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BRIEF COMMUNICATION

Pharmacological perturbation reveals deficits in D2 receptor responses in *Thap1* null mice

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Abstract

The primary dystonia DYT6 is caused by mutations in the transcription factor Thanatos-associated protein 1 (THAP1). To understand THAP1's functions, we generated mice lacking THAP1 in the nervous system. THAP1 loss causes locomotor deficits associated with transcriptional changes. Since many of the genes misregulated involve dopaminergic signaling, we pharmacologically challenged the two striatal canonical dopamine pathways: the direct, regulated by the D1 receptor, and the indirect, regulated by the D2 receptor. We discovered that depleting THAP1 specifically interferes with the D2 receptor responses, pointing to a selective misregulation of the indirect pathway in DYT6 with implications for pathogenesis and treatment.

Introduction

Dystonia is a common movement disorder characterized by the involuntary co-contraction of agonist and antagonist muscles. This leads in varying degrees to abnormal twisting or writhing movements, tremors, and sustained or abnormal postures.[1] Since dystonia often occurs as a result of lesions to the basal ganglia, neuronal deficits are thought to arise from dysfunction within this subcortical neuronal module. Nonetheless, it has been difficult to parse out the specific neuronal networks at the synaptic level.

In less common cases dystonia occurs because of genetic mutations. These syndromes—numbered DYT1 to 29 (based on the order of identifying families with distinct dystonias)[2,3]—hold the promise of providing more nuanced insights into deciphering pathology. For instance, altered genes could point to specific intracellular pathogenic pathways, which in turn could help identify aberrations in specific neuronal circuits. This has certainly been the case with the dopamine-responsive dystonias caused by mutations in genes encoding proteins involved in dopamine metabolism and signaling.[4]

It is still unclear, however, whether deficits in dopamine transmission are generalizable to other dystonias. It is in this context that we focused on the autosomal dominant dystonia DYT6, caused by mutations in Thanatos-associated domain-containing apoptosis-associated protein 1 (*THAP1*).[5] This primary dystonia is characterized by adolescent onset segmental dystonia that affects upper limbs, neck, and larynx.[6]

The preponderance of data suggests that DYT6 is a loss of function disease caused by haploinsufficiency at the *THAP1* locus. With its well-established role as a transcription factor, we sought to understand THAP1's functions through the genes that it regulates. We initially generated constitutive *Thap1* null mice; this strategy, however, does not lead to viable offspring.[7] We therefore generated conditional mice lacking *Thap1* specifically in the nervous system.[7] *Thap1*cKO mice do not display a dystonic phenotype, which is not entirely surprising given that the disease shows incomplete penetrance with only 60% of patients displaying a phenotype;[8,9] moreover, human genetic dystonic syndromes are notoriously difficult to recapitulate in the mouse.[10] Nonetheless, *Thap1*cKO mice are clearly abnormal. Behaviorally they show motor phenotypes of abnormal gait and grasp, at a histological level they display synaptic pathology,[7] and from a molecular perspective they exhibit alterations in gene networks in a few discrete pathways.[7] Since the most significantly dysregulated pathway involved dopaminergic signaling, we used a pharmacologic approach to dissect the functional relevance of these alterations.

Materials and Methods

Generation of *Thap1* null mice

*Thap1*cKO mice were generated by mating *Thap1* floxed mice with nestin-Cre mice in a pure C57BL/6J background.[7] Mice were housed under specific pathogen-free conditions. All experiments were performed in compliance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and with approval by Northwestern University's Institutional Animal Care and Use Committee.

