DOPA (20 mg/kg, i.p.) for 2 weeks. As expected, this treatment induced dyskinetic behaviors in lesioned mice. TG mice displayed more severe axial, limb, and orolingual dyskinetic behaviors than their WT littermates (Fig. 1A–C). The total dyskinetic scores were also greater in TG mice than in WT controls (Fig. 1D).

LID persisted for at least 180 min after L-DOPA was injected, but the greatest difference between TG and WT mice occurred during the first hour (Fig. 2A). LID was present but very mild before a 30-min time point (data not shown) and peaked at 30 and 60 min, at which times the phenotypic differences between TG and WT mice were most pronounced. However, the overall effect during the entire 180 min was still robust; the sum of all observation periods over the entire 180-min period shows that TG mice displayed enhanced dyskinesia compared with that in WT mice (Fig. 2B).

We measured motor coordination using the rotarod test (Solís et al., 2017). Basal motor coordination was indistinguishable between the TG and WT animals (Fig. 2C, prelesion). 6-OHDA lesions decreased the latencies to fall from the rotarod equally in the two genotypes (Fig. 2C, pre-L-DOPA). L-DOPA treatment restored motor coordination equally in WT and TG mice (Fig. 2C, post-L-DOPA).

The repeated administration of L-DOPA results in behavioral sensitization that can be measured by the development of contralateral rotations (Pavón et al., 2006). We assessed this behavior for 15 min beginning 5 min after L-DOPA was injected. Contralateral rotations increased gradually over several days, reaching a plateau at approximately day 9 of treatment. We found no differences in L-DOPA-induced contralateral rotations between the TG and WT mice (Fig. 2D).

3.2. Overexpression of COMT potentiates L-DOPA-induced FosB and pAcH3 expression in the dorsal striatum

LID has been attributed to enhancement of dopamine receptor 1 (D1R) signaling (Darmopil et al., 2009; Santini et al., 2009). We measured D1R-dependent molecular responses, FosB and pAcH3 expression, in DA-depleted striatum. Immunostaining for TH confirmed that DA denervation was similar in TG and WT mice (Fig. 3A, B). However, TG mice had significantly greater densities of $FosB^+$ (Fig. 3C) and $pAcH3^+$ (Fig. 3D) cells than WT mice.

3.3. Overexpression of COMT alters basal levels of DA and its metabolite but not 5-HT or its metabolite in mouse striatum

As one of the three genes in the 190-kb segment (*COMT*) encodes for an enzyme that degrades DA and its metabolites, we next examined striatal monoamine levels. We used HPLC to analyze the levels of DA and its metabolites DOPAC and 3-MT in WT and TG mice that were

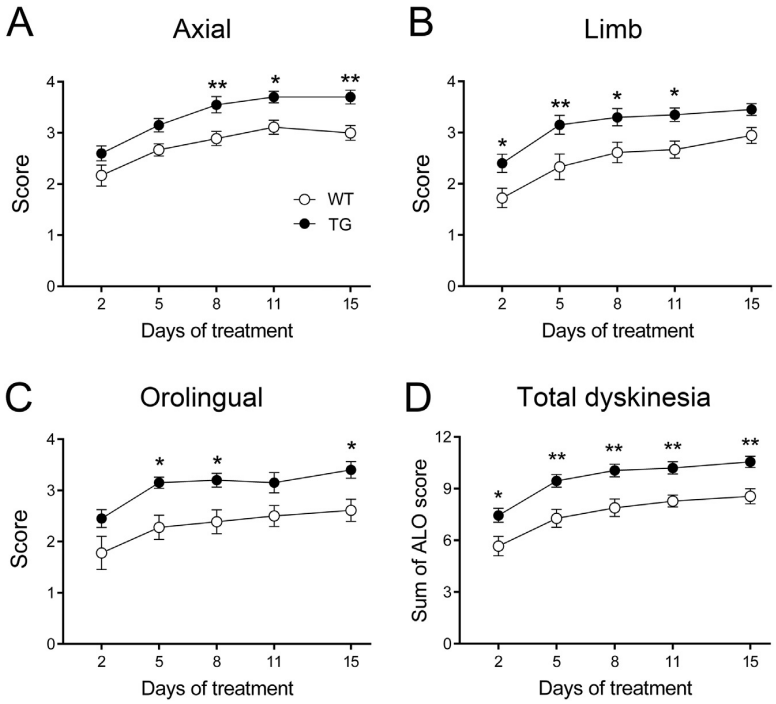


Fig. 1. Dyskinetic responses to L-DOPA are potentiated in COMT-overexpressing mice. Scores for axial (A), limb (B), orolingual (C), and the sum total (ALO) (D) dyskinetic behaviors were evaluated for 4 min, 40 min after L-DOPA injections in mice. A repeated two-way ANOVA indicated significant main effects for axial ([A] genotype, $F_{1,17} = 13.21$, $P = 0.0021$; day, $F_{4,68} = 34.59$, $P < 0.0001$; interaction, $F_{4,68} = 0.63$, not significant [n.s.]), limb ([B] genotype, $F_{1,17} = 10.00$, $P = 0.0057$; day, $F_{4,68} = 37.65$, $P < 0.0001$; interaction, $F_{4,68} = 0.60$, n.s.), orolingual ([C] genotype, $F_{1,17} = 8.73$, $P = 0.0089$; day, $F_{4,68} = 22.46$, $P < 0.0001$; interaction, $F_{4,68} = 0.44$, n.s.), and total ([D] genotype, $F_{1,17} = 13.62$, $P = 0.0018$; day, $F_{4,68} = 78.53$, $P < 0.0001$; interaction, $F_{4,68} = 0.38$, n.s.) dyskinesia. Data are expressed as the means ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$ *vs.* WT as determined by Bonferroni *post hoc* test; $n = 9$–10/group.

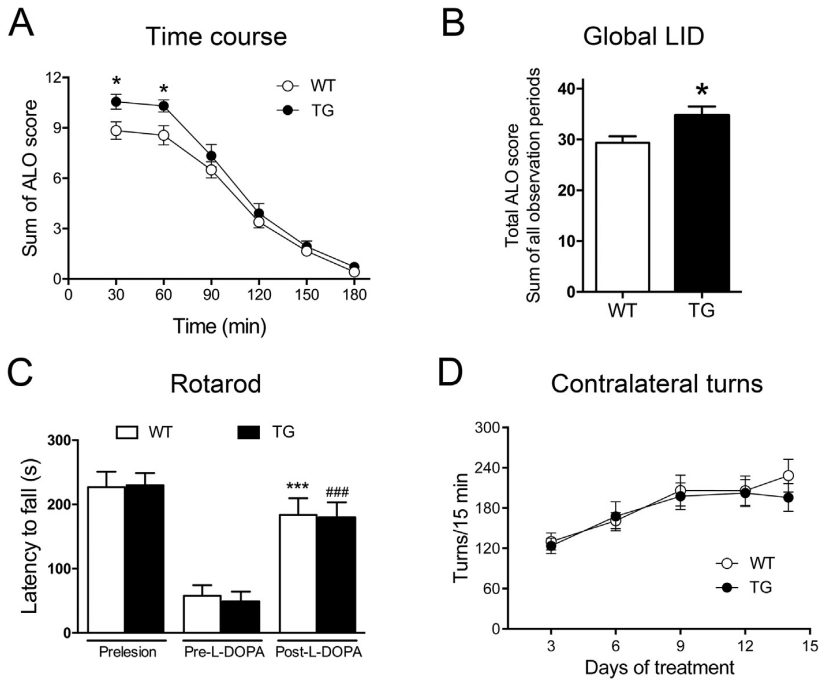


Fig. 2. Overexpression of COMT enhances dyskinesia in mice but not motor coordination or rotational behaviors. (A) Time course of dyskinetic (axial, limb, and orolingual [ALO]) behaviors on day 16, evaluated for 1 min once every 30 min during a 180-min period following L-DOPA treatment. A repeated two-way ANOVA indicated significant main effects (genotype, $F_{1,17} = 5.88$, $P = 0.0268$; time, $F_{5,85} = 191.63$, $P < 0.0001$; interaction, $F_{5,85} = 1.45$, $P = 0.215$), and a genotype effect at each time point, as determined by Bonferroni's tests. $^{*}P < 0.05$ *vs.* WT. (B) Sum of all scores over the 180-min period ($t[17] = 2.425$) $^{*}P = 0.0268$ *vs.* WT. (C) Motor coordination and balance assessed on the rotarod before 6-OHDA lesions (prelesion), 3 weeks after lesions (pre-L-DOPA), and 90 min after L-DOPA was injected on day 14 of the chronic treatment (post-L-DOPA). A two-way ANOVA indicates a significant group effect only (genotype, $F_{1,17} = 0.02$, not significant [n.s.]; group, $F_{2,34} = 60.70$, $P < 0.0001$; interaction, $F_{2,34} = 0.06$, n.s.). $^{***}P < 0.001$ *vs.*TG pre-L-DOPA, $^{###}P < 0.001$ *vs.* WT pre-L-DOPA as determined by Bonferroni *post hoc* tests. (D) Contralateral turns were measured for a period of 15 min, 5 min after L-DOPA was injected during chronic treatment. A repeated two-way ANOVA indicates a significant effect of day only (genotype, $F_{1,17} = 0.46$, n.s.; day, $F_{4,68} = 18.12$, $P < 0.0001$; interaction, $F_{4,68} = 1.10$, n.s.). Data are expressed as the means ± SEM. $n = 9$–10/group.

sham-operated, 6-OHDA-lesioned (Parkinsonian [Park] group), or 6-OHDA lesioned and treated with L-DOPA (dyskinetic [dysk] group). We confirmed that TG mice in this cohort also displayed more severe dyskinesia than their WT littermates (see Supplementary Fig. 2).

The levels of DA in the striatum were significantly lower in sham-operated TG mice than in their WT counterparts (Fig. 4, sham). 6-OHDA lesions induced severe decreases in DA levels in the ipsilateral (I) striatum compared with levels in the contralateral (C) striata in TG and WT mice (Fig. 4, Park). Chronic L-DOPA treatment did not alter DA levels in the striatum ipsilateral to lesions in dyskinetic WT or TG mice, although it increased DA levels in contralateral striatum in TG mice (Fig. 4, dysk).

Regarding DA metabolites, DOPAC levels were not significantly different between TG and WT sham-operated mice (Fig. 4, DOPAC, Sham). 6-OHDA lesions decreased DOPAC levels equally in the ipsilateral striatum of the two genotypes (Fig. 4, DOPAC, Park). Similarly, chronic treatment with L-DOPA increased DOPAC levels equally in the two genotypes (Fig. 4, DOPAC, dysk). Levels of 3-MT were significantly elevated in the striatum of sham-operated TG mice compared with the levels in WT mice. 6-OHDA lesions reduced the levels 3-MT in the striatum ipsilateral to 6-OHDA lesions equally in TG and WT mice (Fig. 4, 3-MT, Park). L-DOPA increased the levels of 3-MT to a greater extent in the striatum ipsilateral to lesions in TG mice than in WT mice (Fig. 4, 3-MT, dysk).

We next assessed the levels of 5-HT and its metabolite 5-HIAA. There were no differences in the levels of 5-HT or 5-HIAA between WT and TG mice under any condition. We found that the striatal 5-HT levels were similar in sham-operated TG and WT mice (Fig. 5, 5-HT, sham). 5-HT levels were augmented equally in the lesioned (DA-depleted) striatum of TG and WT mice (Fig. 5, 5-HT, Park). L-DOPA treatment in 6-OHDA-lesioned mice normalized this effect equally in the two genotypes (Fig. 5, 5-HT, dysk). 5-HIAA striatal levels mirrored 5-HT levels in the sham and Park groups; 5-HIAA levels remained elevated in the striatum ipsilateral to lesions in both WT and TG mice (Fig. 5, 5-HIAA).

4. Discussion

Our findings show that the overexpression of COMT included in a 190-kb segment of 22q11.2 in mice confers a heightened susceptibility to abnormal axial, limb, and orolingual movements. We also observed potentiated expressions of FosB and pAcH3 and DA metabolism in DA-depleted striatum concomitant with lower basal DA levels in mice without 6-OHDA lesions. In contrast, the overexpression of COMT had no effect on the therapeutic actions of L-DOPA in mice as measured by contralateral rotations and motor coordination and by levels of 5-HT and its metabolite.

The constitutive transgenic mouse is ideal for testing our hypothesis that COMT overexpression confers altered susceptibility to dyskinesia. However, the BAC transgene construct could potentially have disrupted or interacted with endogenous murine genes. As such, the observed phenotypes might reflect these secondary effects rather than the effects from overexpressing genes that are encoded in the BAC.

Although the TG mice express TXNRD2, COMT, and ARVCF, it is not clear which of these contributed to the observed phenotypes. The existing evidence suggests that COMT and DA contribute to dyskinesia (de Lau et al., 2012). We previously demonstrated that this TG mouse has 2-fold higher levels of COMT enzymatic activity in the brain including in the striatum (Suzuki et al., 2009). Our data also show that basal

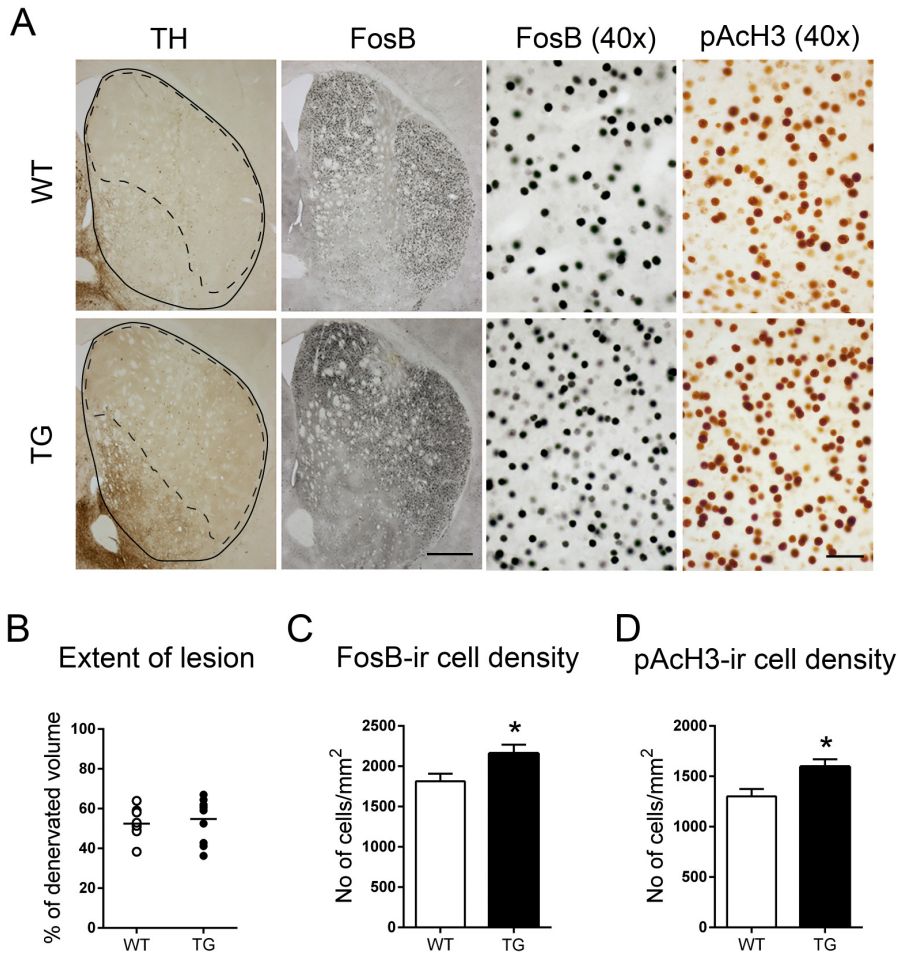


Fig. 3. Induction of FosB and pAcH3 in the striatum of COMT-overexpressing mice. (A) Immunostaining for TH, FosB, and pAcH3. Photomicrographs of adjacent coronal striatal sections of the lesioned striatum at low and high (40×) magnification from WT and TG mice. Scale bar = 100 μm for low-magnification and 50 μm for high-magnification images. The continuous outline represents the dorsal striatum and the dashed outline represents the completely denervated striatum in the low magnification TH pictures. (B) The extent of striatal lesions was assessed by quantifying the percentage of striatal volume that did not stain for TH ($t[17] = 0.53$, n.s.). The densities of FosB-positive (C) ($t[17] = 2.38$, $P = 0.0292$) and pAcH3-positive (D) ($t[17] = 2.80$, $P = 0.0123$) cells in the lesioned striatum. Data are expressed as the means ± SEM. $^{*}P < 0.05$ *vs.* WT mice; $n = 9$–10/group.

levels of DA—an enzymatic target of COMT but not 5-HT—are significantly reduced in the striatum of TG mice. Thus, we surmise that COMT contributed to the observed phenotypes.

There is an interesting dissociation between the motor effects induced by L-DOPA. Although the transgenic mice used here demonstrate more severe LID, the basal locomotor activity is not altered (Suzuki et al.,

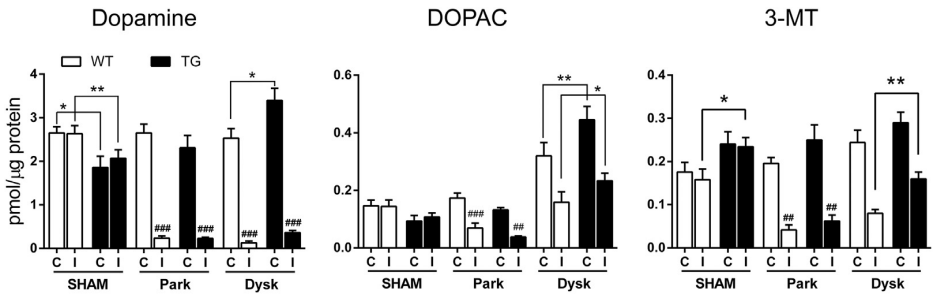


Fig. 4. Levels of DA and its metabolites in lesioned mice after L-DOPA treatment. Levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and 3-methoxytyramine (3-MT) in the striatum contralateral (C) and ipsilateral (I) to 6-OHDA lesions in sham-operated, lesioned and saline-administered (Park), and lesioned and L-DOPA-administered (dysk) mice. Mice were sacrificed 60 min after the last saline or L-DOPA injection, and levels were measured by HPLC. A three-way ANOVA with significant *P* levels adjusted by Bonferroni corrections showed genotype × condition interactions for DA ($F_{2,29} = 10.838$, $P = 0.001$) and DOPAC ($F_{2,28} = 6.326$, $P = 0.005$). For 3-MT levels, significant effects were found according to genotype ($F_{1,29} = 21.205$, $P < 0.001$), condition ($F_{2,29} = 11.01$, $P < 0.001$), and hemisphere ($F_{1,29} = 71.61$, $P < 0.001$) without interactions (genotype × condition, $F_{2,29} = 0.659$, $P = 0.525$; genotype × hemisphere, $F_{1,29} = 0.026$, $P = 0.873$; genotype × condition × hemisphere, $F_{2,29} = 0.588$, $P = 0.562$). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* WT ipsilateral striatum. The data are shown as the means ± SEM. $n = 5$–7/group.

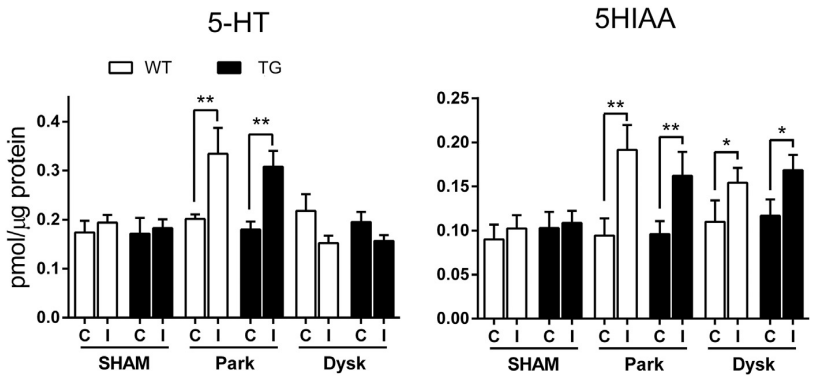


Fig. 5. Levels of serotonin and its metabolite in lesioned mice after L-DOPA treatment. Levels of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the striatum contralateral (C) and ipsilateral (I) to 6-OHDA lesions in sham-operated, lesioned and saline-administered (Park), and lesioned and L-DOPA-administered (dysk) mice. Mice were sacrificed 60 min after the last saline or L-DOPA injection, and levels were measured by HPLC. A three-way ANOVA with significant *P* levels adjusted by Bonferroni corrections showed significant differences for effects of condition ($F_{2,29} = 8.215$, $P = 0.001$) and a condition × hemisphere interaction ($F_{2,29} = 12.61$, $P < 0.001$) for 5-HT; no other effect was significant. For 5-HIAA, no main or interaction effect involving genotype was significant (genotype, $F_{1,28} = 0.018$, not significant [n.s.]; genotype × condition, $F_{2,28} = 0.283$, n.s.; genotype × hemisphere, $F_{1,28} = 0.415$, n.s.; genotype × condition × hemisphere, $F_{2,28} = 0.543$, n.s.). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* contralateral striatum. Data are presented as the means ± SEM. $n = 5$–7/group.

2009) nor were the therapeutic behavioral effects on motor coordination and rotational behavior. TG mice exhibit elevated basal levels of COMT and reduced levels of DA. Thus, L-DOPA treatment likely induces a shorter duration of dopaminergic stimulation in these mice. This condition is known to induce severe dyskinesia (Papathanou et al., 2011; Mulas et al., 2016). On the other hand, continuous L-DOPA administration reduces LID in Parkinson's patients (Timpka et al., 2016) and prolongs the half-life of L-DOPA *via* COMT inhibition to decrease dyskinesia (Espinoza et al., 2012; Müller, 2015). Thus, elevated COMT levels might cause repeated and short stimulation by L-DOPA thereby potentiating the dyskinetic phenotype.

Our interpretations on the lack of phenotypes in contralateral turns and rotarod tests should be considered with caution because the results might have been due to the timing of the testing. Contralateral turns were measured for 15 min starting 5 min after L-DOPA. Mice were examined for motor coordination on the rotarod before and 3 weeks after 6-OHDA lesions and again 90 min after the last injection on day 14. In contrast, dyskinesia was measured 40 min after l-DOPA injection or for 180 min on day 16 of L-DOPA treatment. A significant genotype effect on dyskinetic responses was seen 30 and 60 min after L-DOPA on day 16, but not thereafter. The time points for rotational and motor coordination assessments were chosen to avoid the secondary effects of dyskinesia, which non-selectively interferes with their detection. The data show that the therapeutic effects of L-DOPA (*i.e.*, restoration of motor coordination and contralateral turns) were equally maintained in both WT and TG mice while inducing more severe dyskinesia in TG mice than in WT mice.

Although the post-synaptic mechanisms for potentiated dyskinetic responses in TG mice remain unclear, LID is associated with aberrant expression of protein kinases, transcription factors, and epigenetic modifications (Pavón et al., 2006; Westin et al., 2007; Santini et al., 2009). In particular, the expression of the transcription factor FosB is enhanced in the denervated striatum upon chronic L-DOPA treatment in a D1R-dependent manner (Darmopil et al., 2009; Murer and Moratalla, 2011; Porras et al., 2012; Berthet et al., 2009; Ruiz-DeDiego et al., 2015). H3 activation also contributes to the L-DOPA-induced accumulation of FosB (Feyder et al., 2016), which occurs in the direct striatal pathway (Pavón et al., 2006; Darmopil et al., 2009; Suarez et al., 2016; Solís et al., 2017). Upon repeated injection of L-DOPA, pronounced and continuous LID develops due to the pulsatile stimulation of DA receptors (Bastide et al., 2015). These results from the sensitization (Darmopil et al., 2009) and increased cell-surface expression and trafficking (Porras et al., 2012; Berthet et al., 2009) of D1R consequently causes aberrant expression of FosB and pAcH3 (Ruiz-DeDiego et al., 2015; Solís et al., 2015). A future challenge is to definitively determine that FosB and pAcH3 in the dorsal striatum functionally contribute to heightened LID intensity in TG mice.

COMT catalyzes DA to produce 3-MT (Männistö and Kaakkola, 1999). In accordance with this, we observed lower levels of DA and higher levels of 3-MT in the striatum of TG mice than in their WT counterparts. However, these differences disappeared after the 6-OHDA lesions. In dyskinetic animals, L-DOPA treatment does not increase DA levels in the lesioned striatum (Del-Bel et al., 2014; Solís et al., 2016). Interestingly, dyskinetic TG animals had higher DOPAC and 3-MT levels in the lesioned striatum than dyskinetic WT mice. Previous studies suggested a modulatory effect of striatal 3-MT on dyskinesia due to activation of trace-amine associated receptor 1 (TAAR1) (Rajput et al., 2004; Sotnikova et al., 2010; Espinoza et al., 2012). Therefore, the increase in LID seen in the TG mice might be due to the increase in 3-MT.

TH staining was completely absent (TH-negative) in ~50% of the entire dorsal striatum in WT and TG mice after 6-OHDA lesions. We applied a stringent criterion to judge the absence of TH-positive fibers; an area where weakly or very weakly stained fibers were present was regarded as a TH-positive area. Our HPLC analysis showed ~90% reduction in DA levels in the dorsal striatum. The explanation for this apparent discrepancy is that either dopaminergic cells with weakly TH-labeled fibers were not sufficient to produce DA or that residual TH-positive fibers included dying cells.

Since the serotonergic system is also implicated in the development of LID (Carta et al., 2007), we evaluated whether 5-HT and its metabolite 5-HIAA were modified in TG mice. The 5-HT and 5-HIAA levels were indistinguishable between TG and WT mice, and 5-HT and 5-HIAA levels were increased in both WT and TG mice with DA-depleted striatum. Interestingly, L-DOPA treatment restored 5-HT levels to normal in both TG and WT mice. Thus, 5-HT might not be involved in the potentiated dyskinetic responses in TG mice, although we do not rule out the possibility that it—along with its pre- and post-synaptic components (*e.g.*, 5-HT transporters and receptors)—might exert an inhibitory effect on dyskinesia.

Human studies have identified a low-activity allele of COMT with a methionine at position 108 as well as a high-activity allele with valine at the same position (Männistö and Kaakkola, 1999; Tunbridge, 2010). The role of this COMT polymorphism in dyskinesia in l-DOPA-treated Parkinson's disease patients is not clear. One study found that the high-activity COMT allele was associated with an increased risk of developing LID (de Lau et al., 2012), but others failed to find any significant association (Bialecka et al., 2008; Contin et al., 2005; Watanabe et al., 2003). On the other hand, patients with higher erythrocyte COMT

activity displayed more dyskinesia (Reilly et al., 1980; Rivera-Calimlim and Reilly, 1984).

Our results are consistent with the idea that prolonging the half-life of L-DOPA through COMT inhibition can decrease dyskinesia (Espinoza et al., 2012; Müller, 2015). This work using genetically modified mice strengthens and consolidates clinical studies using COMT inhibitors. To the best of our knowledge, this is the first study to show that over-expressing COMT increases LID.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2017.03.006.

Conflict of interest

The authors declare no competing financial interests.

Acknowledgements

This work was supported by grants from the Spanish Ministerio de Economía y Competitividad (SAF2016-48532-R, PCIN-2015-098) and Sanidad Política Social e Igualdad (ISCIII, CIBERNED CB06/05/0055) and by SECITI from Mexico ref 037-2016 to R.M., and by R01MH99660 and U54HD090260 to N.H. O.S. and J.-R.G.-M. acknowledge CONACYT and SECITI for scholarship. We thank Beatriz Pro and Emilia Rubio for their technical assistance.

References

Bastide, M.F., et al., 2015. Pathophysiology of L-DOPA-induced motor and non-motor complications in Parkinson's disease. Prog. Neurobiol. 132, 96–168.
Berthet, A., Porras, G., Doudnikoff, E., Stark, H., Cador, M., Bezard, E., Bloch, B., 2009. Pharmacological analysis demonstrates dramatic alteration of D1 dopamine receptor neuronal distribution in the rat analog of L-DOPA-induced dyskinesia. J. Neurosci. 29, 4829–4835.
Bialecka, M., Kurzawski, M., Klodowska-Duda, G., Opala, G., Tan, E.K., Drozdzik, M., 2008. The association of functional catechol-O-methyltransferase haplotypes with risk of Parkinson's disease, levodopa treatment response, and complications. Pharmacogenet. Genomics 18, 815–821.
Carta, M., Carlsson, T., Kirik, D., Björklund, A., 2007. Dopamine released from 5-HT terminals is the cause of L-DOPA-induced dyskinesia in parkinsonian rats. Brain 130, 1819–1833.
Cenci, M.A., Lundblad, M., 2007. Ratings of L-DOPA-induced dyskinesia in the unilateral 6-OHDA lesion model of Parkinson's disease in rats and mice. Curr. Protoc. Neurosci. 25 Chapter 9, Unit 9.
Contin, M., Martinelli, P., Mochi, M., Riva, R., Albani, F., Baruzzi, A., 2005. Genetic polymorphism of catechol-O-methyltransferase and levodopa pharmacokinetic–pharmacodynamic pattern in patients with Parkinson's disease. Mov. Disord. 20, 734–739.
Darmopil, S., Martín, A.B., De Diego, I.R., Ares, S., Moratalla, R., 2009. Genetic inactivation of dopamine D1 but not D2 receptors inhibits L-DOPA induced dyskinesia and histone activation. Biol. Psychiatry 66, 603–613.
Del-Bel, E., Padovan-Neto, F.E., Szawka, R.E., da Silva, C.A., Raisman-Vozari, R., Anselmo-Franci, J., Romano-Dutra, A.C., Guimaraes, F.S., 2014. Counteraction by nitric oxide synthase inhibitor of neurochemical alterations of dopaminergic system in 6-OHDA-lesioned rats under L-DOPA treatment. Neurotox. Res. 25, 33–44.
de Lau, L.M., Verbaan, D., Marinus, J., Heutink, P., van Hilten, J.J., 2012. Catechol-O-methyltransferase Val158Met and the risk of dyskinesias in Parkinson's disease. Mov. Disord. 27, 132–135.
Espinoza, S., Manago, F., Leo, D., Sotnikova, T.D., Gainetdinov, R.R., 2012. Role of catechol-O-methyltransferase (COMT)-dependent processes in Parkinson's disease and L-DOPA treatment. CNS Neurol. Disord. Drug Targets 11, 251–263.
Feyder, M., Södersten, E., Santini, E., Vialou, V., LaPlant, Q., Watts, E.L., Spigolon, G., Hansen, K., Caboche, J., Nestler, E.J., Fisone, G., 2016. A role for mitogen- and stress-activated kinase 1 in L-DOPA-induced dyskinesia and ΔFosB expression. Biol. Psychiatry 79, 362–371.
Gogos, J.A., Morgan, M., Luine, V., Santha, M., Ogawa, S., Pfaff, D., Karayiorgou, M., 1998. Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. Proc. Natl. Acad. Sci. U. S. A. 95, 9991–9996.
Hernández, F., Castela, L., Ruiz-DeDiego, I., Obeso, J.A., Moratalla, R., 2017. Striatal activation by optogenetics induces dyskinesias in the 6-hydroxydopamine rat model of Parkinson disease. Mov. Disord.
Hiroi, N., Graybiel, A.M., 1996. Atypical and typical neuroleptic treatments induce distinct programs of transcription factor expression in the striatum. J. Comp. Neurol. 374, 70–83.
Hiroi, N., Martín, A.B., Grande, C., Alberti, I., Rivera, A., Moratalla, R., 2002. Molecular dissection of dopamine receptor signaling. J. Chem. Neuroanat. 23, 237–242.
LeWitt, P.A., 2015. Levodopa therapy for Parkinson's disease: pharmacokinetics and pharmacodynamics. Mov. Disord. 30, 64–72.
Männistö, P.T., Kaakkola, S., 1999. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacol. Rev. 51, 593–628.
Marin, C., Obeso, J.A., 2010. Catechol-O-methyltransferase inhibitors in preclinical models as adjuncts of L-DOPA treatment. Int. Rev. Neurobiol. 95, 191–205.
Mena, M.A., Aguado, E.G., de Yebenes, J.G., 1984. Monoamine metabolites in human cerebrospinal fluid. HPLC/ED method. Acta Neurol. Scand. 69, 218–225.
Muhlack, S., Herrmann, L., Salmen, S., Müller, T., 2014. Fewer fluctuations, higher maximum concentration and better motor response of levodopa with catechol-O-methyltransferase inhibition. J. Neural Transm. 121, 1357–1366.
Mulas, G., Espa, E., Fenu, S., Spiga, S., Cossu, G., Pillai, E., Carboni, E., Simbula, G., Jadžić, D., Angius, F., Spolitu, S., Batetta, B., Lecca, D., Giuffrida, A., Carta, A.R., 2016. Differential induction of dyskinesia and neuroinflammation by pulsatile versus continuous L-DOPA delivery in the 6-OHDA model of Parkinson's disease. Exp. Neurol. 286, 83–92.
Müller, T., 2015. Catechol-O-methyltransferase inhibitors in Parkinson's disease. Drugs 75, 157–174.
Murer, M.G., Moratalla, R., 2011. Striatal signaling in L-DOPA-induced dyskinesia: common mechanisms with drug abuse and long term memory involving D1 dopamine receptor stimulation. Front. Neuroanat. 5, 51.
Nyholm, D., Askmark, H., Aquilonius, S.-M.M., 2011. Stalevo reduction in dyskinesia evaluation in Parkinson's disease results were expected from a pharmacokinetic viewpoint. Ann. Neurol. 69, 424 (author reply 425).
Papathanou, M., Rose, S., McCreary, A., Jenner, P., 2011. Induction and expression of abnormal involuntary movements is related to the duration of dopaminergic stimulation in 6-OHDA-lesioned rats. Eur. J. Neurosci. 33, 2247–2254.
Pavón, N., Martín, A.B., Mendialdua, A., Moratalla, R., 2006. ERK phosphorylation and FosB expression are associated with L-DOPA-induced dyskinesia in hemiparkinsonian mice. Biol. Psychiatry 59, 64–74.
Porras, G., Berthet, A., Dehay, B., Li, Q., Ladepeche, L., Normand, E., Dovero, S., Martinez, A., Doudnikoff, E., Martin-Négrier, M.L., Chuan, Q., Bloch, B., Choquet, D., Boué-Grabot, E., Groc, L., Bezard, E., 2012. PSD-95 expression controls L-DOPA dyskinesia through dopamine D1 receptor trafficking. J. Clin. Invest. 122, 3977–3989.
Rajput, A.H., Fenton, M.E., Di Paolo, T., Sitte, H., Pifl, C., Hornykiewicz, O., 2004. Human brain dopamine metabolism in levodopa-induced dyskinesia and wearing-off. Parkinsonism Relat. Disord. 10, 221–226.
Reilly, D.K., Rivera-Calimlim, L., Van Dyke, D., 1980. Catechol-O-methyltransferase activity: a determinant of levodopa response. Clin. Pharmacol. Ther. 2892, 278–286.
Rivera-Calimlim, L., Reilly, D.K., 1984. Difference in erythrocyte catechol-O-methyltransferase activity between Orientals and Caucasians: difference in levodopa tolerance. Clin. Pharmacol. Ther. 35, 804–809.
Ruiz-DeDiego, I., Mellstrom, B., Vallejo, M., Naranjo, J.R., Moratalla, R., 2015. Activation of DREAM (downstream regulatory element antagonistic modulator), a calcium-binding protein, reduces L-DOPA-induced dyskinesias in mice. Biol. Psychiatry 77, 95–105.
Santini, E., Alcacer, C., Cacciatore, S., Heiman, M., Hervé, D., Greengard, P., Girault, J.A., Valjent, E., Fisone, G., 2009. L-DOPA activates ERK signaling and phosphorylates histone H3 in the striatonigral medium spiny neurons of hemiparkinsonian mice. J. Neurochem. 108, 621–633.
Solís, O., Espadas, I., Del-Bel, E.A., Moratalla, R., 2015. Nitric oxide synthase inhibition decreases L-DOPA-induced dyskinesia and the expression of striatal molecular markers in Pitx3(−/−) aphakia mice. Neurobiol. Dis. 73, 49–59.
Solís, O., García-Sanz, P., Herranz, A.S., Asensio, M.J., Moratalla, R., 2016. L-DOPA reverses the increased free amino acids tissue levels induced by dopamine depletion and rises GABA and tyrosine in the striatum. Neurotox. Res. 30, 67–75.
Solís, O., Garcia-Montes, J.R., González-Granillo, A., Xu, M., Moratalla, R., 2017. Dopamine D3 receptor modulates L-DOPA-induced dyskinesia by targeting D1 receptor-mediated striatal signaling. Cereb. Cortex 27, 435–446.
Sotnikova, T.D., Beaulieu, J.M., Espinoza, S., Masri, B., Zhang, X., Salahpour, A., Barak, L.S., Caron, M.G., Gainetdinov, R.R., 2010. The dopamine metabolite 3-methoxytyramine is a neuromodulator. PLoS One 5, e13452.
Stocchi, F., Rascol, O., Kieburtz, K., Poewe, W., Jankovic, J., Tolosa, E., Barone, P., Lang, A.E., Olanow, W.C., 2010. Initiating levodopa/carbidopa therapy with and without entacapone in early Parkinson disease: the STRIDE PD study. Ann. Neurol. 68, 18–27.
Suárez, L.M., Solís, O., Caramés, J.M., Taravini, I.R., Solís, J.M., Murer, M.G., Moratalla, R., 2014. L-DOPA treatment selectively restores spine density in dopamine receptor D2-expressing projection neurons in dyskinetic mice. Biol. Psychiatry 75, 711–722.
Suarez, L.M., Solis, O., Aguado, C., Lujan, R., Moratalla, R., 2016. L-DOPA oppositely regulates synaptic strength and spine morphology in D1 and D2 striatal projection neurons in dyskinesia. Cereb. Cortex 26, 4253–4264.
Suzuki, G., Harper, K.M., Hiramoto, T., Funke, B., Lee, M., Kang, G., Buell, M., Geyer, M.A., Kucherlapati, R., Morrow, B., Männistö, P.T., Agatsuma, S., Hiroi, N., 2009. Over-expression of a human chromosome 22q11.2 segment including TXNRD2, COMT and ARVCF developmentally affects incentive learning and working memory in mice. Hum. Mol. Genet. 18, 3914–3925.
Timpka, J., Mundt-Petersen, U., Odin, P., 2016. Continuous dopaminergic stimulation therapy for Parkinson's disease - recent advances. Curr. Opin. Neurol. 29, 474–479.
Tunbridge, E.M., 2010. The catechol-O-methyltransferase gene: its regulation and polymorphisms. Int. Rev. Neurobiol. 95, 7–27.
Watanabe, M., Harada, S., Nakamura, T., Ohkoshi, N., Yoshizawa, K., Hayashi, A., Shoji, S., 2003. Association between catechol-O-methyltransferase gene polymorphisms and wearing-off and dyskinesia in Parkinson's disease. Neuropsychobiology 48, 190–193.
Westin, J.E., Vercammen, L., Strome, E.M., Konradi, C., Cenci, M.A., 2007. Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. Biol. Psychiatry 62, 800–810.
Zhao, W.Q., Latinwo, L., Liu, X.X., Lee, E.S., Lamango, N., Charlton, C.G., 2001. L-DOPA upregulates the expression and activities of methionine adenosyl transferase and catechol-O-methyltransferase. Exp. Neurol. 171, 127–138.

