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# Discharging dopamine

*Boosting endogenous tyrosine hydroxylase activity as a treatment for Parkinson's disease*

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# CHAPTER 4

## L-DOPA induced feedback inhibition on tyrosine hydroxylase phosphorylation in the mouse striatum can be attenuated by increasing intracellular cAMP

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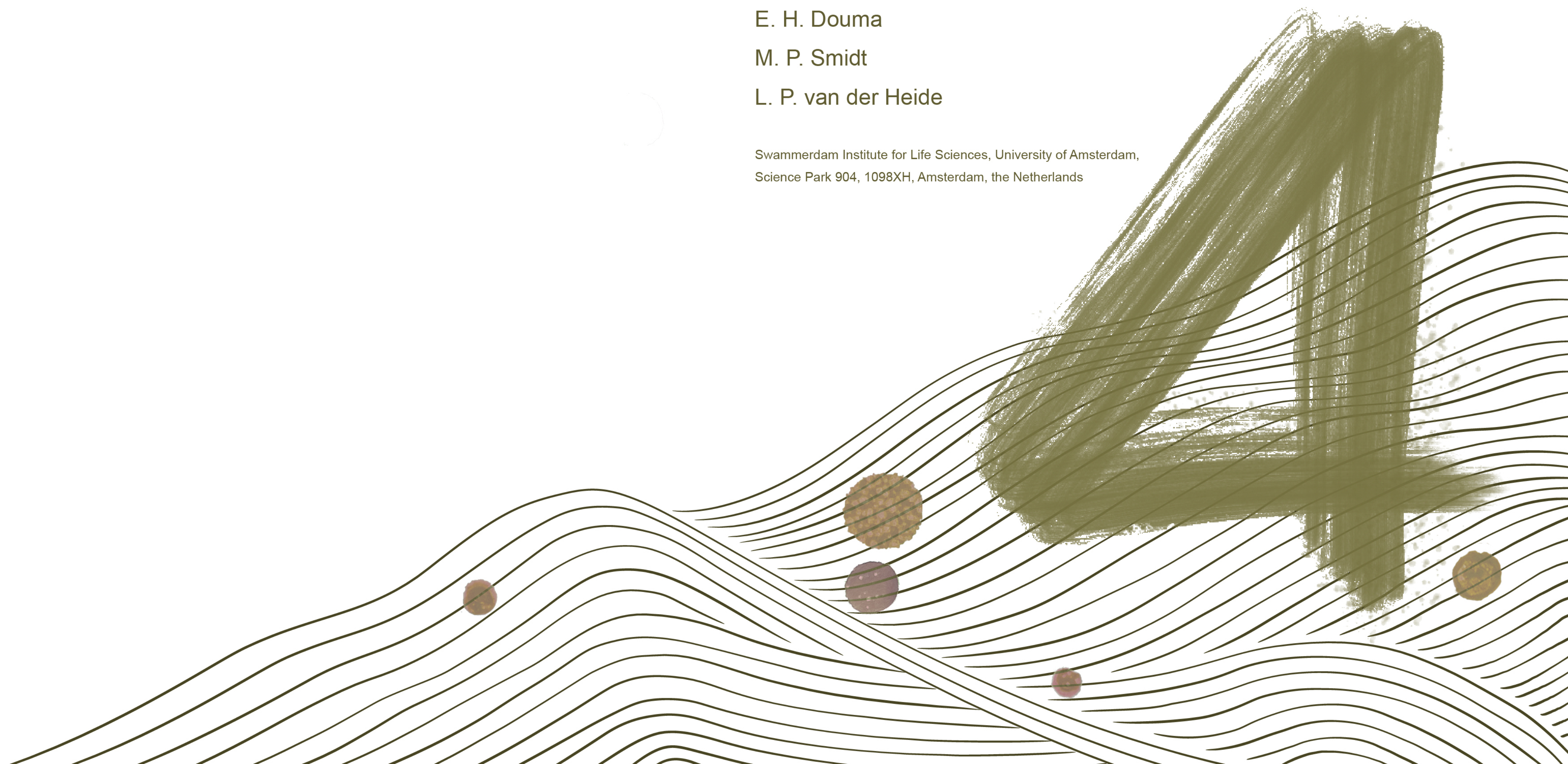

## Abstract

**Parkinson's disease (PD) is the second most prevalent neurodegenerative disease worldwide and is characterized by a variety of motor and non-motor symptoms, caused by the degeneration of dopamine neurons in the Substantia Nigra pars compacta (SNpc). As a consequence, less projection of dopamine fibers together with decreased dopamine release translates into the pathology of PD. Dopamine is produced from L-DOPA by the enzyme aromatic l-amino acid decarboxylase (AADC), while L-DOPA is produced from L-tyrosine by Tyrosine hydroxylase (TH), the rate-limiting enzyme in the dopamine biosynthesis pathway. The current golden standard in treatment of PD is exogenous application of L-DOPA, to supplement the loss of dopamine. L-DOPA passes the blood brain barrier and is effective in the first years of treatment. However, L-DOPA treatment loses its effectivity over time and results in various side-effects such as levodopa-induced dyskinesias. As exogenous L-DOPA application may affect the internal dopamine synthesis machinery, we investigated the effects on Th phosphorylation, which controls the rate of dopamine biosynthesis. TH activity is regulated by phosphorylation of serine residues on the regulatory domain in which phosphorylation of serine at position 40 (Ser40) increases enzymatic activity by lifting the extensively described mechanism of end-product feedback inhibition by catecholamines. Therefore, we quantified phosphorylation of Ser40 after L-DOPA application in the mouse striatum. L-DOPA treatment attenuated Ser40 phosphorylation, in which these inhibitory effects are possible due to the result of feedback inhibition by endogenously produced dopamine, as dopamine treatment also attenuated Ser40 phosphorylation. Interestingly, we show that the downregulation of Th Ser40 phosphorylation by L-DOPA and dopamine could be rescued by enhancing cAMP signaling. These results suggest that activation of cAMP-dependent routes would allow endogenous Th activity to be restored, lowering the effective dose of exogenous L-DOPA. If this approach is used in a clinical setting, patients treated with a lower dose of L-DOPA would experience less side-effects, which improves quality of life.**

## Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide [1] and its prevalence increases with age [2,3]. PD is characterized by a range of motor symptoms, such as bradykinesia, akinesia and tremor at rest [4–7]. PD patients also exhibit a variety of non-motor symptoms, such as cognitive dysfunction, depression and anxiety [8,9]. Motor symptoms are caused by a progressive degeneration of dopamine neurons of the Substantia Nigra pars compacta (SNpc) [10], that project towards the corpus striatum, one of the forebrain centers in charge of motor control [11,12]. Due to this degeneration, there is less projection of dopaminergic fibers, resulting in decreased dopamine release into the striatum.

