




Tetrahydrobiopterin (BH₄) treatment stabilizes tyrosine hydroxylase: Rescue of tyrosine hydroxylase deficiency phenotypes in human neurons and in a knock-in mouse model

Kunwar Jung-KC^{1,2}  | Alba Tristán-Noguero^{3,4,5}  | Altanchimeg Altankhuyag¹ | David Piñol Belenguer^{6,7} | Karina S. Prestegård¹ | Irene Fernandez-Carasa^{6,7} | Arianna Colini Baldeschi^{6,7} | Maria Sigatulina Bondarenko³ | Angeles García-Cazorla^{3,8} | Antonella Consiglio^{6,7,9} | Aurora Martinez^{1,2,10} 

¹Department of Biomedicine, University of Bergen, Bergen, Norway

²K.G. Jebsen Center for Translational Research in Parkinson's Disease, University of Bergen, Bergen, Norway

³Neurometabolic Unit and Synaptic Metabolism Lab, Neurology Department, Institut Pediàtric de Recerca and MetabERN, Hospital Sant Joan de Déu, Barcelona, Spain

⁴Departament de Genètica, Microbiologia i Estadística, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain

⁵Molecular Physiology of the Synapse, Institut de Recerca Sant Pau (IR Sant Pau), Universitat Autònoma Barcelona, Barcelona, Spain

⁶Department of Pathology and Experimental Therapeutics, Bellvitge University Hospital-IDIBELL, Hospitalet de Llobregat, Barcelona, Spain

⁷Institute of Biomedicine of the University of Barcelona (IBUB), Barcelona, Spain

⁸Centro de Investigación Biomédica En Red Enfermedades Raras (CIBERER), Madrid, Spain

⁹Department of Molecular and Translational Medicine, University of Brescia, Brescia, Italy

¹⁰Neuro-SysMed, Department of Neurology, Haukeland University Hospital, Bergen, Norway

Correspondence

Antonella Consiglio, Department of Pathology and Experimental Therapeutics, Bellvitge University Hospital-IDIBELL, Hospitalet de Llobregat, Barcelona, Spain.
Email: consiglio@ub.edu

Alba Tristán-Noguero, Neurometabolic Unit and Synaptic Metabolism Lab, Neurology Department, Institut Pediàtric de Recerca and MetabERN, Hospital Sant Joan de Déu, Barcelona, Spain.
Email: atristan@idibell.onmicrosoft.com

Aurora Martinez, Department of Biomedicine, University of Bergen, Jonas Lies vei 91, 5009 Bergen, Norway.
Email: aurora.martinez@uib.no

Abstract

Proteostatic regulation of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis, is crucial for maintaining proper brain neurotransmitter homeostasis. Variants of the *TH* gene are associated with tyrosine hydroxylase deficiency (THD), a rare disorder with a wide phenotypic spectrum and variable response to treatment, which affects protein stability and may lead to accelerated degradation, loss of TH function and catecholamine deficiency. In this study, we investigated the effects of the TH cofactor tetrahydrobiopterin (BH₄) on the stability of TH in isolated protein and in DAN-differentiated from iPSCs from a human healthy subject, as well as from THD patients with the R233H variant in homozygosity (THDA) and R328W and T399M variants in heterozygosity (THDB). We report an increase in TH and dopamine levels, and an increase in the number of TH+ cells in control and THDA cells. To translate this in vitro effect, we treated with BH₄ a knock-in

Kunwar Jung-KC and Alba Tristán-Noguero contributed equally to this study.

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THD mouse model with *Th* variant corresponding to R233H in patients. Importantly, treatment with BH₄ significantly improved motor function in these mice, as demonstrated by increased latency on the rotarod test and improved horizontal activity (catalepsy). In conclusion, our study demonstrates the stabilizing effects of BH₄ on TH protein levels and function in THD neurons and mice, rescuing disease phenotypes and improving motor outcomes. These findings highlight the therapeutic potential of BH₄ as a treatment option for THDA patients with specific variants and provide insights into the modulation of TH stability and its implications for THD management.

KEYWORDS

dopamine, iPSC-derived neurons, mice model, pharmacological chaperones, tetrahydrobiopterin (BH₄), tyrosine hydroxylase deficiency (THD)

1 | INTRODUCTION

Tyrosine hydroxylase deficiency (THD) is a rare autosomal recessive inherited metabolic disorder caused by variants in tyrosine hydroxylase (*TH*, OMIM 191290).^{1–3} TH is a non-heme iron-dependent enzyme that catalyzes the hydroxylation of L-Tyr to L-Dopa, using molecular oxygen as an additional substrate and tetrahydrobiopterin (BH₄) as a cofactor.⁴ This is the rate-limiting step in the biosynthesis of catecholamines, such as dopamine, norepinephrine and epinephrine,⁵ where the essential role of the cofactor explains the association of variants in genes involved in the synthesis of BH₄ with deficient catecholamine synthesis.⁶

To date, more than 59 disease-causing variants have been identified in the *TH* gene (chromosome 11p15.5, Gene ID: 7054) and its promoter region, and are deposited in the PND database (<http://www.biopku.org/home/pnddb.asp>). The vast majority of THD-related variants are missense,^{7,8} and most affect enzyme activity and TH protein stability and solubility, leading to accelerated degradation, cerebral catecholamine deficiency and a

complex pathophysiology with considerable heterogeneity in phenotype.^{7,9} Human TH exists as four main isoforms (TH-1–4) generated via alternative splicing of pre-mRNA, and TH1 (497 residues) is predominant in the brain and is also the isoform most studied.¹⁰ However, THD variants are customarily provided with the numeration of the longest isoform (TH4 or just TH), but in the PND database, they are listed with the numeration in the TH1 isoform, which we will also include in this work in certain instances. The Arg233His TH variant (p.Arg202His-TH1), p.R233H-TH from now on in the text, is a common missense variant in THD,⁸ leading to conformational instability of TH and altered catalytic efficiency.^{9,11}

Tyrosine hydroxylase deficiency clinical diagnosis is based on the analysis of cerebrospinal fluid (CSF), showing lower catecholamine metabolites (HVA and MHPG), and the diagnosis is confirmed by mutation analysis of the *TH* gene.^{8,12,13} Traditionally, THD has been characterized into two phenotypes. Type A refers to an infantile-onset, progressive, hypokinetic-rigid syndrome associated or not to dyskinetic movements that have in

general a beneficial response to L-Dopa (Dopa responsive dystonia or DRD). Initial symptoms include lower limb dystonia, difficulty walking with diurnal fluctuation of the symptoms to truncal hypotonia, and Parkinsonian symptoms such as hypokinesia, rigidity of extremities, and tremors.⁸ The type B phenotype tends to have an early onset, within the first year of life with a broader range of movement disorders that are often associated with cognitive impairment. Patients present a poor response to L-Dopa, usually hypersensitive to it, developing motor fluctuations and dyskinesia on low doses.^{8,14,15} For the R233H-TH variant, it has been reported that most homozygote patients show a clinical phenotype consistent with type A TH deficiency, with good response to L-Dopa, while about 25%–35% of these patients^{8,16} present a severe phenotype and either null response to L-Dopa or treatment is hindered by unbearable side effects.⁸ Recently, the “International Working Group on Neurotransmitter-Related Disorders” carried out a first standardized evaluation of the patient registry, concluding that the type A/B classification needs to be reconsidered for THD, since intermediate forms, continuum of symptoms, and response to treatment are not clearly differentiating between the two groups.¹³ Thus, the existence of a wide spectrum and overlap of clinical features and variants prevent the preparation of clear genotype–phenotype correlations.^{7–9}