Nitric oxide synthase inhibition decreases l-DOPA-induced dyskinesia and the expression of striatal molecular markers in Pitx3$^{-/-}$ aphakia mice.

Solís O, Espadas I, Del-Bel EA, Moratalla R.

Neurobiol Dis. 2015; 73:49-59. doi: 10.1016/j.nbd.2014.09.010.

Neurobiology of Disease 73 (2015) 49–59

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Nitric oxide synthase inhibition decreases L-DOPA-induced dyskinesia and the expression of striatal molecular markers in *Pitx3*$^{-/-}$ aphakia mice

Oscar Solís [a,b], Isabel Espadas [a,b], Elaine A. Del-Bel [c], Rosario Moratalla [a,b,*]

[a] Instituto Cajal, Consejo Superior de Investigaciones Científicas, CSIC, 28002 Madrid, Spain
[b] CIBERNED, Instituto de Salud Carlos III, Madrid, Spain
[c] Department of Morphology, Physiology and Pathology, School of Odontology, University of Sao Paulo, Campus Ribeirao Preto, Brazil

ARTICLE INFO

Article history:
Received 26 May 2014
Revised 12 September 2014
Accepted 21 September 2014
Available online 30 September 2014

Keywords:
Parkinson disease
L-DOPA
Abnormal involuntary movements
Nitrergic system
NO/cGMP
Pitx3 deficient aphakia mice
FosB
Histone3
Molsidomine
Zaprinast
Striatum

ABSTRACT

Nitric oxide (NO), a gaseous messenger molecule synthesized by nitric oxide synthase (NOS), plays a pivotal role in integrating dopamine transmission in the basal ganglia and has been implicated in the pathogenesis of Parkinson disease (PD). To study the role of the nitrergic system in L-DOPA-induced dyskinesia (LID), we assessed the effect of the pharmacological manipulation of NO levels and NO/cyclic guanosine monophosphate (cGMP) signaling on LID in the *Pitx3*$^{-/-}$ aphakia mouse, a genetic model of PD. To evaluate the effect of decreased NO signaling on the development of LID, *Pitx3*$^{-/-}$ mice were chronically treated with L-DOPA and 7-nitroindazole (7-NI, a neuronal NOS inhibitor). To evaluate its effect on the expression of established LID, 7-NI was administered acutely to dyskinetic mice. The chronic 7-NI treatment attenuated the development of LID in the *Pitx3*$^{-/-}$ mice, and the sub-acute 7-NI treatment attenuated established dyskinesia without affecting the beneficial therapeutic effect of L-DOPA. Moreover, 7-NI significantly reduced FosB and pAcH3 expression in the acutely and chronically L-DOPA-treated mice. We also examined how increasing NO/cGMP signaling affects LID expression by acutely administering molsidomine (an NO donor) or zaprinast (a cGMP phosphodiesterase 5-PDE5 inhibitor) before L-DOPA in mice with established dyskinesia. Paradoxically, the administration of either of these drugs also significantly diminished the expression of established LID; however, the effect occurred at the expense of the antiparkinsonian L-DOPA properties. We demonstrate that targeting the NO/cGMP signaling pathway reduces dyskinetic behaviors and molecular markers, but only the 7-NI treatment preserved the antiparkinsonian effect of L-DOPA, indicating that NOS inhibitors represent a potential therapy to reduce LID.

Introduction

Parkinson disease (PD) is a neurodegenerative disease with both genetic and acquired etiologies. It is characterized by the degeneration of the dopaminergic neurons of the substantia nigra and a decrease of dopamine (DA) in the dorsal striatum (Dauer and Przedborski, 2003). Despite the progress in PD research, current treatment focuses on dopamine replacement therapy through the administration of L-3,4-dihydroxyphenylalanine (L-DOPA). However, repeated treatment with L-DOPA is associated with adverse effects, including a reduction in drug efficacy ("wearing off" and "on–off" fluctuations) and the onset of dyskinesia. L-DOPA-induced dyskinesia (LID) is characterized by excessive and abnormal purposeless movements, which interfere with physiological motor activity. The prevalence of LID in PD patients increases with L-DOPA treatment duration, and once it develops, the LID severity increases over time. This LID can be debilitating and represents a major disadvantage of continued L-DOPA therapy (Gerlach et al., 2011; Jankovic, 2005; Prashanth et al., 2011).

Despite extensive investigation, the mechanisms underlying LID are not completely understood. Several lines of evidence have associated dyskinesia with the expression of FosB, phosphorylation of ERK1/2, and the phosphoacetylation of histone 3 (H3) (Andersson et al., 1999; Cenci and Konradi, 2010; Murer and Moratalla, 2011; Pavón et al., 2006; Santini et al., 2007; Westin et al., 2007). These molecular markers of dyskinesia require the D1, but not the D2, dopamine receptor (Darmopil et al., 2009). A wide range of other neurotransmitter systems and molecular mechanisms have been proposed to participate in the pathogenesis of dyskinesia, including glutamate, serotonin, adenosine, noradrenaline, and the cannabinoid receptor (González-Aparicio and Moratalla, 2014; Huot et al., 2013; Iravani and Jenner, 2011), but their precise contributions to LID remain less well understood. A growing body of evidence also suggests that nitric oxide (NO) plays a role in the maintenance of LID (Del Bel et al., 2005; Padovan-Neto et al., 2011).

The gaseous signaling molecule NO is produced by a subclass of interneurons containing the neuronal NO synthase (nNOS). NO activates the secondary messenger cyclic guanosine monophosphate (cGMP) through activation of soluble guanyl cyclase and plays a crucial role in

* Corresponding author at: Instituto Cajal, CSIC, Avd. Dr. Arce 37, 28002 Madrid, Spain. Fax: +34 91 585 4754.
E-mail address: moratalla@cajal.csic.es (R. Moratalla).
Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2014.09.010
0969-9961/

the integration of glutamate and DA transmission (West and Tseng, 2011). NO signaling may play an important role in the integration of information transmitted to basal ganglia output centers via corticostriatal and striatal efferent pathways (Del Bel et al., 2005). Neuronal NOS inhibitors have received increasing attention as a potential therapeutic approach to ameliorate dyskinesia (Novaretti et al., 2010; Padovan-Neto et al., 2009; Takuma et al., 2012). Behavioral studies have demonstrated that NOS inhibitors reduce locomotor activity and hyperlocomotion induced by cocaine or methamphetamine in mice (Del Bel et al., 2002; Itzhak, 1997; Ohno and Watanabe, 1995). Interestingly, striatal NOS activity is depressed in both parkinsonian animal models (Sancesario et al., 2004) and human PD (Eve et al., 1998). In addition, phosphodiesterase-5 inhibitors have been able to rescue LID and L-DOPA-induced loss of striatal long-term depression (Picconi et al., 2011). These data suggest that NO signaling plays a pivotal role in basal ganglia and nigrostriatal dopaminergic activity.

The Pitx3-deficient aphakia mouse (*Pitx3*$^{-/-}$) is considered a reliable animal model of PD. Pitx3 is a transcription factor implicated in the development of midbrain DA neurons (Hwang et al., 2003; Nunes et al., 2003; van den Munckhof et al., 2003), and Pitx3 polymorphisms have been associated with PD (Fuchs et al., 2009). The *Pitx3*$^{-/-}$ mice present a selective bilateral dopamine depletion in the nigrostriatal system that is associated with impaired spontaneous locomotor activity, showing akinesia (Nunes et al., 2003; van den Munckhof et al., 2003), and thus provide a consistent tool to study drugs with antiparkinsonian activity (Hwang et al., 2005; van den Munckhof et al., 2006). This model is also a good predictor of the ability of L-DOPA to induce dyskinesia and reproduces the biochemical changes induced by L-DOPA in MPTP-treated monkeys, mice, and rats (Ding et al., 2007, 2011; Espadas et al., 2012; Li and Zhou, 2013). Furthermore, the *Pitx3*$^{-/-}$ mouse is the only genetic model of PD that develops dyskinesia following L-DOPA administration (Iderberg et al., 2012).

To further understand the potential role of the nitrergic system in the development and expression of LID, we used the nNOS inhibitor 7-nitroindazole (7-NI), the NO donor molsidomine, and the cGMP phosphodiesterase 5 (PDE5) inhibitor zaprinast to modulate the NO signaling pathway in *Pitx3*$^{-/-}$ mice. We evaluated motor performance using rotarod and locomotor activity tests along with measurements of specific cellular markers known to be strongly associated with LID [FosB, H3 phophoacetylation (pAcH3), and pERK].

Materials and methods

Animals

This study was performed in 4–6 month-old *Pitx3*$^{-/-}$ mice (Nunes et al., 2003). The genotype of all *Pitx3*$^{-/-}$ mice was confirmed with a polymerase chain reaction amplification analysis of tail-tip DNA. All mice were housed under a 12-h dark/light cycle with free access to food and water. The mice weighed 24–30 g at the beginning of the study. Animal care and experimental procedures were conducted in accordance with the European Union Council Directive (86/609/EEC), and the experimental protocols involving animals were approved by the Consejo Superior de Investigaciones Científicas ethics committee.

Drug treatment and experimental design

L-DOPA, benserazide, 7-NI, molsidomine, and zaprinast were purchased from Sigma-Aldrich (Madrid, Spain). L-DOPA (10 mg/kg, i.p.) was administrated with the peripheral dopa decarboxylase inhibitor benserazide (10 mg/kg, i.p.). The dose regimen and route of administration were based on previously published studies (Ding et al., 2007; Giorgi et al., 2008; Lorenc-Koci et al., 2013; Padovan-Neto et al., 2009). Zaprinast (30 mg/kg, s.c.) and 7-NI (30 mg/kg, i.p.) were prepared in 10% DMSO-saline and injected 30 min prior to L-DOPA. Molsidomine (2 mg/kg, i.p.) was dissolved in saline and injected 10 min prior to L-DOPA. All drugs and their respective vehicles were freshly prepared before use and injected in a volume of 10 mL/kg.

7-NI, a nNOS selective inhibitor (Deutsch et al., 1996), was used to investigate the effect of nNOS inhibition on the development and expression of dyskinesia. To assess the effect of 7-NI on the development of LID, 7-NI or the vehicle was injected daily 30 min prior to administering L-DOPA as described above. The *Pitx3*$^{-/-}$ mice were randomized to one of the following 6 treatment groups: (i) vehicle ($n = 8$); (ii) chronic 7-NI ($n = 10$); (iii) vehicle + acute L-DOPA ($n = 10$); (iv) acute L-DOPA + acute 7-NI ($n = 10$); (v) chronic L-DOPA + vehicle ($n = 21$); and (vi) chronic co-treatment of L-DOPA + 7-NI ($n = 15$). To determine whether the effect of 7-NI is lingering, we treated another group of mice with L-DOPA and 7-NI for 13 days and administered L-DOPA alone on Day 14 ($n = 5$, subgroup of group vi).

Moreover, to assess the role of 7-NI in the expression of dyskinesia, a subgroup of Group (v) with established dyskinesia was injected with either 7-NI (subacute; $n = 8$) or the vehicle ($n = 8$, a subgroup of Group v) for 3 consecutive days (Days 15–17) 30 min prior to administering L-DOPA. The mice in each group were sacrificed 1 h after the last L-DOPA treatment to obtain the brains for immunohistochemical assessment.

We also evaluated the effects of an increase in NO/cGMP signaling in already established LID. In this case, *Pitx3*$^{-/-}$ mice that were rendered dyskinetic following chronic L-DOPA treatment were administered either molsidomine ($n = 8$) or zaprinast ($n = 8$) for 3 days (Days 15–17) as described above. Controls ($n = 7$) received the vehicle. Molsidomine, an NO donor, and zaprinast, a PDE5 inhibitor, are used extensively as pharmacological tools to study the NO/cGMP pathway in the striatum (Feelisch, 1998; Giorgi et al., 2008).

Behavioral tests

Dyskinesia

L-DOPA-induced dyskinesia consisted primarily of abnormal stereotypic paw movements that were qualitatively identical to the dyskinetic events described by Ding et al. (2007). The quantification of the dyskinetic events was performed manually (Ding et al., 2007). On odd days, we placed individual mice in home cages and video-recorded their motor activity following the drug administration. A 4-minute video was taken 30 min and 60 min after the L-DOPA injection. Previous studies (Pavón et al., 2006; Ruiz-DeDiego et al., 2014) have demonstrated that the incidence and intensity of abnormal involuntary movements are maximal at 30 and 60 min following L-DOPA administration. On Day 14, we evaluated the LID every 20 min for 160 min after administering L-DOPA. The videos were manually analyzed off-line to calculate the total dyskinesia duration by summing the duration of all three-paw and four-paw dyskinetic events, as described by Ding et al. (2011).

Rotarod test

We used the rotarod (UgoBasile, Rome, Italy) test (González-Aparicio and Moratalla, 2014) to evaluate the motor coordination and balance. All animal groups were habituated to the rod before the sessions, and each mouse was given a 10-min training session on the rod with it set at a constant speed of 10 rpm. If the mouse fell from the rotarod, it was placed back on. We measured the time each animal was able to maintain its balance on the rod during a 3-min session at 10 rpm. Animals were tested ~60 min (the peak of dyskinetic symptoms) and/or ~90 min (to avoid the peak of dyskinetic symptoms) after the L-DOPA injection.

Locomotor activity

On even days during the chronic L-DOPA treatment, horizontal and vertical locomotor activity was assessed during the light phase using Activity Cages (AccuScan Instruments Inc.). The activity was measured over a 30 min period beginning 60 min after administering the L-DOPA. Automated hardware and software (AccuScan) were used to

record the number of beam breaks for each beam, similar to the protocol of González-Aparicio and Moratalla (2014).

Tissue preparation

Animals were sacrificed 1 h after the last L-DOPA injection by an overdose of pentobarbital (Laboratorios Normon, Madrid, Spain), followed by an intracardial injection of 0.5 mL 1% heparin (Rovi, Madrid, Spain) and perfusion with 10 mL saline and 100 mL 4% paraformaldehyde (pH 7.4). The brains were post-fixed for 24 h and transferred to a solution of 0.1 M phosphate buffer (PB) containing 0.02% sodium azide for storage at 4 °C. The brains were then immersed in 3% agarose and sectioned at a thickness of 30 μm using a vibratome (Leica, Wetzlar, Germany).

Immunohistochemistry

Immunostaining was performed on free-floating coronal brain sections using a standard avidin–biotin immunocytochemical protocol (Granado et al., 2008; Darmopil et al., 2008) with the following rabbit antisera: tyrosine hydroxylase (TH; 1:1000; Chemicon, Temecula, California), FosB (1:7500; Santa Cruz Biotechnology, Santa Cruz, California), pERK (1:250; Cell Signaling Technology, Beverly, Massachusetts), phospho-(Ser10)-acetyl (Lys14)-histone 3 (pAcH3; 1:1500; Upstate — Cell Signaling Solutions, Lake Placid, New York), or nNOS (1:3000; gift from Dr. J. Rodrigo, Instituto Cajal, CSIC, Madrid, Spain). The quantification of the TH, nNOS, FosB, pERK, and pAcH3 immunoreactivity was performed using Image J, an image analysis system (Schneider et al., 2012). Immunostaining intensity and the number of immunolabeled nuclei/cells were determined for all animals in each group using two serial rostrocaudal sections per animal from both sides of the dorsolateral striatum for a total of 6 images per animal. Digital images were obtained under a Leica microscope using a 40× objective. The data are presented as the number of stained nuclei per mm^2 in the lesioned striatum. For the nNOS inmunohistochemistry, the images were thresholded at a standardized gray-scale level before measuring the intensity to include only the nNOS cell bodies. The data are expressed as mean gray values (Ares-Santos et al., 2014). Neurolucida (MicroBrightField, Williston, Vermont) was used to trace the soma of the nNOS positive neurons to determine the somal area. The quantifications were done by a researcher blind to the experimental conditions.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Behavioral data was analyzed using repeated-measures two-way analyses of variance (ANOVAs) followed by Bonferroni post hoc tests. The quantifications of the immunostaining intensities were compared by one-way ANOVAs and post hoc Bonferroni tests. The criterion for significance was $p < 0.05$.

Results

Treatment with the nNOS inhibitor 7-NI attenuates the development of LID in the Pitx3$^{-/-}$ mice

We used *Pitx3*$^{-/-}$ mice to confirm the role of nNOS inhibition in the development of L-DOPA-induced dyskinesia, because the bilateral loss of DA neurons and bilateral dyskinetic symptoms in these mice mimic human PD patients more closely than the widely used unilateral 6-OHDA-lesioned mouse model. In accordance with previous reports (Ding et al., 2007; Li and Zhou, 2013), the chronic L-DOPA (10 mg/kg) treatment of *Pitx3*$^{-/-}$ mice resulted in dyskinetic movements, including front paw, hind paw, three-paw, and four-paw dyskinetic movements. As a measure of the intensity of dyskinesia, we quantified only the three- and four-paw dyskinetic movements (Ding et al., 2011) measured 30 and 60 min after administering L-DOPA. Three- and four-paw dyskinetic movements appeared after the first injection of L-DOPA and significantly increased in frequency during the first week of treatment. After this, the frequency of the dyskinetic movements remained stable for the remainder of the chronic treatment.

Systemic administration of the nNOS inhibitor 7-NI (30 mg/kg) 30 min before the daily L-DOPA injection significantly reduced the duration of three/four-paw dyskinetic movements on each test day at 30 min post-L-DOPA administration ($p < 0.05$), except for Days 9 and 13 of treatment. In addition, we found that administering L-DOPA alone on Day 14 to a group of *Pitx3*$^{-/-}$ mice that had been treated with the combination of L-DOPA and 7-NI for 13 days did not increase the dyskinetic score beyond what was observed on the previous day ($p < 0.05$; Figs. 1A and B). Sixty minutes after the L-DOPA administration, the difference was no longer significant, but a trend for 7-NI to attenuate the dyskinetic symptoms was observed (Fig. 1B). *Pitx3*$^{-/-}$ mice treated with the repeated administration of 7-NI alone showed no dyskinetic movements. The global time courses revealed that dyskinesias were present for at least 100 min after administering L-DOPA. The 7-NI significantly reduced the LID after 40 and 60 min, the peak-dose dyskinesia interval (Fig. 1C). In another group of animals, we evaluated the effect of 7-NI on acute L-DOPA-induced dyskinesia and found that the 7-NI pretreatment prevented the LID symptoms both 30 and 60 min after the first L-DOPA injection ($p < 0.05$; Fig. 1D).

7-NI treatment also attenuated the expression of established LID

To further characterize the antidyskinetic effect of 7-NI, we used a crossover design in the chronic L-DOPA-treated mice used in the previous experiment. Thus, on Day 15, we divided the mice into two equivalent groups with one group continuing to receive L-DOPA and the vehicle, while the other group received L-DOPA plus 7-NI. As shown in Fig. 2, on Day 15, the animals treated with the nNOS inhibitor showed a significant reduction in the duration of L-DOPA-induced three/four-paw dyskinesia, both 30 min ($p < 0.05$; Fig. 2A) and 60 min ($p < 0.05$; Fig. 2B) post-treatment, in comparison with the group that received L-DOPA alone, and this decrease was still evident at the end of the experiment (Day 17). Thus, 7-NI was able to attenuate the expression of established LID, in agreement with previous results in the 6-OHDA unilaterally dyskinetic rat model (Padovan-Neto et al., 2009).

Neuronal NOS inhibitor does not affect the therapeutic effect of L-DOPA

To address whether the antidyskinetic action of 7-NI occurred at the expense of its antiparkinsonian effect, we measured locomotor activity 60 min post-L-DOPA and motor coordination on the rotarod 90 min post-L-DOPA. On all evaluation days, the rotarod test revealed no significant differences in the motor performance between the *Pitx3*$^{-/-}$ mice chronically treated with both L-DOPA and 7-NI in comparison to the mice treated with L-DOPA or 7-NI alone ($p > 0.05$; Fig. 3A).

Due to the robust decrease of the established LID after the 7-NI treatment, we next assessed how the subacute administration of 7-NI might affect the motor coordination in *Pitx3*$^{-/-}$ mice chronically treated with L-DOPA. On the second day of the 7-NI treatment (Day 16 of the L-DOPA treatment), we measured the latency on the rotarod at the peak of the dyskinetic effect (60 min), as well as 90 min after the L-DOPA treatment to avoid the peak of dyskinesia. We found that the *Pitx3*$^{-/-}$ mice that were injected with 7-NI prior to L-DOPA significantly improved their motor performance on the rod at 60 min ($p < 0.05$), and showed an increased, though not significant, amount of time spent on the rotarod at 90 min post-L-DOPA administration (Fig. 3B).

We also examined horizontal and vertical activity for 30 min, beginning 60 min after the L-DOPA injection. As expected based on previous studies, the L-DOPA significantly increased horizontal activity relative to saline ($p < 0.001$). The 7-NI had no effect on horizontal activity, with or without L-DOPA. There was no significant difference between L-DOPA administered alone and in conjunction with 7-NI (Fig. 3C).

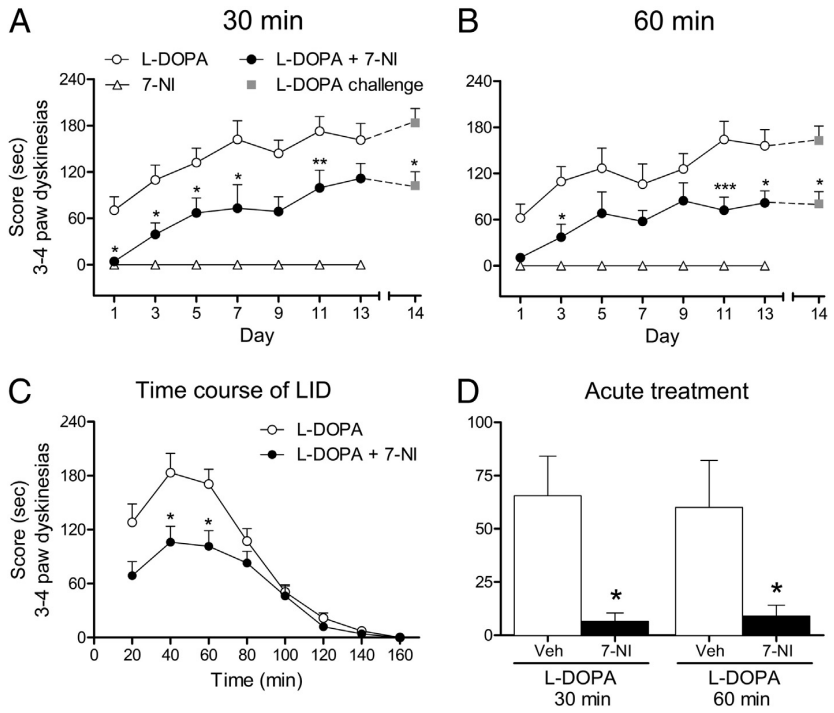


Fig. 1. The effect of 7-NI on the development of L-DOPA-induced dyskinesia in *Pitx3*$^{-/-}$ mice. Chronic 7-NI (30 mg/kg) pretreatment significantly attenuates dyskinesia at 30 min (A) and 60 min (B) post-L-DOPA. On Day 14, following a 24 h drug washout, all animals received only L-DOPA. A two-way ANOVA with repeated measures followed by a Bonferroni test showed significant differences for treatment [$F_{2,262} = 118.44$, $p < 0.001$ for 30 min; $F_{2,262} = 105.90$, $p < 0.001$ for 60 min] and day [$F_{7,262} = 5.96$, $p < 0.001$ for 30 min; $F_{7,262} = 4.15$, $p < 0.01$ for 60 min]. The kinetic profile of dyskinetic symptoms was evaluated once every 20 min over 160 min on Day 15 of the L-DOPA treatment (C). A two-way ANOVA with repeated measures followed by a Bonferroni test showed significant differences for treatment [$F_{1,88} = 13.72$, $p < 0.001$] and time [$F_{7,88} = 24.34$, $p < 0.001$]. The acute co-treatment with 7-NI and L-DOPA was also effective at 30 min and 60 min (D), and a two-way ANOVA followed by a Bonferroni test showed significant differences for treatment [$F_{1,36} = 13.79$, $p < 0.001$]. The data are expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus L-DOPA alone; $n = 10–18$ for each treatment.

There was also no significant difference in horizontal activity between the 7-NI-treated and saline-treated *Pitx3*$^{-/-}$ mice. However, the L-DOPA + 7-NI treatment had a tendency to decrease the vertical activity response in comparison with that of L-DOPA alone ($p > 0.05$; Fig. 3D).

The extent of striatal DA denervation in Pitx3$^{-/-}$ *mice is not affected by the drug treatments*

Previous studies have documented a reduction in DA innervation in the dorsal striatum of *Pitx3*$^{-/-}$ mice (Nunes et al., 2003). To confirm that the drug treatments had no effect on the extent of the dopaminergic denervation, we used TH-immunoreactivity (TH-ir) to assess the percentage of striatal area devoid of TH-ir for each treatment group (Granado et al., 2011). On average, 60% of the striatum was denervated in all treatment groups; we found no significant differences between the groups ($p > 0.05$). This confirms that the difference in the intensity of dyskinesia observed in the different groups was due to the pharmacological treatments and not to differences in the extent of lesions (Supplementary Fig. 1A). In the *Pitx3*$^{-/-}$ mice, the striatal denervated areas are similar and occur in both hemispheres (Supplementary Fig. 1B).

7-NI decreases nNOS immunoreactivity in the striatum

The nNOS-immunoreactive cell bodies were pyriform or oval and had two or three main processes (Fig. 4A). The intensity of the nNOS immunoreactivity (nNOS-ir) in the striatum was greater after chronic L-DOPA treatment than in the saline-treated mice ($p < 0.01$). Repeated treatment with 7-NI prior to L-DOPA induced a significant reduction in the intensity of nNOS immunoreactivity compared to the L-DOPA-alone group ($p < 0.05$), while the 7-NI treatment (Days 15–17) of mice with established dyskinesia did not modify the L-DOPA-induced nNOS-ir increase (Fig. 4B). The morphological reconstruction of the cell body perimeter allowed us to assess the total area of cell bodies, showing that the nNOS-ir somas in the L-DOPA-treated mice were significantly larger than those in the saline-treated mice ($p < 0.05$). This also revealed that the 7-NI treatment had no effect on the size of the nNOS-immunoreactive cell somas when L-DOPA was delivered chronically or acutely on Days 15–17 after the establishment of dyskinesia (Fig. 4C). The nNOS-ir was quantified only in totally denervated striatal areas, as was done in Suárez et al. (2014) (Fig. 4D).

Effect of 7-NI treatment on FosB expression in the denervated striatum of L-DOPA-treated mice

Increased FosB expression in the dorsolateral striatum of parkinsonian animals correlates with the appearance of dyskinesia (Darmopil et al., 2009). Thus, immunohistochemistry was used to examine the effects of nNOS inhibition on L-DOPA-induced FosB expression in the DA-denervated striatum of the *Pitx3*$^{-/-}$ mice (Fig. 5A). The acute L-DOPA treatment induced a small increase in FosB expression in comparison to the saline- and 7-NI-treated animals. Interestingly, the acute co-administration of 7-NI caused a significant decrease in FosB expression in the denervated striatum ($p < 0.05$). In keeping with previous reports (Pavón et al., 2006), the chronic L-DOPA treatment induced significantly higher levels of FosB immunoreactivity in the denervated striatum than did the saline or 7-NI treatments. Co-treating with chronic 7-NI significantly diminished the number of FosB-positive cells compared to the chronic L-DOPA-treated mice ($p < 0.001$). The administration of 7-NI on Days 15–17 also significantly reduced L-DOPA-induced FosB expression, but to a lesser extent ($p < 0.05$; Fig. 5B).

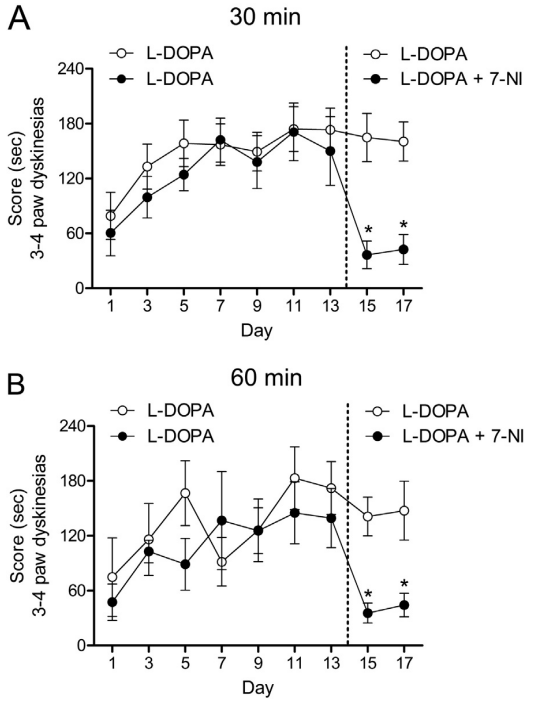


Fig. 2. The effect of nNOS inhibition on established LID. *Pitx3*$^{-/-}$ mice received daily injections of L-DOPA (10 mg/kg) for 14 days to establish the expression of LID. On Days 15–17, the animals received 7-NI (30 mg/kg) or the vehicle prior to the L-DOPA challenge. The 7-NI treatment significantly reduced dyskinesia at 30 (A) and 60 (B) min post-L-DOPA. A two-way ANOVA followed by a Bonferroni test showed significant differences for treatment [$F_{1,106} = 11.32$, $p < 0.001$ for 30 min; $F_{1,78} = 8.03$, $p < 0.01$ for 60 min] and day [$F_{8,106} = 3.62$, $p < 0.001$ for 30 min; $F_{8,78} = 2.42$, $p < 0.05$ for 60 min]. The data are expressed as the mean ± SEM. *$p < 0.05$ versus L-DOPA + vehicle; $n = 8$ for each group.