Pharmacological interventions

Both pharmacological and behavioral studies were performed on 3-month-old mice. Mice were transferred from their home cage to an experimental cage where they were singly housed and allowed to habituate for 15 min. Next, each mouse was injected intraperitoneally (IP) with the experimental drug or vehicle. The dose of drug was calibrated to body weight (*Thap*1cKO mice are ~30% smaller than the wildtype littermates).[7] The following drugs and doses were used: (1) D1 receptor agonist: SKF 81297, 1 mg/kg body weight (cat# S179, Sigma Aldrich); (2) D1 receptor antagonist SCH 23390, 1 mg/kg body weight (cat# D054, Sigma Aldrich); (3) D2 receptor agonist quinpirole, 0.1 mg/kg (cat# Q102, Sigma Aldrich), and (4) D2 receptor antagonist raclopride; 0.1 mg/kg (cat# R121, Sigma Aldrich).[11,12] To control for possible effects of handling and injecting mice, wildtype and *Thap1*cKO mice were also injected with vehicle (0.9% NaCl solution at similar volumes to the experimental drug) (Fig. S1A and B). To minimize the number of experimental mice, the same mice were used with a 4-day drug washout in between different treatments. The drug treatments were administered in the following order: SKF 81297, quinpirole, saline (control), SCH 23390, and raclopride.[11] Since there were no differences based on gender, the data on male and female mice were combined into a single cohort.

Behavioral testing and statistical analyses

Video recordings were taken for each mouse, which were then scored by an independent blinded operator.[13] Active times were defined as intervals when the mouse displayed ambulation, grooming, or exploratory behaviors. The time intervals were configured into 5-min bins. To compare experimental groups, two sets of analysis were performed: in the first, the active time was monitored 15 min prior to injection and 100 min post-injection; in the second, the total active times post-injection were compared. Statistical significance was determined by two-way repeated measures ANOVA and the unpaired Student's *t*-test, respectively. Statistical analyses were carried out with GraphPad Prism 8.

RNA-seq analysis

The transcriptomic data were generated as previously described.[7] Briefly, striatal tissue was dissected from 6-month-old *Thap1*cKO mice and wildtype controls (3 animals per genotype). RNA was extracted with the RNeasy Plus Universal Mini Kit (Qiagen). Sequencing libraries were prepared using the TruSeq Stranded mRNA pipeline (Illumina) and sequenced onto a NextSeq 500 platform (Illumina). After filtering low-quality reads and adaptor sequences, the remaining reads were aligned to the mouse genome (mm10) using STAR (Spliced Transcripts Alignment to a Reference).[14] The HTSeq tool was used to count the aligned reads for each gene,[15] and the Bioconductor package DESeq2 was then used to calculate differentially expressed genes.[16] *p*-values less than 0.05 after false discovery rate (FDR) correction were considered significant.

Results

*Thap1*cKO mice display locomotor deficits providing support for the role of THAP1 in neuronal function.[7] We compared gene expression of *Thap1*cKO mice using the Kyoto Encyclopedia of Genes and Genomes (KEGG analysis). The top pathway dysregulated in the striatum was categorized as "dopaminergic synapse" (log $p = -2.7$). The individual genes included the type 2 dopamine receptor (*Drd2*), along with 18 other genes implicated in dopaminergic signaling.[7] These include adenylate cyclase 5 (*Adcy5*), protein kinase C beta (*Prkcb*), mitogen-activated protein kinase 14 (*Mapk14*), and other enzymes and molecules that regulate dopamine signaling and circuits (Table 1). These results were independently validated by qRT-PCR and where antibodies were available by western blotting as well [D1R, D2R, tyrosine hydroxylase (TH), and dopamine transporter (DAT)]. Interestingly, these expression changes were accompanied by abnormal morphology of striatal medium spiny neurons (MSNs) and reduced dopamine levels.[7]

With these findings, we hypothesized that *Thap1* ablation results in altered dopaminergic transmission in the medium striatal neurons reminiscent to that seen in the dopamine-responsive dystonias. We therefore decided to selectively block or activate the two major classes of dopamine receptors—D1 and D2—implicated in basal ganglia neurotransmission. The D1Rs are expressed on the "direct pathway neurons" (dMSNs) that project directly to the basal ganglia output nuclei [Globus Pallidus Interna (GPi) and Substantia Nigra Pars Reticulata (SNr)], while the D2 receptors are expressed on the "indirect pathway neurons" (iMSNs) that target the globus pallidus externa (GPe).[17]

We first turned to manipulate the D1 receptors. Inhibiting these receptors with the dopamine antagonist SCH 23390 (a halobenzazepine derivative) causes wildtype mice to display a decrease in locomotor activity, while activating them with the dopamine agonist SKF 81297 (a phenyl benzazepine derivative) causes an increase in locomotor activity (Fig. 1A–D). These are well-described outcomes based on prior studies in mice.[18,19] *Thap1*cKO mice showed comparable behavior to wildtype mice suggesting that the functioning of the dopaminergic D1 receptors do not differ between the *Thap1*cKO mice and wildtype controls (Fig. 1A–D).