The first-choice treatment for THD still consists of L-Dopa and carbidopa.^{2,8} However, for those patients with little to no response to this treatment, there are as of today no effective alternatives to improve their cognitive and motor functions. We have previously reported that BH₄, the cofactor of TH and the other amino acid hydroxylases phenylalanine hydroxylase (PAH) and the tryptophan hydroxylases (TPH1 and TPH2), stabilizes purified TH.¹⁷ Furthermore, oral BH₄ supplementation to mice increased TH activity and protein levels in brain extracts, while the *Th*-mRNA level was not affected, establishing that orally administered BH₄ crosses the blood–brain barrier (BBB).¹⁷

For two decades BH₄, which is customarily known as sapropterin when provided as a medication, has been successfully used to treat BH₄-responsive phenylketonuria (PKU) patients, representing about 40% of total PKU patients, who show a blood phenylalanine (Phe) reduction of $\geq 30\%$ upon BH₄ treatment.¹⁸ The BH₄-responsive patients are able to metabolize more Phe and reduce the strictness of the protein-free diet.¹⁹ In this study, we aimed to investigate the potential for repurposing BH₄ for THD treatment. The progress in human induced pluripotent stem cell (iPSC) technologies enables the generation of patient-specific and disease-relevant cell types that can model human diseases and can be used as a source for cell therapy and/or drug screening.²⁰ We recently described an iPSC-based model that recapitulates THD disease

phenotypes in dopaminergic neurons (DAN) derived from iPSCs from a type A patient (homozygous for R233H-TH variant) and a type B patient (heterozygous with R328W-TH and T399M-TH variants), which are L-Dopa responsive and unresponsive, respectively.²¹ For the sake of simplification in this study, the iPSC models would be referred to as THDA (milder) and THDB (severe). Furthermore, there are two *knock-in* mouse models of THD, one with the variant Q382K-*Th*, corresponding to Q412K-TH (i.e., p.Q381K-TH1) in humans, which is representative of DRD,²² and the homozygous for the variant R203H-*Th*, corresponding to R233H-TH in human. Differently to most patients with this variant, the R203H-*Th* mouse model, referred here as Th-Ki, presents with a severe phenotype, not responsive to L-Dopa.¹¹ Thus, the Th-Ki model appears as an appropriate platform for testing novel therapies that could substitute L-Dopa.

2 | RESULTS

2.1 | TH stabilization and BH₄ assayed by differential scanning fluorimetry (DSF)

Differential scanning fluorimetry is also a powerful method to investigate the binding of ligands that promote protein stabilization^{23,24} and we probed the suitability of this method to investigate the effect of the cofactor on TH stability. The thermal unfolding transition of wild-type (WT) purified human TH1 was monitored by measuring an increase in SYPRO Orange fluorescence upon binding to hydrophobic patches of the protein that become exposed upon thermal denaturation.²⁵ As BH₄ is quickly oxidized in the absence of reductant conditions²⁶ it is usually kept reduced with dithiothreitol (DTT) or similar reagents. While these are not deleterious for TH during catalytic conditions, long incubation leads to dissociation of the active site iron²⁷ and TH destabilization, interfering with the DSF assays with BH₄. Indeed, the melting temperature (T_m) of TH control with DMSO is $51.8 \pm 0.1^\circ\text{C}$, whereas is reduced to $48.1 \pm 1.2^\circ\text{C}$ in the presence of DTT (Figure 1A). Nevertheless, we observed $1.1 \pm 0.1^\circ\text{C}$ stabilization of TH in the presence of $10 \mu\text{M}$ BH₄. In order to confirm the stabilization we tested the effect of a stable oxidized analog of the cofactor, i.e., 7,8-dihydrobiopterin (BH₂), which binds at the same site as BH₄, although with lower affinity.²⁸ We measured a stabilization of TH by BH₂ leading to increased T_m for TH (ΔT_m) of 1.1°C at $62.5 \mu\text{M}$ (Figure 1A). To further probe the binding-dependent stabilization of TH we measured the dose-dependent stabilization by BH₂, which provided an EC₅₀ value - the concentration of the compound that gives half-maximal stabilization - of $12.2 \pm 5.8 \mu\text{M}$ (Figure 1B).

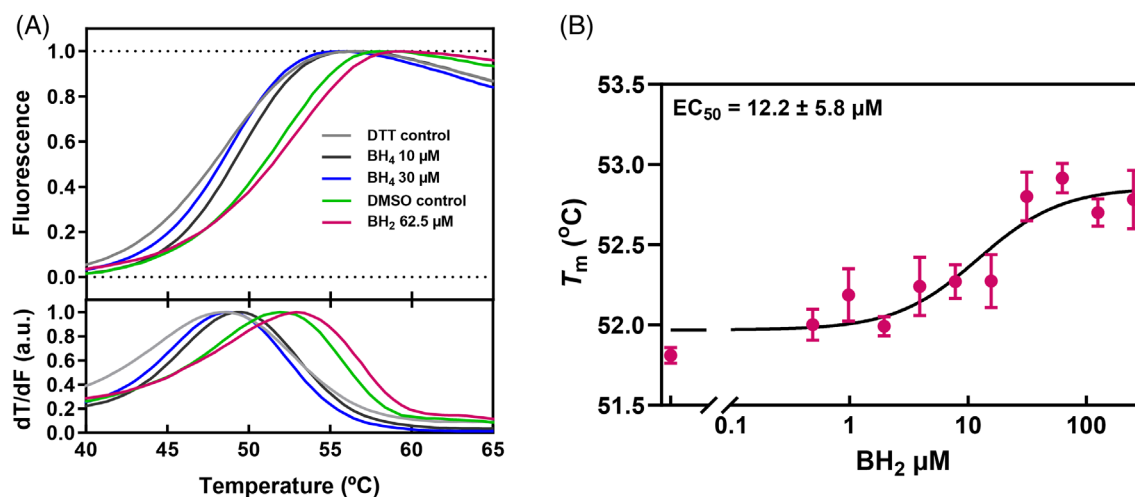


FIGURE 1 The effect of BH₄ and BH₂ on the thermal stability of TH. (A) The thermal melting curve of TH monitored by DSF (upper traces) and first derivative (bottom traces) with BH₄ at 10 and 30 μM (in 5 mM DTT) and BH₂ at 62.5 μM (in DMSO) (Number of replicas at each condition, $N = 3$). The T_m -values for TH-controls and TH with compounds are as follows: DTT control, $T_m = 48.2 \pm 1.2^\circ\text{C}$; BH₄ (10 μM), $T_m = 49.2 \pm 0.1^\circ\text{C}$; BH₄ (30 μM), $T_m = 48.4 \pm 0.0^\circ\text{C}$; DMSO control, $T_m = 51.8 \pm 0.1^\circ\text{C}$; BH₂ (62.5 μM), $T_m = 52.9 \pm 0.2^\circ\text{C}$. (B) Determination of EC₅₀ value ($12.2 \pm 5.8 \mu\text{M}$) for the stabilization of TH by BH₂.

TABLE 1 Information about iPSC lines used in this study.