7-NI attenuated L-DOPA-induced phosphoacetylation of histone 3 (pAcH3)

We have previously shown (Darmopil et al., 2009) that the repeated administration of L-DOPA in hemiparkinsonian animals greatly increased phosphoacetylation of H3. In keeping with these results, we found that *Pitx3*$^{-/-}$ mice chronically treated with L-DOPA showed a significant increase in pAcH3 immunoreactivity (Fig. 6A). Repeated treatment with 7-NI prior to administering L-DOPA caused a significant decrease in pAcH3 expression ($p < 0.05$). The 7-NI treatment (Days 15–17) of mice with established dyskinesia also induced a significant reduction in pAcH3 expression ($p < 0.05$). The acute L-DOPA treatment induced a marked increase in the phosphorylation and acetylation of H3 in comparison to the saline-treated mice, an effect partially reversed by 7-NI ($p < 0.05$; Fig. 6B).

7-NI treatment reduces pERK expression produced by L-DOPA

Our previous studies demonstrated ERK activation in 6-OHDA-lesioned mice after administering L-DOPA (Darmopil et al., 2009; Pavón et al., 2006). In accordance with these results, we found that acute L-DOPA treatment induced a marked increase in pERK in both sides of the striatum of *Pitx3*$^{-/-}$ mice in comparison to the saline-treated animals (Fig. 7A). The 7-NI treatment significantly reduced the acute L-DOPA-induced ERK1/2 phosphorylation ($p < 0.05$), while the chronic L-DOPA treatment significantly increased the number of pERK-positive cells in the dorsal striatum relative to the mice treated with only saline or 7-NI. Interestingly, neither the chronic nor subacute 7-NI treatment had an effect on this increase in pERK expression ($p > 0.05$; Fig. 7B).

Molsidomine and zaprinast decrease the expression of LID, but affect the anti-akinetic effect of L-DOPA

Previous studies have implicated increased NO/cGMP signaling in the regulation of dyskinetic symptoms in 6-OHDA-lesioned rats (Giorgi et al., 2008; Picconi et al., 2011). To examine the role of increased NO signaling, we tested whether molsidomine or zaprinast could reduce established LID in *Pitx3*$^{-/-}$ mice. Daily L-DOPA treatment (14 days) led to the expression of dyskinesia in all treated animals. Molsidomine given before L-DOPA for three consecutive days (Days 15–17) significantly attenuated the LID relative to L-DOPA alone ($p < 0.05$; Fig. 8A). Similarly, the expression of dyskinesia was also significantly reduced in response to zaprinast given prior to L-DOPA for three days (Days 15–17) in comparison to L-DOPA administered alone ($p < 0.05$; Fig. 8A).

To further evaluate molsidomine and zaprinast as potential antidyskinetic drugs, we assessed whether these treatments inhibited the antiparkinsonian effects of the L-DOPA treatment in these animals. Mice were tested on the rotarod 90 min after the injection with L-DOPA to avoid the peak of the dyskinetic symptoms. Surprisingly, the administration of either molsidomine or zaprinast prior to the L-DOPA treatment significantly reduced the time spent on the rod ($p < 0.05$), blocking the antiparkinsonian action of L-DOPA (Fig. 8B).

Discussion

The work described here demonstrates that the development of dyskinesia induced by chronic exposure to L-DOPA can be significantly decreased by the chronic administration of 7-NI, which also attenuates established dyskinesia when administered subacutely in *Pitx3*$^{-/-}$ mice. In both cases, 7-NI blocks LID without affecting the therapeutic antiparkinsonian effect of L-DOPA. The chronic L-DOPA treatment also increases nNOS-ir in totally denervated striatal areas. As seen in other models of PD, the expression of FosB, pAcH3, and pERK was associated with the development and expression of dyskinesia. Neuronal NOS inhibition with 7-NI significantly reduced FosB and pAcH3 expression in mice treated either acutely or chronically with L-DOPA, but diminished pERK expression only after acute L-DOPA administration, with no effect on the pERK levels in the mice treated chronically with L-DOPA. Intriguingly, increasing the NO/cGMP signaling with molsidomine and zaprinast attenuated the expression of LID, but reduced the antiparkinsonian effect of L-DOPA.

Although previous studies have reported the effect of nNOS inhibition on LID in 6-OHDA-lesioned rats, we chose to assess the antidyskinetic effect of 7-NI in *Pitx3*$^{-/-}$ mice that show a bilateral dyskinesia similar to the dyskinetic symptoms in parkinsonian patients (Ding et al., 2011; Espadas et al., 2012; Li and Zhou, 2013). While other genetic models of PD reproduce some aspects of the behavioral and pathological characteristics of PD, the *Pitx3*$^{-/-}$ mice provide the first genetic rodent model in which the animals exhibit a loss of midbrain DA neurons and behavioral deficits that can be reversed by treating with L-DOPA (Hwang et al., 2005; van den Munckhof et al., 2006), similar to those symptoms seen in human PD (Carta et al., 2012). The *Pitx3*$^{-/-}$ mice also share many characteristics of human LID, and chronic L-DOPA treatment leads to the progressive development of dyskinetic movements. In addition, this model reproduces peak-dose dyskinesia. At the beginning of treatment, only a few mice develop LID, but eventually most do, which is very similar to what is seen in the clinic (Ding et al., 2007, 2011; Li and Zhou, 2013). Furthermore, the *Pitx3*$^{-/-}$ mouse is the only genetic model of PD that develops dyskinesia following the administration of L-DOPA (Iderberg et al., 2012). Moreover, the drugs that attenuate dyskinesia in PD patients and other animal models (Lundblad et al., 2005; Schapira, 2009) also diminish LID in *Pitx3*$^{-/-}$ mice (Ding et al., 2007), suggesting similar underlying

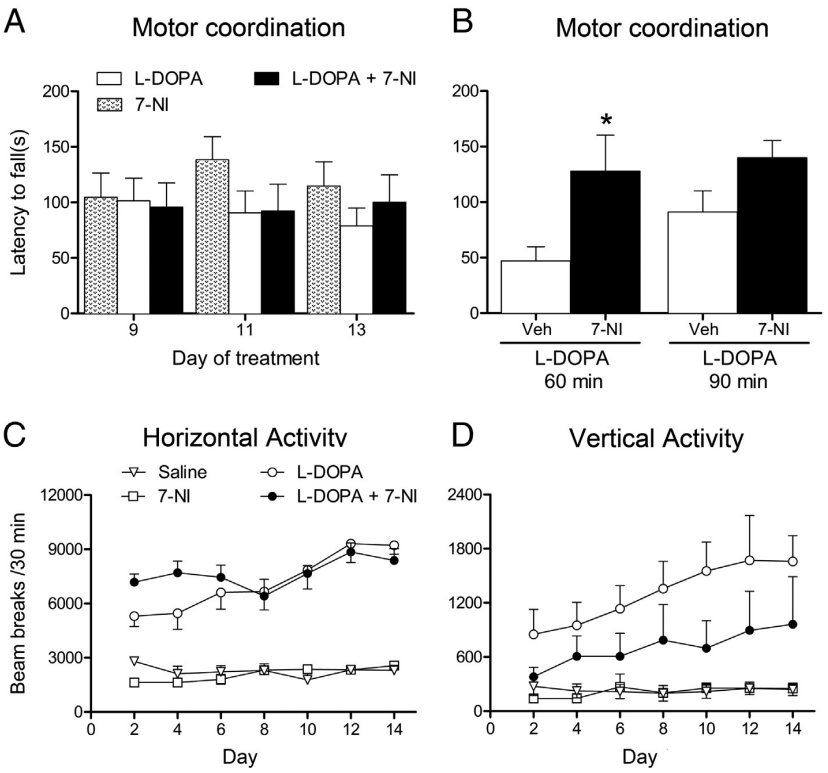


Fig. 3. Neuronal NOS inhibition does not affect the therapeutic effect of L-DOPA. (A) Chronic 7-NI pretreatment does not modify the time on the rotarod at 90 min post-L-DOPA administration. (B) The 7-NI challenge in *Pitx3*$^{-/-}$ mice with established LID improves the latency to fall from the rotarod at 60 and 90 min post-L-DOPA on Day 16 of treatment. A two-way ANOVA followed by a Bonferroni test showed significant differences for treatment [$F_{1,20} = 9.85$, $p < 0.01$] and no significant effect of time or the interaction of treatment × time. (C) Horizontal and (D) vertical locomotor activity measured in a multicage activity meter system 60 min after the L-DOPA or vehicle challenge. The data are expressed as the mean ± SEM. *$p < 0.05$ versus L-DOPA + vehicle.

mechanisms. With regard to the phenotype of *Pitx3*$^{-/-}$ mice, Smidt et al. (2004) demonstrated that substantia nigra pars compacta dopaminergic neurons are absent from embryonic day 12.5 onwards, suggesting a developmental failure. In addition, although *Pitx3*$^{-/-}$ mice are normal in appearance and weight, they show abnormal eye lens development and are blind (Hwang et al., 2003).

The effects of 7-NI on the LID in *Pitx3*$^{-/-}$ mice that we report here are in agreement with the changes observed in the 6-OHDA rat model of dyskinesia (Takuma et al., 2012). The results in both models suggest that 7-NI may affect the development of sensitization to L-DOPA. This contention is further supported by our data where the administration of L-DOPA alone to a group of *Pitx3*$^{-/-}$ mice treated with the combination of L-DOPA and 7-NI for 13 days did not increase the dyskinetic score beyond what was observed on the previous day, suggesting a lingering effect of the 7-NI. In line with previous results in the rat model (Padovan-Neto et al., 2009), we also found that the sub-acute administration of 7-NI consistently diminished the severity of fully established dyskinesia. These antidyskinetic properties of 7-NI are in agreement with the effects of other NOS inhibitors such as L-N^{G}-nitro arginine (L-NOARG) and L-N^{G}nitro arginine methyl ester (L-NAME), which reduce dyskinesia in the 6-OHDA rat model of PD (Padovan-Neto et al., 2011; Takuma et al., 2012). Together, these data add to the general knowledge that NO plays an important role in the development and expression of dyskinesia.

We hypothesized that an overactive nitrergic system contributes to the pathogenesis of LID. In this regard, we showed that L-DOPA induces an increase in nNOS-ir and in the size of the nNOS-immunoreactive neurons in striatal areas that are totally denervated. Previous studies have reported that L-DOPA did not produce an increase in the nNOS levels in the ipsilateral striatum of either the 6-OHDA rat model (Padovan-Neto et al., 2011) or MPTP-treated mice (Chalimoniuk and Langfort, 2007). These discrepancies may be due to different measurement methods; as indicated previously (Suárez et al., 2014), we only measured completely denervated areas, while the other studies measured nNOS levels in the whole striatum.

A growing body of evidence has shown the importance of NO in numerous pathophysiological processes, including the treatment of Parkinson disease and LID (West and Tseng, 2011). Although NO is produced in the nNOS-synthesizing interneurons in the striatum, it affects medium spiny neuron activity, primarily by stimulating the cGMP signaling pathway and through direct interactions with ligand-gated channels. Furthermore, NO stimulates the release of L-DOPA from striatal tissue, which is mediated by cGMP (Sanchez et al., 2002). Previous studies have demonstrated that chronic treatment with L-DOPA induced striatal NO production in freely mobile mice (Itokawa et al., 2006), and recently, Czarnecka et al. (2013) reported that L-DOPA enhanced nNOS protein expression in the ipsilateral substantia nigra of hemiparkinsonian rats. In addition, we showed in a previous study that chronic L-DOPA induces FosB expression in the NOS-positive striatal interneurons (Pavón et al., 2006). The mechanism underlying the ability of nNOS inhibition to attenuate the development and expression of LID remains unclear, but one possible explanation for these results is that 7-NI is reducing DA-glutamate synaptic activity, decreasing the D1/D5 receptor stimulation (Park and West, 2009) by attenuating the striatal NO efflux (Sammut et al., 2006). This effect on DA-glutamate activity could contribute to the effect of 7-NI, because reducing striatal glutamatergic inputs has an antidyskinetic effect (Bido et al., 2011). Indeed, 7-NI reduces the increased phospho-GluA1 AMPA receptor levels induced by chronic

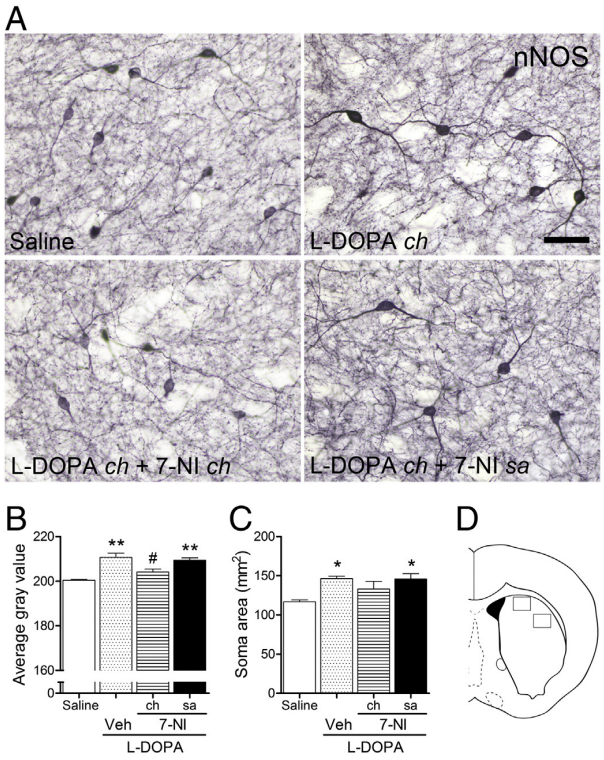


Fig. 4. 7-NI reduces the expression of nNOS in *Pitx3*$^{-/-}$ mouse striatum. Photomicrographs of nNOS positive neurons in striatal sections of mice treated with saline, L-DOPA, the subacute challenge of 7-NI + chronic L-DOPA, and the chronic co-treatment of 7-NI + L-DOPA in *Pitx3*$^{-/-}$ mice (A). Graphs showing the cytoplasmic nNOS immunohistochemical staining in the totally denervated striata, represented as average gray values (B), and the average cell body area of nNOS-ir neurons (C). A one-way ANOVA followed by a Bonferroni test showed significant differences for staining intensity [$F_{3,8} = 13.27$, $p < 0.01$] and cell body area [$F_{3,8} = 5.04$, $p < 0.05$]. The locations of the sampled areas are indicated by black boxes in the modified section from the Paxinos and Franklin (2001) brain atlas at 0.65 mm anterior to bregma (D). Data are expressed as the mean ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus saline; $^{\#}p < 0.05$ versus L-DOPA + vehicle. sa, subacute; ch, chronic. Scale bar = 50 μm.

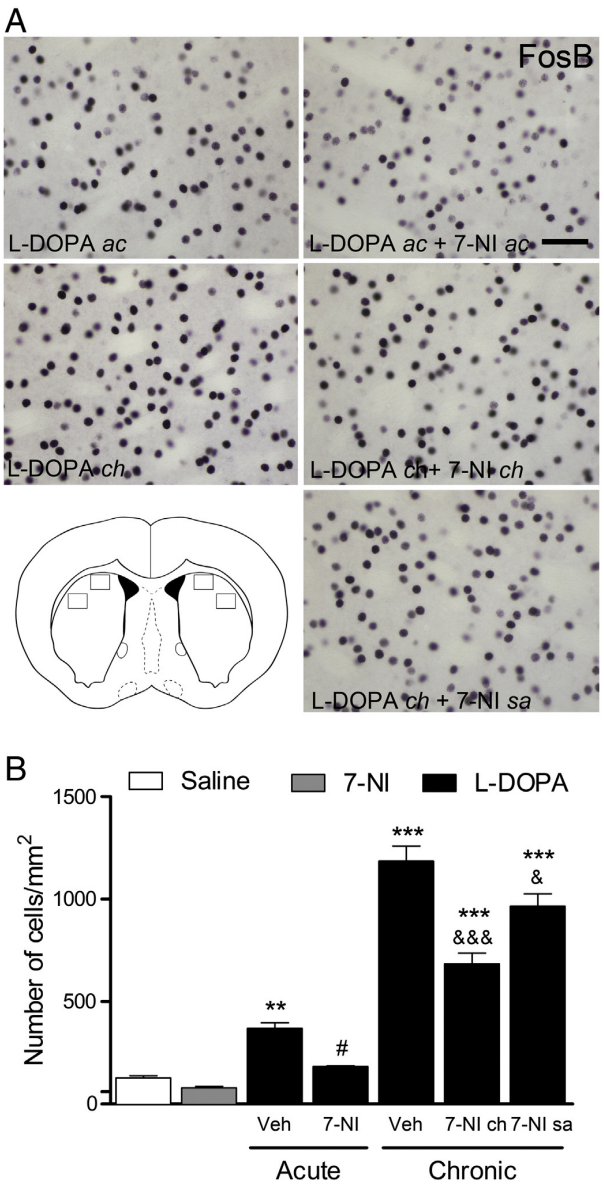
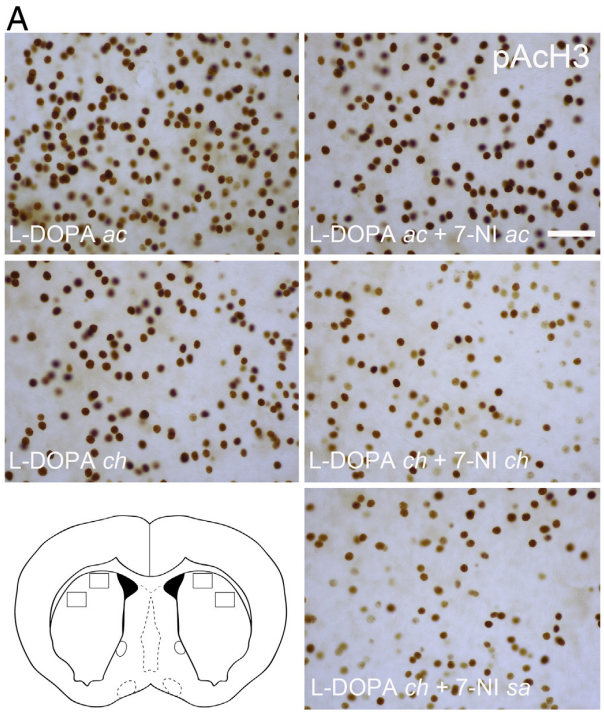


Fig. 5. 7-NI treatment decreases L-DOPA-induced FosB expression in the *Pitx3*$^{-/-}$ mouse striatum. (A) High-power microphotographs of striatal sections from *Pitx3*$^{-/-}$ mice illustrating the effect of subacute or chronic 7-NI treatment on L-DOPA-induced FosB expression. The locations of sampled areas are indicated by black boxes in the modified section from the Paxinos and Franklin (2001) brain atlas at 0.65 mm anterior to bregma. (B) Striatal quantification of FosB-positive cells after different treatments. The 7-NI challenge attenuates the increased expression of FosB-positive cells prior to acute and chronic L-DOPA treatment. Histograms represent the number of FosB-positive nuclei. The mean ± SEM values were analyzed by a one-way ANOVA followed by a Bonferroni test, and significant differences were found [$F_{6,60} = 96.80$, $p < 0.001$]. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus saline; $^{\#}p < 0.05$ versus acute L-DOPA + vehicle; $^{\&}p < 0.05$ and $^{\&\&\&}p < 0.001$ versus chronic L-DOPA + vehicle. ac, acute; sa, subacute; ch, chronic. Mice were sacrificed 1 h after the last L-DOPA injection. Scale bar = 50 μm.

L-DOPA treatment and completely blocks the phosphorylation of DARPP32 (Takuma et al., 2012), a key regulator of information processing in dopaminoceptive neurons that are known to be stimulated by the NO/cGMP signaling pathway (Nishi et al., 2005). In addition, Del-Bel et al. (2014) have shown that the reduction of established LID by an nNOS inhibitor is associated with a marked decrease in dopamine turnover in the lesioned striatum, suggesting a key role for 7-NI in normalizing the dopamine content in the striatum of dyskinetic rats. Nevertheless, we cannot rule out the role of NO with other neurotransmitter systems, because it modulates the activity of serotonin (5-HT) transporters (Kiss and Vizi, 2001), and the blockade of 5-HT neuron activity has been shown to reduce LID (Muñoz et al., 2008).

To exclude the possibility that the antidyskinetic efficacy of nNOS inhibition was due to an attenuation of L-DOPA's therapeutic capacity, we examined the effect of 7-NI on locomotor activity and motor coordination. In this study, the chronic administration of an nNOS inhibitor had no effect on horizontal activity, but did reduce vertical activity in the dyskinetic mice. The dyskinetic symptoms in the *Pitx3*$^{-/-}$ mice are different from those in the 6-OHDA model, but share some similarities to the reserpine model (Ding et al., 2007; Johnston et al., 2005). Indeed, in the reserpine model, antidyskinetic drugs (amantadine, idazoxan) also selectively reduced vertical, but not horizontal activity (Johnston et al., 2005). Our results support the antidyskinetic and antiparkinsonian effects of 7-NI. Interestingly, in established bilateral LID, nNOS inhibition produced a significant improvement in motor performance on the rotarod in comparison to the L-DOPA-treated mice, in accordance with the 6-OHDA rat model (Padovan-Neto et al., 2009). Similarly, 7-NI improved motor performance on the rotarod when chronically co-administered with L-DOPA. These findings indicate that inhibiting nNOS does not alter the antiparkinsonian effect of L-DOPA. One concern regarding the use of



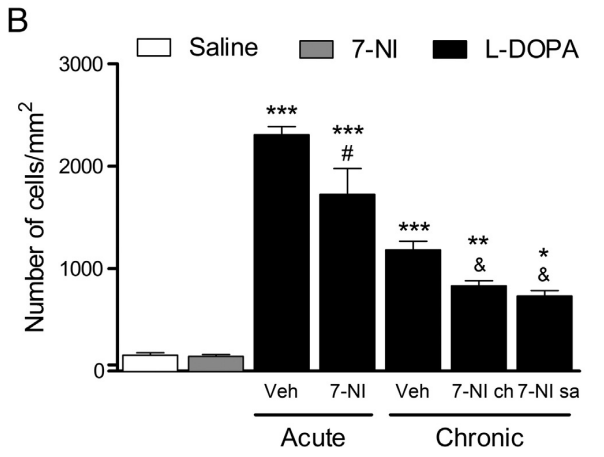


Fig. 6. Treatment with 7-NI decreases L-DOPA-induced pAcH3 expression in the *Pitx3*$^{-/-}$ mouse striatum. (A) High-power microphotographs of the striatal sections of *Pitx3*$^{-/-}$ mice illustrating the effect of subacute or chronic 7-NI treatment on pAcH3 expression induced by L-DOPA. The locations of sampled areas are indicated by black boxes in the modified section from the Paxinos and Franklin (2001) brain atlas at 0.65 mm anterior to bregma. (B) Striatal quantification of pAcH3-positive cells after different treatments; nNOS inhibition decreases acute and chronic L-DOPA-induced pAcH3 inmunoreactivity. Histograms represent the number of pAcH3-positive nuclei. The mean ± SEM values were analyzed by a one-way ANOVA followed by a Bonferroni test, and significant differences were found [$F_{6,60} = 47.21$, $p < 0.001$]. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus saline; #$p < 0.05$ versus acute L-DOPA + vehicle; &$p < 0.05$ versus chronic L-DOPA + vehicle. ac, acute; sa, subacute; ch, chronic. Mice were sacrificed 1 h after the last L-DOPA injection. Scale bar = 50 μm.

Fig. 7. 7-NI treatment decreases acute L-DOPA-induced pERK expression in the *Pitx3*$^{-/-}$ mouse striatum. (A) High-power microphotographs of striatal sections of *Pitx3*$^{-/-}$ mice illustrating the effect of subacute or chronic 7-NI treatment on pERK expression induced by L-DOPA. The locations of sampled areas are indicated by black boxes in the modified section from the Paxinos and Franklin (2001) brain atlas at 0.65 mm anterior to bregma. (B) Striatal quantification of pERK-positive cells after different treatments. The acute 7-NI challenge diminishes the expression in pERK-positive cells induced by the acute L-DOPA challenge. Histograms represent the number of pERK-positive nuclei. The mean ± SEM values were analyzed by a one-way ANOVA followed by a Bonferroni test, and significant differences were found [$F_{6,60} = 22.86$, $p < 0.001$]. **$p < 0.01$ and ***$p < 0.001$ versus saline; #$p < 0.05$ versus acute L-DOPA + vehicle. ac, acute; sa, subacute; ch, chronic. Mice were sacrificed 1 h after the last L-DOPA injection. Scale bar = 50 μm.

NOS inhibition is the development of tolerance to nNOS inhibitors, which would likely be an obstacle for their clinical use (Del Bel et al., 2005). Importantly, for its potential clinical implications, a tolerance for this effect of 7-NI did not develop, consistent with a previous report in 6-OHDA-lesioned rats (Novaretti et al., 2010).

Intriguingly, increasing the NO/cGMP pathway activity also has an antidyskinetic effect (Giorgi et al., 2008; Picconi et al., 2011). In this study, we found an antidyskinetic effect induced by zaprinast, in accordance with previous results in 6-OHDA-lesioned rats (Giorgi et al., 2008). However, this was linked to a decrease of the antiparkinsonian effect of L-DOPA, since zaprinast reduced motor performance on the

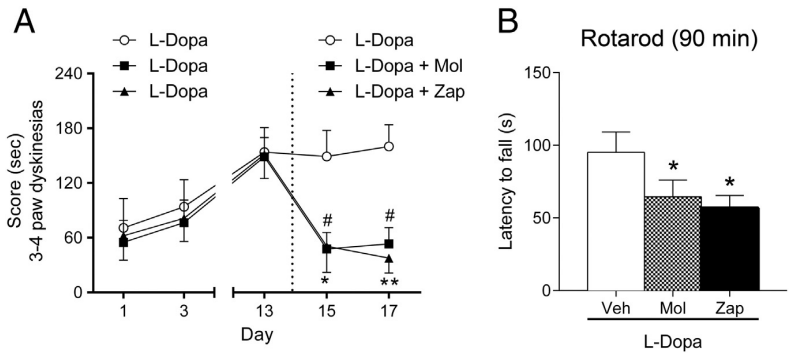


Fig. 8. Effect of increasing NO/cGMP signaling on established L-DOPA-induced dyskinesia (LID) and motor performance. *Pitx3*$^{-/-}$ mice received daily injections of L-DOPA (10 mg/kg) for 14 days to establish the expression of LID. On Days 15–17, the animals received either molsidomine (2 mg/kg), zaprinast (30 mg/kg), or the vehicle (30 mg/kg) prior to the L-DOPA challenge. The PDE inhibitor zaprinast and the NO donor molsidomine decreased established LID (A), but affected the therapeutic effect of L-DOPA (B). Data are expressed as the mean ± SEM. A two-way ANOVA followed by a Bonferroni test showed significant differences for treatment [$F_{5,200} = 4.95$, $p < 0.001$] and day [$F_{4,200} = 11.13$, $p < 0.001$]. #$p < 0.05$ versus L-DOPA + vehicle; *$p < 0.05$ versus L-DOPA + vehicle; n = 7–8 for each group.

rotarod. Nevertheless, Picconi et al. (2011) showed that zaprinast rescues the L-DOPA-induced loss of striatal long-term depression. To better understand the contribution of increasing NO levels, we also assessed the effect of molsidomine and found a reduction in LID, but a compromise of the therapeutic effects of L-DOPA, as it also reduced motor coordination in comparison to mice treated with L-DOPA alone. This reduction in the anti-akinetic effect of L-DOPA could be related to previous research (Lorenc-Koci et al., 2013) showing that the chronic administration of molsidomine reduced the L-DOPA motor effects. The pharmacological manipulation of this signaling pathway and its relationship with dyskinesia are complex, because NO can exert actions both pre- and post-synaptically, and it interacts with several transduction pathways (Garthwaite, 2008). However, more studies are needed to better understand why both treatments, increasing and decreasing NO levels, induce similar antidyskinetic effects.

L-DOPA-induced dyskinesia is associated with a hyper-responsiveness of direct-pathway medium spiny neurons (Darmopil et al., 2009; Suárez et al., 2014). Acute L-DOPA treatment elicits long-lasting alterations in synaptic transmission, while chronic treatment produces further changes that contribute to the development and expression of dyskinesia (Murer and Moratalla, 2011). L-DOPA treatment has also been shown to induce increases in FosB, pAcH3 and pERK expression in medium spiny neurons in the denervated striatum in rodent models of dyskinesia (Andersson et al., 1999; Ding et al., 2011; González-Aparicio and Moratalla, 2014; Pavón et al., 2006; Westin et al., 2007), an effect that is mediated largely by the activation of D1 receptors (Darmopil et al., 2009; Fisone and Bezard, 2011; Santini et al., 2007). Our study shows that the acute and chronic administration of L-DOPA results in a persistent elevation of FosB, pAcH3, and pERK in the dorsal striatum of *Pitx3*$^{-/-}$ mice, further confirming the association between LID and the expression of these proteins. Moreover, significant reductions in FosB immunoreactivity were observed in the striata of mice that received 7-NI before L-DOPA, acutely or chronically, consistent with previous findings in the unilateral 6-OHDA rat model (Padovan-Neto et al., 2013; Takuma et al., 2012). We also found that the chronic or acute 7-NI challenge attenuated the L-DOPA-induced pAcH3 immunoreactivity.

However, while the nNOS inhibition produced a significant decrease in the levels of pERK induced by acute exposure to L-DOPA, it had no effect on pERK induced by chronic L-DOPA exposure. We found that the acute L-DOPA treatment induced higher levels of H3 and ERK activation than the chronic treatment, whereas FosB was higher after the chronic treatment. A possible explanation for these results is that in the acute L-DOPA challenge, H3 activates a plethora of different immediate early genes (IEGs), such as cFos and JunB (Tsankova et al., 2007), that have high expression levels and are lower after chronic treatment (Cenci et al., 1999; Ebihara et al., 2011), whereas FosB protein accumulates during the L-DOPA treatment (Darmopil et al., 2009; Pavón et al., 2006). There is a debate regarding the effects of acute and chronic L-DOPA treatment on ERK activation. Several groups have shown that the acute L-DOPA treatment produces higher levels of pERK than the chronic treatment in both the 6-OHDA mouse model (Santini et al., 2010) and *Pitx3*$^{-/-}$ mice (Ding et al., 2011), suggesting that ERK is associated with the appearance of dyskinesia rather than its expression.

Increased D1 signaling activates the ERK signaling pathway, and the activation of this pathway can promote (via phosphorylation) IEGs such as FosB (Pavón et al., 2006) and the phosphoacetylation of H3 (Santini et al., 2007, 2009). Indeed, the pharmacological inactivation of the ERK pathway abolished L-DOPA-induced H3 activation (Santini et al., 2007). A recent study (Feyder et al., 2014) showed that the genetic inactivation of mitogen- and stress-activated kinase 1, a downstream target of ERK, largely reduced H3 phosphorylation at the FosB promoter. This reduction, however, was not complete indicating that additional kinases participate in the chromatin modifications induced by L-DOPA. In addition, increasing evidence shows that H3 phosphorylation can be independent of ERK activation (Bertran-Gonzalez et al., 2009; Ciccarelli et al., 2013). Moreover, Stipanovich et al. (2008) showed that H3 activation may be regulated by DARPP-32 via inhibition of protein phosphatase 1. Thus, taking into account that the 7-NI treatment reduced L-DOPA-induced DARPP-32 phosphorylation (Takuma et al., 2012), it is possible that in our mice, nNOS inhibition modifies pAcH3 through both the DARPP-32 and ERK pathways. Taken altogether, our study indicates that NO plays an important role in the short- and long-term changes in gene expression and protein activation elicited by acute and chronic L-DOPA exposure.

In summary, we demonstrate that targeting nNOS can modify both the behavioral and molecular consequences of treatment with L-DOPA and show that although increasing NO/cGMP pathway activity attenuates LID, it leads to a reduction in the beneficial effect of L-DOPA. Our results suggest a role for NO in the short- and long-term maladaptive neuroplasticity underlying dyskinesia. These findings may have significant implications in the novel management of LID based on nitrergic pathways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2014.09.010.

Acknowledgments

This work was supported by grants from the Spanish Ministerios de Economía y Competitividad (SAF2013-48532-R) and of Sanidad Política Social e Igualdad (PNSD #2012/071, and ISCIII, CIBERNED CB06/05/0055) and from Comunidad de Madrid ref. S2011/BMD-2336 to RM. OS has a CONACYT-Mexico doctoral scholarship. We thank Ms. Emilia

Rubio and Beatriz Pro for technical assistance; and Dr. Yousef Tizabi for fruitful comments on the manuscript.

References

Andersson, M., Hilbertson, A., Cenci, M.A., 1999. Striatal fosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. Neurobiol. Dis. 6, 461–474.

Ares-Santos, S., Granado, N., Espadas, I., Martinez-Murillo, R., Moratalla, R., 2014. Methamphetamine causes degeneration of dopamine cell bodies and terminals of the nigrostriatal pathway evidenced by silver staining. Neuropsychopharmacology 39, 1066–1080.

Bertran-Gonzalez, J., Håkansson, K., Borgkvist, A., Irinopoulou, T., Brami-Cherrier, K., Usiello, A., Greengard, P., Hervé, D., Girault, J.A., Valjent, E., Fisone, G., 2009. Histone H3 phosphorylation is under the opposite tonic control of dopamine D2 and adenosine A2A receptors in striatopallidal neurons. Neuropsychopharmacology 34, 1710–1720.

Bido, S., Marti, M., Morari, M., 2011. Amantadine attenuates levodopa-induced dyskinesia in mice and rats preventing the accompanying rise in nigral GABA levels. J. Neurochem. 118, 1043–1055.

Carta, A.R., Giuffrida, A., Fisone, G., 2012. Dyskinesia in Parkinson's disease therapy. Park. Dis. 2012, 639080.