We next turned to testing the D2 receptor system. For these experiments, we used the well-characterized D2 antagonist raclopride (a benzamide derivative), and the D2 agonist quinpirole (a quinoline derivative). Both of these drugs cause a decrease in movements either from cataleptic effects (in the case of antagonists) or by activating the indirect locomotor pathway (in the case of agonists).[20] *Thap1*cKO mice differ from wildtype mice in their response to both of these agents, demonstrating that the synaptic alteration to D2 stimulation is significantly altered (Fig. 2A–D). These responses are similar to the impaired response of D2R knockout mice with the same

Table 1. Genes involved in dopaminergic transmission that are differentially expressed in the striatum of *Thap1*cKO mice compared to wildtype littermates.

Gene symbol	Description	Entrez ID	Log2 fold change	FDR adjusted *p*-value
Itpr3	Inositol 1,4,5-triphosphate receptor 3	16440	−0.597	1.83E-19
Ppp2r3a	Protein phosphatase 2 (formerly 2A), regulatory subunit B″, delta	235542	−0.455	6.01E-05
Creb5	cAMP responsive element binding protein 5	231991	−0.442	1.23E-03
Cacna1c	Calcium channel, voltage-dependent, L type, alpha 1C subunit	12288	−0.319	3.17E-09
Kif5a	Kinesin family member 5A	16572	−0.251	4.25E-02
Itpr1	Inositol 1,4,5-trisphosphate receptor 1	16438	−0.241	3.24E-03
Cacna1a	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	12286	−0.217	4.06E-02
Cacna1d	Calcium channel, voltage-dependent, L type, alpha 1D subunit	12289	−0.210	8.31E-05
Caly	calcyon neuron-specific vesicular protein	68566	−0.210	4.63E-04
Cacna1b	Calcium channel, voltage-dependent, N type, alpha 1B subunit	12287	−0.197	4.81E-06
Drd2	Dopamine receptor D2	13489	−0.191	3.38E-02
Mapk14	Mitogen-activated protein kinase 14	26416	−0.174	2.32E-05
Adcy5	Adenylate cyclase 5	224129	−0.172	3.82E-02
Prkcb	Protein kinase C, beta	18751	−0.153	1.06E-06
Gnal	Guanine nucleotide binding protein, alpha stimulating, olfactory type	14680	0.099	1.11E-02
Scn1a	Sodium channel, voltage-gated, type I, alpha	20265	0.157	8.94E-05
Ppp2r5b	Protein phosphatase 2, regulatory subunit B″, beta	225849	0.180	2.76E-02
Slc6a3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	13162	0.382	5.15E-03
Ppp2r3c	Protein phosphatase 2, regulatory subunit B″, gamma	59032	1.270	1.15E-28