In the terminals of nigrostriatal neurons, dopamine is locally synthesized by the enzyme aromatic amino acid decarboxylase (AADC) from the precursor L-3,4-dihydroxyphenylanine (L-DOPA). L-DOPA is in native conditions produced from L-Tyrosine by the rate-limiting enzyme tyrosine hydroxylase (TH) and with the aid of co-factors tetrahydrobiopterin (BH4), oxygen and ferrous iron ($Fe^{2+}$) [13–15]. TH is an evolutionary conserved multidomain protein consisting of an oligomerization-, catalytic- and regulatory domain. The N-terminal regulatory domain contains conserved serine residues that can be phosphorylated by a variety of kinases [16–21]. TH phosphorylation at serine position 40 (Ser40) increases enzymatic activity by lifting the extensively described mechanism of end-product feedback inhibition (Fig. 1). Via this mechanism catecholamines bind almost irreversibly to the ferric iron in the catalytic domain of Th, blocking binding of co-factor BH4, inhibiting the enzyme [22–36]. Phosphorylation of Ser40 causes a conformational change through electrostatic interactions, which allows catecholamines to dissociate [37,38]. Phosphorylation of Ser31 has been implicated to affect localization of intracellular Th and increase stability [39–43]. Interestingly, previous results demonstrated that phosphorylation of Ser40 is a prerequisite for Ser31 to be phosphorylated (Chapter 2, Fig. 7), linking activity of the enzyme to proper intracellular localization and suggesting that Ser40 phosphorylation is the rate-limiting factor in Th activity and functionality.

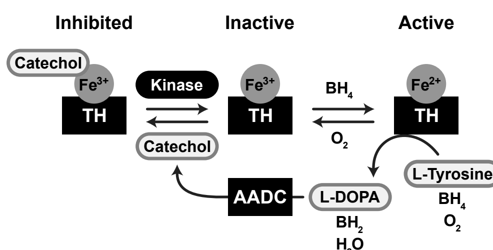


**Figure 1: TH feedback inhibition by catecholamines**

Illustration of the activation and inactivation of TH. In the inactive inhibited state, catecholamines are bound to the ferric iron, and prevent the binding of co-factor BH4. The result of this strict inhibitory control is suppression of the dopamine biosynthesis pathway. A conformational change mediated through kinase phosphorylation of TH induced results in the ability of the catecholamine to dissociate. At once, BH4 binds and leads to the reduction of the ferric iron to the ferrous iron making TH active. L-DOPA can now be synthesized from L-Tyrosine by TH using oxygen. L-DOPA is subsequently converted by the enzyme AADC to the catecholamine dopamine. *Abbreviations: AADC, aromatic amino acid decarboxylase; BH4, tetrahydrobiopterin; TH, Tyrosine hydroxylase.*

To supplement the loss of dopamine, PD patients are orally treated with L-DOPA, which unlike dopamine can pass through the blood brain barrier (BBB). L-DOPA is considered the gold standard treatment paradigm in PD and is administered as soon as a patient is diagnosed [13,44–46]. Via cerebral arteries, L-DOPA is actively transported via the L- amino acid transporter (LAT) to pass the BBB and is distributed all over the brain [47–51]. The first few years of treatment are relative effective (honeymoon period), however, in later stages of the disease patients develop fluctuations in their therapeutic response, and demonstrate side-effects [52,53]. Accordingly, patients depend on incremental adjustments in L-DOPA dosage and dosages per day [54–56].

Although L-DOPA supplementation bypasses the rate-limiting enzyme in dopamine synthesis and as such eliminates the need for Th it is unknown what the effects of L-DOPA are on the regulation of the internal machinery of dopamine neurons. In the current study, we used the previously discussed ex vivo striatal slice approach to investigate effects of L-DOPA on Th phosphorylation. We demonstrate that both L-DOPA and dopamine downregulate the phosphorylation of Th at Ser40 in the mouse striatum. Interestingly, forskolin application rescued phospho-Ser40 levels after exogenous L-DOPA and dopamine exposure, suggesting a cAMP-dependent signaling route to recover Th activity. Altogether, increasing Th Ser40 phosphorylation through cAMP-dependent signaling routes may attenuate the drawbacks of L-DOPA therapy on pre-synaptic Th activity, restoring the suppression of the endogenous DA biosynthesis machinery and improving the therapeutic outcome.

## Materials and methods

### Animal housing

All ex vivo experiments were performed on adult (~3 months old) C57/Bl6/J wild-type mice brain tissue. Animals were housed on a 12-hour light-dark cycle, with food and water provided *ad libitum*. All animal experimentation was supported and granted by the Animals Experimentation Committee of the University of Amsterdam (UvA) according to national and international legislation. Animals are cared for on a daily basis and sacrificed according to rules and regulations of the Dutch and European law.

### Ex vivo slicing and chemical treatment

Mice were euthanized by cervical dislocation and brains were immediately isolated and sliced on a Leica VT100S vibratome in ice-cold slicing buffer (120 mM Choline Chloride, 3.5 mM KCl, 0.5 mM $CaCl_2$, 6 mM $MgSO_4$, 1.25 mM $NaH_2PO_4$, 27.5 mM D-Glucose, 25 mM $NaHCO_3$). Coronal Midbrain (MB) or Corpus Striatum (CS) slices with a thickness of 250 µm were collected using a vibratome (VT1000S; Leica). Subsequently, the slices were microdissected and divided in two hemispheres to have an internal control. After microdissection of the brain areas of interest, the slices were transferred to 32°C carbogenized (95% $O_2$, 5% $CO_2$) artificial cerebrospinal fluid (aCSF; 120 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.25 mM $NaH_2PO_4$, 27.5 mM D-Glucose, 25 mM $NaHCO_3$) for 30 minutes. Subsequently, the slices were put at room temperature (RT) for another 30 minutes, while constantly being carbogenized. In Eppendorf tubes, slices were incubated with specific compounds diluted in RT aCSF, stated in the table on the next page (Table 1).

*Table 1. Chemicals used for pharmacological treatment*

| Name | Concentration (μM) | Vendor | Category number |
|---|---|---|---|
| Forskolin | 10 | Cell Signaling Technology | 3828S |
| L-DOPA | 100 | Sigma-Aldrich | D9628 |
| Dopamine | 10 | Sigma-Aldrich | H8502 |

**Sample preparation**

After treatment, aCSF was removed and slices were lysed in 37°C 1X Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS; Merck Millipore, 10% glycerol; Sigma-Aldrich) and 0.01% w/v bromophenol blue; Sigma-Aldrich) supplemented with 50 mM dithiotretol (DTT; Merck Millipore). All samples were collected, sonicated for 3 min in a Bioruptor sonicator (Diagenode) at maximum potency, heated at 95°C for 5 min, and briefly spun down.

**Inclusion criteria for ex vivo striatal slice analysis**

To adequately compare the level of Th protein between each individual mouse, we excluded samples that did not exceed the threshold of 15% of average total Th levels outside of the regions of interest. This threshold was determined by the average of Th protein content of the ‘axon region’, the area between the striatum and midbrain that reached 9% +- 1% of Th protein compared to the average Th protein in the striatum (Chapter 3, Fig. 2).