Cenci, M.A., Konradi, C., 2010. Maladaptive striatal plasticity in L-DOPA-induced dyskinesia. Prog. Brain Res. 183, 209–233.

Cenci, M.A., Tranberg, A., Andersson, M., Hilbertson, A., 1999. Changes in the regional and compartmental distribution of FosB- and JunB-like immunoreactivity induced in the dopamine-denervated rat striatum by acute or chronic L-dopa treatment. Neuroscience 94, 515–527.

Chalimoniuk, M., Langfort, J., 2007. The effect of subchronic, intermittent L-DOPA treatment on neuronal nitric oxide synthase and soluble guanylyl cyclase expression and activity in the striatum and midbrain of normal and MPTP-treated mice. Neurochem. Int. 50, 821–833.

Ciccarelli, A., Calza, A., Santoru, F., Grasso, F., Concas, A., Sassoè-Pognetto, M., Giustetto, M., 2013. Morphine withdrawal produces ERK-dependent and ERK-independent epigenetic marks in neurons of the nucleus accumbens and lateral septum. Neuropharmacology 70, 168–179.

Czarnecka, A., Lenda, T., Domin, H., Konieczny, J., Smiałowska, M., Lorenc-Koci, E., 2013. Alterations in the expression of nNOS in the substantia nigra and subthalamic nucleus of 6-OHDA-lesioned rats: the effects of chronic treatment with l-DOPA and the nitric oxide donor, molsidomine. Brain Res. 1541, 92–105.

Darmopil, S., Muñetón-Gómez, V.C., de Ceballos, M.L., Bernson, M., Moratalla, R., 2008. Tyrosine hydroxylase cells appearing in the mouse striatum after dopamine denervation are likely to be projection neurones regulated by L-DOPA. Eur. J. Neurosci. 27, 580–592.

Darmopil, S., Martín, A.B., De Diego, I.R., Ares, S., Moratalla, R., 2009. Genetic inactivation of dopamine D1 but not D2 receptors inhibits L-DOPA-induced dyskinesia and histone activation. Biol. Psychiatry 66, 603–613.

Dauer, W., Przedborski, S., 2003. Parkinson's disease: mechanisms and models. Neuron 39, 889–909.

Del Bel, E.A., Souza, A.S., Guimarães, F.S., da-Silva, C.A., Nucci-da-Silva, L.P., 2002. Motor effects of acute and chronic inhibition of nitric oxide synthesis in mice. Psychopharmacology 161, 32–37.

Del Bel, E.A., Guimarães, F.S., Bermúdez-Echeverry, M., Gomes, M.Z., Schiaveto-de-souza, A., Padovan-Neto, F.E., Tumas, V., Barion-Cavalcanti, A.P., Lazzarini, M., Nucci-da-Silva, L.P., de Paula-Souza, D., 2005. Role of nitric oxide on motor behavior. Cell. Mol. Neurobiol. 25, 371–392.

Del-Bel, E., Padovan-Neto, F.E., Szawka, R.E., da-Silva, C.A., Raisman-Vozari, R., Anselmo-Franci, J., Romano-Dutra, A.C., Guimaraes, F.S., 2014. Counteraction by nitric oxide synthase inhibitor of neurochemical alterations of dopaminergic system in 6-OHDA-lesioned rats under L-DOPA treatment. Neurotox. Res. 25, 33–44.

Deutsch, S.I., Rosse, R.B., Paul, S.M., Tomasino, V., Koetzner, L., Morn, C.B., Mastropaolo, J., 1996. 7-Nitroindazole and methylene blue, inhibitors of neuronal nitric oxide synthase and NO-stimulated guanylate cyclase, block MK-801-elicited behaviors in mice. Neuropsychopharmacology 15, 37–43.

Ding, Y., Restrepo, J., Won, L., Hwang, D.Y., Kim, K.S., Kang, U.J., 2007. Chronic 3,4-dihydroxyphenylalanine treatment induces dyskinesia in aphakia mice, a novel genetic model of Parkinson's disease. Neurobiol. Dis. 27, 11–23.

Ding, Y., Won, L., Britt, J.P., Lim, S.A., McGehee, D.S., Kang, U.J., 2011. Enhanced striatal cholinergic neuronal activity mediates L-DOPA-induced dyskinesia in parkinsonian mice. Proc. Natl. Acad. Sci. U. S. A. 108, 840–845.

Ebihara, K., Ishida, Y., Takeda, R., Abe, H., Matsuo, H., Kawai, K., Magata, Y., Nishimori, T., 2011. Differential expression of FosB, c-Fos, and Zif268 in forebrain regions after acute or chronic L-DOPA treatment in a rat model of Parkinson's disease. Neurosci. Lett. 496, 90–94.

Espadas, I., Darmopil, S., Vergaño-Vera, E., Ortiz, O., Oliva, I., Vicario-Abejón, C., Martín, E.D., Moratalla, R., 2012. L-DOPA-induced increase in TH-immunoreactive striatal neurons in parkinsonian mice: insights into regulation and function. Neurobiol. Dis. 48, 271–281.

Eve, D.J., Nisbet, A.P., Kingsbury, A.E., Hewson, E.L., Daniel, S.E., Lees, A.J., Marsden, C.D., Foster, O.J., 1998. Basal ganglia neuronal nitric oxide synthase mRNA expression in Parkinson's disease. Brain Res. Mol. Brain Res. 63, 62–71.

Feelisch, M., 1998. The use of nitric oxide donors in pharmacological studies. Naunyn Schmiedeberg's Arch. Pharmacol. 358, 113–122.

Feyder, M., Södersten, E., Santini, E., Vialou, V., LaPlant, Q.C., Watts, E.L., Spigolon, G., Hansen, K., Caboche, J., Nestler, E., Fisone, G., 2014. A role for mitogen- and stress-activated kinase 1 in L-DOPA-induced dyskinesia and ΔFosB expression. Biol. Psychiatry http://dx.doi.org/10.1016/j.biopsych.2014.07.019.

Fisone, G., Bezard, E., 2011. Molecular mechanisms of l-DOPA-induced dyskinesia. Int. Rev. Neurobiol. 98, 95–122.

Fuchs, J., Mueller, J.C., Lichtner, P., Schulte, C., Munz, M., Berg, D., Wüllner, U., Illig, T., Sharma, M., Gasser, T., 2009. The transcription factor PITX3 is associated with sporadic Parkinson's disease. Neurobiol. Aging 30, 731–738.

Garthwaite, J., 2008. Concepts of neural nitric oxide-mediated transmission. Eur. J. Neurosci. 27, 2783–2802.

Gerlach, M., Riederer, P., Scheller, D., 2011. Mechanisms underlying and medical management of L-Dopa-associated motor complications. J. Neural Transm. 118, 1659–1660.

Giorgi, M., D'Angelo, V., Esposito, Z., Nuccetelli, V., Sorge, R., Martorana, A., Stefani, A., Bernardi, G., Sancesario, G., 2008. Lowered cAMP and cGMP signalling in the brain during levodopa-induced dyskinesias in hemiparkinsonian rats: new aspects in the pathogenetic mechanisms. Eur. J. Neurosci. 28, 941–950.

González-Aparicio, R., Moratalla, R., 2014. Oleoylethanolamide reduces L-DOPA-induced dyskinesia via TRPV1 receptor in a mouse model of Parkinson's disease. Neurobiol. Dis. 62, 416–425.

Granado, N., O'Shea, E., Bove, J., Vila, M., Colado, M.I., Moratalla, R., 2008. Persistent MDMA-induced dopaminergic neurotoxicity in the striatum and substantia nigra of mice. J. Neurochem. 107, 1102–1112.

Granado, N., Ares-Santos, S., Oliva, I., O'Shea, E., Martin, E.D., Colado, M.I., Moratalla, R., 2011. Dopamine D2-receptor knockout mice are protected against dopaminergic neurotoxicity induced by methamphetamine or MDMA. Neurobiol Dis. 42, 391–403.

Huot, P., Johnston, T.H., Koprich, J.B., Fox, S.H., Brotchie, J.M., 2013. The pharmacology of L-DOPA-induced dyskinesia in Parkinson's disease. Pharmacol. Rev. 65, 171–222.

Hwang, D.Y., Ardayfio, P., Kang, U.J., Semina, E.V., Kim, K.S., 2003. Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice. Brain Res. Mol. Brain Res. 114, 123–131.

Hwang, D.Y., Fleming, S.M., Ardayfio, P., Moran-Gates, T., Kim, H., Tarazi, F.I., Chesselet, M.F., Kim, K.S., 2005. 3,4-dihydroxyphenylalanine reverses the motor deficits in Pitx3-deficient aphakia mice: behavioral characterization of a novel genetic model of Parkinson's disease. J. Neurosci. 25, 2132–2137.

Iderberg, H., Francardo, V., Pioli, E.Y., 2012. Animal models of L-DOPA-induced dyskinesia: an update on the current options. Neuroscience 211, 13–27.

Iravani, M.M., Jenner, P., 2011. Mechanisms underlying the onset and expression of levodopa-induced dyskinesia and their pharmacological manipulation. J. Neural Transm. 118, 1661–1690.

Itokawa, K., Ohkuma, A., Araki, N., Tamura, N., Shimazu, K., 2006. Effect of L-DOPA on nitric oxide production in striatum of freely mobile mice. Neurosci. Lett. 402, 142–144.

Itzhak, Y., 1997. Modulation of cocaine- and methamphetamine-induced behavioral sensitization by inhibition of brain nitric oxide synthase. J. Pharmacol. Exp. Ther. 282, 521–527.

Jankovic, J., 2005. Motor fluctuations and dyskinesias in Parkinson's disease: clinical manifestations. Mov. Disord. 20 (Suppl. 11), S11–S16.

Johnston, T.H., Lee, J., Gomez-Ramirez, J., Fox, S.H., Brotchie, J.M., 2005. A simple rodent assay for the in vivo identification of agents with potential to reduce levodopa-induced dyskinesia in Parkinson's disease. Exp. Neurol. 191, 243–250.

Kiss, J.P., Vizi, E.S., 2001. Nitric oxide: a novel link between synaptic and nonsynaptic transmission. Trends Neurosci. 24, 211–215.

Li, L., Zhou, F.M., 2013. Parallel dopamine D1 receptor activity dependence of l-Dopa-induced normal movement and dyskinesia in mice. Neuroscience 236, 66–76.

Lorenc-Koci, E., Czarnecka, A., Lenda, T., Kamińska, K., Konieczny, J., 2013. Molsidomine, a nitric oxide donor, modulates rotational behavior and monoamine metabolism in 6-OHDA lesioned rats treated chronically with L-DOPA. Neurochem. Int. 63, 790–804.

Lundblad, M., Usiello, A., Carta, M., Håkansson, K., Fisone, G., Cenci, M.A., 2005. Pharmacological validation of a mouse model of l-DOPA-induced dyskinesia. Exp. Neurol. 194, 66–75.

Muñoz, A., Li, Q., Gardoni, F., Marcello, E., Qin, C., Carlsson, T., Kirik, D., Di Luca, M., Björklund, A., Bezard, E., Carta, M., 2008. Combined 5-HT1A and 5-HT1B receptor agonists for the treatment of L-DOPA-induced dyskinesia. Brain 131, 3380–3394.

Murer, M.G., Moratalla, R., 2011. Striatal signaling in L-DOPA-induced dyskinesia: common mechanisms with drug abuse and long term memory involving D1 dopamine receptor stimulation. Front. Neuroanat. 5, 51.

Nishi, A., Watanabe, Y., Higashi, H., Tanaka, M., Nairn, A.C., Greengard, P., 2005. Glutamate regulation of DARPP-32 phosphorylation in neostriatal neurons involves activation of multiple signaling cascades. Proc. Natl. Acad. Sci. U. S. A. 102, 1199–1204.

Novaretti, N., Padovan-Neto, F.E., Tumas, V., da-Silva, C.A., Del Bel, E.A., 2010. Lack of tolerance for the anti-dyskinetic effects of 7-nitroindazole, a neuronal nitric oxide synthase inhibitor, in rats. Braz. J. Med. Biol. Res. 43, 1047–1053.

Nunes, I., Tovmasian, L., Silva, R.M., Burke, R.E., Goff, S.P., 2003. Pitx3 is required for development of substantia nigra dopaminergic neurons. Proc. Natl. Acad. Sci. U. S. A. 100, 4245–4250.

Ohno, M., Watanabe, S., 1995. Nitric oxide synthase inhibitors block behavioral sensitization to methamphetamine in mice. Eur. J. Pharmacol. 275, 39–44.

Padovan-Neto, F.E., Echeverry, M.B., Tumas, V., Del-Bel, E.A., 2009. Nitric oxide synthase inhibition attenuates L-DOPA-induced dyskinesias in a rodent model of Parkinson's disease. Neuroscience 159, 927–935.

Padovan-Neto, F.E., Echeverry, M.B., Chiavegatto, S., Del-Bel, E., 2011. Nitric oxide synthase inhibitor improves de novo and long-term l-DOPA-induced dyskinesia in hemiparkinsonian rats. Front. Syst. Neurosci. 5, 40.

Padovan-Neto, F.E., Ferreira, N.R., de Oliveira-Tavares, D., de Aguiar, D., da Silva, C.A., Raisman-Vozari, R., Del Bel, E., 2013. Anti-dyskinetic effect of the neuronal nitric oxide synthase inhibitor is linked to decrease of FosB/deltaFosB expression. Neurosci. Lett. 541, 126–131.

Park, D.J., West, A.R., 2009. Regulation of striatal nitric oxide synthesis by local dopamine and glutamate interactions. J. Neurochem. 111, 1457–1465.
Pavón, N., Martín, A.B., Mendialdua, A., Moratalla, R., 2006. ERK phosphorylation and FosB expression are associated with L-DOPA-induced dyskinesia in hemiparkinsonian mice. Biol. Psychiatry 59, 64–74.
Paxinos, G., Franklin, K.B.J., 2001. The Mouse Brain in Stereotaxic Coordinates. Academic Press, San Diego.
Picconi, B., Bagetta, V., Ghiglieri, V., Paillè, V., Di Filippo, M., Pendolino, V., Tozzi, A., Giampà, C., Fusco, F.R., Sgobio, C., Calabresi, P., 2011. Inhibition of phosphodiesterases rescues striatal long-term depression and reduces levodopa-induced dyskinesia. Brain 134, 375–387.
Prashanth, L.K., Fox, S., Meissner, W.G., 2011. l-Dopa-induced dyskinesia-clinical presentation, genetics, and treatment. Int. Rev. Neurobiol. 98, 31–54.
Ruiz-DeDiego, I., Mellstrom, B., Vallejo, M., Naranjo, J.R., Moratalla, R., 2014. Activation of DREAM (downstream regulatory element antagonistic modulator), a calcium-binding protein, reduces L-DOPA-induced dyskinesias in mice. Biol. Psychiatry http://dx.doi.org/10.1016/j.biopsych.2014.03.023.
Sammut, S., Dec, A., Mitchell, D., Linardakis, J., Ortiguela, M., West, A.R., 2006. Phasic dopaminergic transmission increases NO efflux in the rat dorsal striatum via a neuronal NOS and a dopamine D(1/5) receptor-dependent mechanism. Neuropsychopharmacology 31, 493–505.
Sancesario, G., Giorgi, M., D'Angelo, V., Modica, A., Martorana, A., Morello, M., Bengtson, C.P., Bernardi, G., 2004. Down-regulation of nitrergic transmission in the rat striatum after chronic nigrostriatal deafferentation. Eur. J. Neurosci. 20, 989–1000.
Sanchez, J.J., Abreu, P., Gonzalez, M.C., 2002. Sodium nitroprusside stimulates L-DOPA release from striatal tissue through nitric oxide and cGMP. Eur. J. Pharmacol. 438, 79–83.
Santini, E., Valjent, E., Usiello, A., Carta, M., Borgkvist, A., Girault, J.A., Hervé, D., Greengard, P., Fisone, G., 2007. Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesia. J. Neurosci. 27, 6995–7005.
Santini, E., Alcacer, C., Cacciatore, S., Heiman, M., Hervé, D., Greengard, P., Girault, J.A., Valjent, E., Fisone, G., 2009. L-DOPA activates ERK signaling and phosphorylates histone H3 in the striatonigral medium spiny neurons of hemiparkinsonian mice. J. Neurochem. 108, 621–633.
Santini, E., Sgambato-Faure, V., Li, Q., Savasta, M., Dovero, S., Fisone, G., Bezard, E., 2010. Distinct changes in cAMP and extracellular signal-regulated protein kinase signalling in L-DOPA-induced dyskinesia. PLoS One 5, e12322.
Schapira, A.H., 2009. Neurobiology and treatment of Parkinson's disease. Trends Pharmacol. Sci. 30, 41–47.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675.
Smidt, M.P., Smits, S.M., Bouwmeester, H., Hamers, F.P., van der Linden, A.J., Hellemons, A.J., Graw, J., Burbach, J.P., 2004. Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. Development 131, 1145–1155.
Stipanovich, A., Valjent, E., Matamales, M., Nishi, A., Ahn, J.H., Maroteaux, M., Bertran-Gonzalez, J., Brami-Cherrier, K., Enslen, H., Corbillé, A.G., Filhol, O., Nairn, A.C., Greengard, P., Hervé, D., Girault, J.A., 2008. A phosphatase cascade by which rewarding stimuli control nucleosomal response. Nature 453, 879–884.
Suárez, L.M., Solís, O., Caramés, J.M., Taravini, I.R., Solís, J.M., Murer, M.G., Moratalla, R., 2014. L-DOPA treatment selectively restores spine density in dopamine receptor D2-expressing projection neurons in dyskinetic mice. Biol. Psychiatry 75, 711–722.
Takuma, K., Tanaka, T., Takahashi, T., Hiramatsu, N., Ota, Y., Ago, Y., Matsuda, T., 2012. Neuronal nitric oxide synthase inhibition attenuates the development of L-DOPA-induced dyskinesia in hemi-Parkinsonian rats. Eur. J. Pharmacol. 683, 166–173.
Tsankova, N., Renthal, W., Kumar, A., Nestler, E.J., 2007. Epigenetic regulation in psychiatric disorders. Nat. Rev. Neurosci. 8, 355–367.
van den Munckhof, P., Luk, K.C., Ste-Marie, L., Montgomery, J., Blanchet, P.J., Sadikot, A.F., Drouin, J., 2003. Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons. Development 130, 2535–2542.
van den Munckhof, P., Gilbert, F., Chamberland, M., Lévesque, D., Drouin, J., 2006. Striatal neuroadaptation and rescue of locomotor deficit by L-dopa in aphakia mice, a model of Parkinson's disease. J. Neurochem. 96, 160–170.
West, A.R., Tseng, K.Y., 2011. Nitric oxide-soluble guanylyl cyclase-cyclic GMP signaling in the striatum: new targets for the treatment of Parkinson's disease? Front. Syst. Neurosci. 5, 55.
Westin, J.E., Vercammen, L., Strome, E.M., Konradi, C., Cenci, M.A., 2007. Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. Biol. Psychiatry 62, 800–810.

Dopamine D3 receptor modulates L-dopa-induced dyskinesia by targeting D1 receptor-mediated striatal signaling.

Solís O, Garcia-Montes JR, González-Granillo A, Xu M, Moratalla R.

Cereb Cortex. 2017; 27:435-446. doi: 10.1093/cercor/bhv231.

OXFORD

Cerebral Cortex, January 2017;27: 435–446

doi:10.1093/cercor/bhv231
Advance Access Publication Date: 18 October 2015
Original Article

ORIGINAL ARTICLE

Dopamine D3 Receptor Modulates L-DOPA-Induced Dyskinesia by Targeting D1 Receptor-Mediated Striatal Signaling

Oscar Solís[1,2], Jose Ruben Garcia-Montes[1,2], Aldo González-Granillo[1], Ming Xu[3] and Rosario Moratalla[1,2]

[1]Instituto Cajal, Consejo Superior de Investigaciones Científicas, CSIC, Madrid 28002, Spain, [2]CIBERNED, Instituto de Salud Carlos III, Madrid, Spain and [3]Department of Anesthesia and Critical Care, The University of Chicago, Chicago, IL, USA

Address correspondence to Rosario Moratalla, Cajal Institute, CSIC, Av. Dr. Arce 37, Madrid 28002, Spain. Email: moratalla@cajal.csic.es

Abstract

The dopamine D3 receptor (D3R) belongs to the dopamine D2-like receptor family and is principally located in the ventral striatum. However, previous studies reported D3R overexpression in the dorsal striatum following L-DOPA treatment in parkinsonian animals. This fact has drawn attention in the importance of D3R in L-DOPA-induced dyskinesia (LID). Here, we used D3R knockout mice to assess the role of D3R in LID and rotational sensitization in the hemiparkinsonian model. Mice lacking D3R presented a reduction in dyskinesia without interfering with the antiparkinsonian L-DOPA effect and were accompanied by a reduction in the L-DOPA-induced rotations. Interestingly, deleting D3R attenuated important molecular markers in the D1R-neurons such as FosB, extracellular signal-regulated kinase, and histone-3 (H3)-activation. Colocalization studies in D1R-tomato and D2R-green fluorescent protein BAC-transgenic mice indicated that L-DOPA-induced D3R overexpression principally occurs in D1R-containing neurons although it is also present in the D2R-neurons. Moreover, D3R pharmacological blockade with PG01037 reduced dyskinesia and the molecular markers expressed in D1R-neurons. In addition, this antagonist further reduced dyskinetic symptoms in D1R heterozygous mice, indicating a direct interaction between D1R and D3R. Together, our results demonstrate that D3R modulates the development of dyskinesia by targeting D1R-mediated intracellular signaling and suggest that decreasing D3R activity may help to ameliorate LID.

Key words: abnormal involuntary movements, basal ganglia, behavioral sensitization, Parkinson disease, striatonigral

Introduction

Dopamine (DA) depletion is the main neuropathological feature of Parkinson disease (PD), and the current standard treatment for PD focuses on restoring dopaminergic neurotransmission by L-3,4-dihydroxyphenylalanine (L-DOPA). However, long-term exposure to this drug causes a decrease in therapeutic efficacy and the appearance of L-DOPA-induced dyskinesia (LID) (Obeso et al. 2000). Although the pathophysiology of this side effect remains unclear, it has been shown that prolonged stimulation of D1 dopamine receptor (D1R) results in the development of LID (Darmopil et al. 2009). The resulting increase in the D1R signaling pathway leads to increased activation of the extracellular signal-regulated kinase (ERK) pathway (Pavón et al. 2006; Santini et al. 2007; Westin et al. 2007; Fasano et al. 2010) and the subsequent activation of molecular markers of dyskinesia such as FosB and histone3 (H3) (Andersson et al. 1999; Santini et al. 2009).

The dopamine receptors are divided into 2 major groups, the dopamine D1-like (D1 and D5) receptors and the dopamine D2-like (D2, D3 and D4) receptors (Beaulieu and Gainetdinov 2011). We demonstrated that D1R but not D2R is critical for the

development of LID (Darmopil et al. 2009; Murer and Moratalla 2011). However, previous research has suggested that the dopamine D3 receptor (D3R) may also play a role in LID, since D3R activation can potentiate D1R-mediated signaling in the striatum (Fiorentini et al. 2008; Marcellino et al. 2008) and regulates D1R internalization (Berthet et al. 2009).

The D3R is mainly expressed in the nucleus accumbens, olfactory tubercle, islands of Calleja, and at low levels in the dorsal striatum (Sokoloff et al. 1990; Flores et al. 1996; Xu et al. 1997). L-DOPA treatment induces an increase in D3R expression in the dorsal striatum in rats with 6-hydroxydopamine (6-OHDA) lesion (Bordet et al. 1997; Guillin et al. 2001), and this increase correlates with LID (Guigoni et al. 2005). Other previous studies have also provided evidence supporting an important role for D3R in LID (Bézard et al. 2003). Recent experimental findings drew attention to the potential use of D3R-preferring antagonists in the treatment of LID since these antagonists potentiate the effect of L-DOPA as well as decrease dyskinesia (Kumar et al. 2009; Visanji et al. 2009). However, some studies found no reduction in LID after D3R blockade (Silverdale et al. 2004; Mela et al. 2010). This variability may be due to the lack of specificity of pharmacological approaches, which do not irrefutably distinguish between the D2 and D3 receptor. To address this discrepancy and conclusively stabilize the role of D3R, we determined how eliminating D3R expression impacts the development of LID.

We used D3 knockout ($D3^{-/-}$) mice to study the development of LID as well as rotational responses to repeated L-DOPA in hemiparkinsonian mice. In addition, we investigated FosB, H3, and ERK activation in the striatum. To complete our understanding, we evaluated the effect of pharmacological blockade of D3R in the development and expression of LID in wild-type (WT) and in dopamine D1 heterozygous ($D1^{+/-}$) mice. We also examined the expression of D3R in the dorsolateral striatum in dyskinetic mice using bacterial artificial chromosome (BAC)-transgenic-D1R-tomato (D1R-tomato) and BAC-transgenic D2R-enhanced green fluorescent protein (D2R-GFP) mice.

Materials and Methods

Animals

This study was carried out in female and male mice lacking D3R generated by homologous recombination as described previously (Xu et al. 1997). Wild-type and homozygous $D3^{-/-}$ mutant mice were obtained from mating heterozygous mice. The genotype of $D3^{-/-}$ and WT mice was determined by the Southern blotting method (Xu et al. 1997). D1R-tomato and D2R-GFP mice (Suárez et al. 2014) were used to study D3R localization in the dorsal striatum. WT mice were also used to study the pharmacological blockade of D3R. $D1^{+/-}$ mice were used to further investigate the interaction between D3R and D1R in the dyskinetic response (Table 1). The maintenance of animals followed guidelines from European Union Council Directive (86/609/European Economic Community), and experimental protocols were approved by the CSIC Ethics Committee.

Drugs

L-DOPA, benserazide, and desipramine were purchased from Sigma–Aldrich (Spain), and PG01037 was purchased from Tocris-Bioscience (UK). All drugs were freshly prepared in 0.9% NaCl before use and injected i.p. in a volume of 10 mL/kg.

6-OHDA Lesion and Treatment

For the stereotaxic procedure, animals (weighing 25–30 g) were deeply anesthetized with isofluorane anesthesia. As previously described (Ruiz-DeDiego et al. 2015), striatal lesions were performed by unilateral injection of 6-OHDA hydrobromide (20 mmol/L, containing 0.02% ascorbic acid; Sigma–Aldrich, Spain) into striatum at the following coordinates (millimeter) from bregma and dura: anteroposterior (+0.65), lateral (−2.0), and dorsoventral (−4.0 and −3.5). Before neurotoxin injection, all mice received desipramine (20 mg/kg) to protect norepinephrine

Table 1 Experimental groups

Group	Genotype	*n*/group	IHC	TH[a]
Effect of D3R deletion on LID				
Saline	WT	10	TH, FosB, pAcH3, pERK	43.3 ± 2.9
	$D3^{-/-}$	3		48.0 ± 2.3
L-DOPA	WT	10		46.0 ± 1.9
	$D3^{-/-}$	10		45.2 ± 2.5
Expression of D3R in the striatum				
Intact	D1R-tomato	3	TH, D3R, FosB	NA
Parkinsonian		3		49.8 ± 6.2
Dyskinetic		4		50.4 ± 1.9
Dyskinetic	D2R-GFP	4	TH, D3R	44.9 ± 2.4
Effect of PG01037 on the development of LID				
L-DOPA+ saline	WT	9	TH, FosB, pAcH3	45.0 ± 3.0
L-DOPA+PG01037		9		47.8 ± 1.9
Effect of PG01037 on the expression of LID				
L-DOPA+saline	WT	9	TH, FosB, pAcH3	47.5 ± 2.3
L-DOPA+PG01037		8		45.7 ± 2.7
Interaction between the D1R and D3R on LID				
L-DOPA	WT	10	TH, FosB, pAcH3	47.4 ± 2.2
	$D1^{+/-}$	7		49.3 ± 3.1
L-DOPA+PG01037	WT	6		46.1 ± 2.6
	$D1^{+/-}$	7		48.8 ± 3.1

Note: IHC: immunohistochemistry; NA, not applicable.
[a]Extent of striatal lesions assessed by percentage of striatal volume completely denervated.

neurons. Three weeks after lesion, all mice started a 2-week course of daily i.p. injections of benserazide (10 mg/kg) followed by L-DOPA (10 mg/kg) 20 min later.

To assess the role of D3R blockade in LID, we injected the selective D3R antagonist PG01037 (10 mg/kg) or saline 15 min after L-DOPA in WT mice and in $D1^{+/-}$ mice. PG01037 is a highly selective D3R antagonist ($K_i = 0.7 \pm 0.1$ nM) with 133-fold selectivity for D3R over D2R (Grundt et al. 2005). Pharmacological magnetic resonance imaging studies showed that PG01037 crosses the blood–brain barrier and localizes in D3R-rich regions such as the islands of Calleja and nucleus accumbens (Grundt et al. 2007). This compound displays plasma half-life $t_{(1/2)}$ of 1.83 h and has higher brain concentrations than plasma concentrations (Mason et al. 2010). The PG01037 dose and procedure were chosen according to previous studies (Kumar et al. 2009). To study the effect of pharmacological blockade of D3R on the development of LID, we daily co-administered L-DOPA plus PG01037 for 2 weeks. To assess the effect of D3R blockade on expression of LID, mice were rendered dyskinetic by 2 weeks of daily L-DOPA administration as described earlier and on Days 15–18 received L-DOPA plus PG01037.

Behavioral Assessment

To evaluate LID, mice were observed in clear-glass cylinders and were rated by a trained observer. During each rating period, individual dyskinesia severity scores ranging from 0 (not present) to 4 (severe) were given for axial, limb and orolingual dyskinesia. The 3 dyskinetic movement subtypes were summed to create a single score for data analysis. On Day 13 of treatment, we evaluated the LID every 20 for 160 min after L-DOPA injection. Circling behavior was measured as a measure of behavioral sensitization. All animals were video-recorded by a vertically mounted video camera for 15, 5 min after L-DOPA injection. Rotations were analyzed in Viewer2, Biobserve (GmbH). Only completed contralateral turns (360°) were counted.

Motor coordination was evaluated using the rotarod test following an accelerating protocol with increasing speed from 4 to 40 rpm over a 5-min period as described elsewhere (Ruiz-DeDiego et al. 2015). Animals were trained and evaluated before 6-OHDA lesion (prelesion), before the treatment of L-DOPA (pre-L-DOPA; Day 0), and on Day 14 or 16, 90 min after the L-DOPA injection (post-L-DOPA) to avoid the peak of dyskinetic symptoms. The variable analyzed was the latency to fall from the rotarod. For locomotor response experiments, animals were placed in Activity Cages (AccuScan Instruments, Inc.). The locomotor activity was measured and displayed as total distance (cm) over a 30-min period beginning 60 min after saline (pre-L-DOPA) or L-DOPA (post-L-DOPA) injection as described previously (Solís et al. 2015). All behavioral experiments were carried out with the experimenter blind to genotype and treatment.

Immunohystochemistry

The mice were deeply anesthetized with pentobarbital and perfused transcardially with cold saline, followed by a solution of 4% paraformaldehyde in phosphate-buffered saline 1 h after the last L-DOPA injection. Immunostaining was performed on free-floating coronal brain sections as described previously (Granado et al. 2011; Ares-Santos et al. 2014), with the following rabbit antisera: tyrosine hydroxylase (TH, 1:1000; Chemicon), FosB (1:7500; Santa Cruz Biotechnology), phospho-(Ser10)-acetyl (Lys14)-histone 3 (pAcH3; 1:1500; Upstate—Cell Signaling Solutions), pERK (1:250; Cell Signaling Technology), D3R (1:100; Alomone Labs), and Hoechst (1 mg/mL; Sigma–Aldrich). For immunofluorescence experiments, we used Alexa fluor 488- and 594-conjugated secondary antibodies (1:500; Invitrogen).

The extent of dopaminergic lesions was quantified using Stereoinvestigator (MBF Bioscience), depicting the border of striatal areas with complete loss of TH-immunoreactive fibers with a 4× lens using 7 serial rostrocaudal sections per animal. Quantification of FosB, pAcH3, and pERK immunoreactivity was carried out using Image J (Schneider et al. 2012). Immunostaining intensity and the number of immunolabeled nuclei/cells were determined for all animals in each group using 2 serial rostrocaudal sections per animal from both sides of the lesioned dorsolateral striatum for a total of 6 images per animal. Digital images were obtained under a Leica microscope using a 40× objective. The data are presented as the number of stained nuclei per square millimeter in the lesioned striatum.

Immunofluorescence images from dorsolateral part of the striatum were obtained using sequential laser scanning confocal microscopy (Leica) at 40× and 63× from 3 sections per animal. Neuronal quantification was performed on 2 images for each section. D3R colocalization data are expressed as % of D3R-positive cells in D1R-positive or -negative cells or as % of D3R-positive cells in D2R-positive or -negative cells. Expression of FosB in D1R-positive and D1R-negative striatal neurons was also studied. Images were quantified with the "cell counter" plug-in in ImageJ (NIH). The investigator performing immunohistochemistry quantification was blinded to sample identities.

Statistical Analysis

Behavioral data were analyzed by repeated-measures two-way analyses of variance (ANOVA) followed by Bonferroni *post hoc* test. Immunohistochemistry (FosB, pAcH3, and pERK) data were evaluated by unpaired *t*-test. Statistical comparison of D3R expression in the dorsal striatum was analyzed using two-way ANOVA followed by Bonferroni *post hoc* test. Analysis was performed with GraphPad Prism 5 (La Jolla). Data are expressed as the mean ± standard error of the mean (SEM) unless stated otherwise. The significance level was $P < 0.05$.

Results

Effect of D3R Deletion on Dyskinesia and Behavioral Sensitization Induced by L-DOPA

To explore the effect of D3R genetic inactivation on LID, 6-OHDA-lesioned WT and $D3^{-/-}$ mice were chronically treated with L-DOPA (Fig. 1A). We assessed 3 subtypes of dyskinetic movements (axial, limb, and orolingual) 40 min after L-DOPA injection as had been described in former work (Pavón et al. 2006). Dyskinetic movements appeared in hemiparkinsonian WT mice on the first day of L-DOPA treatment and increased progressively, reaching a plateau at Day 5 that was maintained until the end of treatment, consistent with previous results (Darmopil et al. 2009). Deletion of the D3R resulted in significant lower axial ($P < 0.05$), limb ($P < 0.05$), and orolingual ($P < 0.01$) (Fig. 1B–D) dyskinetic subtypes, as well as total dyskinesia compared with their WT counterparts ($P < 0.01$) (Fig. 1E). In addition, time course analysis carried out on Day 13 revealed that total dyskinesia was significantly attenuated in $D3^{-/-}$ mice assessed during the peak of LID (Fig. 1F). The hemiparkinsonian mice of both genotypes that received saline did not show any dyskinetic symptom. Together these data demonstrate that D3R deletion significantly attenuates LID.