Cell line code	Code previous study ²¹	Subject type	TH gene mutation
Control	CONTROL1#5	Healthy individual	No
THDA	THDA1#17	THD (mild phenotype)	p.R233H
THDB	THDB1#15	THD (severe phenotype)	p.R328W/p.T399M

Despite the fact that BH₂ seems to be effective in entering cells, BH₄ presents higher bioavailability in vivo and has been shown to penetrate the brain.^{12,17,29} Moreover, dihydrofolate reductase, which is mainly involved in folate metabolism and converts inactive BH₂ back to BH₄, is not equally expressed in all organs.³⁰ We therefore used BH₄ in the following studies to test the effect of the cofactor as a modulator of TH protein and activity in DAN differentiated from THD patient-specific iPSC, and further in THD mice.

2.2 | Validation of BH₄ in a THD human iPSC-based model

To test the potential of BH₄ to act as pharmacological chaperone of TH and evaluate its ability to increase the steady-state levels of TH protein and dopamine, we generated dopaminergic neurons (DAN) from two iPSC lines representing THDA (mild form) and THDB (severe form) patients, along with a healthy control iPSC line (Control), all previously generated and fully characterized in our laboratory²¹ (see Table 1 for further details). iPSC differentiation toward DAN fate was performed using a previously established protocol, which enabled the maintenance of differentiated cells for up to 21 days.

Very briefly, neuronal precursor cells (NPCs) derived from Control, THDA, and THDB iPSC lines were seeded on top of mouse feeder PA6 cells and maintained in N2B27 medium (B27 with vitamin A) for 21 days, the time by which neurons are mature. 6-pyruvoyl-tetrahydropterin synthase (PTPS) and dihydropteridine reductase (DHPR) deficiency iPSC cellular models, both recapitulating the features of these BH₄ deficiency disorders, have been shown the rescue of BH₄ deficiencies phenotypes using BH₄ treatment (30 μM dose).³¹

Dopaminergic neurons were treated with BH₄ after 11 days in culture, with the addition of BH₄ three times a week, at each change of the medium. We treated Control, THDA and THDB iPSC-derived DAN with BH₄ (30 μM) during the last 10 days of differentiation (Figure 2A). We investigated whether BH₄ treatment affects TH expression levels by measuring the number of TH-expressing cells in the DAN cultures. After detailed counting analysis, we found more TH-immunoreactive cells in THDA neuronal treated cultures (~47% of all TUJ1 (Tubulin β3)+ cells, p -value < 0.05) when compared to non-treated cultures (Figure 2B,C).

Interestingly, BH₄ treatment rescued the phenotype in THDA neurons, as shown by the increase in TH protein levels nearly two-fold (p -value < 0.01, Figure 3A,B). BH₄ treatment also increased intracellular dopamine levels

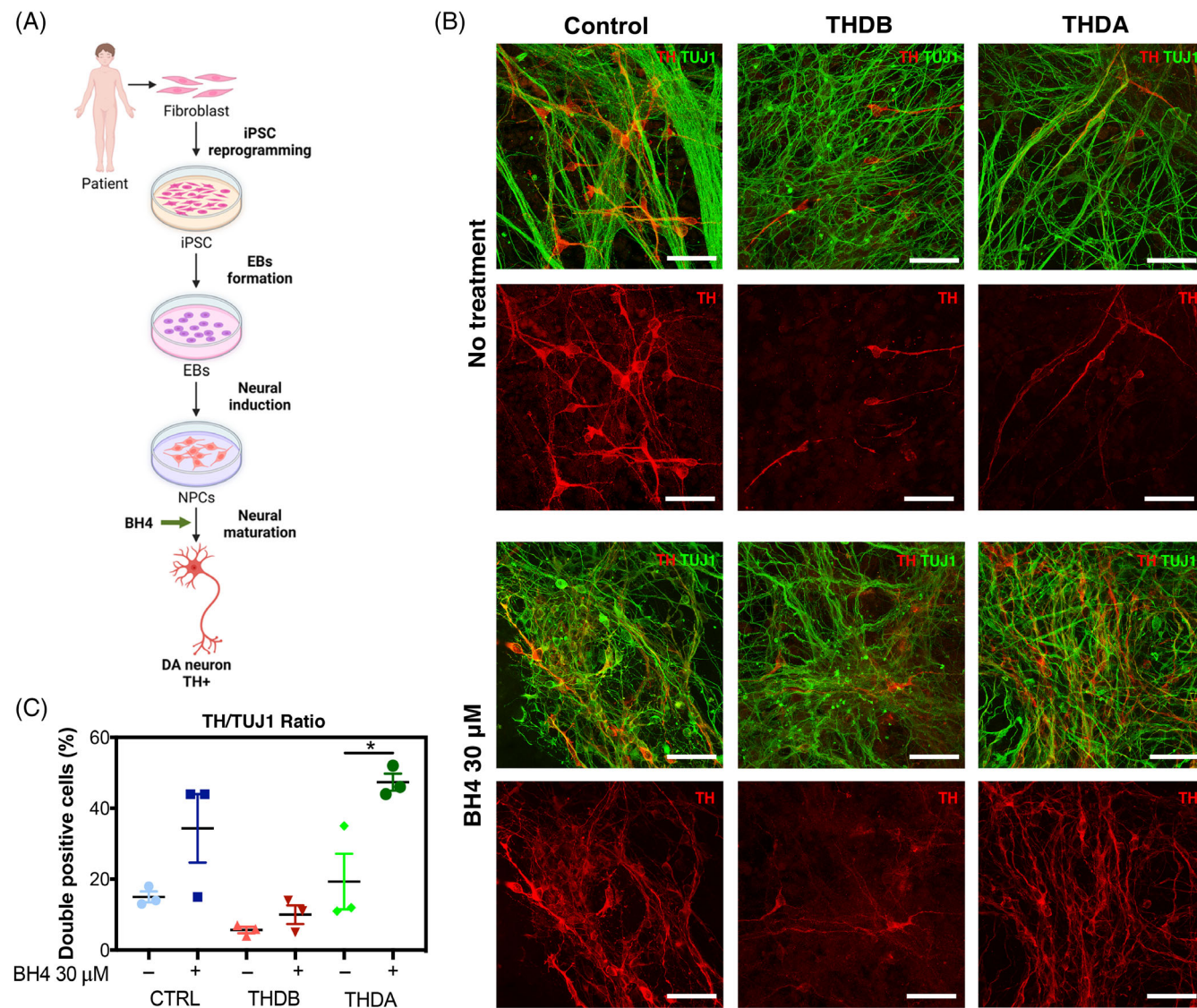


FIGURE 2 BH₄ treatment increases the number of TH positive neurons in DAN-differentiated from THDA-iPSCs. (A) DAN differentiation from iPSC lines and treatment administration. The illustration was created with Biorender. (B) Immunocytochemistry images of all cell lines, i.e., Healthy control (control), THDA and THDB neuronal cultures (TH in red, TUJ1 (Tubulin β3) in green and DAPI in blue). (C) Quantification of TH/TUJ1 ratio in untreated and treated cultures of all cell lines. Scale bars, 50 μm. Number of biological replicates, *N* = 3. Data are expressed as mean ± SEM. Unpaired two-tailed Student's *t* test was used for pairwise comparisons, **p* < 0.05.

both in control and THDA iPSC lines by 10-fold (*p*-value < 0.05, Figure 3C). However, the THD phenotype was not rescued in the THDB neuronal cultures (Figures 2 and 3).

These data demonstrate the efficacy of BH₄ to revert THD traits on control and THDA DAN, so we decided to test the efficacy of BH₄ treatment using the Th-Ki mice model of THD.