**WES™ Protein detection**

Evaluation of protein expression was performed using a Wes™ automated capillary western blot system (Protein Simple, San Jose, CA, USA) according to manufactures instructions and under the default settings. Briefly, 1 part of prepared cell lysate samples were diluted in 9 parts of MilliQ water, combined with the fluorescent master mix (PS-ST01EZ-8; ProteinSimple), and heated at 95°C for 5 min. The samples, biotinylated ladder (PS-ST01EZ-8; ProteinSimple), reagents (including the secondary antibody) from the anti-rabbit detection module (DM-001, ProteinSimple), and primary antibodies were loaded into designated wells in the 12-230 kDa separation module assay plate (PS-PP03; ProteinSimple). Digital images were analyzed using the Compass for SW software (ProteinSimple). Antibodies: rabbit anti-tyrosine hydroxylase (P40101; Pel-Freez); rabbit anti-phospho-tyrosine hydroxylase (Ser40) (2791S; CST); rabbit anti-phospho-tyrosine hydroxylase (Ser31) (13041S; CST). All antibodies were diluted 1:50 in antibody diluent (042-203; ProteinSimple), unless stated otherwise.

**Statistical analysis**

The amount of phospho-protein is corrected for the total amount of that protein and normalized to the control condition. To determine statistical significance for the mouse ex vivo experiments comparing two groups, two-tailed paired student’s *t*-tests were used. For the ex vivo experiments comparing multiple groups, area under the curve (AUC) values were determined for each condition per animal for which outer values were determined by interpolation, and two-tailed paired student’s *t*-tests were used followed by p-value adjustment in multiple comparisons testing. Ex vivo data are expressed as fold change compared to the control condition (control = 1) and presented as minimum to maximum boxplots showing the first quartile, third quartile, and the mean or bar charts showing the mean ± SEM. Differences were considered to be significant at a p-value < 0.05. Asterisks indicate significance (n.s. $p > 0.05$, $^{*}p < 0.05$ and $^{**}p < 0.01$).

## Results

### L-DOPA and dopamine downregulate Th phospho-Ser40 protein levels in the mouse striatum

Catecholamines have a higher affinity to unphosphorylated TH as compared to the co-factor BH4, resulting in feedback inhibition of enzyme activity [33,57,58]. Ser40 phosphorylation is known to lift this catecholamine-induced inhibitory feedback [34,36]. However, the influence of increased levels of L-DOPA, the pre-cursor of dopamine, on the phosphorylation state of Th in striatal dopamine neurons is not clear. For this reason, we collected and microdissected coronal striatal slices along the rostral-caudal axis and exposed the slices to 100 µM L-DOPA for 30 minutes (Fig. 2A). Per collected slice, one microdissected region of interest from one hemisphere (randomly chosen) was exposed to L-DOPA while the counter hemisphere was treated with vehicle. Subsequently, Th phosphorylation levels were examined.

L-DOPA treatment resulted in a significant downregulation of Ser40 phosphorylation levels (Fig. 2A,B; $p < 0.01$, $M = 0.66$). A similar result was obtained after 60 minutes of L-DOPA treatment (Fig. 2C, left panel; $p < 0.01$, $M = 0.60$).

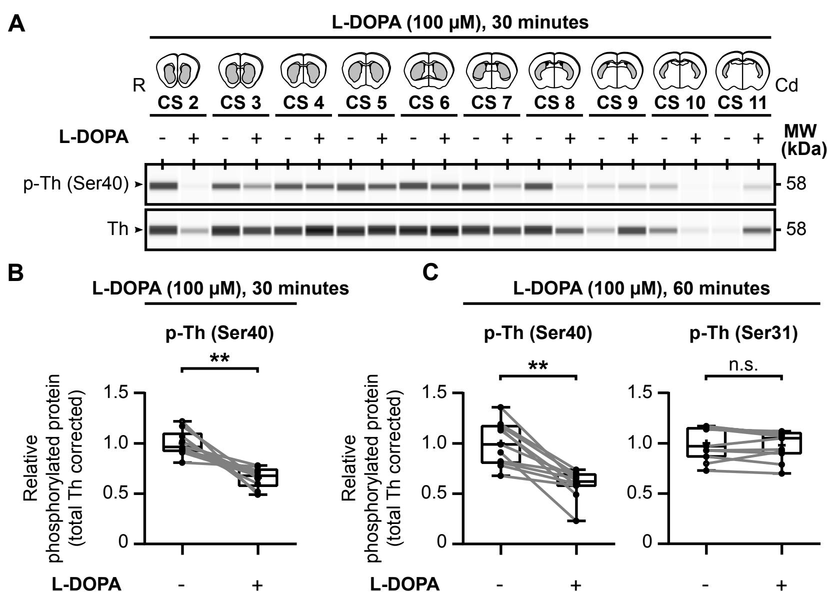


**Figure 2: Th phospho-Ser40 levels are downregulated by L-DOPA**

The effect of exogenous application of L-DOPA on Th phosphorylation was investigated in mouse striatal slices. Striatal slices were exposed to either 100 µM L-DOPA or vehicle for 30 minutes. **A |** Schematic images are shown of each examined brain slice over the rostral-caudal axis with representative western blot images for protein levels of Th and phospho-Th (Ser40). **B |** Quantitative analysis on the effect of 100 µM L-DOPA demonstrate that 30 minutes exposure to 100 µM L-DOPA downregulated phospho-Ser40 levels ($n = 10$). **C |** Quantifications on the effect of 100 µM L-DOPA demonstrates that 60 minutes exposure to 100 µM L-DOPA decreased phospho-Ser40 levels ($n = 11$), while there was no effect on phospho-Ser31 levels ($n = 11$). Data are minimum to maximum boxplots showing the first quartile, third quartile, and the mean, n.s. $p > 0.05$, $^{**}p < 0.01$ (two-tailed paired student's *t*-test).

Additionally, we quantified phospho-Ser31 levels after 60 minutes of L-DOPA exposure but found no significant effects (Fig. 2C, right panel; $p > 0.05$, $M = 0.98$). Thus, L-DOPA downregulates Ser40 phosphorylation, while leaving Ser31 phosphorylation unaffected.

Since L-DOPA can be metabolized into dopamine in nigrostriatal terminals, there is a possibility that dopamine is the actual cause of the downregulation of Ser40 phosphorylation. Therefore, we also investigated the effects of exogenous dopamine exposure on Th phosphorylation in striatal neurons.

Relative Ser40 phosphorylation levels are downregulated by dopamine, after both 30 minutes (Fig. 3A,B, left panel; $p < 0.01$, $M = 0.76$) and 60 minutes of exposure (Fig. 3C, left panel; $p < 0.01$, $M = 0.64$). Again, there were no effects on phospho-Ser31 levels, after both 30 minutes and 60 minutes (Fig. 3BC, right panels; $p > 0.05$, $M = 1.01$ and $M = 0.95$, respectively). To that end, increased dopamine levels also downregulated Ser40 phosphorylation, while leaving phospho-Ser31 levels unaffected. These results incline that L-DOPA derived dopamine and/ or extracellular dopamine signaling are the culprit that downregulate Ser40 phosphorylation.