We evaluated circling behavior as a measure of sensitization (González-Aparicio and Moratalla 2014). Administration of

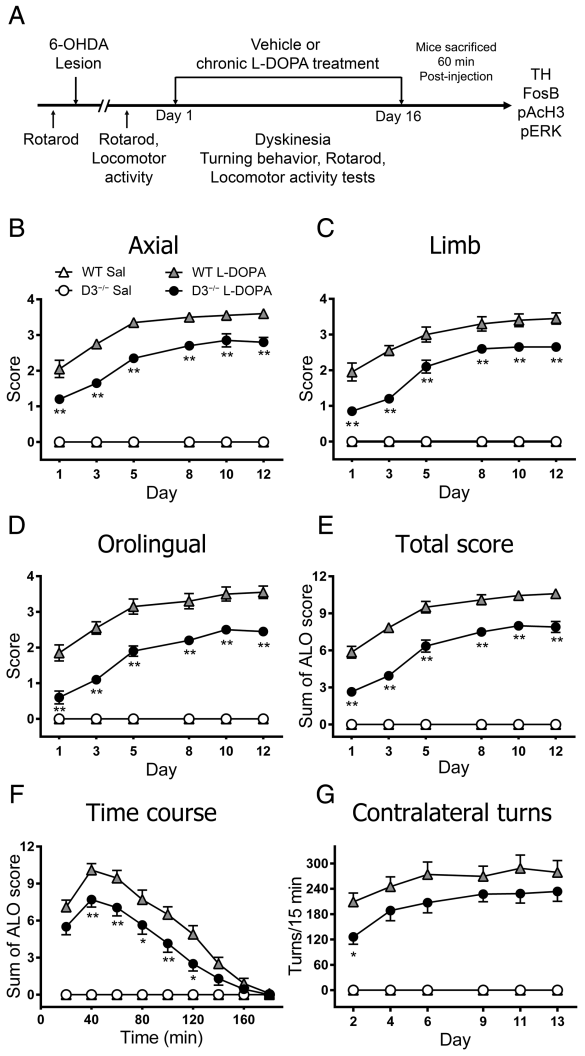


Figure 1. Effect of genetic deletion of D3R on LID and behavioral sensitization. (*A*) Schematic view of the experimental design. The $D3^{-/-}$ mice exhibited a reduction in axial (*B*), limb (*C*), and orolingual (*D*), as well as the cumulated total score (*E*) compared with littermate controls. Dyskinetic movements were evaluated 40 min after L-DOPA. Two-way ANOVA with repeated measures followed by a Bonferroni *post hoc* test showed significant differences for genotype ($F_{3,150} = 414.3$, $P < 0.001$ for axial; $F_{3,150} = 212.6$, $P < 0.001$ for limb; $F_{3,150} = 187.9$, $P < 0.001$ for orolingual; $F_{3,150} = 375.5$, $P < 0.001$ for total score) and day ($F_{5,150} = 39.5$, $P < 0.001$ for axial; $F_{5,150} = 44.1$, $P < 0.001$ for limb; $F_{5,150} = 40.4$, $P < 0.001$ for orolingual; $F_{5,150} = 75.1$, $P < 0.001$ for total score). The kinetic profile of dyskinetic symptoms was evaluated once every 20 min over 180 min on Day 13 of the L-DOPA treatment (*F*). Two-way ANOVA followed by a Bonferroni test showed significant differences for genotype ($F_{3,270} = 294.4$, $P < 0.001$) and time ($F_{5,270} = 39.07$, $P < 0.001$). Contralateral turns in hemiparkinsonian mice evaluated for 15 min on the indicated day (*F*). Two-way ANOVA with repeated measures followed by a Bonferroni test showed significant differences for genotype ($F_{3,150} = 68.6$, $P < 0.001$) and day ($F_{5,150} = 5.6$, $P < 0.001$). Data are expressed as the mean ± S.E.M. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus WT + L-DOPA. $n = 10$ for each group, except in the $D3^{-/-}$ saline group, $n = 3$.

chronic L-DOPA induced contralateral rotations in hemiparkinsonian mice, with $D3^{-/-}$ mice showing significant lower contralateral rotations on the first evaluation day compared with WT littermates ($P < 0.05$). This decrease continued during the next evaluation days, but it did not reach significance ($P > 0.05$; Fig. 1*F*). Saline-treated animals did not develop contralateral rotations.

D3R Deletion Does not Affect the Antiparkinsonian Efficacy of L-DOPA

To rule out the possibility that the antidyskinetic effect in $D3^{-/-}$ mice was due to locomotor impairment, we measured motor coordination and locomotor activity. The $D3^{-/-}$ group animals did not show any significant difference to WT animals in the motor coordination (tested by rotarod) before (prelesion) and after the 6-OHDA lesion (pre-L-DOPA). The lesion affected motor coordination similarly in both genotypes. However, chronic L-DOPA treatment, tested on Day 14 (post-L-DOPA), significantly improved the time on the rotarod ($P < 0.01$) (Fig. 2*A*). Although latency to fall from the rotarod was longer in $D3^{-/-}$ mice, this increase was not statistically significant compared with WT littermates ($P > 0.05$). Locomotor activity in hemiparkinsonian WT and $D3^{-/-}$ mice significantly improves after chronic L-DOPA, tested on Day 15 ($P < 0.0001$). We did not find any difference in the locomotor response between WT and $D3^{-/-}$ mice (Fig. 2*B*). The results suggest that D3R genetic inactivation does not impair L-DOPA's antiparkinsonian effect.

Previous evidence indicated that the extent of the dopaminergic lesion correlates with the severity of dyskinesia (Darmopil et al. 2009). To exclude the possibility of differences in lesion size between WT and $D3^{-/-}$ mice, we measured the volume in the ipsilateral striatum devoid of TH fibers and found no difference in the extent of the lesion between groups ($P > 0.05$; Fig. 2*C*,*D*)

Deleting D3R Regulates Molecular Markers of LID

To explore potential molecular mechanisms underlying the effects of D3R deletion on LID, we measured the expression of FosB, pERK, and pAcH3 in the DA-denervated striatum (Fig. 3*A*). These have been implicated as key determinants in appearance of D1 dopamine receptor supersensitivity in LID (Pavón et al. 2006; Santini et al. 2007; Westin et al. 2007; Darmopil et al. 2009). Immunostaining revealed that repeated treatment with L-DOPA resulted in significant FosB expression in the lesioned striatum in WT mice, as expected. D3R deletion significantly decreased the number of FosB-positive cells ($P < 0.01$; Fig. 3*B*). It has been demonstrated that L-DOPA activates the ERK pathway (Pavón et al. 2006). Thus, we assessed pERK activation after L-DOPA treatment and found that pERK activation in the lesioned striatum was significantly diminished in the $D3^{-/-}$ mice compared with WT ($P < 0.05$; Fig. 3*C*). Next, we examined the effect of chronic L-DOPA treatment on the phosphoacetylation of striatal H3, a downstream target of ERK (Santini et al. 2007, 2009). As shown in Figure 3*D*, there was a significant increase in pAcH3 in the denervated striatum of WT mice. $D3^{-/-}$ mice also showed attenuated pAcH3 expression ($P < 0.05$). Our data indicate that D3R regulates FosB, pERK, and pAcH3 in the denervated striatum of dyskinetic mice.

L-DOPA-Induced D3R-Increased Expression Occurs in the Direct and Indirect Striatal Neurons in the Dorsal Striatum

L-DOPA treatment causes an increase in D3R expression in the DA-denervated dorsal striatum (Bordet et al. 1997; Guigoni et al. 2005). However, to date there is no direct evidence that determines whether this expression occurs in striatonigral D1R or in striatopallidal D2R projection neurons. To address this issue, we used intact (unlesioned), hemiparkinsonian, and dyskinetic D1R-tomato mice to identify direct from indirect pathway neurons. In intact animals, there were some D1R-negative neurons that expressed the D3R but not in the D1R-positive neurons of

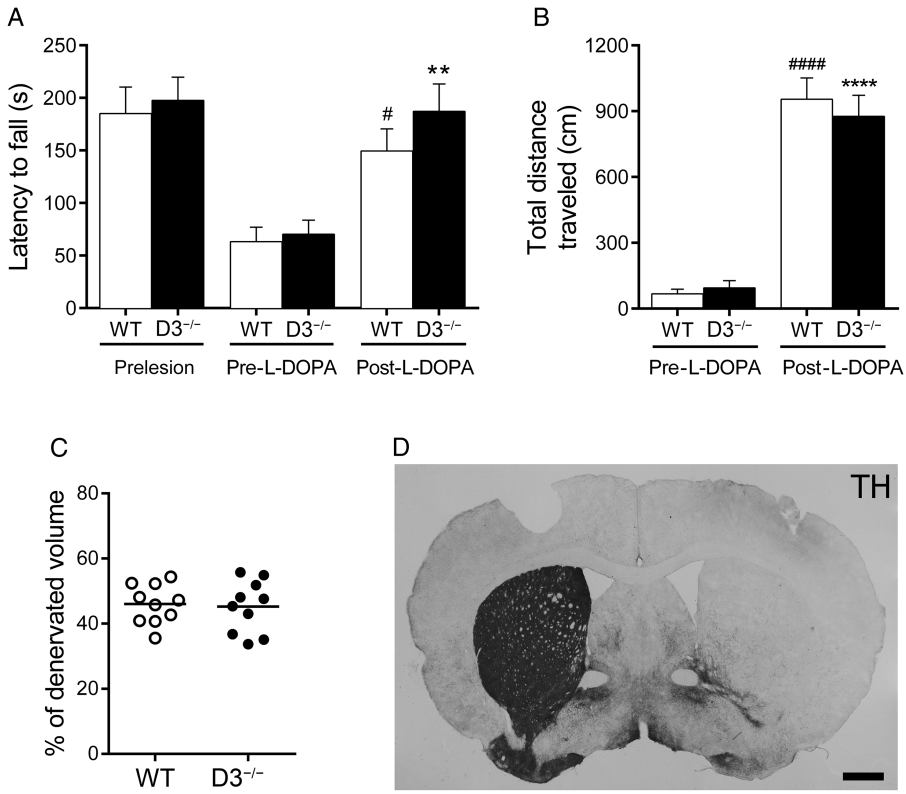


Figure 2. Genetic deletion of D3R does not affect the therapeutic effect of L-DOPA. (*A*) Motor coordination on the rotarod was evaluated in $D3^{-/-}$ and WT mice before 6-OHDA lesion (prelesion), 3 weeks after lesion (pre-L-DOPA), and on Day 14 of the chronic treatment, 90 min after the L-DOPA injection (post-L-DOPA). Two-way ANOVA, followed by Bonferroni's test. $^{**}P < 0.01$ versus $D3^{-/-}$ (pre-L-DOPA) mice; $^{\#}P < 0.05$ versus WT (pre-L-DOPA) mice. (*B*) Total distance traveled (cm) during 30 min was measured in a multicage activity meter system. Two-way ANOVA, followed by Bonferroni's test. $^{****}P < 0.0001$ versus $D3^{-/-}$ (pre-L-DOPA) mice; $^{\#\#\#\#}P < 0.0001$ versus WT (pre-L-DOPA) mice. (*C*) Scatter dot plot of the extent of striatal lesions assessed by percentage of striatal volume completely denervated. (*D*) Coronal section from a dyskinetic $D3^{-/-}$ mouse stained for TH. $n = 10$ for each group. Scale bar = 500 µm.

the dorsal striatum (Fig. 4A). This pattern of expression was similar in the parkinsonian denervated dorsal striatum. Interestingly, we found that L-DOPA increased D3R expression in the hemiparkinsonian mice; this increase occurred in 58.1% of the D1R-positive cells and in 23.6% of the D1R-negative cells of the dorsal striatum (Fig. 4A′). This pattern of D3R-increased distribution in dyskinetic animals was further confirmed using (BAC)-transgenic D2R-GFP mice (Fig. 4B). As expected, D3R expression increased in D2R-negative cells (64%) and in D2R-positive cells (18%) (Fig 4B′). As additional controls, we used L-DOPA-treated $D3^{-/-}$ mice, and no D3R expression was observed (data not shown). Altogether, these results indicate that L-DOPA increases D3R expression in both striatonigral and striatopallidal neurons, although this increase is 2.5- to 3-fold larger in D1R than that in D2R-neurons in dyskinetic animals.

Despite the fact that D3R increases in both types of striatal neurons after L-DOPA, we found that FosB expression was induced in the D1R-positive neurons and was hardly detected in the D1R-negative neurons (Fig. 5), suggesting that FosB activation occurs in the striatonigral neurons, consistent with previous reports (Pavón et al. 2006; Darmopil et al. 2009). In parkinsonian mice, FosB expression was barely detected in the striatum.

Effect of D3R Pharmacological Blockade on LID in WT Mice

To rule out the possibility of compensatory mechanism in $D3^{-/-}$ during development that could be acting on LID, we used the selective D3R-preferring antagonist PG01037 to further investigate the role of D3R on the development and expression of LID. To assess the role of D3R in the development of LID, we administered PG01037 or saline to hemiparkinsonian mice daily for 2 weeks, 15 min after L-DOPA treatment (Fig 6A). Notably, the dyskinetic response of PG01037-treated mice upon initial exposure to L-DOPA was both smaller ($P < 0.01$; Fig. 6B) and of shorter duration ($P < 0.01$; Fig. 6C) than that of mice that received only L-DOPA plus saline over the 2 weeks of treatment. This treatment did not significantly reduce the therapeutic effect of L-DOPA as measured in the rotarod test (Day 14) ($P > 0.05$; Fig 6D). In agreement with the dyskinetic response, PG01037 significantly decreased FosB ($P < 0.05$) and pAcH3 ($P < 0.05$) expression induced by L-DOPA (Fig 6E,F).

We next asked whether D3R pharmacological blockade had a role in the expression of LID. This was measured in mice with established dyskinesia (Fig 7A). We found that co-administration of L-DOPA plus PG01037 on Day 15–17 decreased LID compared with L-DOPA plus saline ($P < 0.05$) (Fig 7B,C). In addition, PG01037 did not significantly affect the antiparkinsonian efficacy of L-DOPA, indicated by the latency to fall in the rotarod test (Day 16) ($P > 0.05$; Fig. 6D). We then investigated FosB and pAcH3 activation and found that PG01037 diminish FosB and pAcH3 ($P = 0.05$) expression induced by L-DOPA, although this decrease was not significant for FosB ($P = 0.08$) (Fig 7D,E). This can be due to the longer half-life of FosB that gradually accumulates following chronic treatment (Hope et al. 1994; Moratalla et al. 1996a, 1996b). D3R antagonist

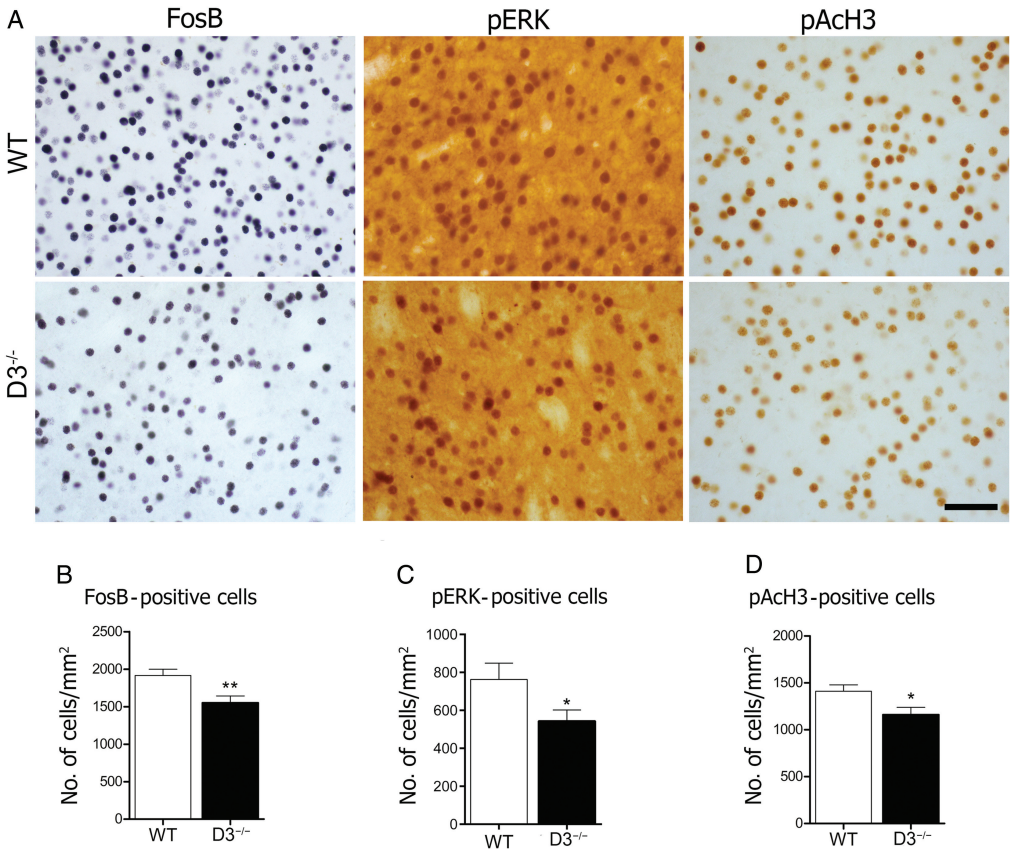


Figure 3. Genetic inactivation of D3R attenuates molecular markers of LID. Photomicrographs of L-DOPA-induced FosB, pERK, and pAcH3 expression in the dopamine-denervated dorsal striatum (*A*). Immunohistochemical analysis shows that the D3R deletion decreases FosB- (*B*), pERK- (*C*), and pAcH3- (*D*) positive cells induced by L-DOPA. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus WT (unpaired *t*-test). $n = 10$ for each group. Scale bar = 50 µm.

can block new FosB expression but is unable to eliminate the accumulated FosB induced by the previous treatment. By contrast, H3 activation is transient, with maximal expression 60 min after injection and returns to baseline after 6 h (data not shown).

Interaction between D1R and D3R in Dyskinetic Mice

In this study, we show that genetic deletion of D3R decreases LID through attenuation of D1R signaling. Previous results of our laboratory demonstrated that the D1R is critical for the development of LID (Darmopil et al. 2009). To see whether D3R interacts with D1R to produce dyskinesias, we test whether D3R antagonist was able to further reduce D1R-mediated LID in $D1^{+/-}$ mice (Fig. 8A). As expected, $D1^{+/-}$ animals and PG01037 treatment in WT mice significantly reduced LID compared with L-DOPA-treated WT group ($P < 0.01$). Interestingly, the pharmacological blockade of D3R synergically reduced LID in $D1^{+/-}$ mice ($P < 0.05$) compared with L-DOPA-treated $D1^{+/-}$ and PG01037 plus L-DOPA WT animals (Fig. 8B,C). In agreement with the behavioral results, we found that $D1^{+/-}$ display lower FosB and pAcH3 expression after L-DOPA compared with WT mice, and this expression was further reduced by PG cotreatment with L-DOPA (Fig. 8D–F). Taken together, our results suggest a direct interaction between the D1R and the D3R in dyskinesia.

Discussion

The aim of this study was to evaluate the role of the specific D3R on the development of LID and the expression of associated molecular markers in the denervated striatum of hemiparkinsonian mice. We demonstrated that LID was decreased in $D3R^{-/-}$ mice compared with their WT counterparts. These behavioral responses were accompanied by a reduction in the expression of L-DOPA-induced molecular markers. Furthermore, we demonstrated that L-DOPA treatment increased D3R expression in both D1R direct and D2R indirect pathway neurons, although the presence of D1R-neuron is 2.5- to 3-fold larger. Moreover, pharmacological blockade of D3R diminished LID, without affecting the antiparkinsonian efficacy of L-DOPA. Finally, our behavioral results using $D1^{+/-}$ hemiparkinsonian mice support the notion that the D3R directly interacts with the D1R in LID.

D3R is highly expressed in the limbic areas of the striatum and barely expressed in the dorsal striatum (Sokoloff et al. 1990; Xu et al. 1997). However, this pattern of expression decreases following DA depletion and increases in the denervated dorsal striatum after L-DOPA treatment (Bordet et al. 1997). Previous research has suggested that D3R is associated with behavioral sensitization and dyskinesia in PD (Bordet et al. 1997; Bézard et al. 2003). In support of this assertion, lentiviral-induced D3R overexpression in the dorsal striatum leads to the appearance of dyskinetic behaviors (Cote et al. 2014). Thus, it has been hypothesized that deleting D3R might ameliorate the motor side effects induced by L-DOPA.

In this study, $D3^{-/-}$ hemiparkinsonian mice exhibited a reduction in L-DOPA-induced behavioral sensitization compared with their WT counterparts. This finding is supported by previous studies that revealed that D3R plays an important role in

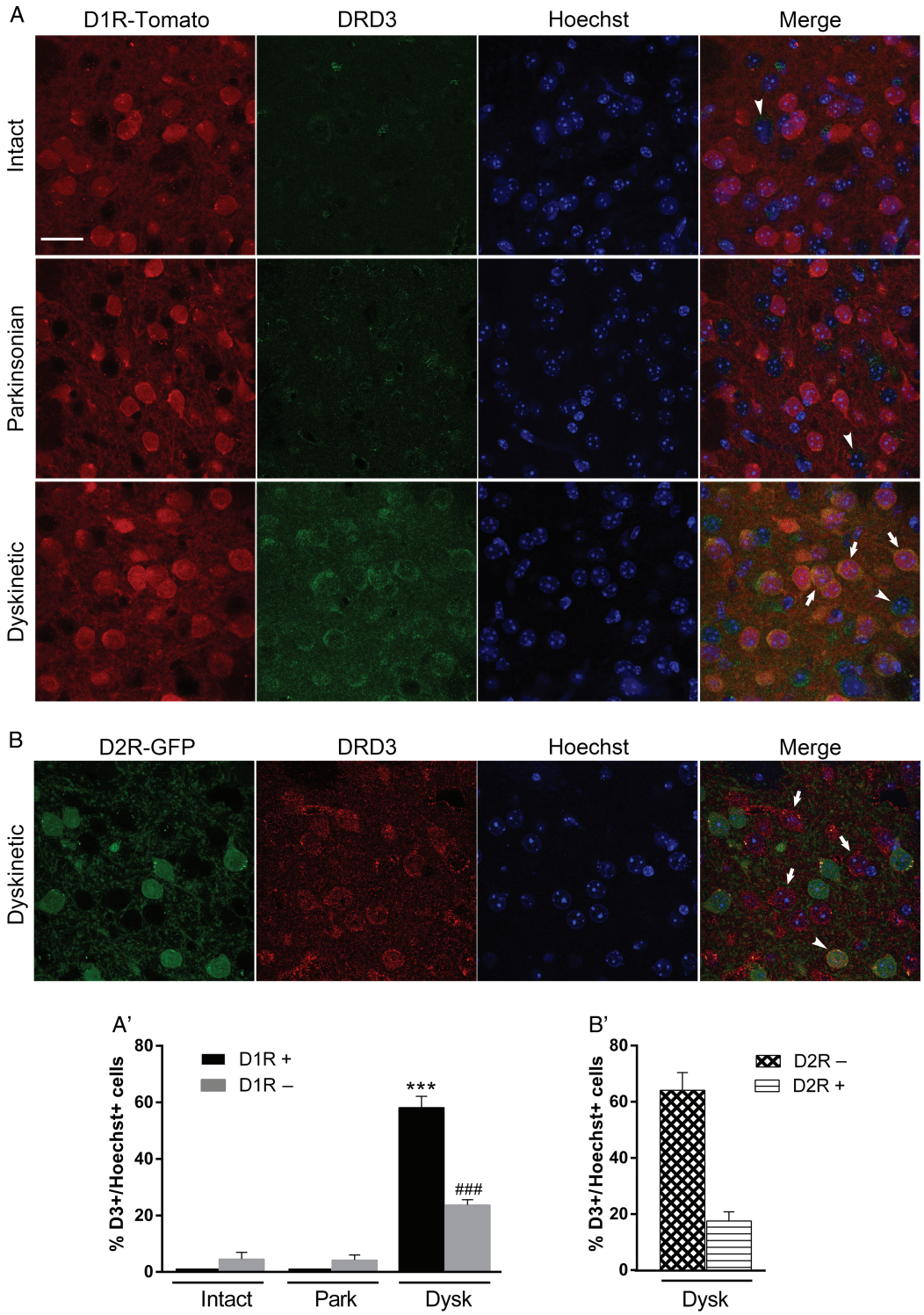


Figure 4. Chronic L-DOPA increases D3R expression in the DA-depleted dorsal striatum. (A) Confocal images of the dorsal striatum of intact, parkinsonian, and dyskinetic D1R-BAC-transgenic mice illustrating D3R expression (green) in D1R-positive neurons (red-tomato) and in D1R-negative neurons. Nuclei are visualized via Hoechst staining. Arrows indicate examples of D3R-positive and D1R-positive neurons; arrow-heads point to D3R-positive and D1R-negative neurons. (A′) Graph showing the percentage of D3R-positive cells in D1R-positive or -negative cells. Data are expressed as the mean ± SEM. Two-way ANOVA, followed by Bonferroni's test. $^{***}P < 0.001$ versus intact D1R-positive neurons; $^{###}P < 0.001$ versus intact D1R-positive neurons. (B) Confocal images of the dorsal striatum of dyskinetic D2R-BAC-transgenic mice illustrating D3R expression (red) in D2R-positive neurons (green-GFP) and in D2R-negative neurons. Nuclei are visualized via Hoechst staining. Arrows indicate examples of D3R-positive and D2R-negative neurons; arrow-heads point to D3R-positive and D2R-positive neurons. (B′) Graph showing the percentage of D3R-positive cells in D2R-positive or negative cells. $n = 3–4$ for each group. Scale bar = 25 µm.

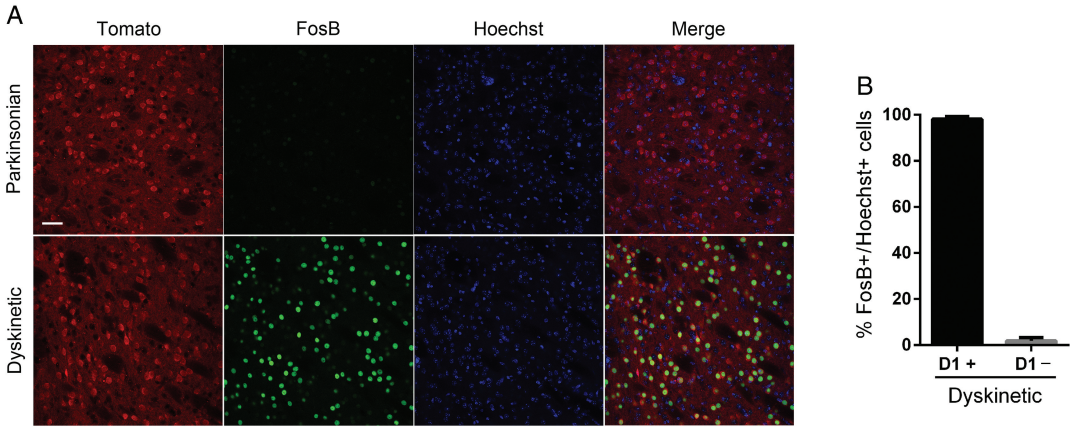


Figure 5. L-DOPA-induced FosB expression occurs in striatonigral projection neurons. (*A*) Confocal images of the dorsal striatum of parkinsonian and dyskinetic D1R-BAC-transgenic mice illustrating FosB expression (green) in D1R-positive neurons (red-tomato). Nuclei are visualized via Hoechst staining. (*B*) Graph showing the percentage of D3R-positive cells in D1R-positive or -negative cells. Data are expressed as the mean ± SEM. $n = 3–4$ for each group. Scale bar = 40 µm.

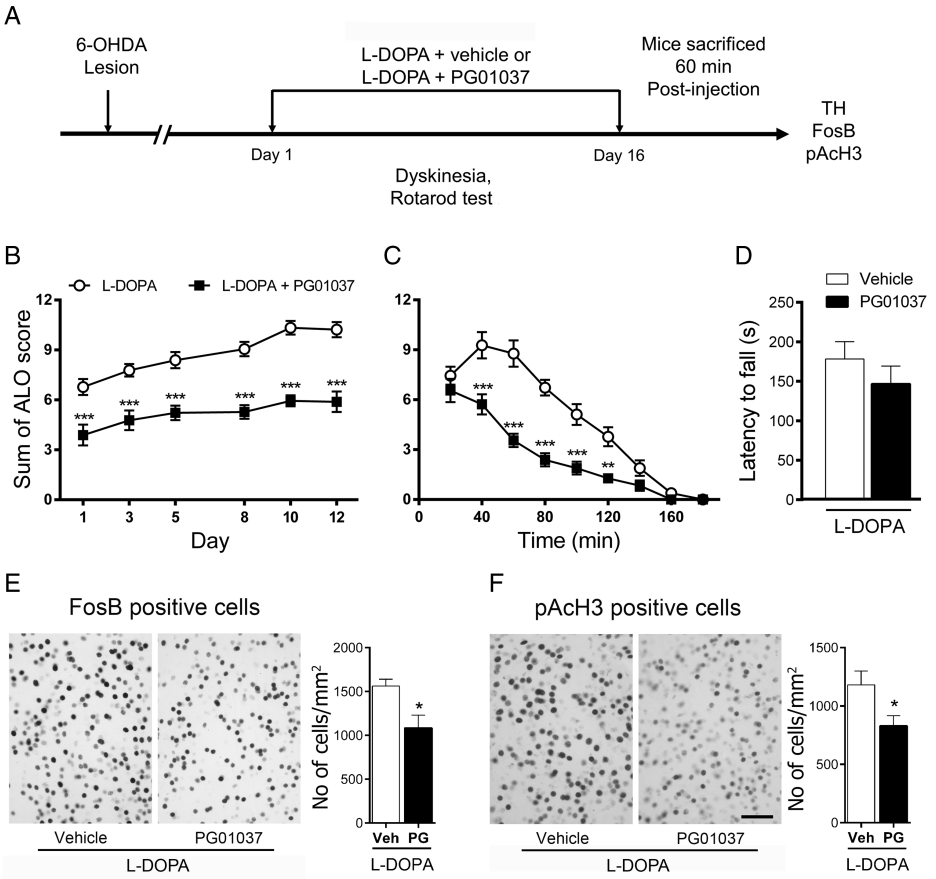


Figure 6. Effect of D3R-preferring antagonist PG01037 on the development of LID. (*A*) Schematic view of the experimental design. (*B* and *C*) The chronic coadministration of L-DOPA with PG01037 decreased the development of LID. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus L-DOPA plus saline, two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test. (*D*) PG01037 cotreatment did not affect the latency to fall from the rotarod. $P = 0.17$ versus L-DOPA plus saline, unpaired *t*-test. Immunohistochemical analysis shows that PG01037 decreases FosB- (*E*) and pAcH3- (*F*) positive cells induced by L-DOPA. $^{*}P < 0.05$ versus WT, unpaired *t*-test. All data are expressed as mean ± S.E.M. $n = 9$ for each group. Scale bar = 50 µm.

behavioral sensitization after L-DOPA treatment (Bordet et al. 1997). Also, D3R antisense oligonucleotides blocked L-DOPA-induced behavioral sensitization (van Kampen and Stoessl 2003). In the current study, we showed a reduction in LID in the $D3^{-/-}$ mice without interfering with the antiparkinsonian effect of L-DOPA. This result is in agreement with previous work that

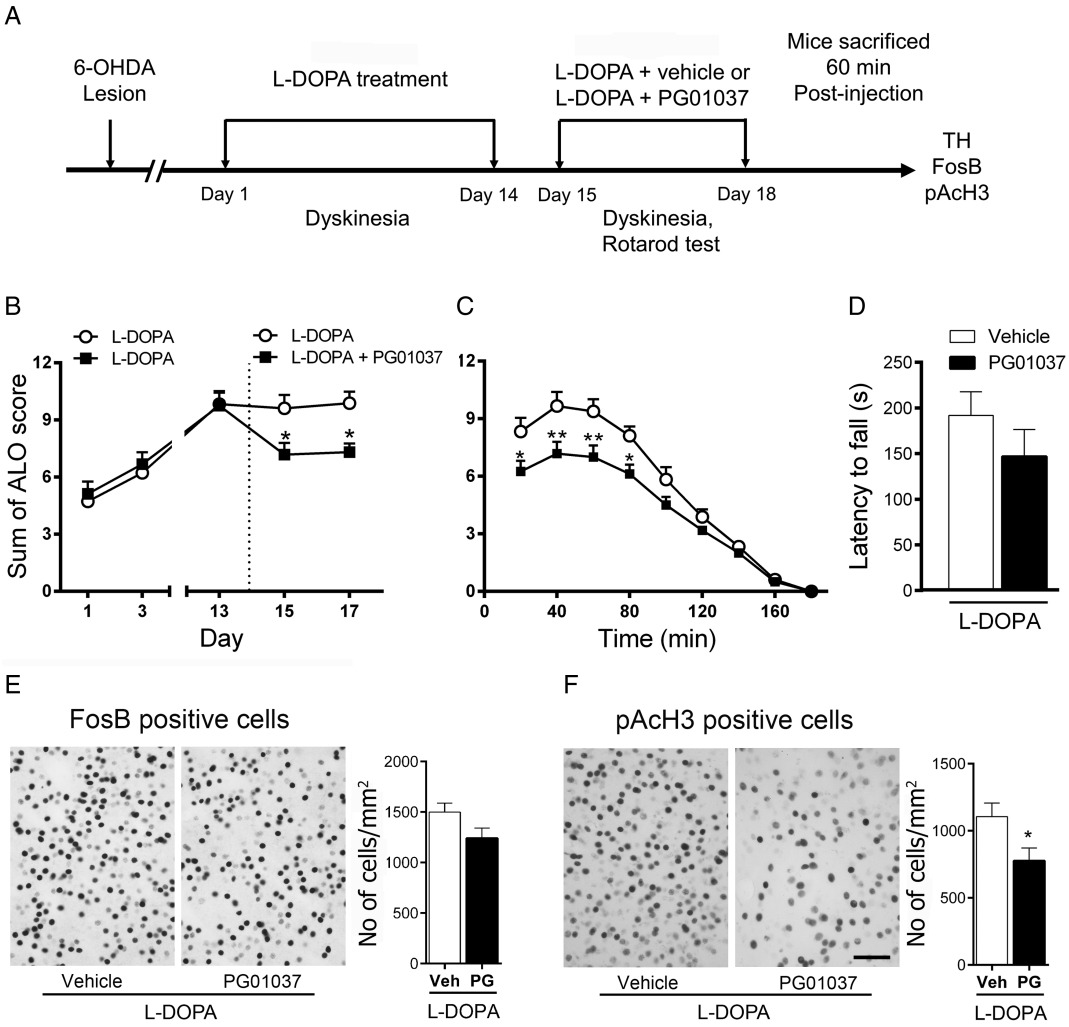


Figure 7. Effect of D3R-preferring antagonist PG01037 on the expression of established LID. (*A*) The time line shows experimental design. (*B* and *C*) The administration of PG01037 diminished the expression of established LID. *$P < 0.05$ and **$P < 0.01$ versus L-DOPA plus saline, two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test. (*D*) PG01037 cotreatment did not affect the latency to fall from the rotarod on established LID. $P = 0.08$ versus L-DOPA plus saline, unpaired *t*-test. Immunohistochemical analysis showed that coadministration of PG01037 does not significantly decrease FosB- (*E*) and pAcH3- (*F*) positive cells in mice with established LID ($P = 0.08$ and $P = 0.22$, respectively), unpaired *t*-test. All data are expressed as mean ± S.E.M. $n = 8–9$ for each group. Scale bar = 50 μm.

showed that D3R-preferring antagonists are effective at decreasing LID in different animal models (Visanji et al. 2009). As a result, we looked at the effect of D3R blockade and observed a reduction in LID without affecting the benefits of L-DOPA, in accordance with previous studies in the dyskinetic rat model (Kumar et al. 2009). However, there has been some controversy regarding pharmacological blockade of D3R and LID with some groups showing no effect (Mela et al. 2010). These conflicting results may be due to different specificities of the D3R antagonists, none of which completely distinguish between the different D2-like receptors. Indeed, recent evidence has also shown an antidyskinetic role of blocking D4R (D2-like) with a D4R-preferring antagonist (Huot et al. 2012).

