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Tyrosine hydroxylase phosphorylation is under the control of serine 40

Jesse Stoop¹  | Erik H. Douma¹  | Marc van der Vlag¹ | Marten P. Smidt² | Lars P. van der Heide² 

¹Macrobian Biotech B.V., Amsterdam, the Netherlands

²Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands

Correspondence

Lars P. van der Heide, Swammerdam Institute for Life Sciences, University of Amsterdam, Room C3.104, Science Park 904, 1098 XH Amsterdam, the Netherlands.
Email: l.p.vanderheide@uva.nl

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Abstract

Tyrosine hydroxylase catalyzes the initial and rate-limiting step in the biosynthesis of the neurotransmitter dopamine. The phosphorylation state of Ser40 and Ser31 is believed to exert a direct effect on the enzymatic activity of tyrosine hydroxylase. Interestingly, some studies report that Ser31 phosphorylation affects Ser40 phosphorylation, while Ser40 phosphorylation has no effect on Ser31 phosphorylation, a process named hierarchical phosphorylation. Here, we provide a detailed investigation into the signal transduction mechanisms regulating Ser40 and Ser31 phosphorylation in dopaminergic mouse MN9D and Neuro2A cells. We find that cyclic nucleotide signaling drives Ser40 phosphorylation, and that Ser31 phosphorylation is strongly regulated by ERK signaling. Inhibition of ERK1/2 with UO126 or PD98059 reduced Ser31 phosphorylation, but surprisingly had no effect on Ser40 phosphorylation, contradicting a role for Ser31 in the regulation of Ser40. Moreover, to elucidate a possible hierarchical mechanism controlling tyrosine hydroxylase phosphorylation, we introduced tyrosine hydroxylase variants in Neuro2A mouse neuroblastoma cells that mimic either phosphorylated or unphosphorylated serine residues. When we introduced a Ser40Ala tyrosine hydroxylase variant, Ser31 phosphorylation was completely absent. Additionally, neither the tyrosine hydroxylase variant Ser31Asp, nor the variant Ser31Ala had any significant effect on basal Ser40 phosphorylation levels. These results suggest that tyrosine hydroxylase is not controlled by hierarchical phosphorylation in the sense that first Ser31 has to be phosphorylated and subsequently Ser40, but, conversely, that Ser40 phosphorylation is essential for Ser31 phosphorylation. Overall our study suggests that Ser40 is the crucial residue to target so as to modulate tyrosine hydroxylase activity.

Abbreviations: Ala, alanine (A); ANOVA, analysis of variance; Asp, aspartic acid (D); ATP, adenosine triphosphate; BACCs, bovine adrenal chromaffin cells; BH4, tetrahydrobiopterin; cAMP, cyclic adenosine monophosphate; CREB, cAMP-regulatory element binding protein; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; DTT, dithiothreitol; ERK, extracellular signal-regulated protein kinase; HiFBS, heat-inactivated fetal bovine serum; L-DOPA, L-3,4-dihydroxyphenylalanine; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; N.D., not detected; PBS, phosphate-buffered saline; PDL, poly-D-lysine; PKA, cAMP-dependent protein kinase A; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; RRID, Research Resource Identifier; SEM, standard error of the mean; Ser, serine; Th, tyrosine hydroxylase; Th-S19A, Th with Ser19 substituted for an alanine; Th-S19D, Th with Ser19 substituted for an aspartic acid; Th-S31A, Th with Ser31 substituted for an alanine; Th-S31D, Th with Ser31 substituted for an aspartic acid; Th-S40A, Th with Ser40 substituted for an alanine; Th-S40D, Th with Ser40 substituted for an aspartic acid; Th-S8A, Th with Ser8 substituted for an alanine; Th-S8D, Th with Ser8 substituted for an aspartic acid; Th-WT, Th wild-type; WT, wild-type.

Jesse Stoop and Erik H. Douma contributed equally to this work.

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KEYWORDS

cell-signaling, crosstalk, Dopamine, phosphorylation, signal transduction, tyrosine hydroxylase

1 | INTRODUCTION

Tyrosine hydroxylase plays a pivotal role in the availability of dopamine, a neurotransmitter that is essential for diverse brain functions such as sensorimotor control, incentive reward processing, and memory (Beninger, 1983; Cools, 2008; Levitt et al., 1965; Nagatsu & Nagatsu, 2016; Nakashima et al., 2009; Udenfriend, 1966). The enzyme catalyzes the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of dopamine. Given that this is the slowest reaction in the dopamine biosynthesis pathway, tyrosine hydroxylase is considered the rate-limiting enzyme for the production of dopamine (Ikeda et al., 1966; Levitt et al., 1965; Nagatsu et al., 1964; Nagatsu & Nagatsu, 2016). Abnormalities in dopamine neurotransmission are related to various neuropsychiatric disorders, including Parkinson's disease, schizophrenia, and mood disorders (Ayano, 2016; Bissonette & Roesch, 2016; Douma & de Kloet, 2020; Stanford & Heal, 2019). Therefore, as a key regulatory enzyme in the regulation of dopamine signaling and, importantly, as it is under stringent control by complex molecular regulatory mechanisms (Tekin et al., 2014), tyrosine hydroxylase is of great therapeutic interest (Salvatore, 2012; Waløen et al., 2017).

Given its major function, it is not surprising that tyrosine hydroxylase is under control of a variety of regulatory mechanisms, a subject described and reviewed continually over the last decades (Daubner et al., 2011; Dickson & Briggs, 2013; Dunkley et al., 2004; Dunkley & Dickson, 2019; Flatmark, 2000; Fujisawa & Okuno, 2005; Johnson et al., 2018; Kumer & Vrana, 1996; Lenartowski & Goc, 2011; Nagatsu et al., 2019; Nagatsu & Nagatsu, 2016; Nakashima et al., 2009; Sumi-Ichinose et al., 2010; Tank et al., 2008; Tekin et al., 2014; Zigmond et al., 1989). The predominant mechanism of tyrosine hydroxylase regulation is by phosphorylation of its serine residues in the N-terminal regulatory domain of the protein. The serine residues 8 (Ser8), 19 (Ser19), 31 (Ser31), and 40 (Ser40) are subject to phosphorylation mediated by a variety of protein kinases, and involve multiple signaling cascades. Conversely, protein phosphatase 2A (PP2A) is the predominant tyrosine hydroxylase phosphatase as it dephosphorylates Ser40, Ser31, and Ser19 (Dunkley et al., 2004; Dunkley & Dickson, 2019). It is believed that the phosphorylation of Ser8 or Ser19 has no direct influence on the enzymatic activity of tyrosine hydroxylase (Haycock et al., 1998; Sutherland et al., 1993), while it is described that the phosphorylation of Ser40 or Ser31 promotes tyrosine hydroxylase activity directly (Dunkley & Dickson, 2019).