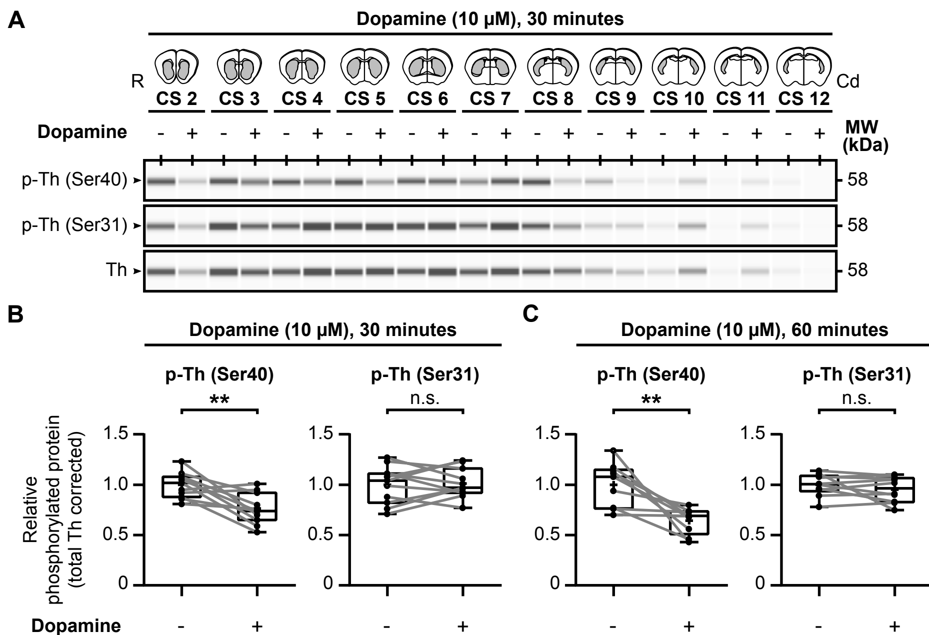


**Figure 3: Th phospho-Ser40 levels are downregulated by dopamine in the mouse striatum**

The effect of pharmacological treatment with dopamine on Th phosphorylation was investigated in mouse striatal slices. Striatal slices were exposed to either 10 μM dopamine or vehicle for 30 minutes. **A |** Schematic images are shown of each examined brain slice over the rostral-caudal axis with representative western blot images for protein levels of Th, phospho-Th (Ser40), and phospho-Th (Ser31). **B |** Quantitative analysis of the effect of 10 μM dopamine demonstrates that 30 minutes exposure to 10 μM dopamine downregulated phospho-Ser40 levels ($n = 11$), while there was no effect on phospho-Ser31 levels ($n = 11$). **C |** Quantifications of 60 minutes exposure to 10 μM dopamine downregulated phospho-Ser40 levels ($n = 9$), while there was no effect on phospho-Ser31 levels ($n = 10$). Data are minimum to maximum boxplots showing the first quartile, third quartile, and the mean, n.s. $p > 0.05$, $^{**}p < 0.01$ (two-tailed paired student's *t*-test).

**Downregulation of Th phospho-Ser40 levels by L-DOPA and dopamine can be rescued with forskolin**

Since Ser40 phosphorylation is regulated by cAMP-dependent signaling routes, we investigated the reversibility of the L-DOPA-induced downregulation of Ser40 phosphorylation in the striatum. To do so, we increased cAMP levels using the adenylyl cyclase activator forskolin. First, we pre-incubated the striatal slices with 100 μM L-DOPA, and subsequently added 10 μM forskolin (Fig. 4).

Again, L-DOPA significantly decreased phospho-Ser40 levels (Fig. 4C; $p < 0.01$, $M_{Vehicle} = 1.00$ vs $M_{L\text{-}DOPA} = 0.61$) and as expected forskolin significantly increased Ser40 phosphorylation levels (Fig. 4C; $p < 0.01$, $M_{Vehicle} = 1.00$ vs $M_{Forskolin} = 1.74$). Interestingly, L-DOPA pre-incubation with subsequent forskolin exposure significantly upregulated phospho-Ser40 levels (Fig. 4C; $p < 0.05$, $M_{L\text{-}DOPA} = 0.61$ vs $M_{L\text{-}DOPA+Forskolin} = 1.18$).

**Figure 4: Th phospho-Ser40 levels are downregulated by L-DOPA and can be rescued with forskolin**

To investigate if the L-DOPA downregulation on Th Ser40 phosphorylation could be reversed through elevations of cAMP, striatal slices were treated with L-DOPA and subsequently treated with forskolin. **A |** Ex vivo pharmacology protocol in which microdissected striatal slices were incubated with 100 μM L-DOPA for 30 minutes, followed by vehicle or 10 μM forskolin exposure for another 30 minutes. **B |** Schematic images are shown of each examined brain slice over the rostral-caudal axis with representative western blot images for protein levels of Th, phospho-Th (Ser40), and phospho-Th (Ser31). **C |** Quantitative analysis of the effect of forskolin on total Th-corrected phospho-Ser40 levels in striatal slices pre-incubated with either L-DOPA or vehicle. L-DOPA decreases and forskolin increases phospho-Ser40 levels ($n = 5$ animals). If slices were exposed to L-DOPA with 30 minutes subsequent exposure to forskolin, phospho-Ser40 levels were recovered to baseline levels. For each animal, the area under the curve was calculated per condition (two-tailed paired student's *t*-test statistical analysis with Bonferroni correction, $n = 5$ animals). Data are bar charts showing the mean ± SEM, *$p < 0.05$ and **$p < 0.01$.

In conclusion, L-DOPA downregulates Ser40 phosphorylation and subsequent activation of cAMP-dependent signaling routes reverses this effect by recovering Ser40 phosphorylation levels.

Finally, we investigated if the observed dopamine induced downregulation of Th Ser40 phosphorylation was reversible in a similar manner as compared to the combinatory L-DOPA experiment. Performing a similar experimental set-up, we pre-incubated the striatal slices with dopamine, and subsequently exposed the samples to forskolin to induce cAMP signaling (Fig. 5). Similarly, exposure to forskolin after dopamine pre-incubation significantly upregulated Ser40 phosphorylation (Fig. 5C, left panel; $p < 0.01$, $M_{Dopamine+Forskolin} = 1.94$), while there was no effect on the level of Ser31 phosphorylation (Fig. 5C, right panel; $p > 0.05$, $M = 0.99$). Thus, the downregulating effects of dopamine on Th Ser40 phosphorylation could be rescued by activation of the cAMP second messenger system via forskolin. Therefore, the silencing effects of L-DOPA and dopamine on Th activity could both be halted by enforcing the level of cAMP.