2.3 | Effect of oral supplementation of BH₄ to Th-Ki mice

The Th-Ki mice, homozygous for the variant R203H-Th, equivalent to the human variant in the THDA DAN, have

been exhaustively characterized earlier and shown to exhibit moderate growth retardation and hypotension with gradual loss of brain catecholamines leading to early motor dysfunction and diurnal fluctuations of the motor deficit.¹¹ We investigated the effect of BH₄ treatment on the motor outcome of the WT (nine mice) and Th-Ki mice (eight mice). Motor function tests such as rotarod and horizontal bar tests (catalepsy) were performed in mice aged between 13 and 16 months. The data are presented as violin plots (Figure 4A,C) to analyze the distribution of the data and scatter dot plots (Figure 4B,D) to analyze the effect of BH₄ treatment. Consistent with the previous results,¹¹ the Th-Ki mice showed significantly reduced latency (40.25 ± 22.3 s; mean ± SD) compared to the WT mice (93.4 ± 45.8 s; mean ± SD) on the rotarod,

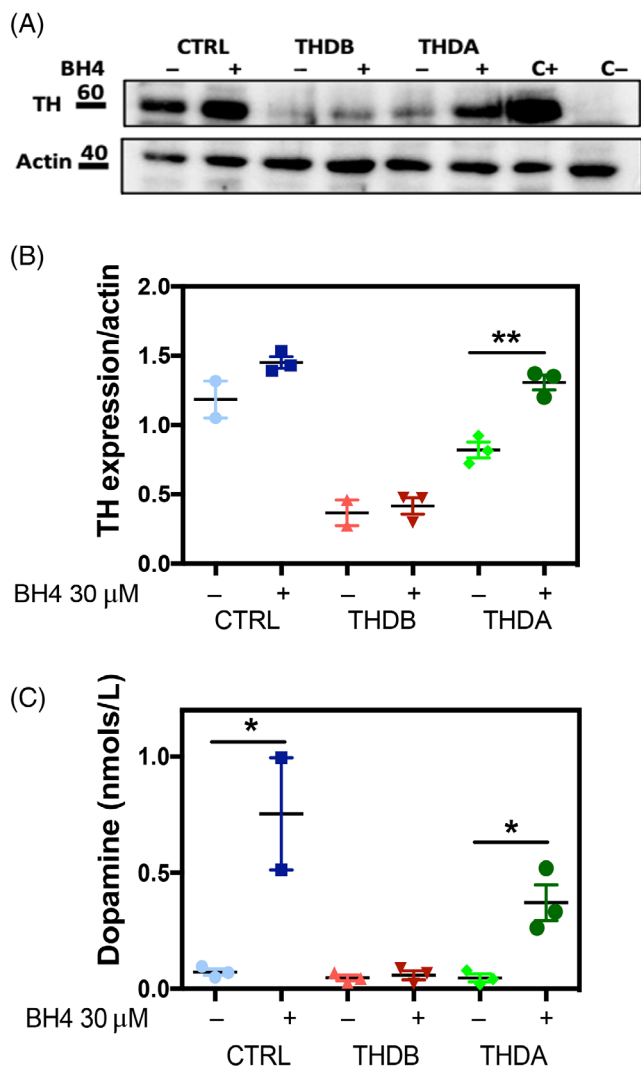


FIGURE 3 BH₄ Treatment increases dopamine levels in DAN-differentiated from healthy control-iPSCs and rescues the THD phenotype in DAN-differentiated from THDA-iPSCs. (A) Western blot of TH protein levels before and after BH₄ treatment in Healthy control (control), THDA and THDB; positive control (C+); Mesencephalon, negative control (C-); Fibroblasts. (B) Quantification of western blot results (normalized by beta-Actin levels). (C) Elisa quantification of intracellular dopamine levels (nmol/l) in control, THDA and THDB lines before and after treatment. Number of biological replicates, $N = 3$. Data are expressed as mean \pm SEM. Unpaired two-tailed Student's t test or Mann-Whitney U test was used for pairwise comparisons, ** $p < 0.01$, * $p < 0.05$.

indicating impaired motor coordination (Figure 4B). However, upon treatment with BH₄, Th-Ki mice showed improvement in the latency (62.75 ± 11.6 s; mean \pm SD; p -value = 0.01; Figure 4B). Moreover, the median value for the Th-Ki (67.5 s) after BH₄ treatment was close to that for WT before BH₄ treatment (76 s), as depicted on the violin plot (Figure 4A). In addition, the higher

density of the data around median value on violin plot indicates consistency in the increased latency of Th-Ki mice on rotarod upon BH₄ treatment.

Th-Ki mice are known to exhibit hypokinetic and bradykinetic phenotypes,¹¹ which we observed as well. Th-Ki mice showed significantly reduced (50 ± 16.4 s; mean \pm SD) horizontal activity (catalepsy) compared to WT (5.7 ± 6.8 s; mean \pm SD; p -value = 0.0003; Figure 4D). Therefore, we wanted to investigate whether BH₄ treatment can improve the symptoms using a catalepsy test. Importantly, Th-Ki after BH₄ treatment showed significantly improved horizontal activity by 60% (20.25 ± 13.2 s; mean \pm SD) compared to before the treatment (50 ± 16.4 s; p -value = 0.007; Figure 4D). WT mice, before and after BH₄ treatment, were rather consistent in the horizontal activity as shown by the data cluster around the median value on the violin plot. In addition to improved horizontal activity, Th-Ki mice were also consistent in their horizontal activity after BH₄ treatment, as depicted by the shape and the length of the violin plot (Figure 4C), indicating improved motor outcome overall.

Some studies have reported a significant correlation between body weight and rotarod performance^{32,33} and we observed a slight weight loss, for both WT and Th-Ki mice after BH₄ treatment (Figure 5A,B), hence we wanted to rule out the possibility that improved rotarod performance was not due to weight loss but the treatment with BH₄ (Figure 5). The average body weight before and after BH₄ treatment was 38.1 and 37.1 g, respectively, for WT, and 26.5 and 25.2 g, for Th-Ki (Figure 5A,B, inset). Besides, repetitive orogastric gavage has been reported to cause stress and may affect food intake and decrease body weight.³⁴ Despite a slight weight loss, we did not observe a significant correlation between body weight and rotarod performance (Figure 5C-F) hence concluding the improved motor outcome upon BH₄ treatment.