The modulation of dopamine synthesis is predominantly achieved via the regulation of Ser40 phosphorylation. In the presence of catecholamines, tyrosine hydroxylase phosphorylation at Ser40 lifts the well-defined feedback inhibition on tyrosine hydroxylase by these catecholamines that inactivate the enzyme

and are competitive with tetrahydrobiopterin (BH₄), cofactor for tyrosine hydroxylase in the enzymatic reaction of L-tyrosine to L-DOPA (Andersson et al., 1988; Fujisawa & Okuno, 2005; Nagatsu & Nagatsu, 2016; Okuno & Fujisawa, 1985; Toshiharu et al., 1972; Udenfriend et al., 1965). Therefore, the phosphorylation of Ser40 is the predominant source of tyrosine hydroxylase activation. Ser40 phosphorylation is mainly increased by cyclic nucleotides signaling and by a range of protein kinases, from which cAMP-dependent protein kinase A (PKA) is the most frequent and prominent described kinase (Almas et al., 1992; Dunkley et al., 2004; Dunkley & Dickson, 2019; Fujisawa & Okuno, 2005; Funakoshi et al., 1991).

Ser31 phosphorylation is linked to promote tyrosine hydroxylase activity as well, although to a considerably lower extent than Ser40 (Daubner et al., 1992; Halloran & Vulliet, 1994; Salvatore et al., 2001; Sutherland et al., 1993) and especially in the somatodendritic compartments of dopaminergic cells (Salvatore, 2014; Salvatore et al., 2016, 2018; Salvatore & Pruetz, 2012). In addition to tyrosine hydroxylase activity, Ser31 phosphorylation is linked to processes such as stability (Moy & Tsai, 2004) and subcellular localization (Jorge-Finnigan et al., 2017). Extracellular signal-regulated protein kinase (ERK) is described as the major protein kinase to regulate Ser31 phosphorylation (Dunkley & Dickson, 2019; Halloran & Vulliet, 1994; Kansy et al., 2004; Lehmann et al., 2006; Moy & Tsai, 2004; Salvatore et al., 2001; Sutherland et al., 1993). Moreover, cell stimulation by potassium chloride (KCl)-mediated depolarization activates ERK in a Ca²⁺-dependent manner resulting in an increase of Ser31 phosphorylation (Frodin et al., 1995; Kansy et al., 2004; Lindgren et al., 2002; Mitchell et al., 1990; Pavlovič-Šurjančev et al., 1992; Rosenblum et al., 2000; Salvatore et al., 2001).

Interestingly, some studies report that Ser31 phosphorylation affects Ser40 phosphorylation, a process coined as hierarchical phosphorylation (Gordon et al., 2009; Lehmann et al., 2006). Lehmann et al. (2006) reported that phosphorylation at Ser31 produced a ninefold increase in Ser40 phosphorylation, while Ser40 phosphorylation had no effect on Ser31 phosphorylation (Lehmann et al., 2006). Work from the same group found that down-regulation of Ser31 phosphorylation by inhibiting the phosphorylation of ERK leads to a 50% decrease in basal levels of Ser40 phosphorylation (Gordon et al., 2009). Moreover, ERK activation is linked to depolarization-dependent activation of tyrosine hydroxylase (Griffiths & Marley, 2001; Lindgren et al., 2002). Therefore, it is proposed that Ser31 phosphorylation provides a mechanism for sustained activation of tyrosine hydroxylase by the facilitation of Ser40 rephosphorylation before dopamine binds to tyrosine hydroxylase and inactivates the enzyme (Lehmann et al., 2006).

Here, we provide a detailed investigation into the signal transduction mechanisms that regulate Ser40 and Ser31 phosphorylation in dopaminergic mouse MN9D cells, as well as in Neuro2A cells. We found that cyclic nucleotide signaling drives Ser40 phosphorylation,



predominantly, but not exclusively, by PKA signaling, and that Ser31 phosphorylation is strongly regulated by ERK signaling and is down-regulated by PKA activity. Moreover, to elucidate hierarchical tyrosine hydroxylase phosphorylation, we introduced tyrosine hydroxylase variants in Neuro2A mouse neuroblastoma cells that mimic either phosphorylated or unphosphorylated serine residues. When we introduced a Ser40Ala tyrosine hydroxylase variant, we observed no detectable levels of Ser31 phosphorylation. Additionally, neither the tyrosine hydroxylase variant Ser31Asp, nor the variant Ser31Ala had effect on basal Ser40 phosphorylation levels. These results suggest that tyrosine hydroxylase is not hierarchically phosphorylated in the sense that first Ser31 is phosphorylated and subsequently Ser40, but, conversely, that Ser40 phosphorylation is essential for Ser31 phosphorylation.

2 | MATERIALS AND METHODS

2.1 | MN9D and Neuro2A cell culture

The dopaminergic MN9D cell line (Choi et al., 1991) was a kind gift from Dr. Thomas Perlmann (Hermanson et al., 2003) and cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; D6429, Sigma-Aldrich) supplemented with 200 nM L-glutamine (25030081, ThermoFisher Scientific), 1 unit/mL Penicillin/Streptomycin (15140163, ThermoFisher Scientific), and 10% (v/v) heat-inactivated fetal bovine serum (HiFBS; S181B, Biowest). While the MN9D cells are grown and maintained on poly-D-lysine (PDL)-coated 10-cm dishes, the Neuro2A (ATCC; mouse neuroblastoma cells) cell line was grown and maintained on uncoated 10-cm dishes in DMEM similar as described above except for the HiFBS (10082147, ThermoFisher Scientific). Both cell lines are cultured in a 37°C incubator with a humidified atmosphere of 95% air and 5% CO₂. For passaging, cells were not allowed to exceed 90% confluency and were not used above passage 30. Cultures were rinsed with phosphate-buffered saline (PBS; 10010023, ThermoFisher Scientific) and incubated with 1 mL 1X trypsin (15400054, ThermoFisher Scientific) in PBS (10010023, ThermoFisher Scientific) for 5 min. For experiments, cells were seeded in either PDL-coated or -uncoated 24-well plates for MN9D cells and Neuro2A cells, respectively. Both the MN9D (RRID:CVCL_M067) and Neuro2A (RRID:CVCL_0470) cell lines are not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee. The cells were not authenticated as the passage number was low and/or the cells were purchased from the company directly.