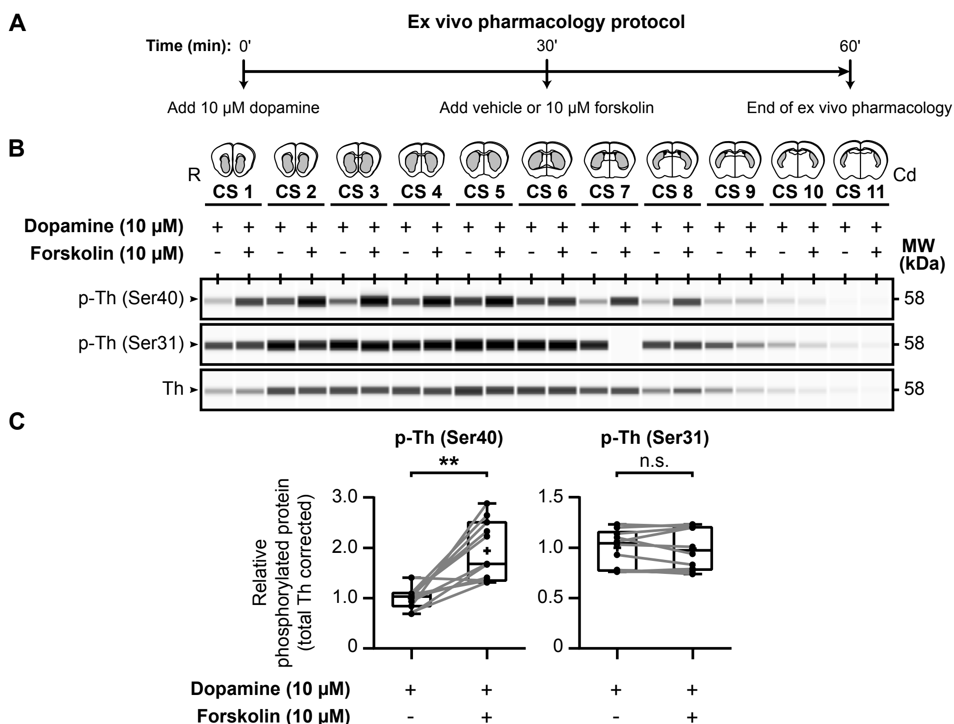

**Figure 5: Th phospho-Ser40 levels are downregulated by dopamine and can be rescued with forskolin**

To investigate if the downregulation effect of dopamine on Th Ser40 phosphorylation could be reversed through increased cAMP signaling, striatal slices were treated with dopamine and subsequently treated with forskolin. **A |** Ex vivo pharmacology protocol in which microdissected striatal slices were incubated with 10 μM dopamine for 30 minutes, followed by exposure to either 10 μM forskolin or vehicle for another 30 minutes. **B |** Schematic images are shown of each examined brain slice over the rostral-caudal axis with representative western blot images for protein levels of Th, phospho-Th (Ser40), and phospho-Th (Ser31). **C |** Quantitative analysis of the effect of 10 μM forskolin on total Th-corrected phospho-Ser40 and phospho-Ser31 levels in 30 minutes dopamine pre-incubated slices. If striatal slices are exposed to dopamine, with 30 minutes subsequent exposure to forskolin, phospho-Ser40 levels are increased ($n = 11$) while phospho-Ser31 levels remain unaffected ($n = 10$). Data are minimum to maximum boxplots showing the first quartile, third quartile, and mean, n.s. $p > 0.05$, $^{**}p < 0.01$ (two-tailed paired student's *t*-test).

## Discussion

L-DOPA is considered the gold standard in PD therapy, but its effects wear down and sustained treatment leads to L-DOPA induced dyskinesias (LIDs) [52,53]. Here we show that in the mouse striatum exogenous L-DOPA and dopamine application affects the internal dopamine machinery as it downregulates the phosphorylation of Th Ser40, the phosphorylation site that is required for catalytic activity and function. Interestingly, this negative feedback mechanism could be reversed by enhancing cAMP-dependent signaling.

It has previously been suggested that L-DOPA affects the activity of TH [27,35,59]. Gordon *and colleagues* demonstrated that after exposing PC12 cells with L-DOPA and or forskolin, baseline TH activity was decreased in situ. However, no significant effect on TH Ser40 phosphorylation was found [27]. Additionally, it has been shown that binding of dopamine to the catalytic domain of TH inhibits PKA-mediated Ser40 phosphorylation, by increasing the Km for PKA, which suggests that binding leads to a state in which dopamine suppresses the phosphorylation of Th at Ser40 [35,38]. Our data demonstrated that L-DOPA and dopamine both downregulated Th Ser40 phosphorylation levels in the striatum, optionally through feedback inhibition on TH activity by directly interacting with the Th protein, that could reduce the Km for the protein kinase (Fig. 6A).

Additionally, phosphorylation of TH in the striatum is regulated by upstream inhibitory feedback pathways. This is mediated through activation of auto-receptors, present in the presynaptic membrane (Fig. 6A). Different types of dopamine receptors (D1 to D5) are located throughout the brain and periphery. Dopamine receptors are G-coupled protein receptors, that activate heterotrimeric inhibitory or stimulatory G proteins. The D1-like family (D1 and $D_5$) mainly couple to $G_S$, that increase cAMP levels by inducing AC activation [60–63]. D1-like receptors (D1R) are found exclusively postsynaptic on dopamine-receptive cells, such as GABA-ergic medium spiny neurons (MSNs) in the striatum [64]. The D2-like family ($D_2$, $D_3$, and $D_4$) mainly couple to $G_{i/o}$ proteins that inhibit cAMP production by downregulating AC activity [62,65,66]. D2-like receptors (D2R) are expressed both postsynaptic on dopamine-receptive cells and presynaptic on TH-positive dopaminergic neurons in the striatum [64,67,68]. Activation of D2Rs results in downregulation of intracellular cAMP levels which lead to reduced activity of cAMP-dependent receptors, such as PKA. This may in turn lead to alterations in phosphorylation levels of TH and suppression of its catalytic activity. Indeed, in rat striatal slices, D2R activation by Quinpirole has been shown to inhibit TH Ser40 phosphorylation and activity, whereas it did not alter Ser31 phosphorylation [69,70]. Also, the increase of TH Ser40 phosphorylation by forskolin was downregulated in response of D2R activation [70].

In our ex vivo model, L-DOPA derived dopamine signaling may have downregulated the activity of ACs, through D2R feedback inhibition and thereby decreased the level of Ser40 phosphorylation. Researchers demonstrated that a peak of L-DOPA derived dopamine release is within a timespan of minutes [71–73]. Our experimental timeline consisted of a timespan of 30-60 minutes, which could allow sufficient production of dopamine and release into the synapse to occur. Indeed, our results demonstrate that L-DOPA and dopamine exposure downregulated phosphorylation of Th Ser40, suggesting this likely mechanism of D2R-AC coupled Th feedback inhibition (Fig. 6A).

Since it is unclear if both L-DOPA and dopamine molecules are responsible for the downregulation of Ser40 phosphorylation or that it is merely depending on L-DOPA derived dopamine, additional experiments are necessary to proof the mechanism. Possibly, by using an AADC inhibitor in our model to block the conversion of L-DOPA to dopamine, the role of L-DOPA could be established in more detail. Secondly, with the use of dopamine receptor antagonists, we could halt the exogenous D2R-AC feedback inhibition loop which blocks the L-DOPA derived dopamine that is released into the synapse from signaling. Following this set-up, we could determine if L-DOPA or dopamine directly intracellularly downregulate Ser40 phosphorylation.