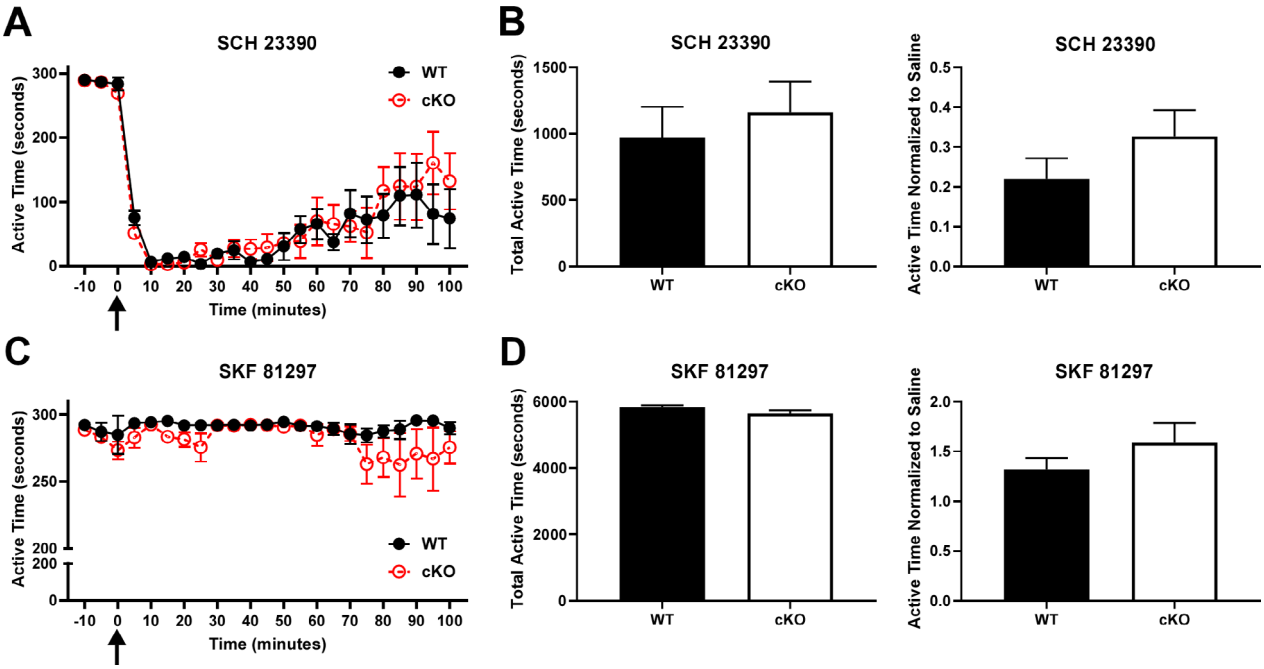

Figure 1. Drug challenges of the D1R dopaminergic circuitry in 3-month-old *Thap1*cKO mice. (A and B) Treatment with D1R antagonist, SCH 23390 1 mg/kg. (A) Active time plotted as time course (statistics: repeated measures ANOVA $F_{(22,276)} = 0.46$, $p = 0.98$). (B) Active time plotted as cumulative total time in the left panel (statistics: unpaired *t*-test $p = 0.58$); total active time normalized to saline-injected controls in the right panel (statistics: unpaired *t*-test $p = 0.21$). (C and D) Treatment with D1R agonist, SKF 81297 1 mg/kg. (C) Active time plotted as time course (statistics: repeated measures ANOVA $F_{(22,276)} = 0.83$, $p = 0.69$). (D) Active time plotted as cumulative total time in the left panel (statistics: unpaired *t*-test $p = 0.09$); total active time normalized to saline-injected controls in the right panel (statistics: unpaired *t*-test $p = 0.23$). For all experiments: 15-min baseline followed by 100-min recording. Time 0 corresponds to the time of IP injection of drug (indicated by arrow). Averaged values were plotted with error bars representing SEM, $n = 8$–9.

drugs.[21] Incidentally, we noticed that *Thap1*cKO mice appear more sensitive to D2 receptor activation than blockade. This difference could be explained by the higher lipophilicity of the agonist quinpirole that then binds the D2 receptors more efficiently compared to the antagonist raclopride.[22]

Discussion

Several lines of evidence, particularly from the acquired ataxias, implicate the striatum as a key anatomical substrate in the etiology of dystonia. In the molecular era, genetic dystonic syndromes such as DYT6 promise to deliver insights into specific synaptic circuits. Starting from its known functions as a transcription factor, we identified the genes that *Thap1* regulates in the striatum; these include several in the dopamine signaling pathway, a pathway that is central to striatal function. Moreover, abnormal dopamine signaling has already been implicated in dystonia pathogenesis, especially in the context of the dopamine-responsive dystonias—a subset of DYT syndromes with mutations in genes encoding tyrosine hydroxylase (*TH*) or regulating the synthesis of its cofactor tetrahydropterin (*GCH* encoding GTP-cyclohydrolase, *SPR* encoding sepiapterin reductase, and *PTS* encoding pyruvoyl-tetrahydropterin synthase). In addition to the dopamine-responsive dystonias, there are other DYT syndromes where mutations occur in genes that encode proteins involved in other aspects of dopamine metabolism and signaling such as *SLC18A2* (encoding intracellular vesicular monoamine transporter 2; mutated in DYT9)[23] and *GNAL* (encoding the alpha subunit of a GTP binding protein that couples dopamine receptor signaling to the adenylate cyclase messenger system; mutated in DYT25).[24]