2.2 | Neuro2A transient transfections for tyrosine hydroxylase over-expression

For transient transfections of Neuro2A cells, cells were grown to approximately 75% confluency in serum-rich DMEM, which was replaced to serum-free DMEM prior to the transfection. A mixture

of DNA plasmid and lipofectamine 2000 reagent (11668019, Invitrogen) was prepared according to the manufacturer's instructions and incubated with the cells. After approximately 8 h, the serum-free DMEM was replaced for fresh serum-rich DMEM and incubated for approximately 24 h. Subsequently, serum-rich DMEM was replaced with serum-free DMEM, and the cells were cultured for an additional approximately 16 h before the *in vitro* experiments were executed. The DNA plasmids used encode either mouse wild-type tyrosine hydroxylase or site-directed mutagenesis variants thereof. In these tyrosine hydroxylase mutants, the Ser40, Ser31, Ser19, or Ser8 residues are replaced for either an alanine (A) or aspartic acid (D), mimicking the unphosphorylated or stoichiometrically phosphorylated variants, respectively, of these serine residues. Tyrosine hydroxylase (Th) wild-type (WT) is described as Th-WT, while the alanine (A) mutants for serine (S) 8, 19, 31 and 40 are described as Th-S8A, Th-S19A, Th-S31A, and Th-S40A, and Th aspartic acid (D) mutants for serine 8, 19, 31 and 40 are described as Th-S8D, Th-S19D, Th-S31D, and Th-S40D. All the plasmids had a pcDNA3.1+ backbone and a BamHI/SacII cloning site. Plasmids were generated by GenScript (Leiden, the Netherlands).

2.3 | *In vitro* chemical treatment and sample preparation

In vitro experiments were executed in approximately 16 h serum-deprived DMEM (0% for Neuro2A cells and 0.5% HiFBS for MN9D cells) in order to limit growth factor interference. All reactions were carried out at 37°C in the same incubator as used for culturing. H-89 (10010556), PD98059 (10006726), and U0126 (70970) were purchased from Cayman chemical company. Calyculin A (9902), forskolin (38285), and okadaic acid (59345) were purchased from Cell Signaling Technology. Dibutyryl cAMP (D0627), 8-Br-cAMP (B7880), and pCPT-cAMP (C3912) were purchased from Sigma-Aldrich. Except for 8-Br-cAMP and dibutyryl cAMP (in H₂O), all are chemicals dissolved in dimethyl sulfoxide (DMSO). Vehicle conditions were all treated with the appropriate amount of either DMSO or H₂O. After treatment, cells were washed with 1X PBS pH 7.4 and lysed in 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] in MilliQ water supplemented with 50 mM dithiothreitol (DTT; [Merck Millipore]). Samples were collected, sonicated for 3 min in a Bioruptor sonicator (Diagenode) at maximum potency, boiled at 95°C for 5–10 min, and briefly spun down.

2.4 | Capillary-based western blot analysis (Wes by ProteinSimple)

The evaluation of protein expression was performed using the Wes™ automated capillary western blot system (Protein Simple) according to the manufacturer's instructions and under the default settings. Briefly, prepared cell lysate samples were diluted with 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 12–230kDa separation module assay plate (PS-PP03, ProteinSimple). The assay plate and a 25-capillary cartridge are inserted into the Wes™ machine. The machine automatically separates the proteins by size and performs the immunoprobing, incubations, washing steps, and detection. Digital images were analyzed using the Compass software (ProteinSimple). Proteins were probed using the following antibodies: rabbit anti-tyrosine hydroxylase (P40101, Pel-Freez; [RRID:AB_2313713](#)); rabbit anti-phospho-tyrosine hydroxylase (Ser40) (2791S, CST; [RRID:AB_2201522](#)); rabbit anti-phospho-tyrosine hydroxylase (Ser31) (13041S, CST; [RRID:AB_2798096](#)); rabbit anti-β-actin (4970S, CST; [RRID:AB_2223172](#)); rabbit anti-CREB (4820S, CST; [RRID:AB_1903940](#)); rabbit anti-phospho-CREB (Ser133) (9198S, CST; [RRID:AB_2561044](#)); rabbit anti-p44/42 MAPK (Erk1/2) (4695S, CST; [RRID:AB_390779](#)); rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (4370, CST; [RRID:AB_2315112](#)). Antibodies were diluted in antibody diluent (1:50; 042-203, ProteinSimple).

2.5 | Statistical analysis

The amount of phospho-protein levels are corrected for the total amount of that protein, phosphorylated or not, and normalized

to the untreated/control condition. One-way analysis of variance (ANOVA) testing was utilized to determine statistical significance, and followed by the Bonferroni's multiple comparisons post hoc testing. The data are expressed as fold change compared to the control condition (control = 1) and presented as mean ± SEM. Differences were considered to be significant at a *p*-value < 0.05. Each “n” represents an individual sample. Asterisks indicate significance (**p* < 0.05 or ***p* < 0.01) with *n* ≥ 4. Data were assessed neither for normality nor for outliers, and all statistical testing was carried out using GraphPad Prism (version 10.0.1; Graphpad Software).

3 | RESULTS

3.1 | Tyrosine hydroxylase phosphorylation-specific antibody validation

We validated the specificity and selectivity of the phospho-specific antibodies targeting tyrosine hydroxylase Ser31 and Ser40 phosphorylation. We used an in vitro approach with Neuro2A cells, a cell line that endogenously does not contain detectable tyrosine hydroxylase protein levels (Figure 1).

These Neuro2A cells were transfected with plasmid DNA encoding either wild-type tyrosine hydroxylase (Th-WT) or tyrosine hydroxylase mutants. In these mutants, only Ser40 or Ser31 is substituted for an alanine (Th-S40A and Th-S31A, respectively). The phospho-specific antibody targeting Ser40 phosphorylation successfully detected Ser40-WT, but no signal was detected for S40A. The antibody targeting total tyrosine hydroxylase levels, independent of phosphorylation, detects levels of tyrosine hydroxylase for both tyrosine hydroxylase variants (Figure 1a,b). Similarly, the antibody targeting Ser31 phosphorylation is specific to Ser31 phosphorylation as it detects phospho-Ser31 in the Ser31-WT condition, but returns no signal for the Th-S31A condition. Again, both tyrosine hydroxylase variants show signal for total tyrosine hydroxylase that is independent of phosphorylation (Figure 1c,d). Thus, the phospho-specific antibodies targeting Ser40 and Ser31 phosphorylation are specific to Ser40 and Ser31 phosphorylation, respectively.