3 | DISCUSSION

Tyrosine hydroxylase deficiency is a rare disorder caused by variants in the *TH* gene leading to catecholamine deficiency manifesting both motor and non-motor symptoms clinically. Variants associated with THD are linked to instability, misfolding, and premature degradation of mutant TH and loss-of-function phenotype, resulting in reduced TH protein and activity and therefore dopamine deficiency.^{9,35,36} In the case of the p.R233H-TH variant, loss-of-function has been demonstrated with recombinant mutant protein,¹¹ in an iPSC-based cellular model²¹ and in vivo in the Th-Ki mouse.¹¹ Current therapy for THD includes oral administration of L-Dopa and carbidopa,

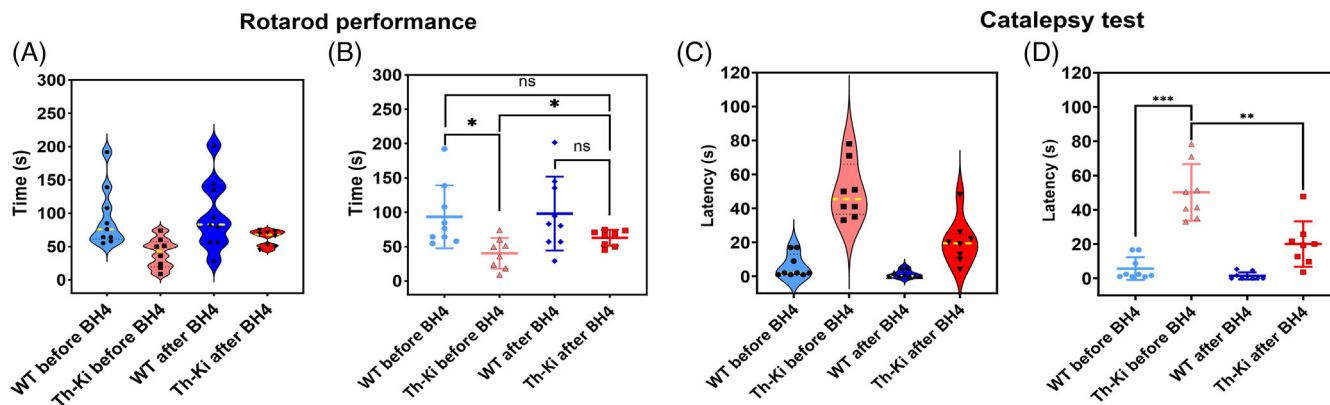


FIGURE 4 Motor function test of Th-WT and Th-Ki mice. (A and B) Rotarod test to determine the motor coordination of Th-WT and Th-Ki mice (WT before BH₄ vs. Th-Ki before BH₄, p -value = 0.03; Th-Ki before BH₄ vs. Th-Ki after BH₄, p -value = 0.01). (C and D) Horizontal bar test to assess catalepsy (WT before BH₄ vs. Th-Ki before BH₄, p -value = 0.0003; Th-Ki before BH₄ vs. Th-Ki after BH₄, p -value = 0.007). Data were analyzed and represented by violin plots (A, C) and scatter dot plots (B, D). Median values were represented by a yellow dotted line on the violin plots (A, C), and data in the scatter dot plots are presented as mean \pm SD. p -values were represented by asterisks (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ and the data were considered significant when the p -value was ≤ 0.05 .

which alleviates some of the symptoms. Otherwise, not all patients respond to L-Dopa treatment,^{14,37} therefore, additional personalized treatment options are needed. The objective of this work was to assess the stabilizing effect of BH₄ and to evaluate its pharmacological chaperone potential. The synthetic form of BH₄, sapropterin, is an approved drug for other applications, such as BH₄-deficiencies and BH₄-responsive PKU, and has been shown to increase TH stability and/or TH levels in a previous study.¹⁷

The concept of developing therapies based on pharmacological chaperones has been explored in various inborn errors of metabolism, especially if the main pathological mechanism is the variant-associated protein instability and misfolding, such as e.g. in PKU, cystic fibrosis, and lysosomal storage disorders.^{38–40} Therefore, small molecule compounds that can stabilize TH protein and increase TH activity in vitro and in vivo appear relevant for THD treatment as alternatives or supplements to L-Dopa. Here we show that in the DSF assays BH₄ and its inactive analog BH₂ exhibited a low but significant ΔT_m increase (1.1 ± 0.1) at low concentration in the case of BH₄ (10 μ M), and higher with BH₂ (62.4 μ M), due to its lower affinity for TH than BH₄.²⁸ The degree of thermal stabilization upon ligand binding may depend on a large number of factors such as the binding affinity, the flexibility of the ligand-binding site and the degree of conformational change upon binding.^{41–43} Nevertheless, despite the relatively low thermal stabilization observed upon BH₄ binding to TH, ligand binding is also customarily associated with enhanced resistance to proteolytic degradation, an effect of high relevance to achieve a larger half-life and higher steady-state levels in cells.⁴⁴ Thus, once TH protein stabilization was proven, BH₄ was

tested in a relevant cellular model, a THD model based on human induced pluripotent stem cell (iPSC).²¹

Human iPSCs are of great value in the biomedical field as they can be used to model human diseases, as a source for cell therapy and to perform drug screening studies.^{20,45} BH₄ rescued TH protein and dopamine levels in DAN-differentiated from control and THDA-iPSCs (homozygous for the R233H-TH variant) but not in THDB-iPSCs (heterozygous, R328W-TH and T399M-TH variants). R328 forms an essential H-bond and electrostatic network, and T399 interacts with I42 in the regulatory N-terminal helix.⁴⁶ Variants in these residues are very deleterious and give rise to highly unstable TH and very low residual activity, <0.2% for p.R328W-TH and <5% for p.T399M-TH,⁹ compared with approx. 14% for p.R233H-TH, which is why BH₄ may not be able to impart a chaperone effect on THDB neurons. For WT-TH, BH₄ exerted a folding-aided chaperone effect, which in turn stimulates hydroxylating activity, increased catecholamine synthesis and dopamine-mediated TH stabilization, as also shown in previous studies.¹⁷ Altogether, the results support that the p.R233H-TH variant, which a priori was found to be unstable and present low affinity for dopamine,¹¹ may upon BH₄ treatment and consequent increase of dopamine synthesis, recover feed-back inhibitory binding, achieving further stabilization. We have earlier shown that BH₄ supplementation also facilitates dopamine binding and regulation to achieve increased amounts of TH levels.¹⁷

The increase in TH protein levels due to BH₄ administration has been debated in the scientific community. Oral administration of BH₄ at similar concentrations as used in this work, i.e., 100 mg/kg/day, led to increased