Pharmacological challenges have been central to understand functional deficits in the circuitry of some of these dystonias in mice. For instance, the dopamine-responsive *TH* dystonia model demonstrates deficits in both the D1 and D2 pathways,[11] while the DYT25 mouse shows deficits in the D1, but not the D2 receptor. *Thap1*cKO mice differ from both of these mice as they have deficits in the D2 receptor, but not the D1 receptor signaling pathway. At the electrophysiological level, these findings are consistent with the aberrant responses to D2 receptor antagonism observed in vitro in another DYT6 mouse model.[25]

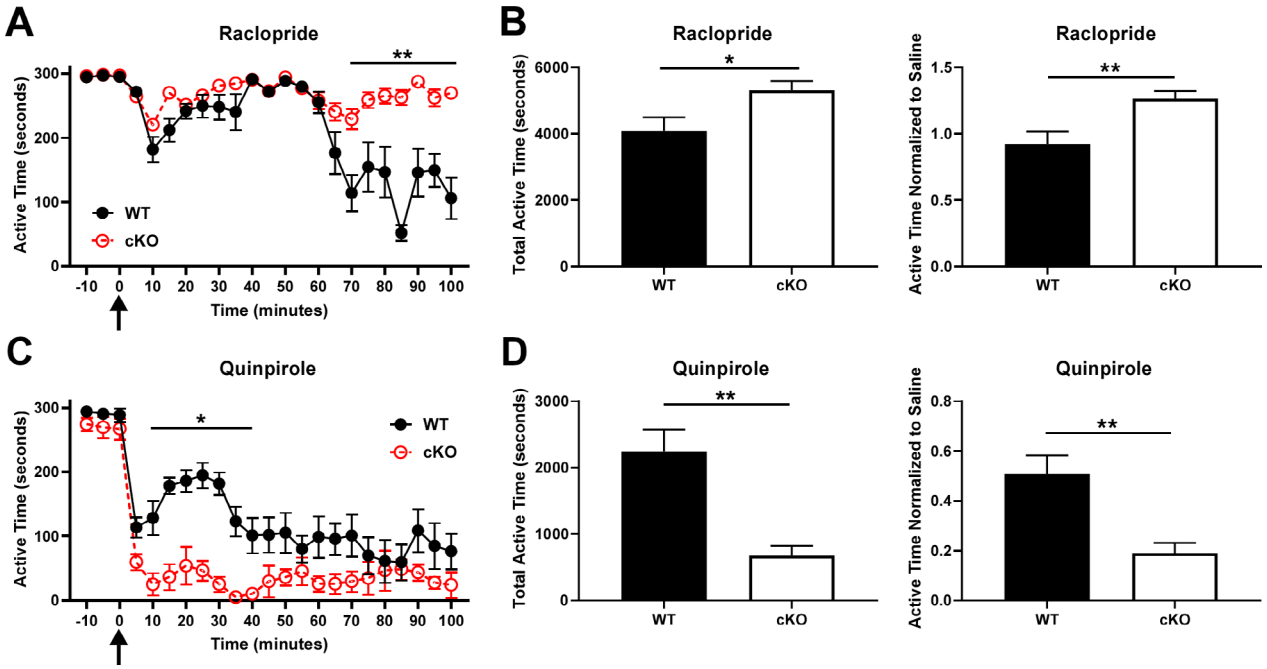

Figure 2. Drug challenges of the D2R dopaminergic circuitry in 3-month-old *Thap1*cKO mice. (A and B) Treatment with D2R antagonist, raclopride 0.1 mg/kg. (A) Active time plotted as time course (statistics: repeated measures ANOVA $F_{(22,276)} = 11.33$, $p = 0.001$). (B) Active time plotted as cumulative total time in the left panel (statistics: unpaired *t*-test $p = 0.029$); total active time normalized to saline-injected controls in the right panel (statistics: unpaired *t*-test $p = 0.008$). (C and D) D2R agonist, quinpirole 1 mg/kg. (C) active time plotted as time course (statistics: repeated measures ANOVA $F_{(22,276)} = 4.834$, $p = 0.014$). (D) Active time plotted as cumulative total time in the left panel (statistics: unpaired *t*-test $p = 0.002$); total active time normalized to saline-injected controls in the right panel (statistics: unpaired *t*-test $p = 0.005$). For all experiments: 15-min baseline followed by 100-min recording. Time 0 corresponds to the IP injection of drug (indicated by arrow). Averaged values were plotted with error bars representing SEM, $n = 8–9$. *$p < 0.05$; **$p < 0.01$.

These findings are also reminiscent of the aberrant D2 responses documented in the more common subtype DYT1, where pathogenic mutations in torsin-1A (*TOR1A*) are similarly connected with decreased D2R levels.[26,27] A comprehensive functional characterization of DYT syndromes is far from complete. At least one other DYT subtype—DYT8, caused by mutations in paroxysmal nonkinesigenic dyskinesia (*PNKD*)—is characterized by impaired D2R signaling; although the mechanistic link to dopamine metabolism in this dystonia is still unclear.[28]