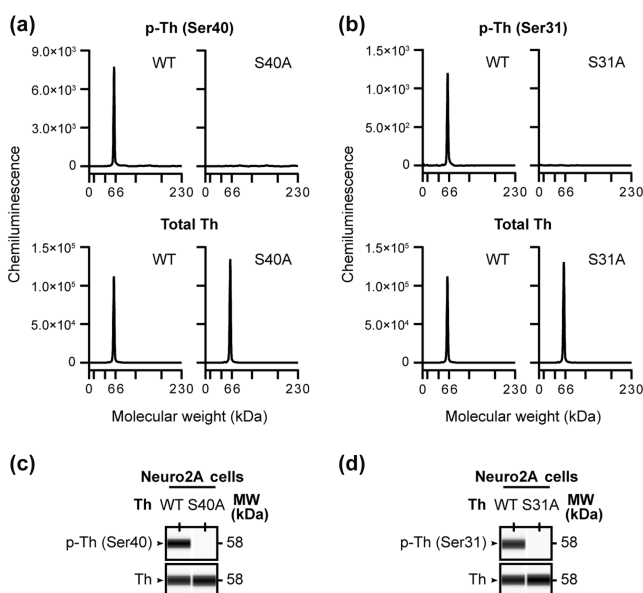


FIGURE 1 Validation of phosphorylated tyrosine hydroxylase antibodies. (a, b) Electropherograms of chemiluminescence signals representing phosphorylated Th protein as well as total Th levels in Th-transfected Neuro2a cells (Th-WT, S40A, and S31A). (c, d) Blot view images representing the electropherograms of (a) and (b) in a traditional western blot-like overview.

3.2 | Tyrosine hydroxylase phosphorylation is regulated via the cyclic AMP second messenger system

It is well described that tyrosine hydroxylase is regulated by the cAMP messenger system (Harris et al., 1974; Joh et al., 1978; Lovenberg et al., 1975; Morgenroth et al., 1975; Roskoski & Roskoski, 1987). Using the validated antibodies, we investigated the influence of cAMP signaling on tyrosine hydroxylase phosphorylation in MN9D cells. The MN9D cell line was originally created by fusing

mouse dopaminergic midbrain with the N18TG2 neuroblastoma cell line (Choi et al., 1991) as such it expresses tyrosine hydroxylase protein endogenously.

Indirectly, rapid activation of cAMP was facilitated by the use of forskolin (Figure 2). Forskolin increases cAMP levels via activation of adenylyl cyclase (Alasbahi & Melzig, 2012) and is known to affect tyrosine hydroxylase phosphorylation in other systems (Bowyer et al., 1992; Cheah et al., 1999). We demonstrate that forskolin increased Ser40 phosphorylation levels and decreased Ser31 phosphorylation levels in MN9D cells (Figure 2a–c). Cells were probed for tyrosine hydroxylase protein levels after exposure to either forskolin for 60 min at different concentrations (1, 3, 10, 30, or 100 μ M; Figure 2, left panels) or 10- μ M forskolin at different time points (5, 15, 30, 60, or 120 min; Figure 2, right panels). Forskolin increases phospho-Ser40 levels (Figure 2b, left panel; $F(5, 18) = 145.9, p < 0.01, M > 2.79$) and decreases phospho-Ser31 levels (Figure 2c, left panel; $F(5, 18) = 299.4, p < 0.01, M < 0.65$) in a dose-dependent manner. Up-regulation of phospho-Ser40 (Figure 2b, right panel; $F(5, 18) = 69.2, p < 0.01, M > 4.24$) and down-regulation of phospho-Ser31 (Figure 2c, right panel; $F(5, 18) = 135.5, p < 0.01, M < 0.40$) are demonstrated as early as after 5 min and persists thereafter at all other inspected time points.

Next, to test the direct influence of cAMP signaling on tyrosine hydroxylase phosphorylation, we exposed MN9D cells to cell-permeable analogues of cAMP (Figure 3).

First, we incubated MN9D cells with 500- μ M dibutyryl cAMP (Haycock, 1990; Roskoski & Roskoski, 1987; Zhang et al., 2014) at different time points (5, 15, 30, 60, or 120 min; Figure 3a–c). Similar to forskolin, dibutyryl cAMP induced a potent increase in phospho-Ser40 levels (Figure 3b; $F(5, 18) = 220.6, p < 0.01, M > 2.06$) and decreased phospho-Ser31 levels (Figure 3c; $F(5, 18) = 19.6, p < 0.01, M < 1.06$). For Ser31 phosphorylation, post hoc analysis showed significant decreases after at least 30 min of incubation with dibutyryl cAMP ($p < 0.03, M < 0.675$). In addition to dibutyryl cAMP (Figure 3d–f, left panels), the effect of the cell-permeable cAMP analogues 8-Br-cAMP (Figure 3d–f, middle panels) and pCPT-cAMP (Figure 3d–f, right panels) was tested at two different time points: 30 and 60 min. Both time points returned significant increases in phospho-Ser40 levels and decreases in phospho-Ser31 levels for all cAMP analogues. For Ser40 phosphorylation, dibutyryl cAMP (Figure 3e, left panel; $F(2, 11) = 635.7, p < 0.01, M > 11.5$) showed the most robust increases, while 8-Br-cAMP (Figure 3e, middle panel; $F(2, 11) = 316.5, p < 0.01, M > 4.12$) and pCPT-cAMP (Figure 3e, right panel; $F(2, 11) = 223.9, p < 0.01, M > 4.26$) showed similar increases. Moreover, dibutyryl cAMP (Figure 3f, left panel; $F(2, 11) = 42.9, p < 0.01, M < 0.54$), 8-Br-cAMP (Figure 3f, middle panel; $F(2, 11) = 25.1, p < 0.01, M < 0.60$), and pCPT-cAMP (Figure 3f, right panel; $F(2, 11) = 297.6, p < 0.01, M < 0.44$) all down-regulated phospho-Ser31 levels. The reduction in tyrosine hydroxylase as observed in Figure 3a upon increased incubation