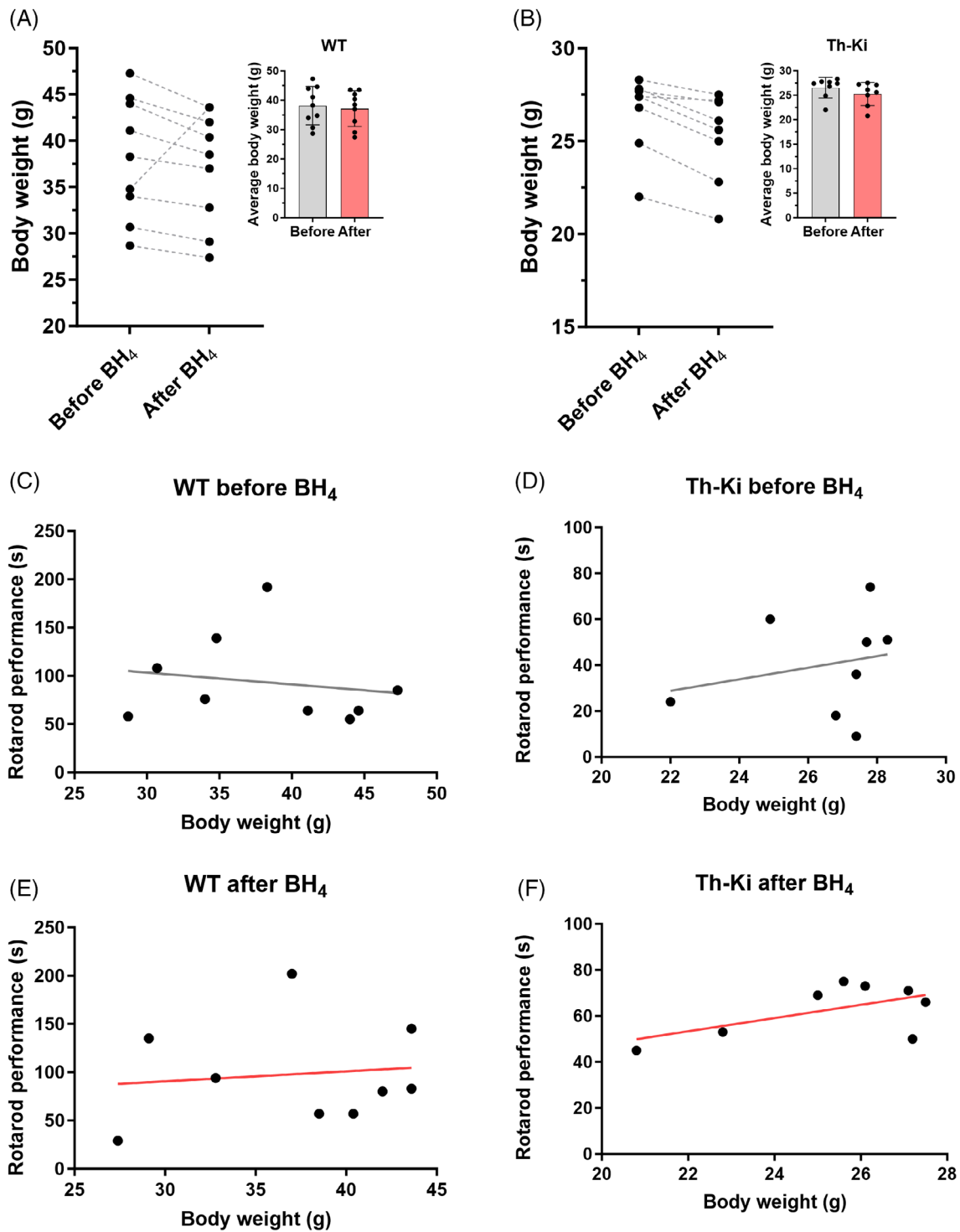


FIGURE 5 Correlation between body weight and rotarod performance. (A and B) Comparison of the body weight of each mouse before and after BH₄ treatment of WT and Th-Ki mice, respectively. The inset is the average body weight of both WT and Th-Ki before and after BH₄ treatment. (C–F) Rotarod performance in relation to body weight was analyzed by Pearson correlation coefficient (r). (C and E) The body weight of Th-WT mice before and after BH₄ treatment (WT-before BH₄ treatment – Pearson $r = -0.17$, p -value = ns; WT-after BH₄ treatment – Pearson $r = 0.11$, p -value = ns), and (D and F) Th-Ki before and after BH₄ treatment (Th-Ki-before BH₄ treatment – Pearson $r = 0.23$, p -value = ns; Th-Ki-before BH₄ treatment – Pearson $r = 0.58$, p -value = ns) was not significantly correlated with the rotarod performance. ns, not significant.

levels of bipterin in the brain and increased TH protein and activity,¹⁷ but it has also been reported that BH₄ administration through acute peripheral injection (50 mg/kg) also led to increased levels of BH₄ in the brain and enhanced AMPA-induced DA release in the nucleus accumbens without affecting dopamine-related proteins, including TH.⁴⁷ In any case, we observed increased TH protein levels, increased numbers of TH-immunoreactive cells and higher dopamine levels, in vitro in the DAN-differentiated from THDA-iPSCs when treated with BH₄, which was followed by in vivo evaluation in Th-Ki mice. Th-Ki mice with the variant equivalent to the common R233H-TH in THD patients appear normal at birth but exhibit reduced TH protein levels, catecholamine, weight loss, and motor dysfunction early in life, abnormalities which were not rescued by the standard L-Dopa treatment.¹¹ Interestingly, in our study, we found that BH₄ administration (100 mg/kg) for 4 days improved motor function in Th-Ki mice as observed by increased latency in rotarod, similar to basal WT performance. Furthermore, the horizontal activity (catalepsy) was significantly improved in Th-Ki mice when treated with BH₄. 30% of the patients with the R233His-TH variant in homozygosity are non-responsive to L-Dopa treatment, which could be due to defective stabilization by catecholamines and mislocalization of TH toward the synaptic terminals, as observed in Th-Ki mice, that are not L-Dopa responsive either.¹¹

Tyrosine hydroxylase is highly regulated and has many interacting partners that facilitate its stability, transport, and dopamine synthesis.⁴⁸ It is therefore necessary to investigate the underlying molecular mechanisms involved in the stabilization of mutant TH by BH₄. A stabilizing effect of BH₄ has also been reported for the TH-homologous BH₄-dependent enzyme PAH, and BH₄ treatment, given as sapropterin (Kuvan) starting at doses of 2–5 mg/kg/daily dose, and adjusting to 20–30 mg/kg/daily dose, is today a safe and successful treatment for more than 30% of PKU patients.¹⁹ Indeed, it has been proposed that in addition to increasing residual PAH activity in BH₄-responsive PKU patients, BH₄ may also improve neurocognitive functioning and thus also be given to BH₄ unresponsive PKU patients who do not show any effect on their hyperphenylalaninemia.⁴⁹ In addition to the enzyme stabilizing effect, there are several mechanisms underlying responsiveness to BH₄ for some PAH variants, such as the hyperbolic relationship between the activity and the concentration of the cofactor/co-substrate BH₄, as well as the protection of the enzyme from rapid oxidative inactivation.⁵⁰ Altogether, our results may indicate a similar multifactorial effect of BH₄ supplementation on TH and THD-associated mutants. However, it seems certain that higher doses of BH₄ may be needed for

the present brain indication than for BH₄-responsive PKU, mostly targeting the liver.

While the motor function tests in Th-Ki mice showed promising results, it is important to further evaluate the broader effects of BH₄ treatment on other aspects of THD pathology. For instance, assessing the impact of BH₄ on other symptoms, such as dystonia, postural hypotension, and fluctuations in brain catecholamine levels,^{8,51} would provide a more comprehensive evaluation of its therapeutic potential. Additionally, long-term studies are needed to determine the sustained efficacy and safety of BH₄ treatment over extended treatment periods. Furthermore, it would be valuable to explore the potential combination therapy approaches involving BH₄ with other pharmacological agents. In fact, combinations of repurposed drugs are currently being examined for some neurological diseases such as Alzheimer's disease, Parkinson's disease, and epilepsy.⁵² Given the multi-symptom nature of THD and the complex interactions involved in dopamine synthesis and regulation, a combination of therapies targeting different aspects of the disease may lead to synergistic effects and improved outcomes.

Otherwise, this study has some limitations. The most important is that we have observed a rescue of the phenotype only in neurons and mice harboring the variant p.R233H-TH. Considering the genetic diversity in THD patients, other variants should be included. Importantly, we think that the effect of the variant in the TH enzymatic features is of paramount importance in the response to pharmacological chaperones. Based on the different responses to BH₄ treatment of DAN-differentiated from THDA- and THDB- iPSCs it seems that responsiveness may be expected for TH variants with residual activity $\geq 14\%$ (for p.R233H-TH, relative to WT-TH), but also between 5% (for p.T399M-TH) and 14%, when expressed in a recombinant prokaryote expression system.⁹ This will include most type A variants, but also some type B.⁹ Nevertheless, the effect on each specific variant should be tested, if possible, in iPSC-derived neuronal lines. This will provide additional insights into the potential benefit of BH₄ as a treatment option for THD and subsequent enrolling of patients in clinical trials.