In summary, L-DOPA application results in a continuous suppression of the endogenous dopamine biosynthesis pathway by attenuating phosphorylation of the main residue linked to Th activity. Treated PD patients are continuously exposed to increasing L-DOPA levels that enforce both the off-target effects induced by extrastriatal dopamine production by non-dopaminergic AADC-containing neurons [51–53,74–78], and the inhibition of the endogenous dopamine biosynthesis pathway through feedback inhibition of Th. We were able to rescue the L-DOPA and/or dopamine induced inhibition of Ser40 phosphorylation by boosting cAMP-dependent signaling. To that end, we propose a mode of reversing the feedback mechanisms using cAMP-dependent signaling routes to enhance Th activity and recover endogenous synthesis of dopamine (Fig. 6B). Our findings could be used to develop a therapeutic that enhances intracellular cAMP levels to recover TH activity specifically in dopamine nigrostriatal neurons in PD patients. Through increasing Th activity in striatal neurons and therefore increase dopamine production and signaling, together with the mitigation of the feedback inhibitory effects of L-DOPA, the therapeutic dose of L-DOPA therapy could be reduced, and effectiveness of treatment prolonged.

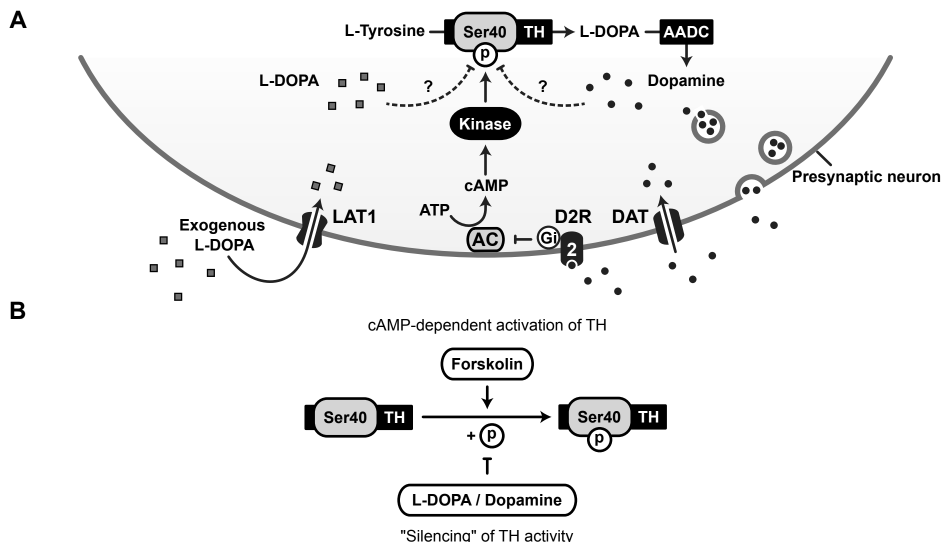


**Figure 6: Cyclic nucleotide-dependent rescue of feedback inhibition by catecholamines on TH activity**

**A |** Illustration of the autoregulatory feedback inhibition of dopamine and L-DOPA in striatal neurons. Elevated extracellular dopamine levels in the synaptic cleft are regulated by reuptake of dopamine by DAT. During treatment, exogenous L-DOPA molecules are transported into the cell by LAT1. Both intracellular dopamine and L-DOPA may inhibit phosphorylation of TH Ser40 directly. Indirectly, extracellular dopamine binds to G-protein coupled Gi inhibitory D2R. D2R inhibits AC activation, which downregulates the intracellular levels of cAMP. Reduced cAMP signaling decreases the ability of protein kinases to phosphorylate TH at Ser40. **B |** Hypothesized model of dopamine and L-DOPA-induced "silencing" of TH activity, an effect which can be negated due to manipulations of cyclic nucleotide levels. When neurons are exposed to L-DOPA or dopamine, TH activity is "silenced" or downregulated due to decreased levels of phospho-Ser40. This effect, however, can be "rescued" by cyclic nucleotide-dependent manipulation using forskolin, which increases phospho-Ser40 levels. *Abbreviations: AADC, aromatic amino acid decarboxylase; AC, Adenylyl cyclase; DAT, Dopamine transporter; D2R, Dopamine 2 Receptor; Gi, large guanine-nucleotide-binding regulatory protein α inhibitory subunit; LAT1, L-Type amino acid transporter 1; TH, Tyrosine hydroxylase.*

## References

1. Tysnes, O. B. & Storstein, A. Epidemiology of Parkinson's disease. Journal of Neural Transmission 124, 901–905 (2017).
2. de Lau, L. M. & Breteler, M. M. Epidemiology of Parkinson's disease. Lancet Neurology 5, 525–535 (2006).
3. Rajput, A. H. Frequency and Cause of Parkinson's Disease. Canadian Journal of Neurological Sciences 19, 103–107 (1992).
4. Sveinbjornsdottir, S. The clinical symptoms of Parkinson's disease. Journal of Neurochemistry 139, 318–324 (2016).
5. Jankovic, J. Parkinson's disease: Clinical features and diagnosis. Journal of Neurology, Neurosurgery and Psychiatry 79, 368–376 (2008).
6. Moustafa, A. A. et al. Motor symptoms in Parkinson's disease: A unified framework. Neuroscience and Biobehavioral Reviews 68, 727–740 (2016).
7. Kalia, L. v. & Lang, A. E. Parkinson's disease. Lancet 386, 896–912 (2015).
8. Han, J. W. et al. Psychiatric Manifestation in Patients with Parkinson's Disease. Journal of Korean Medical Science 33, (2018).
9. Zesiewicz, T. A., Sullivan, K. L. & Hauser, R. A. Nonmotor symptoms of Parkinson's disease. Expert Review of Neurotherapeutics 6, 1811–1822 (2006).
10. Tagliaferro, P. & Burke, R. E. Retrograde Axonal Degeneration in Parkinson Disease. Journal of Parkinson's Disease 6, 1–15 (2016).
11. Gerfen, C. R. & Surmeier, D. J. Modulation of Striatal Projection Systems by Dopamine. Annual Review of Neuroscience 34, 441–466 (2011).
12. Björklund, A. & Dunnett, S. B. Dopamine neuron systems in the brain: an update. Trends in Neurosciences 30, 194–202 (2007).
13. Fitzpatrick, P. F. The aromatic amino acid hydroxylases. Advances in Enzymology and Related Areas of Molecular Biology 74, 235–294 (2000).
14. Blaschko, H. Biochemistry of catecholamines — The biochemical method. Journal of Chromatography A 103, 404 (1975).
15. Roberts, K. M. & Fitzpatrick, P. F. Mechanisms of tryptophan and tyrosine hydroxylase. IUBMB Life 65, 350–357 (2013).
16. Gonçalves, C.-A. et al. Tyrosine Hydroxylase Phosphorylation in Digitonin-Permeabilized Bovine Adrenal Chromaffin Cells: The Effect of Protein Kinase and Phosphatase Inhibitors on Ser19 and Ser40 Phosphorylation. Journal of Neurochemistry 69, 2387–2396 (1997).
17. Kumar, G. K., Kim, D.-K., Lee, M.-S., Ramachandran, R. & Prabhakar, N. R. Activation of tyrosine hydroxylase by intermittent hypoxia: involvement of serine phosphorylation. Journal of Applied Physiology 95, 536–544 (2003).
18. Nakashima, A. et al. Role of N-terminus of tyrosine hydroxylase in the biosynthesis of catecholamines. Journal of Neural Transmission 116, 1355–1362 (2009).
19. Dunkley, P. R., Bobrovskaya, L., Graham, M. E., von Nagy-Felsobuki, E. I. & Dickson, P. W. Tyrosine hydroxylase phosphorylation: regulation and consequences. Journal of Neurochemistry 91, 1025–1043 (2004).
20. Dunkley, P. R. & Dickson, P. W. Tyrosine hydroxylase phosphorylation in vivo. Journal of Neurochemistry 149, 706–728 (2019).
21. Haycock, J. W. Phosphorylation of tyrosine hydroxylase in situ at serine 8, 19, 31, and 40. Journal of Biological Chemistry 265, 11682–11691 (1990).