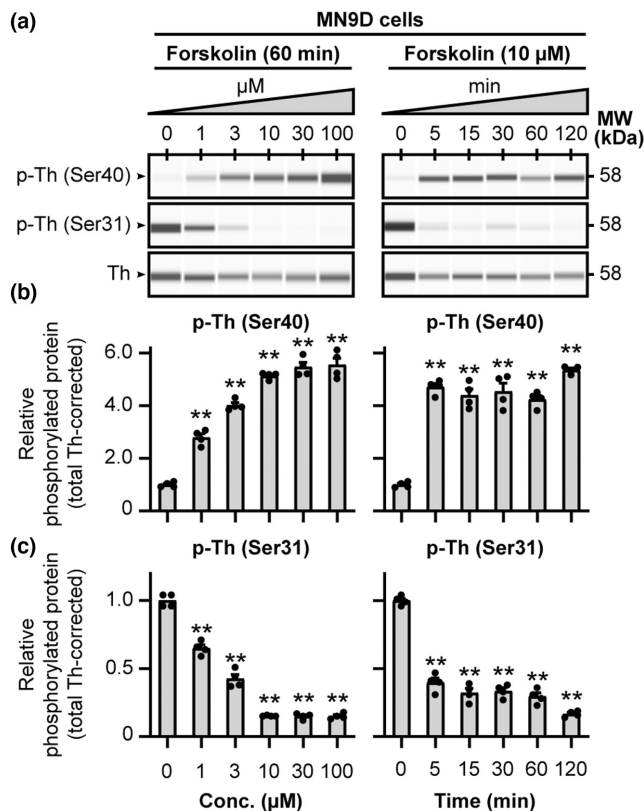


FIGURE 2 Forskolin alters phosphorylation of tyrosine hydroxylase in a dose-dependent manner in MN9D cells. MN9D cells were exposed to forskolin for 60 min at different concentrations (left panels) or for different times at 10 μ M (right panels). Cell lysate was probed with Th, phospho-Th (Ser40), and phospho-Th (Ser31) antibodies (a). Quantitative analysis of total Th-corrected (b) phospho-Th (Ser40) and (c) phospho-Th (Ser31) levels. $n = 4$ for each condition where each sample represents an independent cell culture preparation from the same passage number.

with dibutyryl cAMP is accompanied by a similar decrease in actin, reflecting a lower amount of cells under those conditions.

3.3 | Protein phosphatase inhibition via okadaic acid and calyculin A increases tyrosine hydroxylase phosphorylation

The phosphorylation state of the tyrosine hydroxylase serine residues is also regulated by protein phosphatases (Dunkley et al., 2004; Dunkley & Dickson, 2019; Leal et al., 2002). The main phosphatase responsible for tyrosine hydroxylase dephosphorylation in dopaminergic cells is PP2A, while protein phosphatase 1 (PP1) has negligible activity toward tyrosine hydroxylase (Berresheim & Kuhn, 1994; Bevilaqua et al., 2003; Haavik et al., 1989; Leal et al., 2002). Inhibiting protein phosphatases can shift the balance to an increased phosphorylated state, as the phosphorylation state of the tyrosine

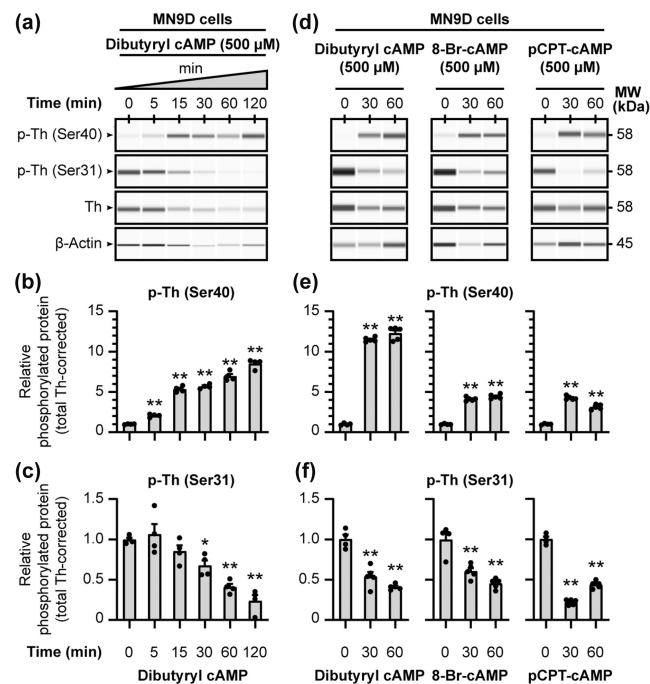


FIGURE 3 Secondary messenger signaling affects tyrosine hydroxylase protein phosphorylation in MN9D cells. (a–c) Time-response experiments for the effects of 500- μ M dibutyl cAMP on Th, phospho-Th (Ser40), phospho-Th (Ser31), and β -actin protein levels in MN9D cells. Quantitative analysis of total Th-corrected levels of (b) phospho-Th (Ser40) and (c) phospho-Th (Ser31). (d–f) The effect of 30 and 60 min exposure to 500- μ M dibutyl cAMP (left panels), 500- μ M 8-Br-cAMP (middle panels), or 500- μ M pCPT-cAMP (right panels) on Th, phospho-Th (Ser40), phospho-Th (Ser31), and β -actin protein levels. Quantitative analysis of total Th-corrected levels of (e) phospho-Th (Ser40) and (f) phospho-Th (Ser31). $n \geq 4$ for each condition where each sample represents an independent cell culture preparation from the same passage number.

hydroxylase serine residues is determined by the balanced and competing activity rates of phosphorylating protein kinases and dephosphorylating protein phosphatases. We used okadaic acid and calyculin A (Cohen et al., 1989; Ishihara et al., 1989), two phosphatase inhibitors specific for PP1 and PP2A, to inhibit protein phosphatase influence on the state of tyrosine hydroxylase phosphorylation in our MN9D dopaminergic cell model. Although both compounds show specificity to PP1 and PP2A, the IC_{50} values (Cohen et al., 1989; Ishihara et al., 1989) suggest stronger efficacy for PP2A inhibition over PP1 inhibition (Figure 4a).

We exposed MN9D cells to okadaic acid (Figure 4b–d) or calyculin A (Figure 4e–g) for 60 min at different concentrations (0.1, 1, 10, 100, or 1000 nM). Both okadaic acid ($F(5, 17) = 833.4$, $p < 0.01$, $M > 0.94$) and calyculin A ($F(5, 18) = 240$, $p < 0.01$, $M > 1.18$) increase phospho-Ser40 levels, although calyculin A increases phospho-Ser40 to a higher degree. Post hoc analysis showed that okadaic acid only increases phospho-Ser40 levels at 1 μ M (Figure 4c; $p < 0.01$, $M = 6.07$), while 10, 100, and 1 μ M of calyculin A are effective (Figure 4f; $p < 0.01$, $M > 2.35$). Moreover, okadaic acid had no effect on

Ser31 phosphorylation (Figure 4d; $F(5, 17) = 0.97$, $p = 0.47$) while calyculin A does (Figure 4g; $F(5, 18) = 145.4$, $p < 0.01$, $M > 1.01$). Post hoc analysis demonstrated that all inspected calyculin A concentrations (Figure 4g; $p < 0.01$, $M > 1.47$) except for 10 nM ($p > 0.99$, $M = 1.01$) are effective in increasing phospho-Ser31 levels.