Thus, we posit dystonias to be caused by either an early reduction in dopamine or an imbalance between D1 and D2 activity. This very narrow requirement could help explain why dystonia is such a common movement disorder seen as part of other genetic causes of nigrostriatal dopamine neuron degeneration including mutations in leucine-rich repeat kinase 2 (*LRRK2*), parkin (*PRKN*), PTEN-induced kinase 1 (*PINK1*), and alpha-synuclein (*SNCA*). It could also help explain why [^{11}C]-raclopride-PET shows differing deficits on D2 or D1 receptors based on the specific syndrome.[29] The episodic nature of some dystonia syndromes, including DYT6,[30] could also be explained by well-described diurnal fluctuations of dopamine levels on the direct and indirect pathways when they are out of balance, as our data would suggest. Regardless, the different etiologies of striatal dysfunction along with the need for a tight synchronization of these pathways makes non-dopamine-responsive dystonias difficult to treat.

One limitation of our study is that since our conditional knockout approach resulted in THAP1 deficiency in the whole central nervous system, the pharmacological interventions could have an impact on non-striatal networks as well. Indeed, the drugs could affect non-motor pathways affecting executive function, motivation, and wakefulness that could indirectly affect the overall mobility and exploratory behavior. To overcome these limitations and achieve a complete understanding of DYT6 pathogenesis, a deeper knowledge of the affected neuronal sub-populations is necessary. These additional studies will likely require synergistic approaches combining more specific Cre-mediated conditional knockout tools alongside chemogenetic or optogenetic manipulation of neuronal activity.

Conflict of Interest

P.O. received support from the NIH (1R01NS062051, 1R01NS082351, and R56NS108639). He has received funding from the following sources for clinical trials: Biohaven Pharmaceuticals, NIH U01NS104326 (site PI), and the National Ataxia Foundation (CRC-SCA natural history study). A.D. reports grants and other support from the NIH (R56NS121112), the International Progressive MS Alliance (PA-2001-36176), the Conrad N. Hilton Foundation (17323), the National Multiple Sclerosis Society (RG-1901-33219), and FISM-Fondazione Italiana Sclerosi Multipla (2014/B/1 and 2017/B/3).

Author Contributions

P.O. conceived and supervised the study. N.M.F., M.M.P., and P.S. carried out the experiments. N.M.F., A.D., and P.O. analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Saline vehicle control injection in 3-month-old mice. A and B: Saline control, 5 mL/kg. A: Active time plotted as time course (statistics: repeated measures ANOVA $F_{(22,230)} = 1.457$, $p = 0.152$). B: Active time plotted as cumulative total time (statistics: unpaired *t*-test $p = 0.45$). For all experiments: 15-minute baseline followed by 100-minute recording. Time 0 corresponds to the IP injection of vehicle control (indicated by arrow). Averaged values were plotted with error bars representing SEM, $n = 8–9$.