In conclusion, this study provides evidence for the stabilizing effects of BH₄ on TH protein and for the first time the potential of BH₄ as an effective pharmacological therapy for the treatment of THD. The findings contribute to our understanding of TH modulation and offer new possibilities for improving the management of THD patients with specific variants. Future investigations should explore the molecular mechanisms underlying BH₄ stabilization and conduct clinical trials to assess the efficacy and safety of BH₄ treatment in THD patients.

4 | MATERIALS AND METHODS

4.1 | Antibodies

A list of the antibodies used in this work is presented in Table S1.

4.2 | DSF assay of binding of BH₄ and BH₂ to TH

Validation of compound binding and TH stabilization was performed using differential scanning fluorimetry (DSF), with BH₄ (Schircks Laboratories) and BH₂ (Schircks Laboratories). The final assay volume was 20 μ L with TH (0.05 mg/mL), two different concentrations of each compound, and controls (1% DMSO or 5 mM DTT) in 20 mM Na-Hepes pH 7, 200 mM NaCl. Protein unfolding was monitored by using a fluorescent probe SYPRO Orange 5 \times (Sigma) at excitation (λ_{ex}) = 465 nm and emission (λ_{em}) = 610 nm in a Light-Cycler[®] 480 (Roche Life Science). Unfolding curves were recorded at a scan rate of 2°C/min with measurements taken every 0.2°C intervals from 20 to 99°C, with four acquisitions per degree including a 10-s hold at 20°C before and after the experiment. Data were processed by using HTSDSF explorer²⁵ and analyzed using GraphPad Prism 9 software. The mean and the standard deviation (SD) of the reference melting temperature (T_m) were calculated from the controls. Dose–response effect of the compound was validated by using a range of compound concentrations. T_m values were calculated from the denaturation curves of 3 samples at each concentration.

4.3 | Description of the THD iPSC lines used in the study

Induced pluripotent stem cells (iPSC) lines were generated in a previous study from two THD patients²¹ (see Table 1 for further details). One of the two THD patients has a type A phenotype (homozygous for the *TH* variant R233H), while the other one has a type B phenotype (compound heterozygous for the *TH* variant R328W and T399M).

4.4 | iPSC differentiation to DA neurons

In this study, three different already established iPSCs lines: Healthy control (control), THDA, and THDB²¹ were differentiated into dopaminergic neurons using a 30-day protocol based on DAN patterning factors and co-

culture with mouse PA6 feeding cells to provide trophic factor support, with minor modifications.⁵³ Specifically, iPSCs were cultured in mTeSR commercial medium until they reached 80% confluence and then mechanically aggregated to form embryoid bodies (EBs), without using lentiviral vectors to express LMX1A transcriptional factor. EBs were cultured for 10 days in suspension in N2B27 medium, consisting of neurobasal medium (GIBCO), DMEM/F12 medium (GIBCO), 0.5 \times N2 supplement (GIBCO), 0.5 \times B27 supplement with vitamin A (GIBCO), 2 mM ultraglutamine (Lonza) and penicillin–streptomycin (Lonza). In this step, N2B27 was supplemented with FGF-8 (100 ng/mL, Peprotech), bFGF (10 ng/mL; Peprotech), and SHH (100 ng/mL, Peprotech). Neural progenitor cells (NPCs) were then seeded on top of PA6 for 21 days and maintained in N2B27 medium, as described.^{21,53}

4.5 | Treatment of dopaminergic neurons with BH₄

For the BH₄ treatment studies, NPCs were seeded on top of mouse PA6 cells and maintained in N2B27 medium, as described above. DAN-differentiated from iPSCs were treated after 11 days in culture with 30 μ M of BH₄. The compound was added three times a week at each change of the medium during 10 days of treatment.

4.6 | Protein extraction and Western blotting

To obtain the total extract of protein from the DAN-differentiated from iPSCs, cells were initially washed with cold PBS with PMSF 0.2 mM and Protease Inhibitor (1 \times) and then mechanically detached from the plate using the same buffer to be collected in a 1.5 mL Eppendorf. After the collection, cells were centrifuged for 5 min at 376 g to discard the PBS and resuspended in RIPA protein extraction buffer (50 mM Tris–HCL pH 7.4 + 1% Triton X-100 + 150 mM NaCl + 2 mM EDTA + Sodium deoxycolate 0.5% + SDS 0.1%) supplemented with protease inhibitor (Roche). Resuspended pellets were then sonicated for 10 s at 10% amplitude using the Branson Digital Sonifier[®] ultrasonic cell disruptor (Branson Ultrasonics Corporation) to lysate the cells. After the sonication, the pellets were subjected to another centrifugation, 5 min at 376 g to discard all the debris and retain only the extracted protein in the supernatant. All the steps were done at 4°C. The supernatant was used for the protein quantification using the Bradford method (Biorad).

For the Western Blotting analysis, we used 15 μg of the total extract that we denatured in the loading buffer (LSB) for 5 min at 95°C to prepare the samples to be loaded into the SDS gel. Samples were run into an 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (Sigma). The membrane was blocked with 5% non-fat milk in 0.1 M Tris-buffered saline (pH 7.4)/Tween 0.1% (TBS-Tween) for 1 h at room temperature and incubated overnight in TBS-Tween containing rabbit anti-TH (1:1000; Santa Cruz cat. no. sc-14007) at 4°C. After incubation with peroxidase-tagged secondary antibodies (1:2000; GE Healthcare, cat. no. NA931V for anti-mouse and cat. no. NA934 for anti-rabbit) for 1 h at room temperature, membranes were revealed with ECL-plus chemiluminescence western blot kit (Amershan-cytiva).

The intensity of the protein band was quantified using Fiji[®] software. The optical density value of each band was corrected by the value of beta-actin (1:2000; Proteintech).

4.7 | Intracellular dopamine ELISA

Cells were harvested with cold PBS and centrifuged for 5 min at 135 g. The pellet obtained was diluted in 135 μL of sodium metabisulfite and EDTA (Sigma) to prevent catecholamine degradation. Next, the pellet was sonicated with a 10% amplitude 3 times for 60 s (10 s pulse alternating with 10 s rest in ice). Around 115 μL of the 135 μL were stored for ELISA studies, and the remaining volume was used to determine protein concentration by the Bradford method. Intracellular dopamine was measured using an ELISA kit for dopamine (LDN) in duplicates. This determination is a two-step process: first dopamine is extracted, acylated, and activated and then enzymatically converted and detected with a competitive ELISA assay. The corresponding dopamine levels were normalized to the previously determined protein concentration.

4.8 | Immunocytochemistry

Induced pluripotent stem cell-derived neurons were fixed using 4% paraformaldehyde for 20 min at room temperature, washed three times for 15 min with DPBS, and permeabilized with 0.1% Triton X-100 in Tris-buffered saline (TBS). Cells were then blocked for 2 h with 0.1% Triton X-100 with 3% donkey serum. Primary antibodies (rabbit anti-TH (1:250; Santa Cruz) and mouse anti-TUJ1 (Tubulin β 3) (1:500; Covance)), prepared in blocking solution, were incubated for 48 h at 4°C, while secondary

antibodies (1:200; Alexa Fluor Series, Jackson Laboratories) were incubated for 2 h at room temperature after washing (Table S1). Cells were stained with DAPI phenylindole (Invitrogen, 1:5000) for 10 min to visualize nuclei, mounted with PVA:DABCO, and stored at 4°C until imaged. Samples were imaged using a Carl Zeiss LSM880 confocal microscope. We used Fiji[®] is Just ImageJ[™] to quantify the TH/TUJ1 ratio in untreated and BH₄ treated cultures at day 30. An average of five images was quantified for each condition in at least three differentiations.