22. Nagatsu, T. & Nagatsu, I. Tyrosine hydroxylase (TH), its cofactor tetrahydrobiopterin (BH4), other catecholamine-related enzymes, and their human genes in relation to the drug and gene therapies of Parkinson's disease (PD): historical overview and future prospects.
Journal of Neural Transmission 123, 1255–1278 (2016).
23. Toshiharu, N., Kimiko, M., Ikuko, N., Sadao, M. & Takashi, S. Pteridines as cofactor or inhibitor of tyrosine hydroxylase.
Biochemical Pharmacology 21, 1945–1953 (1972).
24. Udenfriend, S., Zaltzman-Nirenberg, P. & Nagatsu, T. Inhibitors of purified beef adrenal tyrosine hydroxylase.
Biochemical Pharmacology 14, 837–845 (1965).
25. Andersson, K. K. et al. Purification and characterization of the blue-green rat phaeochromocytoma (PC12) tyrosine hydroxylase with a dopamine-Fe(III) complex. Reversal of the endogenous feedback inhibition by phosphorylation of serine-40.
Biochemical Journal 284, 687–695 (1992).
26. Daubner, S. C., Le, T. & Wang, S. Tyrosine hydroxylase and regulation of dopamine synthesis.
Archives of Biochemistry and Biophysics 508, 1–12 (2011).
27. Gordon, S. L. et al. The low affinity dopamine binding site on tyrosine hydroxylase: The role of the N-terminus and in situ regulation of enzyme activity.
Neurochemical Research 34, 1830–1837 (2009).
28. Nakashima, A. et al. Intracellular stability of tyrosine hydroxylase: phosphorylation and proteasomal digestion of the enzyme.
Advances in pharmacology, 68, 3-11 (2013).
29. Sura, G. R., Daubner, S. C. & Fitzpatrick, P. F. Effects of phosphorylation by protein kinase A on binding of catecholamines to the human tyrosine hydroxylase isoforms.
Journal of Neurochemistry 90, 970–978 (2004).
30. Tekin, I., Roskoski, R., Carkaci-Salli, N. & Vrana, K. E. Complex molecular regulation of tyrosine hydroxylase.
Journal of Neural Transmission 121, 1451–1481 (2014).
31. Valpuesta, J. et al. The Structure of Human Tyrosine Hydroxylase Reveals the Mechanism for Feedback Inhibition by Dopamine.
Res. Sq (2020).
32. Dickson, P. W. & Briggs, G. D. Tyrosine hydroxylase. Regulation by feedback inhibition and phosphorylation.
Advances in Pharmacology vol. 68 (2013).
33. Briggs, G. D., Bulley, J. & Dickson, P. W. Catalytic domain surface residues mediating catecholamine inhibition in tyrosine hydroxylase.
Journal of Biochemistry 155, 183–193 (2014).
34. Andersson, K. K., Cox, D. D., Que, L., Flatmark, T. & Haavik, J. Resonance Raman studies on the blue-green-colored bovine adrenal tyrosine 3-monooxygenase (tyrosine hydroxylase). Evidence that the feedback inhibitors adrenaline and noradrenaline are coordinated to iron.
Journal of Biological Chemistry 263, 18621–18626 (1988).
35. Almås, B., le Bourdelles, B., Flatmark, T., Mallet, J. & Haavik, J. Regulation of recombinant human tyrosine hydroxylase isozymes by catecholamine binding and phosphorylation. Structure/activity studies and mechanistic implications.
European journal of biochemistry 209, 249–55 (1992).
36. Fujisawa, H. & Okuno, S. Regulatory mechanism of tyrosine hydroxylase activity.
Biochemical and Biophysical Research Communications 338, 271–276 (2005).
37. Ramsey, A. J. & Fitzpatrick, P. F. Effects of phosphorylation of serine 40 of tyrosine hydroxylase on binding of catecholamines: Evidence for a novel regulatory mechanism.
Biochemistry 37, 8980–8986 (1998).

38. Teresa Bueno-Carrasco, M. et al. Structural mechanism for tyrosine hydroxylase inhibition by dopamine and reactivation by Ser40 phosphorylation.
Nature Communications 2022 13:1 13, 1–16 (2022).
39. Jorge-Finnigan, A. et al. Phosphorylation at serine 31 targets tyrosine hydroxylase to vesicles for transport along microtubules.
Journal of Biological Chemistry 292, 14092–14107 (2017).
40. Halloran, S. M. & Vulliet, P. R. THE JOURNAL OF BIOLOGICAL CHEMISTRY Microtubule-associated Protein
Kinase-2 Phosphorylates and Activates Tyrosine Hydroxylase following Depolarization of Bovine Adrenal Chromaffin Cells*.
Journal of Biological Chemistry 269, 30960–30965 (1994).
41. Salvatore, M. F., Waymire, J. C. & Haycock, J. W. Depolarization-stimulated catecholamine biosynthesis: Involvement of protein kinases and tyrosine hydroxylase phosphorylation sites in situ.
Journal of Neurochemistry 79, 349–360 (2001).
42. Sutherland, C. et al. Phosphorylation and activation of human tyrosine hydroxylase in vitro by mitogen-activated protein (MAP) kinase and MAP-kinase-activated kinases 1 and 2.
European Journal of Biochemistry 217, 715–722 (1993).
43. Moy, L. Y. & Tsai, L. H. Cyclin-dependent Kinase 5 phosphorylates serine 31 of tyrosine hydroxylase and regulates its stability.
Journal of Biological Chemistry 279, 54487–54493 (2004).
44. Armstrong, M. J. & Okun, M. S. Diagnosis and Treatment of Parkinson Disease: A Review.
Journal of the American Medical Association 323, 548–560 (2020).
45. Ahlskog, J. E. Medical treatment of Parkinson's disease.
Comprehensive Therapy 15, 53–59 (1989).
46. Hufton, S. E., Jennings, I. G. & Cotton, R. G. H. Structure and function of the aromatic amino acid hydroxylases.
Biochemical Journal 311, 353–366 (1995).
47. Uchino, H. et al. Transport of Amino Acid-Related Compounds Mediated by L-Type Amino Acid Transporter 1 (LAT1): Insights Into the Mechanisms of Substrate Recognition.
Molecular Pharmacology 61, 729–737 (2002).
48. Kido, Y. et al. Molecular and functional identification of large neutral amino acid transporters LAT1 and LAT2 and their pharmacological relevance at the blood-brain barrier.
Journal of Pharmacy and Pharmacology 53, 497–503 (2001).
49. Upadhya, M. A., Shelkar, G. P., Subhedar, N. K. & Kokare, D. M. CART modulates the effects of levodopa in rat model of Parkinson's disease.
Behavioural Brain Research 301, 262–272 (2016).
50. Chagraoui, A. et al. L-DOPA in Parkinson's Disease: Looking at the "False" Neurotransmitters and Their Meaning.
International Journal of Molecular Sciences 2020, Vol. 21, Page 294 21, 294 (2019).
51. de Deurwaerdère, P., di Giovanni, G. & Millan, M. J. Expanding the repertoire of L-DOPA's actions: A comprehensive review of its functional neurochemistry.
Progress in Neurobiology 151, 57–100 (2017).
52. Carta, M. & Tronci, E. Serotonin system implication in L-DOPA-induced dyskinesia: From animal models to clinical investigations.
Frontiers in Neurology 5 MAY, (2014).
53. Jenner, P. Molecular mechanisms of L-DOPA-induced dyskinesia.
Nature Reviews Neuroscience 9, 665–677 (2008).