3.4 | PKA signaling is involved in Ser40 phosphorylation and cross-talks with ERK signaling

cAMP and PKA signaling (Figure 3) are involved in the regulation of both Ser40 and Ser31 phosphorylation (Funakoshi et al., 1991; Gonçalves et al., 1997; Harada et al., 1996; Haycock, 1996; Salvatore et al., 2001; Sutherland et al., 1993), whereas ERK is the major protein kinase responsible for the phosphorylation of Ser31 (Gelain et al., 2007; Håkansson et al., 2004; Knowles et al., 2011; Luke & Hexum, 2008; Núñez et al., 2007; Salvatore et al., 2001; Sutherland et al., 1993). However to which degree these two pathways interact is more elusive. Therefore we investigated the possibility that PKA is involved in the regulation of both Ser40 and as well as Ser31 phosphorylation (Figure 5).

To do so, we used kinase inhibitors targeting PKA (H-89) and ERK (U0126 and PD98059). Despite the given fact that kinase inhibitors generally target multiple substrates (Bain et al., 2003, 2007; Davies et al., 2000), they provide a useful and commonly used tool to test the involvement of PKA (Bobrovskaya et al., 2004, 2007; Cheah et al., 1999; Chitre et al., 2020; Dang et al., 2015; Gelain et al., 2007; Gonçalves et al., 1997; He & Ron, 2008; Kawahata et al., 2015; Knowles et al., 2011; Zhang et al., 2014) or ERK (Chen et al., 2021; Gordon et al., 2009; Kansy et al., 2004; Knowles et al., 2011; Kobori et al., 2004; Moy & Tsai, 2004; Rosenblum et al., 2000; Salvatore et al., 2001; Zhang et al., 2014) in cellular processes.

First, we investigated the effect of H-89 on tyrosine hydroxylase phosphorylation (Figure 5). MN9D cells were pre-incubated for 45 min in the presence or absence of 30- μ M H-89, followed by 60 min exposure to either vehicle or 10- μ M forskolin (Figure 5a,b). As expected, forskolin treatment without H-89 pre-incubation increases relative Ser40 levels (Figure 5c; $p < 0.01$, $M = 1.00$ vs. 4.53) and decreases relative Ser31 levels (Figure 5d; $p < 0.01$, $M = 1.00$ vs. 0.23). These effects of forskolin are still present when pre-incubated with H-89, but less prominent for both Ser40 (Figure 5c; $p < 0.01$, $M = 1.86$ vs. 2.93) and Ser31 (Figure 5d; $p < 0.01$, $M = 0.34$ vs. 0.20).

Moreover, protein levels of cAMP-regulatory element binding (CREB) were measured (Figure 5e). PKA activity stimulates CREB phosphorylation (Brindle et al., 1995) and can therefore be used to confirm if PKA inhibition via H-89 was successful. Forskolin significantly increased the levels of CREB phosphorylation (Figure 5e; $p < 0.01$, $M = 1.00$ vs. 29.16). Interestingly, basal levels of CREB phosphorylation appear relatively low, suggesting low cAMP-dependent signaling which corresponds to low basal Ser40 phosphorylation. H-89 pre-incubation dramatically blocked phospho-CREB levels (Figure 5e; $p < 0.01$, $M = 29.16$ vs. 3.49), confirming the activity of the inhibitor.