4.9 | Mouse husbandry and genotyping

All animal studies were approved by the Norwegian Food Safety Authority and performed at the Laboratory Animal Facility, University of Bergen. International and national guidelines and standards on the use of animals in research were followed. Mice were housed in a temperature-controlled (21°C and 50% air humidity) environment with 12 h light/dark cycles. In between experiments, food (standard chow pellets) and water were available ad libitum.

The preparation and phenotyping of the Th-Ki mouse used in this work with the variant p.R203H-Th, equivalent to the THD-associated human variant p.R233H-TH, has previously been described.¹¹ Continuous backcrossing of the colony to avoid genomic drift was achieved by breeding vendor-bought wild-type (WT) mice (C57BL/6J from Charles River, males, and females every second time) with heterozygous Th-Ki/WT mice every 6 months. Heterozygous siblings from this backcrossing were bred to produce homozygous Th-Ki mice for experiments. Th-WT siblings were used as control counterparts. To maintain the strain's genetic integrity, we made sure to never go further than three generations of inbreeding before using new breeding animals from the backcrossing.

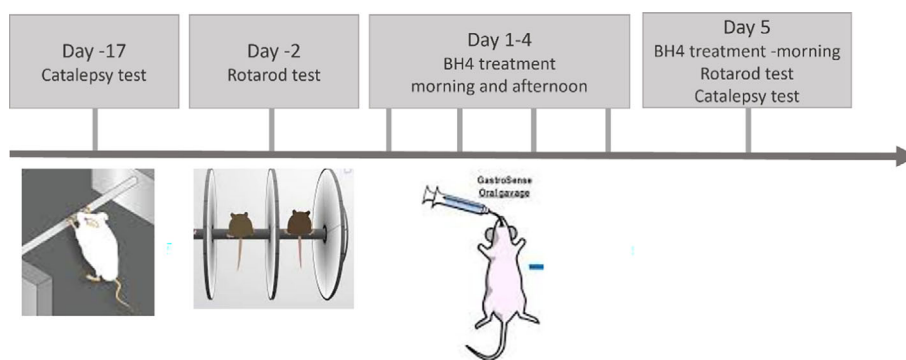
The mice used for experiments were all between 13 and 16 months of age. The Th-WT/WT group consisted of two females and seven males and ranged in weight from 28 to 47 g, while the Th-Ki group consisted of one female and seven males and ranged in weight from 22 to 28 g. The Th-Ki mice were smaller in size and weight than their WT counterparts, as described in Korner et al.¹¹

Ear biopsies were collected for genotyping and DNA was extracted and purified using the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's instructions. DNA was then amplified by standard PCR (Primers from Sigma: TH_33: GAGCTCCCAGAATTGACAGC (5'-3'), TH_34: GATCACACTCCACCATATCAAGG (5'-3')) and the following PCR product was incubated for 15 min at 37°C with the endonuclease Hae

II digestion enzyme (BioNordica). The fractions were resolved in 2.5% agarose gel electrophoresis and bands were visualized in a ChemiDoc XRS (Bio-Rad Laboratories) imaging system.

4.10 | BH₄ treatment

Both groups of mice were treated with BH₄ (Schircks Laboratories) two times a day (9:00 am and 3:00 pm) for 4 days with a daily dose of 100 mg/kg. The doses were administered orally by gavage, and the gavage needle was first dipped in sugar syrup (0.5 g/mL) for animal welfare. On the fifth day, the mice received only the morning dose (50 mg/kg).



4.11 | Motor function tests

For practical reasons, the behavioral tests were performed between 9:00 am and 2:00 pm before the BH₄ treatment and between 4:00 and 8:00 pm after the BH₄ treatment.

The motor function of Th-Ki and Th-WT mice were tested on a rotarod instrument (Harvard Apparatus) consisting of a rotating 5 cm grooved rod positioned 25 cm above a lever that stopped the timer at the time of fall. The mice first had a training session and then the test was performed with an accelerated speed from 4 to 40 rpm over 10 min. Time and rpm at the time of fall were recorded and each mouse was tested three times with a 45-min break in between tests. The average of three trials was used for analysis.

Differences in spontaneous catalepsy between Th-WT and Th-Ki mice were determined using the horizontal bar test where the time of immobility (immobile trunk and limbs) was assessed as described in Noain et al.⁵⁴ To avoid immobility due to fear or stress, the mice were first habituated to the experimental context and were allowed a “test-run” a week in advance. On testing day, three trials were conducted on each mouse (without break) and the cut-off time was 180 s. The mice were placed with

their front paws on the horizontal bar elevated 4 cm up and their back legs on the working bench. Tests were videoed and the time was recorded from the videos. The time started when the mouse had been placed and stopped when both paws left the rod. The average of the three trials was used for analysis.

4.12 | Data analysis and statistics

Number of independent experiments (*N*) is indicated in each figure legend. Normalized means across multiple experiments were used to generate graphs of each individual's mean. Statistical analyses of the obtained data were performed using ANOVA, Kruskal–Wallis, or Student's

t/Mann–Whitney U-tests according to the normality of comparisons and were plotted using Prism version 7.00 for Mac and version 9 for Windows (GraphPad Software, La Jolla, CA, USA) with SEM error bars. The body weight and rotarod performance correlation were analyzed by Pearson correlation coefficient (*r*). ***p* < 0.01 and **p* < 0.05.

AUTHOR CONTRIBUTIONS

Kunwar Jung-KC: Planning, conducting and reporting. Alba Tristán-Noguero: Planning, conducting and reporting. Altanchimeg Altankhuyag: Conducting. David Piñol Belenquer: Conducting and reporting. Karina S. Prestegård: Conducting. Irene Fernandez-Carasa: Conducting. Arianna Colini Baldeschi: Planning and conducting. Maria Sigatulina Bondarenko: Conducting. Angeles García-Cazorla: Planning. Antonella Consiglio: Planning and reporting. Aurora Martinez: Planning and reporting.

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CONFLICT OF INTEREST STATEMENT

Dr. Garcia Cazorla has received honoraria for research support and lectures from PTC Therapeutics; she has received honoraria for lectures from Biomarin, Immedica and Recordati Rare Diseases Foundation, and is a co-founder of the Hospital Sant Joan de Déu start-up “Neuroprotect Life Sciences”. Dr. Martinez is co-founder and CSO of Pluvia Biotech, a start-up from the University of Bergen, and A. Altankhuyag and Dr. Prestegård are employees at Pluvia Biotech, working on separate projects than the work presented here. K.J.-KC, A.T.N., D.P.B., I.F.C., A.C.B., M.S.B., and A.C. declare no competing interests.

ETHICS STATEMENT

All institutional and national guidelines for the care and use of laboratory animals were followed.


INFORMED CONSENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Institutional and National) and with the Helsinki Declaration of 1975, as revised in 2000 (5th). Informed consent was obtained from all patients for being included in the study.

ORCID

Kunwar Jung-KC  <https://orcid.org/0000-0002-6745-7318>

Alba Tristán-Noguero  <https://orcid.org/0000-0002-0170-9762>

Aurora Martinez  <https://orcid.org/0000-0003-1643-6506>

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SUPPORTING INFORMATION

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

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