54. Olanow, C. W., Obeso, J. A., & Stocchi, F. Continuous dopamine-receptor treatment of Parkinson's disease: scientific rationale and clinical implications.
The Lancet Neurology, 5(8), 677-687 (2006).
55. Miller, D. W. & Abercrombie, E. D. Role of High-Affinity Dopamine Uptake and Impulse Activity in the Appearance of Extracellular Dopamine in Striatum After Administration of Exogenous L-DOPA.
Journal of Neurochemistry 72, 1516–1522 (1999).
56. Abercrombie, E. D., Bonatz, A. E., & Zigmond, M. J. Effects of L-DOPA on extracellular dopamine in striatum of normal and 6-hydroxydopamine-treated rats.
Brain research, 525, 36-44 (1990).
57. Gordon, S. L., Quinsey, N. S., Dunkley, P. R. & Dickson, P. W. Tyrosine hydroxylase activity is regulated by two distinct dopamine-binding sites.
Journal of Neurochemistry 106, 1614–1623 (2008).
58. Briggs, G. D., Gordon, S. L. & Dickson, P. W. Mutational analysis of catecholamine binding in tyrosine hydroxylase.
Biochemistry 50, 1545–1555 (2011).
59. Haavik, J. L-DOPA Is a Substrate for Tyrosine Hydroxylase.
Journal of Neurochemistry 69, 1720–1728 (1997).
60. Monsma, F. J., Mahan, L. C., McVittie, L. D., Gerfen, C. R. & Sibley, D. R. Molecular cloning and expression of a D1 dopamine receptor linked to adenylyl cyclase activation.
Proceedings of the National Academy of Sciences 87, (1990).
61. Tong, J. et al. Decreased Striatal Dopamine D1 Receptor- Stimulated Adenylyl Cyclase Activity in Human Methamphetamine Users.
American Journal of Psychiatry, 160(5), 896-903 (2003).
62. Snyder, S. H. What dopamine does in the brain.
Proceedings of the National Academy of Sciences 108, 18869–18871 (2011).
63. Corvol, J. C., Studler, J. M., Schonn, J. S., Girault, J. A. & Hervé, D. Gαolf is necessary for coupling D1 and A2a receptors to adenylyl cyclase in the striatum.
Journal of Neurochemistry 76, 1585–1588 (2001).
64. Beaulieu, J. M. & Gainetdinov, R. R. The physiology, signaling, and pharmacology of dopamine receptors.
Pharmacological Reviews 63, 182–217 (2011).
65. Obadiah, J. et al. Adenylyl Cyclase Interaction with the D2 Dopamine Receptor Family; Differential Coupling to Gi, Gz, and Gs.
Cellular and Molecular Neurobiology 1999 19:5 19, 653–664 (1999).
66. Ferré, S. et al. Adenosine–cannabinoid receptor interactions. Implications for striatal function.
British Journal of Pharmacology 160, 443–453 (2010).
67. Sokoloff, P. et al. The dopamine D3 receptor: a therapeutic target for the treatment of neuropsychiatric disorders.
CNS & Neurological Disorders-Drug Targets 5, 25–43 (2006).
68. Rondou, P., Haegeman, G. & van Craenenbroeck, K. The dopamine D4 receptor: biochemical and signalling properties.
Cellular and molecular life sciences 67, 1971–1986 (2010).
69. Salah, R., Kuhn, D. & Galloway, M. Dopamine autoreceptors modulate the phosphorylation of tyrosine hydroxylase in rat striatal slices.
Journal of Neurochemistry 52, 1517–1522 (1989).
70. Lindgren, N. et al. Dopamine D2 receptors regulate tyrosine hydroxylase activity and phosphorylation at Ser40 in rat striatum.
European Journal of Neuroscience 13, 773–780 (2001).

71. Spencer, S. E. & Wooten, G. F. Altered pharmacokinetics of L-dopa metabolism in rat striatum deprived of dopaminergic innervation.
Neurology 34, 1105–1108 (1984).
72. Staal, R. G. W., Mosharov, E. v. & Sulzer, D. Dopamine neurons release transmitter via a flickering fusion pore.
Nature Neuroscience 7, 341–346 (2004).
73. Pothos, E. N., Davila, V. & Sulzer, D. Presynaptic recording of quanta from midbrain dopamine neurons and modulation of the quantal size.
Journal of Neuroscience 18, 4106–4118 (1998).
74. Carta, M. & Bezard, E. Contribution of pre-synaptic mechanisms to l-DOPA-induced dyskinesia.
Neuroscience 198, 245–251 (2011).
75. Carta, M., Carlsson, T., Kirik, D. & Björklund, A. Dopamine released from 5-HT terminals is the cause of L-DOPA-induced dyskinesia in parkinsonian rats.
Brain 130, 1819–1833 (2007).
76. Cenci, M. A. Dopamine dysregulation of movement control in l-DOPA-induced dyskinesia.
Trends in Neurosciences 30, 236–243 (2007).
77. Cenci, M. A. L-DOPA-induced dyskinesia: cellular mechanisms and approaches to treatment.
Parkinsonism and Related Disorders 13, S263–S267 (2007).
78. Angela Cenci, M. Presynaptic mechanisms of L-DOPA-induced dyskinesia: The findings, the debate, the therapeutic implications.
Frontiers in Neurology 5, 1–15 (2014).


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