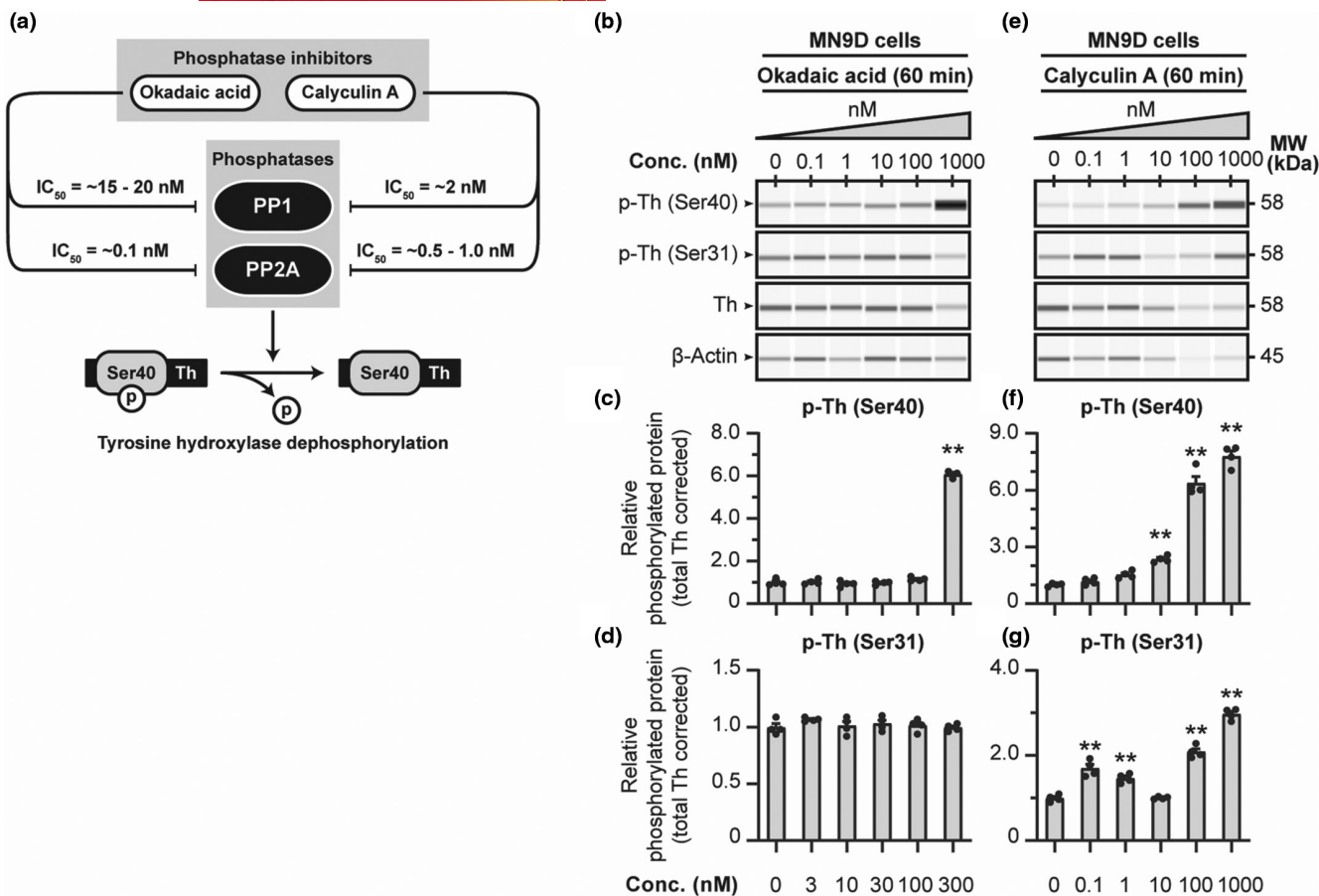


FIGURE 4 PP2A and PP1 inhibition manipulates tyrosine hydroxylase phosphorylation in MN9D cells. (a) Schematic of the hypothesized effects of okadaic acid and calyculin A on the dephosphorylation of Th. Okadaic acid and calyculin A inhibit the phosphatases PP1 and PP2A with higher specificity for PP2A (Cohen et al., 1989; Ishihara et al., 1989). (b–d) Dose–response experiment for the effect of 60min exposure to okadaic acid on Th, phospho-Th (Ser40), phospho-Th (Ser31), and β -actin protein levels. Quantitative analysis of total Th-corrected levels of (c) phospho-Th (Ser40) and (d) phospho-Th (Ser31). (e–g) Dose–response experiment for the effect of 60min exposure to calyculin A on Th, phospho-Th (Ser40), phospho-Th (Ser31), and β -actin protein levels. Quantitative analysis of total Th-corrected levels of (f) phospho-Th (Ser40) and (g) phospho-Th (Ser31). $n \geq 3$ for each condition where each sample represents an independent cell culture preparation from the same passage number.

Next, we investigated the effects of H-89 on the phosphorylation of ERK, as phosphorylation of ERK is linked to its activity and the phosphorylation of tyrosine hydroxylase at Ser31. ERK1/2 phosphorylation can easily be detected, which suggests high basal activity of this pathway, which also corresponds to high basal levels of tyrosine hydroxylase Ser31 phosphorylation. The effects of H-89 on ERK phosphorylation are highly comparable to the effects of H-89 on tyrosine hydroxylase Ser31 phosphorylation. First, forskolin exposure without H-89 pre-incubation mediated a significant decrease in ERK phosphorylation (Figure 5f; $p < 0.01$, $M = 1.00$ vs. 0.30). Just like Ser31 phosphorylation, the effects of forskolin are still present although less prominent when pre-incubated with H-89 (Figure 5f; $p = 0.03$, $M = 0.43$ vs. 0.15). Moreover, H-89 without subsequent forskolin exposure significantly lowers ERK phosphorylation (Figure 5f; $p < 0.01$, $M = 1.00$ vs. 0.43), while there is no difference in forskolin-induced effects between pre-incubation with or without H-89 (Figure 5f; $p = 0.43$, $M = 0.30$ vs. 0.15).

3.5 | Ser31 phosphorylation is regulated by ERK and does not influence Ser40 phosphorylation

We demonstrated that forskolin decreased phosphorylation of ERK (Figure 5f). As this decrease is similar to the demonstrated decrease in tyrosine hydroxylase Ser31 phosphorylation (Figure 5d) and ERK is the major protein kinase responsible for the phosphorylation of Ser31 (Gelain et al., 2007; Håkansson et al., 2004; Knowles et al., 2011; Luke & Hexum, 2008; Núñez et al., 2007; Salvatore et al., 2001; Sutherland et al., 1993), we investigated a more direct link between ERK and Ser31 phosphorylation. In the same setup, we tested whether Ser31 phosphorylation contributes to the hierarchical phosphorylation of Ser40 (Gordon et al., 2009). The ERK kinase activity inhibitors U0126 and PD98059 were used to investigate the influence of ERK signaling on tyrosine hydroxylase phosphorylation. MN9D cells were pre-incubated for 45 min in the presence or absence of either 10- μ M U0126 or 50- μ M

FIGURE 5 Inhibition of PKA signaling affects tyrosine hydroxylase protein phosphorylation in MN9D cells. (a) Schematic of the experimental setup to investigate how H-89 modulates phospho-Th (Ser40) levels, either with or without exposure to forskolin. MN9D cells were pre-incubated for 45 min in the presence or absence of 30- μ M H-89, followed by 60 min exposure to either vehicle or 10- μ M forskolin. (b) The effect of the presence or absence of H-89 on the effect of forskolin on Th, phospho-Th (Ser40), phospho-Th (Ser31), Creb, phospho-Creb (Ser133), Erk1/2, phospho-Erk1/2 (Thr202/Tyr20), and β -actin protein levels. (c) Quantitative analysis of phospho-Th (Ser40) levels corrected to total Th levels. With or without H-89 and forskolin. (d) Quantitative analysis of total Th-corrected phospho-Th (Ser31) levels. (e) Quantitative analysis of phospho-Creb (Ser133) levels corrected to total Creb levels. (f) Quantitative analysis of total Erk1/2-corrected phospho-Erk1/2 (Thr202/Tyr204) levels. $n=4$ for each condition where each sample represents an independent cell culture preparation from the same passage number.

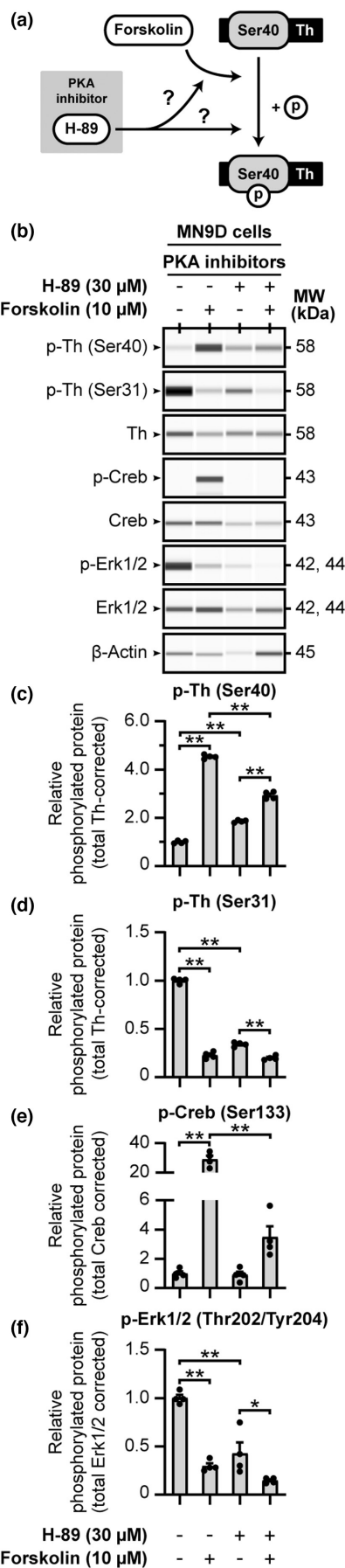
PD98059, followed by 60-min exposure to either vehicle or 10- μ M forskolin (Figure 6a,b).