Previous evidence has shown that D3R stimulation potentiates D1R-mediated behavioral responses and adenyl cyclase-mediated intracellular responses (Fiorentini et al. 2008; Marcellino et al. 2008). Indeed, it was described that D3R contributes to D1R-agonist-induced c-Fos expression in the striatum (Jung and Schmauss 1999). In addition, D3R stimulation enhances the transmitter release induced by D1R activation in the substantia nigra reticulata (Cruz-Trujillo et al. 2013). Taken together, these data suggest a synergic effect of D1R and D3R. The precise mechanisms underlying LID reduction in $D3^{-/-}$ mice in our study remain unclear. Possible mechanisms include loss of synergy between D1R and D3R that may act on direct pathway neurons to promote the activation of immediate early gene expression. Nevertheless, it is noteworthy that intracellular signaling in the striatum is oppositely regulated by D1R and D3R following cocaine challenge (Zhang et al. 2004). The paradoxical difference of the antagonistic effect of D3R in the normosensitive striatum versus the synergic role of D3R in the lesioned striatum is currently uncertain. A likely explanation is suggested by work showing that the D3R responsiveness is increased in the medium spiny neurons in the DA-depleted striatum (Prieto et al. 2011), and this enhanced activity of D3R could induce synergic cross talk with D1R following chronic L-DOPA treatment (Farré et al. 2014). In line with this, we found that D3R blockade in $D1^{+/-}$ mice, the dyskinetic response was further reduced compared

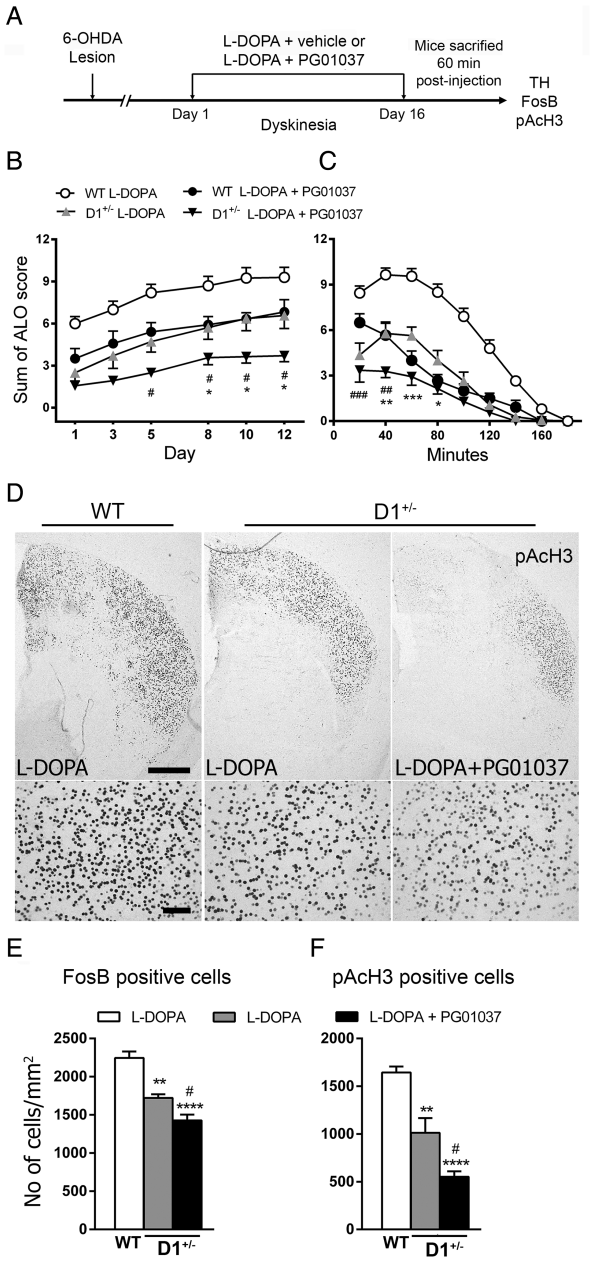


Figure 8. Effect of coadministration of PG01037 plus L-DOPA in $D1^{+/-}$ mice. (*A*) The time line shows experimental design. (*B*) Total dyskinetic score was evaluated 40 min after L-DOPA administration at the indicated days. Two-way ANOVA followed by a Bonferroni test showed significant differences for genotype ($F_{3,234} = 165.5$, $P < 0.001$) and day ($F_{8,234} = 108.6$, $P < 0.001$). (*C*) Time course of dyskinetic symptoms evaluated once every 20 min during 180 min on Day 13 of L-DOPA treatment. Two-way ANOVA followed by a Bonferroni test showed significant differences for genotype ($F_{3,156} = 71.04$, $P < 0.001$) and time ($F_{5,156} = 13.86$, $P < 0.001$). (*D*) Immunostaining for pAcH3. Photomicrographs at high and low magnifications of coronal sections from the dopamine-denervated striatum of L-DOPA-treated WT, $D1^{+/-}$ mice, and L-DOPA plus PG01037-treated mice. Scale bar = 500 µm for low-magnification and 100 µm for high-magnification images. (*E*,*F*) Immunohistochemical analysis shows that the D3R blockade decreases FosB- and pAcH3-positive cells induced by L-DOPA in $D1^{+/-}$ mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus $D1^{+/-}$ + L-DOPA; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$. All data are expressed as mean ± S.E.M. $n = 6$–10 for each group.

with dyskinetic animals that received PG01037 and those observed in $D1^{+/-}$ mice, suggesting a direct interaction between the D1R and the D3R in LID.

Previous studies have shown that L-DOPA-induced D3R mRNA was preferentially expressed in dynorphin mRNA-containing neurons in the denervated dorsal striatum (Bordet et al. 2000). In the present work, using BAC-transgenic -D1R-tomato and -D2R-GFP mice, we directly demonstrated that L-DOPA-induced D3R expression occurs in both types of neurons, although in D1R-neurons, D3R expression is 2.5- to 3-fold larger than that in D2R-neurons. The colocalization of D1R and D3R may allow interaction through heterodimerization and/or overactivation of adenyl cyclase (Fiorentini et al. 2008; Marcellino et al. 2008; Farré et al. 2014) and ERK signaling pathways (Guitart et al. 2014). It is widely accepted that L-DOPA treatment increases D1R signaling, leading to increased pERK levels (Pavón et al. 2006; Westin et al. 2007), which promote phosphoacetylation of H3 (Darmopil et al. 2009; Santini et al. 2009) at the FosB promoter (Feyder et al. 2014). Our data suggest that the capacity of L-DOPA to activate this transduction pathway is partially dependent on activation of dopamine D3 receptor, since we found a decrease in FosB, pERK, and pAcH3 expressed in D1R (Darmopil et al. 2009).

An important question that persists concerns the role of the D2R striatopallidal pathway in dyskinesia. Recent evidence has shown modest L-DOPA-induced increases in gene expression in D2R indirect pathway neurons (Heiman et al. 2014). Moreover, we demonstrated that L-DOPA selectively restores the number of the dendritic spines in the D2R medium spiny neurons, suggesting that these neurons play an important role in LID (Suárez et al. 2014). Interestingly, in the present study, we observed that L-DOPA increases D3R expression in the striatopallidal neurons in hemiparkinsonian mice. Although the role of D3R in these neurons in LID is currently unknown, previous studies showed an upregulation of D2R signaling in the striatopallidal neurons that is partially mediated by the D3R in parkinsonian mice, indicating that D3R contributes to D2R-supersensitive signaling (Prieto et al. 2011). Since D2R can be responsible for the abnormal expression of long-term depression in dyskinesia (Thiele et al. 2014), it is possible that D3R could participate in this abnormal plasticity in D2R-containing neurons. However, future experiments are needed to investigate these possibilities.

To the best of our knowledge, the present study is the first to investigate the effect of genetic inactivation of D3R in parkinsonian animal models of LID. We found that D3R deletion reduces dyskinesia and expression of associated molecular markers, supporting the therapeutic relevance of D3R as a potential target for treating or preventing LID. In addition, we provide direct evidence for the first time of the expression of the D3R in the striatonigral and striatopallidal projection neurons in LID.

Funding

This work was supported by grants from the Spanish Ministerios de Economía y Competitividad (SAF2013-48532-R) and of Sanidad Política Social e Igualdad (PNSD #2012/071), and Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CB06/05/0055) and Comunidad de Madrid ref. S2011/BMD-2336 to R.M. This work was supported by the National Institutes of Health (R21 DA036921A1 to M.X.). O.S. and J.R.G.M. acknowledge Consejo Nacional de Ciencia y Tecnología-Mexico and Secretaría de Ciencia, Tecnología e Innovación-Mexico, Distrito Federal for scholarship.

Notes

We thank Beatriz Pro and Emilia Rubio for excellent technical assistance. *Conflict of Interest*: None declared.

References

Andersson M, Hilbertson A, Cenci MA. 1999. Striatal fosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. Neurobiol Dis. 6:461–474.

Ares-Santos S, Granado N, Espadas I, Martinez-Murillo R, Moratalla R. 2014. Methamphetamine causes degeneration of dopamine cell bodies and terminals of the nigrostriatal pathway evidenced by silver staining. Neuropsychopharmacology. 39:1066–1080.

Beaulieu JM, Gainetdinov RR. 2011. The physiology, signaling, and pharmacology of dopamine receptors. Pharmacol Rev. 63:182–217.

Berthet A, Porras G, Doudnikoff E, Stark H, Cador M, Bezard E, Bloch B. 2009. Pharmacological analysis demonstrates dramatic alteration of D1 dopamine receptor neuronal distribution in the rat analog of L-DOPA-induced dyskinesia. J Neurosci. 29:4829–4835.

Bézard E, Ferry S, Mach U, Stark H, Leriche L, Boraud T, Gross C, Sokoloff P. 2003. Attenuation of levodopa-induced dyskinesia by normalizing dopamine D3 receptor function. Nat Med. 9:762–767.

Bordet R, Ridray S, Carboni S, Diaz J, Sokoloff P, Schwartz JC. 1997. Induction of dopamine D3 receptor expression as a mechanism of behavioral sensitization to levodopa. Proc Natl Acad Sci. 94:3363–3367.

Bordet R, Ridray S, Schwartz JC, Sokoloff P. 2000. Involvement of the direct striatonigral pathway in levodopa-induced sensitization in 6-hydroxydopamine-lesioned rats. Eur J Neurosci. 12:2117–2123.

Cote SR, Chitravanshi VC, Bleickardt C, Sapru HN, Kuzhikandathil EV. 2014. Overexpression of the dopamine D3 receptor in the rat dorsal striatum induces dyskinetic behaviors. Behav Brain Res. 263:46–50.

Cruz-Trujillo R, Avalos-Fuentes A, Rangel-Barajas C, Paz-Bermúdez F, Sierra A, Escartín-Perez E, Aceves J, Erlij D, Florán B. 2013. D3 dopamine receptors interact with dopamine D1 but not D4 receptors in the GABAergic terminals of the SNr of the rat. Neuropharmacology. 67:370–378.

Darmopil S, Martín AB, De Diego IR, Ares S, Moratalla R. 2009. Genetic inactivation of dopamine D1 but not D2 receptors inhibits L-DOPA–induced dyskinesia and histone activation. Biol Psychiat. 66:603–613.

Farré D, Muñoz A, Moreno E, Reyes-Resina I, Canet-Pons J, Dopeso-Reyes IG, Rico AJ, Lluís C, Mallol J, Navarro G, et al. 2014. Stronger dopamine D1 receptor-mediated neurotransmission in dyskinesia. Mol Neurobiol. doi:10.1007/s12035-014-8936-x.

Fasano S, Bezard E, D'Antoni A, Francardo V, Indrigo M, Qin L, Doveró S, Cerovic M, Cenci MA, Brambilla R. 2010. Inhibition of Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) signaling in the striatum reverts motor symptoms associated with l-dopa–induced dyskinesia. Proc Natl Acad Sci. 107: 21824–21829.

Feyder M, Södersten E, Santini E, Vialou V, LaPlant Q, Watts EL, Spigolon G, Hansen K, Caboche J, Nestler EJ, et al. 2014. A role for mitogen- and stress-activated kinase 1 in L-DOPA-induced dyskinesia and ΔFosB expression. Biol Psychiatry. doi:10.1016/j.biopsych.2014.07.019.

Fiorentini C, Busi C, Gorruso E, Gotti C, Spano P, Missale C. 2008. Reciprocal regulation of dopamine D1 and D3 receptor function and trafficking by heterodimerization. Mol Pharmacol. 74:59–69.

Flores G, Barbeau D, Quirion R, Srivastava LK. 1996. Decreased binding of dopamine D3 receptors in limbic subregions after neonatal bilateral lesion of rat hippocampus. J Neurosci. 16:2020–2026.

González-Aparicio R, Moratalla R. 2014. Oleoylethanolamide reduces L-DOPA-induced dyskinesia via TRPV1 receptor in a mouse model of Parkinsońs disease. Neurobiol Dis. 62:416–425.

Granado N, Ares-Santos S, Oliva I, O'Shea E, Martin ED, Colado MI, Moratalla R. 2011. Dopamine D2-receptor knockout mice are protected against dopaminergic neurotoxicity induced by methamphetamine or MDMA. Neurobiol Dis. 42:391–403.

Grundt P, Carlson EE, Cao J, Bennett CJ, McElveen E, Taylor M, Luedtke RR, Newman AH. 2005. Novel heterocyclic trans olefin analogues of N-{4-[4-(2,3-dichlorophenyl)piperazin-1-yl] butyl}arylcarboxamides as selective probes with high affinity for the dopamine D3 receptor. J Med Chem. 48:839–848.

Grundt P, Prevatt KM, Cao J, Taylor M, Floresca CZ, Choi JK, Jenkins BG, Luedtke RR, Newman AH. 2007. Heterocyclic analogues of N-(4-(4-(2,3-dichlorophenyl)piperazin-1-yl)butyl)arylcarboxamides with functionalized linking chains as novel dopamine D3 receptor ligands: potential substance abuse therapeutic agents. J Med Chem. 50:4135–4146.

Guigoni C, Aubert I, Li Q, Gurevich VV, Benovic JL, Ferry S, Mach U, Stark H, Leriche L, Håkansson K, et al. 2005. Pathogenesis of levodopa-induced dyskinesia: focus on D1 and D3 dopamine receptors. Parkinsonism Relat Dis. 11(Suppl 1):S25–S29.

Guillin O, Diaz J, Carroll P, Griffon N, Schwartz JC, Sokoloff P. 2001. BDNF controls dopamine D3 receptor expression and triggers behavioural sensitization. Nature. 411:86–89.

Guitart X, Navarro G, Moreno E, Yano H, Cai NS, Sanchez-Soto M, Kumar-Barodia S, Naidu YT, Mallol J, Cortes A, et al. 2014. Functional selectivity of allosteric interactions within G protein-coupled receptor oligomers: the dopamine D1-D3 receptor heterotetramer. Mol Pharmacol. 86:417429.

Heiman M, Heilbut A, Francardo V, Kulicke R, Fenster RJ, Kolaczyk ED, Mesirov JP, Surmeier DJ, Cenci MA, Greengard P. 2014. Molecular adaptations of striatal spiny projection neurons during levodopa-induced dyskinesia. Proc Natl Acad Sci USA. 111:4578–4583.

Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS, Nestler EJ. 1994. Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. Neuron. 13:1235–1244.

Huot P, Johnston TH, Koprich JB, Aman A, Fox SH, Brotchie JM. 2012. L-745,870 reduces L-DOPA-induced dyskinesia in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned macaque model of Parkinson's disease. J Pharmacol Exp Ther. 342:576–585.

Jung MY, Schmauss C. 1999. Decreased c-fos responses to dopamine D(1) receptor agonist stimulation in mice deficient for D(3) receptors. J Biol Chem. 274:29406–29412.

Kumar R, Riddle L, Griffin SA, Grundt P, Newman AH, Luedtke RR. 2009. Evaluation of the D3 dopamine receptor selective antagonist PG01037 on L-dopa-dependent abnormal involuntary movements in rats. Neuropharmacology. 56:944–955.

Marcellino D, Ferré S, Casadó V, Cortés A, Le Foll B, Mazzola C, Drago F, Saur O, Stark H, Soriano A, et al. 2008. Identification of dopamine D1-D3 receptor heteromers. Indications for a role of synergistic D1-D3 receptor interactions in the striatum. J Biol Chem. 283:26016–26025.

Mason CW, Hassan HE, Kim KP, Cao J, Eddington ND, Newman AH, Voulalas PJ. 2010. Characterization of the transport, metabolism, and pharmacokinetics of the dopamine D3 receptor-selective fluorenyl- and 2-pyridylphenyl amides developed for treatment of psychostimulant abuse. J Pharmacol Experim Ther. 333:854–864.

Mela F, Millan MJ, Brocco M, Morari M. 2010. The selective D(3) receptor antagonist, S33084, improves parkinsonian-like motor dysfunction but does not affect L-DOPA-induced dyskinesia in 6-hydroxydopamine hemi-lesioned rats. Neuropharmacology. 58:528–536.

Moratalla R, Elibol B, Vallejo M, Graybiel AM. 1996a. Network-level changes in expression of inducible Fos-Jun proteins in the striatum during chronic cocaine treatment and withdrawal. Neuron. 17:147–156.

Moratalla R, Vallejo M, Elibol B, Graybiel AM. 1996b. D1-class dopamine receptors influence cocaine-induced persistent expression of Fos-related proteins in striatum. Neuroreport. 8:1–5.

Murer MG, Moratalla R. 2011. Striatal signaling in L-DOPA-induced dyskinesia: common mechanisms with drug abuse and long term memory involving D1 dopamine receptor stimulation. Front Neuroanat. 5:51.

Obeso JA, Olanow CW, Nutt JG. 2000. Levodopa motor complications in Parkinson's disease. Trends Neurosci. 23:S2–S7.

Pavón N, Martín AB, Mendialdua A, Moratalla R. 2006. ERK Phosphorylation and FosB Expression Are Associated with L-DOPA-Induced Dyskinesia in Hemiparkinsonian Mice. Biol Psychiatry. 59:64–74.

Prieto GA, Perez-Burgos A, Palomero-Rivero M, Galarraga E, Drucker-Colin R, Bargas J. 2011. Upregulation of D2-class signaling in dopamine-denervated striatum is in part mediated by D3 receptors acting on Ca V 2.1 channels via PIP2 depletion. J Neurophysiol. 105:2260–2274.

Ruiz-DeDiego I, Mellstrom B, Vallejo M, Naranjo JR, Moratalla R. 2015. Activation of DREAM (downstream regulatory element antagonistic modulator), a calcium-binding protein, reduces L-DOPA-induced dyskinesias in mice. Biol Psychiatry. 77:95–105.

Santini E, Alcacer C, Cacciatore S, Heiman M, Hervé D, Greengard P, Girault JA, Valjent E, Fisone G. 2009. l DOPA activates ERK signaling and phosphorylates histone H3 in the striatonigral medium spiny neurons of hemiparkinsonian mice. J Neurochem. 108:621–633.

Santini E, Valjent E, Usiello A, Carta M, Borgkvist A, Girault JA, Hervé D, Greengard P, Fisone G. 2007. Critical Involvement of cAMP/DARPP-32 and Extracellular Signal-Regulated Protein Kinase Signaling in l-DOPA-Induced Dyskinesia. J Neurosci. 27:6995–7005.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 9:671–675.

Silverdale MA, Nicholson SL, Ravenscroft P, Crossman AR, Millan MJ, Brotchie JM. 2004. Selective blockade of D(3) dopamine receptors enhances the anti-parkinsonian properties of ropinirole and levodopa in the MPTP-lesioned primate. Exp Neurol. 188:128–138.

Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC. 1990. Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature. 347:146–151.

Solís O, Espadas I, Del-Bel EA, Moratalla R. 2015. Nitric oxide synthase inhibition decreases l-DOPA-induced dyskinesia and the expression of striatal molecular markers in Pitx3(-/-) aphakia mice. Neurobiol Dis. 73:49–59.

Suárez LM, Solís O, Caramés JM, Taravini IR, Solís JM, Murer MG, Moratalla R. 2014. L-DOPA treatment selectively restores spine density in dopamine receptor D2-expressing projection neurons in dyskinetic mice. Biol Psychiatry. 75:711–722.

Thiele SL, Chen B, Lo C, Gertler TS, Warre R, Surmeier JD, Brotchie JM, Nash JE. 2014. Selective loss of bi-directional synaptic plasticity in the direct and indirect striatal output pathways accompanies generation of parkinsonism and l-DOPA induced dyskinesia in mouse models. Neurobiol Dis. 71:334–344.

van Kampen JM, Stoessl AJ. 2003. Effects of oligonucleotide antisense to dopamine D3 receptor mRNA in a rodent model of behavioural sensitization to levodopa. Neuroscience. 116:307–314.

Visanji NP, Fox SH, Johnston T, Reyes G, Millan MJ, Brotchie JM. 2009. Dopamine D3 receptor stimulation underlies the development of L-DOPA-induced dyskinesia in animal models of Parkinson's disease. Neurobiol Dis. 35:184–192.

Westin JE, Vercammen L, Strome EM, Konradi C, Cenci MA. 2007. Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. Biol Psychiatry. 62:800–810.

Xu M, Koeltzow TE, Santiago GT, Moratalla R, Cooper DC, Hu XT, White NM, Graybiel AM, White FJ, Tonegawa S. 1997. Dopamine D3 receptor mutant mice exhibit increased behavioral sensitivity to concurrent stimulation of D1 and D2 receptors. Neuron. 19:837–848.

Zhang L, Lou D, Jiao H, Zhang D, Wang X, Xia Y, Zhang J, Xu M. 2004. Cocaine-induced intracellular signaling and gene expression are oppositely regulated by the dopamine D1 and D3 receptors. J Neurosci. 24:3344–3354.

DISCUSIÓN

Los mecanismos exactos que subyacen a las discinesias inducidas por L-DOPA son aún desconocidos; sin embargo, se ha demostrado que el substrato anatómico son las neuronas estriatales completamente denervadas, y que el D1R es fundamental para el desarrollo de las mismas (Pavón *et al.*, 2006). Tanto la inactivación genética del D1R (Darmopil *et al.*, 2009) como de DARPP-32 en neuronas que contienen al D1R (Bateup *et al.*, 2010), evitan el desarrollo de las discinesias. Debido a que el sistema dopaminérgico interacciona con otros sistemas de neurotransmisión, modificaciones pre o postsinápticas en la señalización del D1R modulan las discinesias. Por tanto, son múltiples los sistemas que se ven involucrados en este efecto secundario motor (Murer & Moratalla, 2011; Bastide *et al.*, 2015). En este trabajo, estudiamos el papel de diferentes dianas moleculares que podrían participar en el desarrollo de las discinesias a diferentes niveles de la cascada de señalización dependiente del D1R. En primer lugar, analizamos las alteraciones de los niveles estriatales de diferentes aminoácidos en el pico de las discinesias. Posteriormente, se estudiaron los efectos de la COMT, que juegan un papel importante en la regulación de los niveles de dopamina estriatal. También, evaluamos el rol del sistema nitrérgico en las discinesias, ya que el óxido nítrico regula la actividad sináptica de las neuronas estriatales. Y, finalmente, se estudió el papel del D3R, que induce un efecto sinérgico sobre la señalización del D1R en las discinesias.

Desregulación en los niveles estriatales de aminoácidos en ratones discinéticos

Como se ha dicho anteriormente, los mecanismos involucrados en las discinesias no están completamente dilucidados, se han asociado con la disfunción de varios sistemas de neurotransmisión, incluyendo el dopaminérgico, glutamatérgico, serotonérgico, colinérgico, GABAérgico y endocanabinoide (Bastide *et al.*, 2015), así como con alteraciones en diferentes aminoácidos cerebrales, como GABA, glutamato y taurina (Rinne *et al.*, 1988; Yuan *et al.*, 2013; Tong *et al.*, 2015).

Se ha sugerido que el sistema glutamatérgico juega un papel importante en la EP y las discinesias (Calabresi *et al.*, 2010). En nuestro trabajo hemos encontrado que los nive-

les estriatales de glutamato en animales parkinsonianos o discinéticos no difieren de los controles, lo que coincide con estudios anteriores (Tanaka *et al.*, 1986). Es posible que este resultado pudiera estar relacionado con un rápido metabolismo de este neurotransmisor para evitar su excitotoxicidad (Schousboe & Waagepetersen, 2006). La degradación del glutamato está asociada al ciclo glutamato-glutamina, en el que el glutamato se convierte en glutamina en los astrocitos (Bak *et al.*, 2006) y su actividad puede medirse por el ratio glutamato-glutamina (Shen, 2013). En este sentido nuestros datos muestran que los ratones parkinsonianos presentan niveles elevados de glutamina, lo que indicaría un mayor recambio y una mayor neurotransmisión glutamatérgica. El tratamiento con L-DOPA restaura el contenido de glutamina estriatal a niveles normales, lo que indica una normalización de la neurotransmisión glutamatérgica con el aporte de dopamina exógena. Estos resultados concuerdan con estudios previos de nuestro laboratorio que muestran la restauración de los niveles de glutamina utilizando técnicas de espectroscopia de resonancia magnética nuclear en un modelo de ratón con depleción dopaminérgica mediante reserpina y tratamiento con L-DOPA (Rodrigues *et al.*, 2007).

En cuanto a los niveles de GABA en el estriado, encontramos que mientras que en ratones parkinsonianos no se alteran, hay un aumento en ratones discinéticos. El GABA también está ligado al ciclo de glutamato-glutamina (Bak *et al.*, 2006), y es sintetizado por la descarboxilasa del ácido glutámico a partir de glutamato (Schousboe & Waagepetersen, 2007), por lo tanto, es posible que los niveles de GABA se mantengan debido a que la glutamina está siendo metabolizada hacia GABA. De hecho, la actividad de la enzima glutamato descarboxilasa (GAD de sus siglas en inglés), encargada de la síntesis de GABA, está aumentada en el estriado de animales lesionado con 6-OHDA (Segovia *et al.*, 1991). Esto también concuerda con que las neuronas gabaérgicas que contienen el D1R estén hiperactivadas en ratones discinéticos (Pavón *et al.*, 2006), y con el incremento de la liberación de GABA en la SNr (Rangel-Barajas *et al.*, 2011).

La taurina y la glicina son aminoácidos inhibitorios que participan en la plasticidad sináptica del estriado mediante la modulación de la transmisión glutamatérgica y GABAérgica (Chepkova *et al.*, 2002; Han *et al.*, 2004). Los niveles de taurina y glicina están

incrementados en el estriado de ratones parkinsonianos, y se restablecen después de la administración de L-DOPA. Es posible que este aumento después de la denervación dopaminérgica sea una respuesta homeostática para contrarrestar la actividad glutamatérgica después de la lesión. De hecho, la excitabilidad de las neuronas estriatales también está aumentada en ratones parkinsonianos (Suarez *et al.*, 2016). La disminución de taurina y glicina después de la L-DOPA también podría ser parte de este mecanismo compensatorio, ya que la actividad glutamatérgica y la excitabilidad estriatal se normaliza en animales discinéticos (Fieblinger *et al.*, 2014; Suárez *et al.*, 2014).

Uno de los cambios más notorios en los niveles de aminoácidos, es el aumento del contenido de tirosina en el estriado denervado de ratones discinéticos en comparación con los animales SHAM y parkinsonianos. En condiciones normales, la tirosina se convierte en L-DOPA con ayuda de la enzima TH (Nagatsu *et al.*, 1964). Aunque el papel de la tirosina en los animales discinéticos no está claro, estudios de nuestro laboratorio demuestran que la L-DOPA induce un aumento de las neuronas estriatales inmunorreactivas a la TH en el modelo 6-OHDA (Darmopil *et al.*, 2008). Este aumento es dosis dependiente y se prolonga durante días después de la retirada de la L-DOPA (Espadas *et al.*, 2012). Por otra parte, es posible que las neuronas catecolaminérgicas restantes aumenten más el contenido de tirosina para producir DA. Estos hallazgos no establecen definitivamente si los diferentes aminoácidos estudiados en el estriado participan en la fisiopatología de las discinesias. Sin embargo, destacan la importancia general de los aminoácidos y deben ser considerados al desarrollar tratamientos para las discinesias (Solís *et al.*, 2016).

Papel de la COMT en las discinesias inducidas por L-DOPA

Se ha sugerido que la degradación de dopamina podría tener un papel importante en el desarrollo de las discinesias. Nuestros resultados demuestran que los ratones que sobrexpresan la COMT (Tg-COMT) presentan más discinesias que los WT, y estas, correlacionan con un aumento en la expresión de FosB y pAcH3. Por otro lado, medimos los niveles de DA y sus metabolitos (DOPAC y 3-MT) en homogeneizados estriatales, y observamos un aumento de 3-MT en los ratones Tg-COMT discinéticos.

La enzima COMT participa en el catabolismo de las catecolaminas en el sistema nervioso central, y su actividad puede modular el comportamiento (Müller, 2015). En condiciones normales, la proteína COMT tiene un papel importante en la corteza frontal y, en menor medida, en el estriado (Gogos *et al.*, 1998). Sin embargo, se ha demostrado que el tratamiento con L-DOPA aumenta tanto la expresión como la actividad de la COMT en el estriado (Zhao *et al.*, 2001). Además, la inhibición de la COMT es una de las estrategias empleadas para aumentar la biodisponibilidad de la L-DOPA en el cerebro en pacientes con EP (Müller, 2015). Nuestros resultados demuestran que los ratones Tg-COMT, cuya actividad COMT en el estriado es 2 veces mayor que la de los WT (Suzuki *et al.*, 2009), desarrollan más discinesias. Estos resultados se correlacionan con el aumento de los marcadores moleculares FosB y pAcH3 y sugieren que la señalización del D1R esta sobreactivada en los ratones Tg-COMT. Aunque los mecanismos subyacentes al aumento de las discinesias en los animales Tg-COMT siguen siendo desconocidos, se ha demostrado que agonistas dopaminérgicos de acción corta inducen discinesias más severas que los agonistas de acción prolongada (Papathanou *et al.*, 2011). Por lo tanto, es posible que nuestros resultados se deban a que la estimulación del D1R es más corta en los Tg-COMT que, en los WT, debido a la mayor degradación de la dopamina.

Otra explicación podría estar relacionada con el aumento significativo de uno de los metabolitos de la DA, el 3-MT, por la acción de la COMT, ya que éste modula la señalización dopaminérgica (Espinoza *et al.*, 2012). Además, la inyección intracerebro ventricular de 3-MT en un modelo de ratón con deficiencia de dopamina, induce movimientos involuntarios anormales a través de la activación de los receptores asociados a aminas traza (TAAR, del inglés *trace amine-asociated receptors*), que aumentan la vía de señalización mediada por el D1R (Sotnikova *et al.*, 2010). Además, en cerebros *post mortem* de pacientes con la EP, la 3-MT está aumentada en el putamen (Rajput *et al.*, 2004). En nuestro estudio, encontramos mayores niveles de 3-MT en el estriado denervado de los ratones Tg-COMT, lo que sugiere que la 3-MT podría estar aumentando los síntomas. Por otra parte, nuestros resultados coinciden con la idea de que la prolongación de la vida media de la L-DOPA, mediante la inhibición de COMT, puede atenuar las discinesias (Müller, 2015). De hecho, trabajos realizados en modelos animales, demuestran que el tratamiento con

inhibidores de la COMT, reduce las discinesias (Smith *et al.*, 2005; Marin & Obeso, 2010). Nuestro estudio con animales genéticamente modificados, aporta nuevos datos para la comprensión del papel que juega la COMT en el desarrollo de las discinesias, y apoya el uso clínico de inhibidores centrales de COMT como tratamiento para las discinesias (Solís *et al.*, 2017a).

Papel del sistema nitrérgico en las discinesias

Por otro lado, debido a que en trabajos anteriores en nuestro laboratorio habiamos observado un aumento en la expresión de la nNOS en el estriado denervado de ratones discinéticos (Pavón *et al.*, 2006), estudiamos el papel del sistema nitrérgico en las discinesias en los ratones aphakia ($Pitx3^{-/-}$). En primer lugar, disminuimos la señalización del ON, inhibimos la nNOS y encontramos una atenuación del desarrollo y la expresión de las discinesias, sin afectar a la eficacia terapéutica de la L-DOPA. Este efecto fue acompañado por una reducción de los marcadores moleculares, FosB y pAcH3. En segundo lugar, aumentamos la señalización del ON mediante la administración de un donador de ON (molsidomina) o un inhibidor de la fosfodiesterasa 5 (zaprinast), en ratones $Pitx3^{-/-}$ tratados con L-DOPA. Ambos tratamientos condujeron a una reducción de las discinesias, aunque en detrimento de los efectos antiparkinsonianos de la L-DOPA.

Estudios previos sugieren que el sistema nitrérgico desempeña una función clave en la fisiología normal de los ganglios basales, así como en condiciones patológicas en la enfermedad de Parkinson y las discinesias (Calabrese *et al.*, 2007; Del-Bel *et al.*, 2015). El estudio con muestras postmortem de pacientes con EP, reveló un aumento de nNOS en los ganglios basales (Eve *et al.*, 1998), similar al que encontramos nosotros en animales discinéticos, lo que sugiere una mayor actividad del sistema nitrérgico en las discinesias. Además, también hemos encontrado que las interneuronas estriatales nNOS-positivas de las áreas estriatales denervadas, expresan FosB tras la administración de L-DOPA (Pavón *et al.*, 2006). Estos resultados se confirmaron posteriormente en el modelo de 6-OHDA en rata (Padovan-Neto *et al.*, 2015).

La disminución de la señalización del ON atenúa el desarrollo y la expresión de las discinesias, sin afectar a la eficacia terapéutica de la L-DOPA. Estos resultados coinciden con los reportados previamente en el modelo de 6-OHDA (Padovan-Neto *et al.*, 2009; Takuma *et al.*, 2012). Sin embargo, los mecanismos mediante los cuales la inhibición de la nNOS reduce tanto el desarrollo como la expresión de las discinesias no se conocen completamente. Sin embargo, en nuestro estudio demostramos que la coadministración de 7-NI y L-DOPA reduce la expresión de FosB y pAcH3 en comparación al tratamiento con L-DOPA, lo que indica que la inhibición de nNOS produce una disminución de la señalización del D1R. Curiosamente, además de la disminución de la señalización del D1R, estudios recientes ponen de manifiesto que el tratamiento con 7-NI previene las discinesias a través de la inhibición de la activación de las células gliales (Bortolanza *et al.*, 2015). Queda, por tanto, pendiente de confirmar si en nuestros resultados sólo interviene el D1R o también está implicada la glía.

Otro resultado importante de nuestro estudio es que el aumento de la señalización ON, mediante la administración de un donador del ON o un inhibidor de la PDE5, también reduce las discinesias. Esto está en línea con estudios anteriores en el modelo de 6-OHDA, que muestran una atenuación de las discinesias tras el aumento de la señalización del ON (Giorgi *et al.*, 2008; Picconi *et al.*, 2011). Sin embargo, ninguno de estos estudios evaluó el impacto de esta manipulación sobre la eficacia antiparkinsoniana de la L-DOPA. Por el contrario, nosotros demostramos que la Molsidomina o el Zaprinast, que aumentan la señalización GMPc, disminuye no sólo las discinesias sino también la eficacia terapéutica de la L-DOPA. En general, nuestros resultados incrementan el conocimiento actual del papel del sistema nitrérgico en las discinesias y dan apoyo a un mecanismo mediante el cual la inhibición de la nNOS reduce los marcadores moleculares asociados que contribuyen a disminuir las discinesias. No obstante, el papel del ON en las discinesias es complejo, debido a su acción pre y postsináptica, y su interacción con diferentes vías de transducción (Garthwaite, 2008). Por este motivo, harían falta más estudios para comprender mejor por qué ambos tratamientos, ya sea aumentando o disminuyendo los niveles de ON, tienen efectos antidiscinéticos (Solís *et al.*, 2015).

Papel del receptor dopaminérgico D3 en las discinesias

Este estudio pone de manifiesto la importancia del D3R en las discinesias, ya que éstas, están atenuadas tras la inactivación genética del D3R. De acuerdo con estos resultados, encontramos que el bloqueo farmacológico del D3R también disminuye el desarrollo de las discinesias. Además, hemos demostrado que los ratones transgénicos D1R-tomato y D2R-GFP, a los que se han inducido discinesias, tienen una sobrexpresión del D3R en el estriado dorsal en las neuronas que contienen el D1R y el D2R.

Con el fin de establecer el papel específico del D3R en el desarrollo de las discinesias, utilizamos ratones $D3^{-/-}$. Estos animales presentan menos discinesias que los WT, sin que esto altere el efecto terapéutico de la L-DOPA. Nuestros resultados concuerdan con estudios previos, en los que antagonistas del D3R como S33084, ST198 o PG01037 disminuyen las discinesias, aunque en el caso del ST198, esta reducción se acompaña con un detrimento de la eficacia terapéutica de la L-DOPA (Bézard *et al.*, 2003; Kumar *et al.*, 2009; Visanji *et al.*, 2009). Otros estudios, sin embargo, usando ST198 en ratas no encuentran ningún efecto antidiscinético (Silverdale *et al.*, 2004; Mela *et al.*, 2010). Es posible que estos resultados controvertidos se deban a una falta de selectividad de los antagonistas, ya que podrían estar actuando no sólo sobre los D3R, sino sobre otros receptores de la familia D2, por lo que el uso de animales transgénicos, inactivando la expresión del D3R se hizo indispensable para establecer esta selectividad.

Para excluir la posibilidad de que la reducción de las discinesias observadas fuese debida a la participación de mecanismos compensatorios durante el desarrollo de los ratones $D3^{-/-}$, realizamos un experimento en el que bloqueamos farmacológicamente este receptor en animales WT. Observamos que, tal y como ocurrió en los ratones $D3^{-/-}$, el bloqueo con el antagonista del D3R reduce el desarrollo de las discinesias. Asimismo, evaluamos el efecto del antagonista del D3R en las discinesias previamente establecidas, y encontramos que el bloqueo farmacológico del D3R también era capaz de reducir estos movimientos anormales.

En nuestro laboratorio hemos demostrado que, en las discinesias, las vías de PKA y ERK están aumentadas de manera aberrante en las neuronas estriatales que contienen el D1R (Pavón *et al.*, 2006). Esta sobrexpresión anormal de diferentes marcadores moleculares tales como FosB, pAcH3 y pERK en animales discinéticos se bloquea con la inactivación del D1R (Darmopil *et al.*, 2009; Ruiz-DeDiego *et al.*, 2015a). Curiosamente, la inactivación del D3R que disminuye las discinesias, también disminuye la expresión de FosB, pAcH3 y pERK, lo que sugiere una disminución de la señalización del D1R debido a una posible interacción D1R-D3R. Estudios realizados en células transfectadas que coexpresan el D1R y el D3R, revelan una interacción sinérgica entre ambos receptores mediante la cual, el D3R aumenta la señalización del D1R, al incrementar la activación de la vía de PKA (Fiorentini *et al.*, 2008; Marcellino *et al.*, 2008) y ERK (Guitart *et al.*, 2014). La heterodimerización de estas dos proteínas se ha demostrado recientemente en el estriado dorsal de ratas discinéticas, donde su activación provoca un aumento en la vía de señalización del D1R (Farré *et al.*, 2015). Para estudiar si esta interacción D1R-D3R también tenía lugar en ratones, y si estaba implicada en el mecanismo antidiscinético del D3R, diseñamos un estudio utilizando ratones $D1^{+/-}$. Estos ratones con menor cantidad de D1R, tienen menos discinesias de por si, y el bloqueo farmacológico del D3R disminuye aún más el desarrollo de las mismas. Este resultado demuestra que el efecto antidiscinético del D3R está mediado a través de su interacción con el D1R.

Estudios pioneros del laboratorio de Sokoloff mostraron, mediante giros contralaterales, que la sensibilización conductual inducida por L-DOPA está correlacionada con la sobrexpresión del D3R en el estriado dorsal de ratas lesionadas con 6-OHDA, y que esta sobrexpresión, se produce en las neuronas D1R positivas (Bordet *et al.*, 1997, 2000). Utilizando ratones transgénicos D1R-tomato y D2R-GFP, encontramos que el D3R apenas se expresa en el estriado dorsal de animales SHAM y parkinsonianos, pero la L-DOPA aumenta significativamente su expresión en ratones discinéticos. Aunque esta sobrexpresión ocurre fundamentalmente en las neuronas D1R positivas, también ocurre, en menor proporción, en las D2R positivas. Mientras que nuestros resultados consolidan el papel del D3R con el D1R en las neuronas de la vía directa en ratones discinéticos el papel de del D3R en las neuronas que contienen el D2R sigue siendo desconocido. No obstante,

se ha demostrado que existe una supersensibilidad del D2R en las neuronas de la vía indirecta en el estriado lesionado, debida en parte al D3R (Prieto *et al.*, 2011). Dado que el D2R puede ser responsable de la expresión anormal de la depresión a largo plazo en las discinesias (Thiele *et al.*, 2014), es posible que el D3R pudiese estar participando en esta plasticidad anormal en las neuronas que contienen al D2R.

En resumen, hemos demostrado que la inactivación del D3R reduce las discinesias inducidas por L-DOPA, mediante la atenuación de la vía de señalización del D1R. Adicionalmente, el D3R se sobrexpresa en el estriado dorsal principalmente en las neuronas de la vía directa, y en las neuronas de la vía indirecta en menor medida (Solís et al., 2017b).

Observaciones Finales

Como se ha discutido anteriormente, el D1R es crucial para el desarrollo de las discinesias inducidas por L-DOPA. En este escenario, su actividad es modulada por diferentes sistemas de neurotransmisión. Los resultados de esta tesis aportan información relevante sobre diferentes dianas farmacológicas que directa o indirectamente regulan la vía de señalización del D1R en las discinesias (Fig. 9). Por todo lo anterior, la COMT, el ON y el D3R se presentan como dianas terapéuticas de interés para aliviar las discinesias inducidas por L-DOPA en la enfermedad de Parkinson.

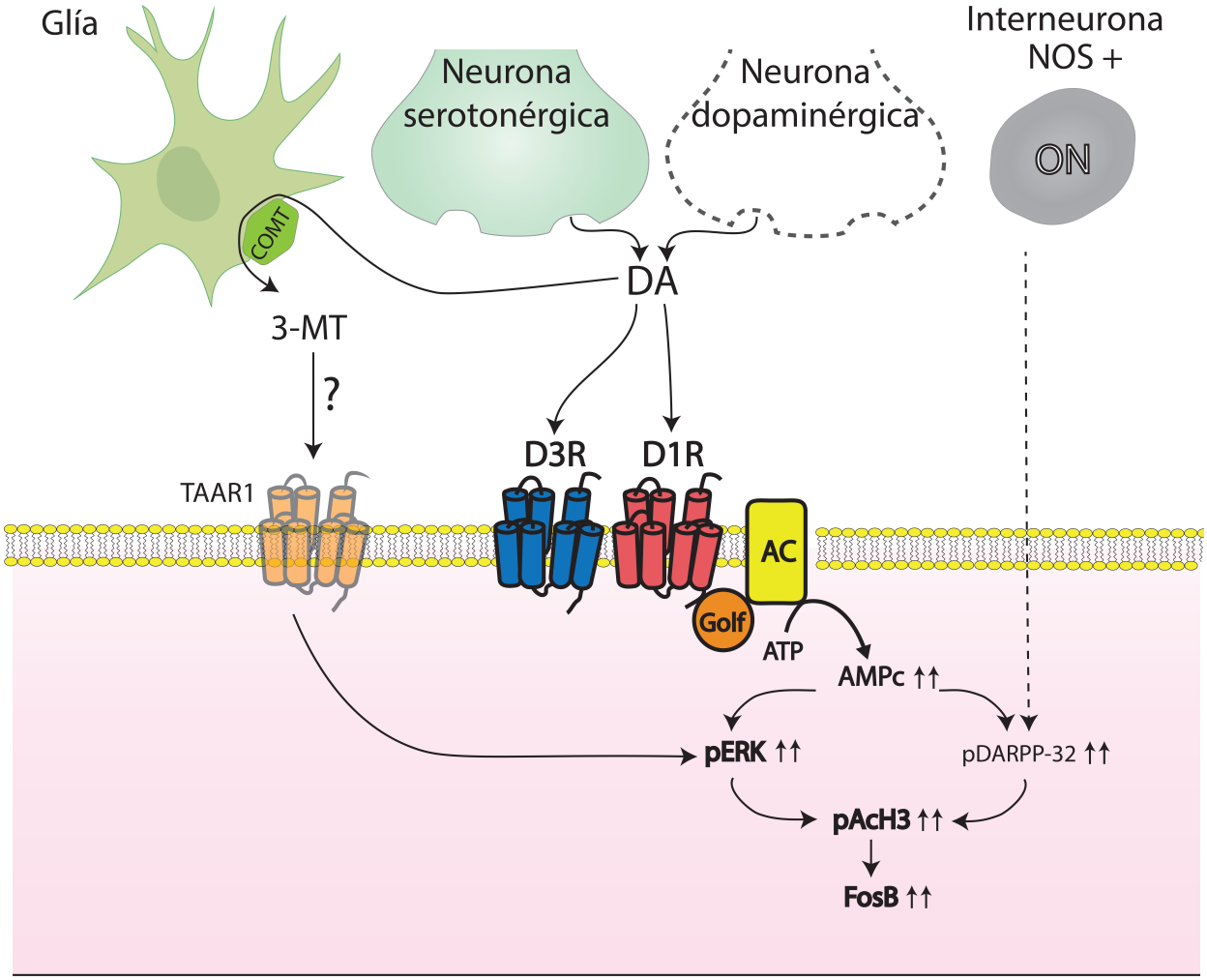


Figura 9. Representación esquemática que ilustra la regulación de la vía de señalización del D1R en las discinesias. La activación del D1R por la dopamina incrementa el AMPc y los marcadores moleculares relacionados con las discinesias (pERK, pAcH3 y FosB), y son modulados por el óxido nitrico, la catecol-O-metil transferasa y el receptor dopaminérgico D3.

CONCLUSIONES

De los resultados obtenidos en la presente Tesis Doctoral se pueden extraer las siguientes conclusiones:

1. La depleción de dopamina estriatal, aunque no altera los niveles de glutamato ni de GABA, aumenta la glutamina indicando una mayor transmisión glutamatérgica, que se revierte con la administración de L-DOPA.

2. La L-DOPA aumenta los niveles de GABA y tirosina en ratones discinéticos.

3. La sobrexpresión genética de la COMT disminuye los niveles de dopamina y aumenta los de su metabolito 3-MT.

4. La sobrexpresión de COMT aumenta las discinesias inducidas por L-DOPA y los marcadores moleculares FosB y pAcH3.

5. En los ratones Tg-COMT, la L-DOPA aumenta la 3-MT en el estriado denervado y este aumento podría estar relacionado con el mayor desarrollo de discinesias.

6. El tratamiento crónico con L-DOPA incrementa la inmunoreactividad de la nNOS en el estriado denervado del ratón aphakia.

7. La inhibición farmacológica de la nNOS disminuye el desarrollo y la expresión de las discinesias inducidas por L-DOPA sin interferir con el efecto terapéutico de la misma.

8. El incremento de la señalización en la vía del óxido nítrico, mediante la administración de molsidomina o zaprinast, disminuye las discinesias a expensas del efecto terapéutico de la L-DOPA.

9. La inactivación genética del D3R disminuye el desarrollo de las discinesias, así como los marcadores moleculares asociados a este efecto secundario.

10. El bloqueo farmacológico del D3R disminuye el desarrollo y la expresión de las discinesias inducidas por L-DOPA.

11. La administración de L-DOPA incrementa la expresión del D3R en ratones discinéticos, principalmente en las neuronas estriatales de la vía directa, y en menor proporción, en las neuronas de la vía indirecta.

12. El D3R modula las discinesias inducidas por L-DOPA mediante la vía de señalización dependiente del receptor D1.

BIBLIOGRAFÍA

Albin RL, Young AB, Penney JB (1989) The functional anatomy of basal ganglia disorders. Trends in Neurosciences 12:366–375.

Albin RL, Young AB, Penney JB (1995) The functional anatomy of disorders of the basal ganglia. Trends in Neurosciences 18:63–64.

Alcacer C, Andreoli L, Sebastianutto I, Jakobsson J, Fieblinger T, Cenci MA (2017) Chemogenetic stimulation of striatal projection neurons modulates responses to Parkinson's disease therapy. The Journal of Clinical Investigation.

Andén NE, Dahlström A, Fuxe K, Larsson K (1966) Functional role of the nigro-neostriatal dopamine neurons. Acta pharmacologica et toxicologica 24:263–274.

Andersson M, Hilbertson A, Cenci MA (1999) Striatal fosB expression is causally linked with l-DOPA-induced abnormal involuntary movements and the associated upregulation of striatal prodynorphin mRNA in a rat model of Parkinson's disease. Neurobiology of Disease 6:461–474.

Andlin-Sobocki P, Jönsson B, Wittchen HU, Olesen J (2005) Cost of disorders of the brain in Europe. European Journal of Neurology 12:1–27.

Bak LK, Schousboe A, Waagepetersen HS (2006) The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. Journal of Neurochemistry 98:641–653.

Bastide MF et al. (2015) Pathophysiology of L-dopa-induced motor and non-motor complications in Parkinson's disease. Progress in Neurobiology 132:96–168.

Bateup HS, Santini E, Shen W, Birnbaum S, Valjent E, Surmeier DJ, Fisone G, Nestler EJ, Greengard P (2010) Distinct subclasses of medium spiny neurons differentially regulate striatal motor behaviors. Proceedings of the National Academy of Sciences of the United

States of America 107:14845–14850.

Beaudoin-Gobert M, Epinat J, Météreau E, Duperrier S, Neumane S, Ballanger B, Lavenne F, Liger F, Tourvielle C, Bonnefoi F, Costes N, Bars DL, Broussolle E, Thobois S, Tremblay L, Sgambato-Faure V (2015) Behavioural impact of a double dopaminergic and serotonergic lesion in the non-human primate. Brain: A Journal of Neurology 138:2632–2647.

Beaulieu J-M, Espinoza S, Gainetdinov RR (2015) Dopamine receptors - IUPHAR Review 13. British Journal of Pharmacology 172:1–23.

Beaulieu J-M, Gainetdinov RR (2011) The physiology, signaling, and pharmacology of dopamine receptors. Pharmacological Reviews 63:182–217.

Berthet A, Porras G, Doudnikoff E, Stark H, Cador M, Bezard E, Bloch B (2009) Pharmacological analysis demonstrates dramatic alteration of D1 dopamine receptor neuronal distribution in the rat analog of L-DOPA-induced dyskinesia. The Journal of Neuroscience 29:4829–4835.

Bézard E, Ferry S, Mach U, Stark H, Leriche L, Boraud T, Gross C, Sokoloff P (2003) Attenuation of levodopa-induced dyskinesia by normalizing dopamine D3 receptor function. Nature Medicine 9:762–767.

Birkmayer W, Hornykiewicz O (1961) [The L-3,4-dioxyphenylalanine (DOPA)-effect in Parkinson-akinesia]. Wiener Klinische Wochenschrift 73:787–788.

Björklund A, Dunnett SB (2007) Dopamine neuron systems in the brain: an update. Trends in Neurosciences 30:194–202.

Boileau I, Guttman M, Rusjan P, Adams JR, Houle S, Tong J, Hornykiewicz O, Furukawa Y, Wilson AA, Kapur S, Kish SJ (2009) Decreased binding of the D3 dopamine receptor-preferring ligand [11C]-(+)-PHNO in drug-naive Parkinson's disease. Brain: A Jour-

nal of Neurology 132:1366–1375.

Bordet R, Ridray S, Carboni S, Diaz J, Sokoloff P, Schwartz JC (1997) Induction of dopamine D3 receptor expression as a mechanism of behavioral sensitization to levodopa. Proceedings of the National Academy of Sciences of the United States of America 94:3363–3367.

Bordet R, Ridray S, Schwartz JC, Sokoloff P (2000) Involvement of the direct striatonigral pathway in levodopa-induced sensitization in 6-hydroxydopamine-lesioned rats. The European Journal of Neuroscience 12:2117–2123.

Bortolanza M, Cavalcanti-Kiwiatkoski R, Padovan-Neto FE, da-Silva CA, Mitkovski M, Raisman-Vozari R, Del-Bel E (2015) Glial activation is associated with l-DOPA induced dyskinesia and blocked by a nitric oxide synthase inhibitor in a rat model of Parkinson's disease. Neurobiology of Disease 73:377–387.

Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AMG (2007) Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. Nature Reviews Neuroscience 8:766–775.

Calabresi P, Di Filippo M, Ghiglieri V, Tambasco N, Picconi B (2010) Levodopa-induced dyskinesias in patients with Parkinson's disease: filling the bench-to-bedside gap. Lancet Neurology 9:1106–1117.

Carlsson A, Lindqvist M, Magnusson T (1957) 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. Nature 180:1200.

Carta M, Bezard E (2011) Contribution of pre-synaptic mechanisms to L-DOPA-induced dyskinesia. Neuroscience 198:245–251.

Carta M, Carlsson T, Kirik D, Björklund A (2007) Dopamine released from 5-HT termi-

nals is the cause of L-DOPA-induced dyskinesia in parkinsonian rats. Brain: A Journal of Neurology 130:1819–1833.

Cenci MA, Lee CS, Björklund A (1998) L-DOPA-induced dyskinesia in the rat is associated with striatal overexpression of prodynorphin- and glutamic acid decarboxylase mRNA. European Journal of Neuroscience 10:2694–2706.

Chalimoniuk M, Stepień A (2004) Influence of the therapy with pergolide mesylate plus L-DOPA and with L-DOPA alone on serum cGMP level in PD patients. Polish Journal of Pharmacology 56:647–650.

Chepkova AN, Doreulee N, Yanovsky Y, Mukhopadhyay D, Haas HL, Sergeeva OA (2002) Long-lasting enhancement of corticostriatal neurotransmission by taurine. The European Journal of Neuroscience 16:1523–1530.

Chinta SJ, Andersen JK (2005) Dopaminergic neurons. The International Journal of Biochemistry & Cell Biology 37:942–946.

Collier TJ, Kanaan NM, Kordower JH (2011) Ageing as a primary risk factor for Parkinson's disease: evidence from studies of non-human primates. Nature Reviews Neuroscience 12:359–366.

Cote SR, Chitravanshi VC, Bleickardt C, Sapru HN, Kuzhikandathil EV (2014) Overexpression of the dopamine D3 receptor in the rat dorsal striatum induces dyskinetic behaviors. Behavioural Brain Research 263:46–50.

Cotzias GC, Van Woert MH, Schiffer LM (1967) Aromatic amino acids and modification of parkinsonism. The New England Journal of Medicine 276:374–379.

Cui G, Jun SB, Jin X, Pham MD, Vogel SS, Lovinger DM, Costa RM (2013) Concurrent activation of striatal direct and indirect pathways during action initiation. Nature 494:238–

242.

Darmopil S, Martín AB, De Diego IR, Ares S, Moratalla R (2009) Genetic inactivation of dopamine D1 but not D2 receptors inhibits L-DOPA-induced dyskinesia and histone activation. Biological Psychiatry 66:603–613.

Darmopil S, Muñetón-Gómez VC, de Ceballos ML, Bernson M, Moratalla R (2008) Tyrosine hydroxylase cells appearing in the mouse striatum after dopamine denervation are likely to be projection neurones regulated by L-DOPA. The European Journal of Neuroscience 27:580–592.

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889–909.

Del Bel EA, Guimarães FS, Bermúdez-Echeverry M, Gomes MZ, Schiaveto-de-souza A, Padovan-Neto FE, Tumas V, Barion-Cavalcanti AP, Lazzarini M, Nucci-da-Silva LP, de Paula-Souza D (2005) Role of nitric oxide on motor behavior. Cellular and Molecular Neurobiology 25:371–392.

Del-Bel E, Padovan-Neto FE, Bortolanza M, Tumas V, Aguiar AS, Raisman-Vozari R, Prediger RD (2015) Nitric oxide, a new player in L-DOPA-induced dyskinesia? Frontiers in bioscience (Elite edition) 7:168–192.

Duty S, Jenner P (2011) Animal models of Parkinson's disease: a source of novel treatments and clues to the cause of the disease. British Journal of Pharmacology 164:1357–1391.

Ehringer H, Hornykiewicz O (1960) [Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system]. Klinische Wochenschrift 38:1236–1239.

Engelborghs S, Marescau B, De Deyn PP (2003) Amino acids and biogenic amines in ce-

rebrospinal fluid of patients with Parkinson's disease. Neurochemical Research 28:1145–1150.

Engeln M, Bastide MF, Toulmé E, Dehay B, Bourdenx M, Doudnikoff E, Li Q, Gross CE, Boué-Grabot E, Pisani A, Bezard E, Fernagut P-O (2016) Selective Inactivation of Striatal FosB/ΔFosB-Expressing Neurons Alleviates L-DOPA-Induced Dyskinesia. Biological Psychiatry 79:354–361.

Errico F, Rossi S, Napolitano F, Catuogno V, Topo E, Fisone G, D'Aniello A, Centonze D, Usiello A (2008) D-aspartate prevents corticostriatal long-term depression and attenuates schizophrenia-like symptoms induced by amphetamine and MK-801. The Journal of Neuroscience 28:10404–10414.

Espadas I, Darmopil S, Vergaño-Vera E, Ortiz O, Oliva I, Vicario-Abejón C, Martín ED, Moratalla R (2012) L-DOPA-induced increase in TH-immunoreactive striatal neurons in parkinsonian mice: insights into regulation and function. Neurobiology of Disease 48:271–281.

Espinoza S, Manago F, Leo D, Sotnikova TD, Gainetdinov RR (2012) Role of catechol-O-methyltransferase (COMT)-dependent processes in Parkinson's disease and L-DOPA treatment. CNS & Neurological Disorders Drug Targets 11:251–263.

Evans JR, Mason SL, Williams-Gray CH, Foltynie T, Brayne C, Robbins TW, Barker RA (2011) The natural history of treated Parkinson's disease in an incident, community based cohort. Journal of Neurology, Neurosurgery, and Psychiatry 82:1112–1118.

Eve DJ, Nisbet AP, Kingsbury AE, Hewson EL, Daniel SE, Lees AJ, Marsden CD, Foster OJ (1998) Basal ganglia neuronal nitric oxide synthase mRNA expression in Parkinson's disease. Brain research Molecular Brain Research 63:62–71.

Farré D, Muñoz A, Moreno E, Reyes-Resina I, Canet-Pons J, Dopeso-Reyes IG, Rico AJ,

Lluís C, Mallol J, Navarro G, Canela EI, Cortés A, Labandeira-García JL, Casadó V, Lanciego JL, Franco R (2015) Stronger Dopamine D1 Receptor-Mediated Neurotransmission in Dyskinesia. Molecular Neurobiology 52:1408–1420.

Feyder M, Södersten E, Santini E, Vialou V, LaPlant Q, Watts EL, Spigolon G, Hansen K, Caboche J, Nestler EJ, Fisone G (2016) A Role for Mitogen- and Stress-Activated Kinase 1 in L-DOPA-Induced Dyskinesia and ΔFosB Expression. Biological Psychiatry 79:362–371.

Fieblinger T, Graves SM, Sebel LE, Alcacer C, Plotkin JL, Gertler TS, Chan CS, Heiman M, Greengard P, Cenci MA, Surmeier DJ (2014) Cell type-specific plasticity of striatal projection neurons in parkinsonism and L-DOPA-induced dyskinesia. Nature Communications 5:5316.

Fiorentini C, Busi C, Gorruso E, Gotti C, Spano P, Missale C (2008) Reciprocal regulation of dopamine D1 and D3 receptor function and trafficking by heterodimerization. Molecular Pharmacology 74:59–69.

Fiorentini C, Savoia P, Bono F, Tallarico P, Missale C (2015) The D3 dopamine receptor: From structural interactions to function. European Neuropsychopharmacology 25:1462–1469.

Gagnon D, Petryszyn S, Sanchez MG, Bories C, Beaulieu JM, De Koninck Y, Parent A, Parent M (2017) Striatal neurons expressing D1 and D2 receptors are morphologically distinct and differently affected by dopamine denervation in mice. Scientific reports 7:41432.

Garthwaite J (2008) Concepts of neural nitric oxide-mediated transmission. The European Journal of Neuroscience 27:2783–2802.

Gerfen CR, Bolam JP (2017) The Neuroanatomical Organization of the Basal Ganglia. In: Handbook of Basal Ganglia Structure and Function, Second Edition, pp 3–32 Handbook of Behavioral Neuroscience. Elsevier.

Gerfen CR, Surmeier DJ (2011) Modulation of striatal projection systems by dopamine. Annual Review of Neuroscience 34:441–466.

Giorgi M, D'Angelo V, Esposito Z, Nuccetelli V, Sorge R, Martorana A, Stefani A, Bernardi G, Sancesario G (2008) Lowered cAMP and cGMP signalling in the brain during levodopa-induced dyskinesias in hemiparkinsonian rats: new aspects in the pathogenetic mechanisms. The European Journal of Neuroscience 28:941–950.

Goedert M, Spillantini MG, Del Tredici K, Braak H (2013) 100 years of Lewy pathology. Nature Reviews Neurology 9:13–24.

Gogos JA, Morgan M, Luine V, Santha M, Ogawa S, Pfaff D, Karayiorgou M (1998) Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behavior. Proceedings of the National Academy of Sciences of the United States of America 95:9991–9996.

González-Aparicio R, Moratalla R (2014) Oleoylethanolamide reduces L-DOPA-induced dyskinesia via TRPV1 receptor in a mouse model of Parkinson´s disease. Neurobiology of Disease 62:416–425.

Grace AA (2008) Physiology of the normal and dopamine-depleted basal ganglia: insights into levodopa pharmacotherapy. Movement Disorders 23:S560–S569.

Grossman MH, Emanuel BS, Budarf ML (1992) Chromosomal mapping of the human catechol-O-methyltransferase gene to 22q11.1-q11.2. Genomics 12:822–825.

Guitart X, Navarro G, Moreno E, Yano H, Cai N-S, Sánchez-Soto M, Kumar-Barodia S, Naidu YT, Mallol J, Cortés A, Lluís C, Canela EI, Casadó V, McCormick PJ, Ferré S (2014) Functional selectivity of allosteric interactions within G protein-coupled receptor oligomers: the dopamine D1-D3 receptor heterotetramer. Molecular Pharmacology 86:417–429.

Guix FX, Uribesalgo I, Coma M, Muñoz FJ (2005) The physiology and pathophysiology of nitric oxide in the brain. Progress in Neurobiology 76:126–152.

Han NL, Clements JD, Lynch JW (2004) Comparison of taurine- and glycine-induced conformational changes in the M2-M3 domain of the glycine receptor. The Journal of Biological Chemistry 279:19559–19565.

Herrera-Marschitz M, Arbuthnott G, Ungerstedt U (2010) The rotational model and microdialysis: Significance for dopamine signalling, clinical studies, and beyond. Progress in Neurobiology 90:176–189.

Hirsch L, Jette N, Frolkis A, Steeves T, Pringsheim T (2016) The Incidence of Parkinson's Disease: A Systematic Review and Meta-Analysis. Neuroepidemiology 46:292–300.

Hornykiewicz O (1963) [The tropical localization and content of noradrenalin and dopamine (3-hydroxytyramine) in the substantia nigra of normal persons and patients with Parkinson's disease]. Wiener Klinische Wochenschrift 75:309–312.

Huot P, Johnston TH, Koprich JB, Fox SH, Brotchie JM (2013) The pharmacology of L-DOPA-induced dyskinesia in Parkinson's disease. Pharmacological Reviews 65:171–222.

Hwang D (2003) Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice. Molecular Brain Research 114:123–131.

Iderberg H, Francardo V, Pioli EY (2012) Animal models of L-DOPA-induced dyskinesia: an update on the current options. Neuroscience 211:13–27.

Itokawa K, Ohkuma A, Araki N, Tamura N, Shimazu K (2006) Effect of L-DOPA on nitric oxide production in striatum of freely mobile mice. Neuroscience Letters 402:142–144.

Jenner P (2008) Molecular mechanisms of L-DOPA-induced dyskinesia. Nature Reviews

Neuroscience 9:665–677.

Jin X, Tecuapetla F, Costa RM (2014) Basal ganglia subcircuits distinctively encode the parsing and concatenation of action sequences. Nature Neuroscience 17:423–430.

Kravitz AV, Freeze BS, Parker PRL, Kay K, Thwin MT, Deisseroth K, Kreitzer AC (2010) Regulation of parkinsonian motor behaviours by optogenetic control of basal ganglia circuitry. Nature 466:622–626.

Kumar R, Riddle L, Griffin SA, Grundt P, Newman AH, Luedtke RR (2009) Evaluation of the D3 dopamine receptor selective antagonist PG01037 on L-dopa-dependent abnormal involuntary movements in rats. Neuropharmacology 56:944–955.

Lees AJ, Tolosa E, Olanow CW (2015) Four pioneers of L-dopa treatment: Arvid Carlsson, Oleh Hornykiewicz, George Cotzias, and Melvin Yahr. Movement Disorders 30:19–36.

Lévesque D, Martres MP, Diaz J, Griffon N, Lammers CH, Sokoloff P, Schwartz JC (1995) A paradoxical regulation of the dopamine D3 receptor expression suggests the involvement of an anterograde factor from dopamine neurons. Proceedings of the National Academy of Sciences of the United States of America 92:1719–1723.

Männistö PT, Kaakkola S (1999) Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacological Reviews 51:593–628.

Marcellino D, Ferré S, Casadó V, Cortés A, Le Foll B, Mazzola C, Drago F, Saur O, Stark H, Soriano A, Barnes C, Goldberg SR, Lluis C, Fuxe K, Franco R (2008) Identification of dopamine D1-D3 receptor heteromers. Indications for a role of synergistic D1-D3 receptor interactions in the striatum. The Journal of Biological Chemistry 283:26016–26025.

Marin C, Obeso JA (2010) Catechol-O-methyltransferase inhibitors in preclinical models as adjuncts of L-dopa treatment. International Review of Neurobiology 95:191–205.

Martínez-Lapiscina EH, Pulido-Fuentes L, Erro-Aguirre ME (2012) Manejo terapéutico de las fluctuaciones motoras en la enfermedad de Parkinson. Revista de Neurologia 54:S25–S32.

Mela F, Millan MJ, Brocco M, Morari M (2010) The selective D(3) receptor antagonist, S33084, improves parkinsonian-like motor dysfunction but does not affect L-DOPA-induced dyskinesia in 6-hydroxydopamine hemi-lesioned rats. Neuropharmacology 58:528–536.

Moratalla R, Solís O, Suárez LM (2017) Morphological Plasticity in the Striatum Associated With Dopamine Dysfunction. In: Handbook of Basal Ganglia Structure and Function, Second Edition, pp 755–770 Handbook of Behavioral Neuroscience. Elsevier.

Morin N, Jourdain VA, Di Paolo T (2014) Modeling dyskinesia in animal models of Parkinson disease. Experimental Neurology 256:105–116.

Mosharov EV, Borgkvist A, Sulzer D (2015) Presynaptic effects of levodopa and their possible role in dyskinesia. Movement Disorders 30:45–53.

Müller T (2015) Catechol-O-methyltransferase inhibitors in Parkinson's disease. Drugs 75:157–174.

Murer MG, Moratalla R (2011) Striatal Signaling in L-DOPA-Induced Dyskinesia: Common Mechanisms with Drug Abuse and Long Term Memory Involving D1 Dopamine Receptor Stimulation. Frontiers in Neuroanatomy 5:51.

Nagatsu T, Levitt M, Udenfriend S (1964) Tyrosine hydroxylase. the initial step in norepinephrine biosynthesis. The Journal of Biological Chemistry 239:2910–2917.

Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP (2003) Pitx3 is required for development of substantia nigra dopaminergic neurons. Proceedings of the National Academy of Sciences of the United States of America 100:4245–4250.

Obeso JA, Olanow CW, Nutt JG (2000) Levodopa motor complications in Parkinson's disease. Trends in Neuroscience 23:S2-7

Padovan-Neto FE, Cavalcanti-Kiwiatkoviski R, Carolino ROG, Anselmo-Franci J, Del Bel E (2015) Effects of prolonged neuronal nitric oxide synthase inhibition on the development and expression of L-DOPA-induced dyskinesia in 6-OHDA-lesioned rats. Neuropharmacology 89:87–99.

Padovan-Neto FE, Echeverry MB, Tumas V, Del-Bel EA (2009) Nitric oxide synthase inhibition attenuates L-DOPA-induced dyskinesias in a rodent model of Parkinson's disease. Neuroscience 159:927–935.

Papathanou M, Rose S, McCreary A, Jenner P (2011) Induction and expression of abnormal involuntary movements is related to the duration of dopaminergic stimulation in 6-OHDA-lesioned rats. The European Journal of Neuroscience 33:2247–2254.

Park DJ, West AR (2009) Regulation of striatal nitric oxide synthesis by local dopamine and glutamate interactions. Journal of Neurochemistry 111:1457–1465.

Parkinson J (2002) An essay on the shaking palsy. 1817. The Journal of Neuropsychiatry and Clinical Neurosciences 14:223–236; discussion 222.

Pavón N, Martín AB, Mendialdua A, Moratalla R (2006) ERK phosphorylation and FosB expression are associated with L-DOPA-induced dyskinesia in hemiparkinsonian mice. Biological Psychiatry 59:64–74.

Peñas E, Gálvez M, Marín M, Pérez-Olivares M (2015) Libro Blanco del Párkinson: aprox-

imación, análisis y propuesta de futuro, 1st ed. España: Real Patronato sobre Discapacidad, Ministerio de Sanidad, Servicios Sociales e Igualdad.

Picconi B, Bagetta V, Ghiglieri V, Paillè V, Di Filippo M, Pendolino V, Tozzi A, Giampà C, Fusco FR, Sgobio C, Calabresi P (2011) Inhibition of phosphodiesterases rescues striatal long-term depression and reduces levodopa-induced dyskinesia. Brain: A Journal of Neurology 134:375–387.

Plenz D, Wickens JR (2017) The Striatal Skeleton: Medium Spiny Projection Neurons and Their Lateral Connections. In: Handbook of Basal Ganglia Structure and Function, Second Edition, pp 121–136 Handbook of Behavioral Neuroscience. Elsevier.

Prashanth LK, Fox S, Meissner WG (2011) l-Dopa-induced dyskinesia-clinical presentation, genetics, and treatment. International Review of Neurobiology 98:31–54.

Prieto GA, Perez-Burgos A, Palomero-Rivero M, Galarraga E, Drucker-Colin R, Bargas J (2011) Upregulation of D2-class signaling in dopamine-denervated striatum is in part mediated by D3 receptors acting on Ca V 2.1 channels via PIP2 depletion. Journal of Neurophysiology 105:2260–2274.

Rangel-Barajas C, Coronel I, Florán B (2015) Dopamine Receptors and Neurodegeneration. Aging and disease 6:349–368.

Rangel-Barajas C, Silva I, Lopéz-Santiago LM, Aceves J, Erlij D, Florán B (2011) L-DOPA-induced dyskinesia in hemiparkinsonian rats is associated with up-regulation of adenylyl cyclase type V/VI and increased GABA release in the substantia nigra reticulata. Neurobiology of Disease 41:51–61.

Rascol O, Dubois B, Caldas AC, Senn S, Del Signore S, Lees A, Parkinson REGAIN Study Group (2006) Early piribedil monotherapy of Parkinson's disease: A planned seven-month report of the REGAIN study. Movement Disorders 21:2110–2115.

Rascol O, Nutt JG, Blin O, Goetz CG, Trugman JM, Soubrouillard C, Carter JH, Currie LJ, Fabre N, Thalamas C, Giardina WW, Wright S (2001) Induction by dopamine D1 receptor agonist ABT-431 of dyskinesia similar to levodopa in patients with Parkinson disease. Archives of Neurology 58:249–254.

Rinne JO, Halonen T, Riekkinen PJ, Rinne UK (1988) Free amino acids in the brain of patients with Parkinson's disease. Neuroscience Letters 94:182–186.

Rivera A, Cuéllar B, Girón FJ, Grandy DK, de la Calle A, Moratalla R (2002) Dopamine D4 receptors are heterogeneously distributed in the striosomes/matrix compartments of the striatum. Journal of Neurochemistry 80:219–229.

Rivera A, Trías S, Peñafiel A, Angel Narváez J, Díaz-Cabiale Z, Moratalla R, de la Calle A (2003) Expression of D4 dopamine receptors in striatonigral and striatopallidal neurons in the rat striatum. Brain Research 989:35–41.

Rodrigues TB, Granado N, Ortiz O, Cerdán S, Moratalla R (2007) Metabolic interactions between glutamatergic and dopaminergic neurotransmitter systems are mediated through D(1) dopamine receptors. Journal of Neuroscience Research 85:3284–3293.

Ruiz-DeDiego I, Mellstrom B, Vallejo M, Naranjo JR, Moratalla R (2015a) Activation of DREAM (downstream regulatory element antagonistic modulator), a calcium-binding protein, reduces L-DOPA-induced dyskinesias in mice. Biological Psychiatry 77:95–105.

Ruiz-DeDiego I, Naranjo JR, Hervé D, Moratalla R (2015b) Dopaminergic regulation of olfactory type G-protein α subunit expression in the striatum. Movement Disorders 30:1039–1049.

Sagi Y, Heiman M, Peterson JD, Musatov S, Scarduzio M, Logan SM, Kaplitt MG, Surmeier DJ, Heintz N, Greengard P (2014) Nitric oxide regulates synaptic transmission between spiny projection neurons. Proceedings of the National Academy of Sciences of the

United States of America 111:17636–17641.

Santini E, Alcacer C, Cacciatore S, Heiman M, Hervé D, Greengard P, Girault JA, Valjent E, Fisone G (2009) L-DOPA activates ERK signaling and phosphorylates histone H3 in the striatonigral medium spiny neurons of hemiparkinsonian mice. Journal of Neurochemistry 108:621–633.

Santini E, Valjent E, Usiello A, Carta M, Borgkvist A, Girault JA, Hervé D, Greengard P, Fisone G (2007) Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesia. The Journal of Neuroscience 27:6995–7005.

Schendzielorz N, Oinas J-P, Myöhänen TT, Reenilä I, Raasmaja A, Männistö PT (2013) Catechol-O-methyltransferase (COMT) protein expression and activity after dopaminergic and noradrenergic lesions of the rat brain. Plos One 8:e61392.

Schousboe A, Waagepetersen HS (2006) Glial modulation of GABAergic and glutamat ergic neurotransmission. Current Topics in Medicinal Chemistry 6:929–934.

Schousboe A, Waagepetersen HS (2007) GABA: homeostatic and pharmacological aspects. Progress in Brain Research 160:9–19.

Segovia J, Armstrong DM, Benzing WC, Hornby PJ (1991) Striatal glutamic acid decarboxylase immunoreactivity is increased after dopaminergic deafferentation: densitometric analysis. Neuroscience Letters 122:252–256.

Sergeeva OA, Fleischer W, Chepkova AN, Warskulat U, Häussinger D, Siebler M, Haas HL (2007) GABAA-receptor modification in taurine transporter knockout mice causes striatal disinhibition. The Journal of Physiology 585:539–548.

Sergeeva OA, Haas HL (2001) Expression and function of glycine receptors in striatal

cholinergic interneurons from rat and mouse. Neuroscience 104:1043–1055.

Shen J (2013) Modeling the glutamate-glutamine neurotransmitter cycle. Frontiers in neuroenergetics 5:1.

Silverdale MA, Nicholson SL, Ravenscroft P, Crossman AR, Millan MJ, Brotchie JM (2004) Selective blockade of D(3) dopamine receptors enhances the anti-parkinsonian properties of ropinirole and levodopa in the MPTP-lesioned primate. Experimental Neurology 188:128–138.

Smith LA, Jackson MJ, Al-Barghouthy G, Rose S, Kuoppamaki M, Olanow W, Jenner P (2005) Multiple small doses of levodopa plus entacapone produce continuous dopaminergic stimulation and reduce dyskinesia induction in MPTP-treated drug-naive primates. Movement Disorders 20:306–314.

Sokoloff P, Giros B, Martres MP, Bouthenet ML, Schwartz JC (1990) Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. Nature 347:146–151.

Solís O, Espadas I, Del-Bel EA, Moratalla R (2015) Nitric oxide synthase inhibition decreases l-DOPA-induced dyskinesia and the expression of striatal molecular markers in Pitx3(-/-) aphakia mice. Neurobiology of Disease 73:49–59.

Solís O, García-Montes JR, Garcia-Sanz P, Herranz AS, Asensio MJ, Kang G, Hiroi N, Moratalla R (2017a) Human COMT over-expression confers a heightened susceptibility to dyskinesia in mice. Neurobiology of Disease 102:133–139.

Solís O, Garcia-Montes JR, González-Granillo A, Xu M, Moratalla R (2017b) Dopamine D3 Receptor Modulates l-DOPA-Induced Dyskinesia by Targeting D1 Receptor-Mediated Striatal Signaling. Cerebral Cortex 27:435–446.

Solís O, García-Sanz P, Herranz AS, Asensio MJ, Moratalla R (2016) L-DOPA Reverses the Increased Free Amino Acids Tissue Levels Induced by Dopamine Depletion and Rises GABA and Tyrosine in the Striatum. Neurotoxicity Research 30:67–75.

Solis O, Limón DI, Flores-Hernández J, Flores G (2007) Alterations in dendritic morphology of the prefrontal cortical and striatum neurons in the unilateral 6-OHDA-rat model of Parkinson's disease. Synapse 61:450–458.

Sotnikova TD, Beaulieu JM, Espinoza S, Masri B, Zhang X, Salahpour A, Barak LS, Caron MG, Gainetdinov RR (2010) The dopamine metabolite 3-methoxytyramine is a neuromodulator. Plos One 5:e13452.

Suarez LM, Solis O, Aguado C, Lujan R, Moratalla R (2016) L-DOPA Oppositely Regulates Synaptic Strength and Spine Morphology in D1 and D2 Striatal Projection Neurons in Dyskinesia. Cerebral Cortex 26:4253–4264.

Suárez LM, Solís O, Caramés JM, Taravini IR, Solís JM, Murer MG, Moratalla R (2014) L-DOPA treatment selectively restores spine density in dopamine receptor D2-expressing projection neurons in dyskinetic mice. Biological Psychiatry 75:711–722.

Suzuki G, Harper KM, Hiramoto T, Funke B, Lee M, Kang G, Buell M, Geyer MA, Kucherlapati R, Morrow B, Männistö PT, Agatsuma S, Hiroi N (2009) Over-expression of a human chromosome 22q11.2 segment including TXNRD2, COMT and ARVCF developmentally affects incentive learning and working memory in mice. Human Molecular Genetics 18:3914–3925.

Takuma K, Tanaka T, Takahashi T, Hiramatsu N, Ota Y, Ago Y, Matsuda T (2012) Neuronal nitric oxide synthase inhibition attenuates the development of L-DOPA-induced dyskinesia in hemi-Parkinsonian rats. European Journal of Pharmacology 683:166–173.

Tanaka Y, Niijima K, Mizuno Y, Yoshida M (1986) Changes in gamma-aminobutyrate, glu-

tamate, aspartate, glycine, and taurine contents in the striatum after unilateral nigrostriatal lesions in rats. Experimental Neurology 91:259–268.

Tepper JM, Koós T (2017) Gabaergic interneurons of the striatum. In: Handbook of basal ganglia structure and function, second edition, pp 157–178 Handbook of behavioral neuroscience. Elsevier.

Thiele SL, Chen B, Lo C, Gertler TS, Warre R, Surmeier JD, Brotchie JM, Nash JE (2014) Selective loss of bi-directional synaptic plasticity in the direct and indirect striatal output pathways accompanies generation of parkinsonism and l-DOPA induced dyskinesia in mouse models. Neurobiology of Disease 71:334–344.

Tong Q, Xu Q, Xia Q, Yuan Y, Zhang L, Sun H, Shan H, Zhang K (2015) Correlations between plasma levels of amino acids and nonmotor symptoms in Parkinson's disease. Journal of Neural Transmission 122:411–417.

Ungerstedt U (1968) 6-Hydroxy-dopamine induced degeneration of central monoamine neurons. European Journal of Pharmacology 5:107–110.

Van den Munckhof P, Luk KC, Ste-Marie L, Montgomery J, Blanchet PJ, Sadikot AF, Drouin J (2003) Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons. Development 130:2535–2542.

Visanji NP, Fox SH, Johnston T, Reyes G, Millan MJ, Brotchie JM (2009) Dopamine D3 receptor stimulation underlies the development of L-DOPA-induced dyskinesia in animal models of Parkinson's disease. Neurobiology of Disease 35:184–192.

Westin JE, Vercammen L, Strome EM, Konradi C, Cenci MA (2007) Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. Biological Psychiatry 62:800–810.

Xu M, Koeltzow TE, Santiago GT, Moratalla R, Cooper DC, Hu XT, White NM, Graybiel AM, White FJ, Tonegawa S (1997) Dopamine D3 receptor mutant mice exhibit increased behavioral sensitivity to concurrent stimulation of D1 and D2 receptors. Neuron 19:837–848.

Yuan YS, Zhou XJ, Tong Q, Zhang L, Zhang L, Qi ZQ, Ge S, Zhang KZ (2013) Change in plasma levels of amino acid neurotransmitters and its correlation with clinical heterogeneity in early Parkinson's disease patients. CNS Neuroscience & Therapeutics 19:889–896.

Zhang L, Yuan Y, Tong Q, Jiang S, Xu Q, Ding J, Zhang L, Zhang R, Zhang K (2016) Reduced plasma taurine level in Parkinson's disease: association with motor severity and levodopa treatment. The International journal of neuroscience 126:630–636.

Zhao WQ, Latinwo L, Liu XX, Lee ES, Lamango N, Charlton CG (2001) L-dopa upregulates the expression and activities of methionine adenosyl transferase and catechol-O-methyltransferase. Experimental Neurology 171:127–138.

OTRAS PUBLICACIONES

L-DOPA treatment selectively restores spine density in dopamine receptor D2-expressing projection neurons in dyskinetic mice.

Suárez LM, Solís O, Caramés JM, Taravini IR, Solís JM, Murer MG, Moratalla R.

Biol Psychiatry. 2014; 75:711-722. doi: 10.1016/j.biopsych.2013.05.006.

ARCHIVAL REPORT

L-DOPA Treatment Selectively Restores Spine Density in Dopamine Receptor D2–Expressing Projection Neurons in Dyskinetic Mice

Luz M. Suárez, Oscar Solís, Jose M. Caramés, Irene R. Taravini, Jose M. Solís, Mario G. Murer, and Rosario Moratalla

Background: L-3,4-dihydroxyphenylalanine (L-DOPA)–induced dyskinesia is an incapacitating complication of L-DOPA therapy that affects most patients with Parkinson's disease. Previous work indicating that molecular sensitization to dopamine receptor D_1 (D1R) stimulation is involved in dyskinesias prompted us to perform electrophysiological recordings of striatal projection "medium spiny neurons" (MSN). Moreover, because enhanced D1R signaling in drug abuse induces changes in spine density in striatum, we investigated whether the dyskinesia is related to morphological changes in MSNs.

Methods: Wild-type and bacterial artificial chromosome transgenic mice (D1R-tomato and D2R–green fluorescent protein) mice were lesioned with 6-hydroxydopamine and subsequently treated with L-DOPA to induce dyskinesia. Functional, molecular, and structural changes were assessed in corticostriatal slices. Individual MSNs injected with Lucifer-Yellow were detected by immunohistochemistry for three-dimensional reconstructions with Neurolucida software. Intracellular current-clamp recordings with high-resistance micropipettes were used to characterize electrophysiological parameters.

Results: Both D1R-MSNs and D2R-MSNs showed diminished spine density in totally denervated striatal regions in parkinsonian mice. Chronic L-DOPA treatment, which induced dyskinesia and aberrant FosB expression, restored spine density in D2R-MSNs but not in D1R-MSNs. In basal conditions, MSNs are more excitable in parkinsonian than in sham mice, and excitability decreases toward normal values after L-DOPA treatment. Despite this normalization of basal excitability, in dyskinetic mice, the selective D1R agonist SKF38393 increased the number of evoked action potentials in MSNs, compared with sham animals.

Conclusions: Chronic L-DOPA induces abnormal spine re-growth exclusively in D2R-MSNs and robust supersensitization to D1R-activated excitability in denervated striatal MSNs. These changes might constitute the anatomical and electrophysiological substrates of dyskinesia.

Key Words: Behavioral sensitization, dyskinesia, L-DOPA, medium spiny neuron, Parkinson's disease, striatum, three-dimensional neuronal reconstruction

An almost complete loss of dopaminergic fibers in the motor region of the striatum results in the profound akinesia that characterizes advanced Parkinson's disease (PD) (1). At this stage most patients need chronic L-3,4-dihydroxyphenylalanine (L-DOPA) therapy, and many will develop L-DOPA-induced dyskinesia (2). Knowledge gained in recent years about the molecular mechanisms underlying dyskinesias has not yet resulted in improved therapies.

This might be due in part to the occurrence of structural changes in the striatal microcircuit. Postmortem studies have shown decreased total length of medium spiny neuron (MSN) dendrites in patients with advanced PD treated with L-DOPA (3). Subsequent studies revealed a decrease in spine density in MSNs of L-DOPA-treated patients (4,5). In parallel, Ingham *et al.* (6,7) showed a reduction of spine density in MSNs in rats with unilateral nigrostriatal lesion, a finding confirmed in additional animal models of PD (8–10). Although pruning of dendritic spines in striatal neurons at late stages of PD and in animal models of PD has been repeatedly reported, it is not clear whether it is modified by L-DOPA therapy.

Importantly, sensitization of dopamine receptor D_1 (D1R) signaling cascade in MSNs is causally related to the dyskinesias (11–17). A very similar D1R sensitization occurs in drug abuse (18,19), but enhanced D1R signaling in addiction induces an increase in spine density in nucleus accumbens MSNs (20–25). Thus, D1R sensitization might induce similar structural changes in dyskinesias and drug abuse. Here we ask whether the dyskinesias are related to changes in the dendritic arbor of striatal MSNs. Moreover, because molecular sensitization to D1R stimulation is associated with an enhanced metabolic response of striatum to D1 agonists (15), we tested the hypothesis that striatal MSNs are more excitable in dyskinetic mice. We used a mouse model of parkinsonism induced by unilateral injection of 6-hydroxydopamine (6-OHDA) in the adult striatum (16,26). The restricted pattern of nigrostriatal degeneration affecting the motor district of the striatum in this animal model allowed us to correlate morphological and functional changes in MSN to the degree of striatal denervation and to test whether the dyskinesias are associated with any additional alteration of MSN dendrites.

From the Instituto Cajal (LMS, OS, JMC, RM), Consejo Superior de Investigaciones Científicas, CSIC; CIBERNED (LMS, OS, JMC, RM), Instituto de Salud Carlos III; Hospital Universitario Ramón y Cajal (JMS), IRYCIS, Madrid Spain; Instituto de Investigaciones Farmacológicas (IRT), Consejo Nacional de Investigaciones Científicas y Técnicas; and the Departamento de Fisiología y Biofísica (MGM), Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.

Address correspondence to Rosario Moratalla, Ph.D., Consejo Superior de Investigaciones Científicas, CSIC, Functional and System Neurobiology, Cajal Institute (CSIC), Avenida Doctor Arce 37, Madrid 28002, Spain; E-mail: moratalla@cajal.csic.es.

Received Dec 3, 2012; revised May 6, 2013; accepted May 8, 2013.

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2013.05.006

BIOL PSYCHIATRY 2014;75:711–722

L-DOPA Oppositely Regulates Synaptic Strength and Spine Morphology in D1 and D2 Striatal Projection Neurons in Dyskinesia.

Suarez LM, Solis O, Aguado C, Lujan R, Moratalla R.

Cereb Cortex. 2016; 26:4253-4264. doi: 10.1093/cercor/bhw263.

OXFORD

Cerebral Cortex, November 2016;26: 4253–4264

doi: 10.1093/cercor/bhw263
Advance Access Publication Date: 9 September 2016
Original Article

ORIGINAL ARTICLE

L-DOPA Oppositely Regulates Synaptic Strength and Spine Morphology in D1 and D2 Striatal Projection Neurons in Dyskinesia

Luz M Suarez[1,2], Oscar Solis[1,2], Carolina Aguado[3], Rafael Lujan[3], and Rosario Moratalla[1,2]

[1]Instituto Cajal, Consejo Superior de Investigaciones Científicas, CSIC, 28002 Madrid, Spain, [2]CIBERNED, Instituto de Salud Carlos III, 28002 Madrid, Spain, and [3]Instituto de Investigación en Discapacidades Neurológicas (IDINE), Dept. Ciencias Medicas, Facultad de Medicina, Universidad de Castilla-La Mancha, Campus Biosanitario, Albacete, Spain, Spain.

Address correspondence to Rosario Moratalla, Cajal Institute (CSIC), Av Dr. Arce 37, 28002 Madrid, Spain. Email: moratalla@cajal.csic.es

Abstract

Dopamine depletion in Parkinson's disease (PD) produces dendritic spine loss in striatal medium spiny neurons (MSNs) and increases their excitability. However, the synaptic changes that occur in MSNs in PD, in particular those induced by chronic L-3,4-dihydroxyphenylalanine (L-DOPA) treatment, are still poorly understood. We exposed BAC-transgenic D1-tomato and D2-eGFP mice to PD and dyskinesia model paradigms, enabling cell type-specific assessment of changes in synaptic physiology and morphology. The distinct fluorescence markers allowed us to identify D1 and D2 MSNs for analysis using intracellular sharp electrode recordings, electron microscopy, and 3D reconstructions with single-cell Lucifer Yellow injections. Dopamine depletion induced spine pruning in both types of MSNs, affecting mushroom and thin spines equally. Dopamine depletion also increased firing rate in both D1- and D2-MSNs, but reduced evoked-EPSP amplitude selectively in D2-MSNs. L-DOPA treatment that produced dyskinesia differentially affected synaptic properties in D1- and D2-MSNs. In D1-MSNs, spine density remained reduced but the remaining spines were enlarged, with bigger heads and larger postsynaptic densities. These morphological changes were accompanied by facilitation of action potential firing triggered by synaptic inputs. In contrast, although L-DOPA restored the number of spines in D2-MSNs, it resulted in shortened postsynaptic densities. These changes in D2-MSNs correlated with a decrease in synaptic transmission. Our findings indicate that L-DOPA-induced dyskinesia is associated with abnormal spine morphology, modified synaptic transmission, and altered EPSP-spike coupling, with distinct effects in D1- and D2-MSNs.

Key words: L-DOPA-induced dyskinesia, medium-spiny-neurons, neuronal excitability, Parkinson's disease, striatum

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by rigidity and slowness of movements caused by the loss of dopaminergic neurons in the substantia nigra (SN). The predominant treatment for PD is administration of L-3,4-dihydroxyphenylalanine (L-DOPA) that initially reverses the motor signs of the disease. Over time however, the combination of disease progression and chronic pulsatile L-DOPA administration

Morphological plasticity in the striatum associated with dopamine dysfunction.

Moratalla R, Solís O, Suárez LM.

Handbook of Basal Ganglia Structure and Function, 2a Ed.
Chapter 37, pp 755–770.
Handbook of Behavioral Neuroscience. Elsevier.

CHAPTER

37

Morphological Plasticity in the Striatum Associated With Dopamine Dysfunction

R. Moratalla[1,2], *O. Solís*[1,2] *and L.M. Suárez*[1,2]

[1]Instituto Cajal, Consejo Superior de Investigaciones Científicas, Madrid, Spain
[2]CIBERNED, Instituto de Salud Carlos III, Madrid, Spain

OUTLINE

I. INTRODUCTION

Dendritic spines are highly specialized sites where neurons receive excitatory inputs and control synaptic strength and plasticity. Since 1891 when Ramón y Cajal described dendritic spines as morphologically distinct neuronal elements (Ramón y Cajal, 1891), analysis of spine development, morphogenesis, and plasticity have been at the vanguard of research to study dynamic changes in spine morphology related to pathological or physiological regulation of neuronal plasticity.

The striatum is composed of GABAergic projection neurons called medium spiny neurons (MSNs) and of GABAergic and cholinergic interneurons (see chapter: The Neuroanatomical Organization of the Basal Ganglia). MSNs represent more than 95% of all striatal neurons and are a unique neuronal population that feature many dendritic spines (Kawaguchi, 1997). Moreover, MSNs form the two output pathways of the striatum and represent integrative centers for striatal information. Neurons of these two pathways are distinguished based on their axonal projections, dopamine

H. Steiner and K. Tseng (Eds): Handbook of Basal Ganglia Structure and Function, Second edition.
DOI: http://dx.doi.org/10.1016/B978-0-12-802206-1.00037-4

Nurr1 blocks the mitogenic effect of FGF-2 and EGF, inducing olfactory bulb neural stem cells to adopt dopaminergic and dopaminergic-GABAergic neuronal phenotypes.

Vergaño-Vera E, Díaz-Guerra E, Rodríguez-Traver E, Méndez-Gómez HR, Solís Ó, Pignatelli J, Pickel J, Lee SH, Moratalla R, Vicario-Abejón C.

Dev Neurobiol. 2015; 75:823-841 doi: 10.1002/dneu.22251.

Nurr1 Blocks the Mitogenic Effect of FGF-2 and EGF, Inducing Olfactory Bulb Neural Stem Cells to Adopt Dopaminergic and Dopaminergic-GABAergic Neuronal Phenotypes

Eva Vergaño-Vera,[1,2] Eva Díaz-Guerra,[1,2] Eva Rodríguez-Traver,[1,2] Héctor R. Méndez-Gómez,[1,2] Óscar Solís,[1,2] Jaime Pignatelli,[1,2] James Pickel,[3] Sang-Hun Lee,[4] Rosario Moratalla,[1,2] Carlos Vicario-Abejón[1,2]

[1] Instituto Cajal, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain

[2] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain

[3] Transgenic Core, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland, USA

[4] Department of Biochemistry and Molecular Biology, College of Medicine, Hanyang university, Seoul, Korea

Received 11 October 2013; revised 30 October 2014; accepted 25 November 2014

ABSTRACT: **The transcription factor Nurr1 is expressed in the mouse olfactory bulb (OB), although it remains unknown whether it influences the generation of dopaminergic neurons (DA) (DA neurons) in cells isolated from this brain region. We found that expressing Nurr1 in proliferating olfactory bulb stem cells (OBSCs) produces a marked inhibition of cell proliferation and the generation of immature neurons immunoreactive for tyrosine hydroxylase (TH) concomitant with marked upregulations of *Th*, *Dat*, *Gad*, and *Fgfr2* transcripts. In long-term cultures, these cells develop neurochemical and synaptic markers of mature-like mesencephalic DA neurons, expressing GIRK2, VMAT2, DAT, calretinin, calbindin, synapsin-I, and SV2. Concurring with the increase in both *Th* and *Gad* expression, a subpopulation of induced cells was both TH- and GAD-immunoreactive indicating that they are dopaminergic-GABAergic neurons. Indeed, these cells could mature to express VGAT, suggesting they can uptake and store GABA in vesicles. Remarkably, the dopamine D1 receptor agonist SKF-38393 induced c-Fos in TH^+ cells and dopamine release was detected in these cultures under basal and KCl-evoked conditions. By contrast, cotransducing the Neurogenin2 and Nurr1 transcription factors produced a significant decrease in the number of TH-positive neurons. Our results indicate that Nurr1 overexpression in OBSCs induces the formation of two populations of mature dopaminergic neurons with features of**

Correspondence to: C. Vicario-Abejón (cvicario@cajal.csic.es)

Contract grant sponsor: Spanish Ministerio de Economía y Competitividad (MINECO: BFU2010-1963 and SAF2013-47596-R) (to C.V.-A.).

Contract grant sponsor: Spanish Ministerio de Economía y Competitividad (BFU2010-20664 and SAF2013-48532-R) (to R.M.).

Contract grant sponsor: Ministerio de Sanidad Política Social e Igualdad (Instituto de Salud Carlos III, ISCIII: CIBERNED CB06/05/065) (to C.V.-A.).

Contract grant sponsor: Ministerio de Sanidad Política Social e Igualdad (Instituto de Salud Carlos III, ISCIII: CIBERNED CB06/05/0055) (to R.M.).

Contract grant sponsor: Comunidad de Madrid (CM: S2011/BMD-2336) (to R.M. and C.V.-A.).

Contract grant sponsor: CM, CONACYT (Gobierno de Mexico), and MINECO (to H.R.M.-G., O.S., and E.R.-T.).

Eva Vergaño-Vera, Eva Díaz-Guerra, and Eva Rodríguez-Traver contributed equally to this work as first authors. Rosario Moratalla, and Carlos Vicario-Abejón contributed equally to this work as senior authors.

Published online 10 December 2014 in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/dneu.22251

Role of Nurr1 in the Generation and Differentiation of Dopaminergic Neurons from Stem Cells.

Rodríguez-Traver E, Solís O, Díaz-Guerra E, Ortiz Ó, Vergaño-Vera E, Méndez-Gómez HR, García-Sanz P, Moratalla R, Vicario-Abejón C.

Neurotox Res. 2016; 30:14-31 doi: 10.1007/s12640-015-9586-0.

Neurotox Res (2016) 30:14–31
DOI 10.1007/s12640-015-9586-0

ORIGINAL ARTICLE

Role of Nurr1 in the Generation and Differentiation of Dopaminergic Neurons from Stem Cells

Eva Rodríguez-Traver[1,2] · Oscar Solís[1,2] · Eva Díaz-Guerra[1,2] · Óscar Ortiz[1] · Eva Vergaño-Vera[1,2] · Héctor R. Méndez-Gómez[1,2] · Patricia García-Sanz[1,2] · Rosario Moratalla[1,2] · Carlos Vicario-Abejón[1,2]

Received: 15 September 2015 / Revised: 27 November 2015 / Accepted: 6 December 2015 / Published online: 17 December 2015

Abstract NURR1 is an essential transcription factor for the differentiation, maturation, and maintenance of midbrain dopaminergic neurons (DA neurons) as it has been demonstrated using knock-out mice. DA neurons of the substantia nigra pars compacta degenerate in Parkinson's disease (PD) and mutations in the *Nurr1* gene have been associated with this human disease. Thus, the study of NURR1 actions in vivo is fundamental to understand the mechanisms of neuron generation and degeneration in the dopaminergic system. Here, we present and discuss findings indicating that NURR1 is a valuable molecular tool for the in vitro generation of DA neurons which could be used for modeling and studying PD in cell culture and in transplantation approaches. Transduction of Nurr1 alone or in combination with other transcription factors such as Foxa2, Ngn2, Ascl1, and Pitx3, induces the generation of DA neurons, which upon transplantation have the capacity to survive and restore motor behavior in animal models of PD. We show that the survival of transplanted neurons is increased when the Nurr1-transduced olfactory bulb stem cells are treated with GDNF. The use of these and other factors with the induced pluripotent stem cell (iPSC)-based technology or the direct reprogramming of astrocytes or fibroblasts into human DA neurons has produced encouraging results for the study of the cellular and molecular mechanisms of neurodegeneration in PD and for the search of new treatments for this disease.

Keywords NURR1 · GDNF · Dopaminergic neurons · Parkinson's disease · Differentiation · Stem cells

Abbreviations

AADC	L-aromatic amino acid decarboxylase
ALDH2	Aldehyde dehydrogenase 2
ALDH1A1	Aldehyde dehydrogenase 1 family member A1
ASCL1	Achaete-scute complex homolog 1
BDNF	Brain-derived neurotrophic factor
bHLH	Basic helix-loop-helix
CA	Catecholamine
CNS	Central nervous system
D1R	Dopamine 1 class receptor
D2R	Dopamine 2 class receptor
DA neurons	Dopaminergic neurons
DAT	Dopamine transporter
EN	Engrailed genes
ESCs	Embryonic stem cells
FGF8	Fibroblast growth factor family member 8
Fgfr2	Fibroblast growth factor receptor 2
FOXA2	Forkhead box protein A2
FP	Floor plate
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GDNF	Glial cell line-derived neurotrophic factor
GIRK2	G-protein-regulated inward-rectifier potassium channel 2
hiPSCs	Human iPSCs
iDA neurons	Induced DA neurons

Rosario Moratalla and Carlos Vicario-Abejón are co-senior authors.

✉ Carlos Vicario-Abejón
cvicario@cajal.csic.es

[1] Instituto Cajal, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Doctor Arce 37, 28002 Madrid, Spain

[2] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Madrid, Spain