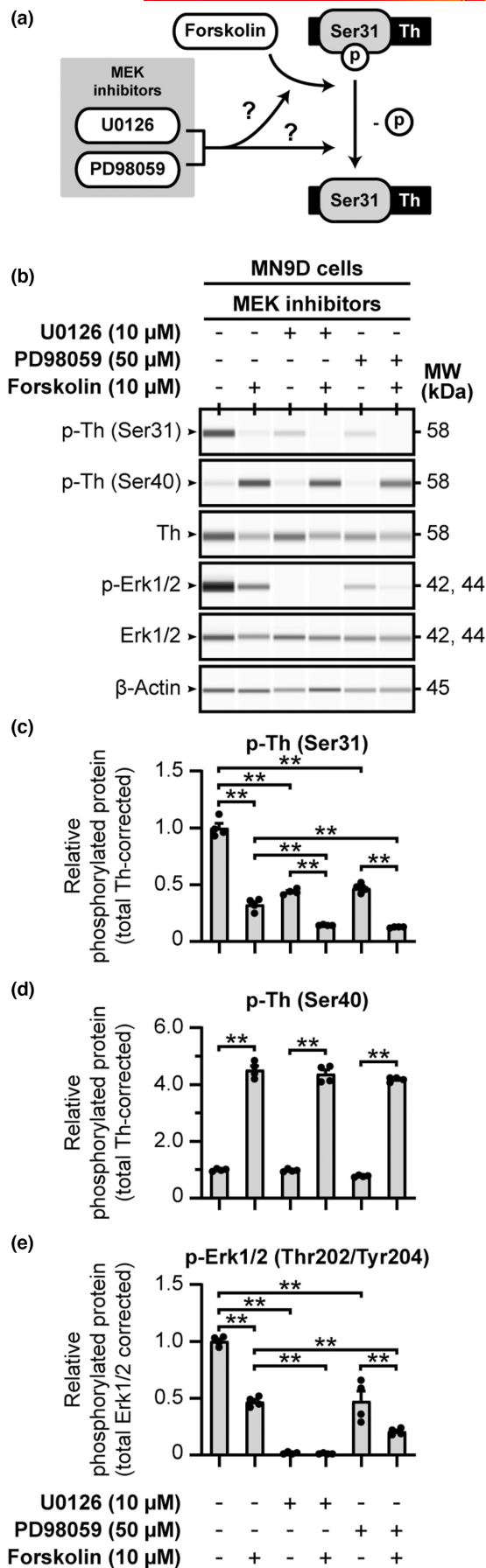
ERK inhibition via either U0126 (Figure 6c; $p < 0.01$, $M = 1.00$ vs. 0.44) or PD98059 (Figure 6c; $p < 0.01$, $M = 1.00$ vs. 0.47) substantially decreased Ser31 levels. Moreover, the forskolin-induced decreases in Ser31 phosphorylation were further down-regulated by both U0126 (Figure 6c; $p < 0.01$, $M = 0.33$ vs. 0.14) and PD98059 (Figure 6c; $p < 0.01$, $M = 0.33$ vs. 0.13). Both inhibitors had no effect on Ser40 phosphorylation (Figure 6d). Without (Figure 6d; $p < 0.01$, $M = 1.00$ vs. 4.52) or with either U0126 (Figure 6d; $p < 0.01$, $M = 0.98$ vs. 4.38) or PD98059 (Figure 6d; $p < 0.01$, $M = 0.78$ vs. 4.18), Ser40 levels increased after exposure to forskolin. In addition, Ser40 levels remain unchanged by pre-incubation with U0126 (Figure 6d; $p > 0.99$, $M = 1.00$ vs. 0.98) and PD98059 (Figure 6d; $p = 0.65$, $M = 1.00$ vs. 0.78). The forskolin-induced increases in Ser40 levels are similar after U0126 (Figure 6d; $p > 0.99$, $M = 4.52$ vs. 4.38) and PD98059 (Figure 6d; $p = 0.09$, $M = 4.52$ vs. 4.18) pre-incubation as well. The effectiveness of the inhibitors was confirmed as U0126 and PD98059 almost diminishes phospho-ERK levels to undetectable levels, while PD98059 roughly halves phospho-ERK levels (Figure 6e).

Together, we confirmed the involvement of phospho-ERK in the phosphorylation of tyrosine hydroxylase at Ser31, while both the decreased levels in phospho-Ser31 and phospho-ERK had no consequences for Ser40 phosphorylation.

3.6 | Phosphorylation of tyrosine hydroxylase at Ser40 is required for Ser31 phosphorylation

Ser40 phosphorylation is considered to be the key mechanism to increase tyrosine hydroxylase activity. The function of Ser31 phosphorylation is, however, less clear. It is proposed that one of the roles of Ser31 phosphorylation can be the promotion of Ser40 phosphorylation in a hierarchical way (Gordon et al., 2009; Lehmann





et al., 2006). Thus, higher Ser31 phosphorylation levels will lead to increased Ser40 phosphorylation levels. However, these effects were found via a kinase-induced Ser31 phosphorylation setup and therefore might be biased by the lack of specificity of the kinases. Therefore, we tested this hypothesis by investigating variants of tyrosine hydroxylase with Ser40 or Ser31 residues that mimic a non-phosphorylated or phosphorylated state.

To do so, we introduced either Th-WT or mutants thereof in Neuro2A cells, a cell line that endogenously does not contain detectable levels of tyrosine hydroxylase. In these tyrosine hydroxylase mutants, Ser40 or Ser31 was replaced for either an alanine (A) or aspartic acid (D). The replacement of Ser40 or Ser31 for the physicochemical innocuous amino acid alanine (Th-S40A and Th-S31A, respectively) mimics complete unphosphorylated conditions (Bevilaqua et al., 2001; Daubner et al., 1992; Jorge-Finnigan et al., 2017; Nakashima et al., 2002; Won et al., 2021), while the replacement of Ser40 or Ser31 to aspartic acid (Th-S40D and Th-S31D, respectively) is used as phospho-mimic (McCulloch et al., 2001; Nakashima et al., 2002). The effects of the phosphorylation state of one residue on the other are explored under basal conditions and after 60 min exposure to 10- μ M forskolin.

Figure 7 shows the effects of introducing Ser40 mutants (Figure 7a-c) and Ser31 mutants (Figure 7d-f) on relative Ser40 and Ser31 phosphorylation levels. When Th-S40A or Th-S40D are introduced no phospho-Ser40 signal can be detected (N.D., not detected) with the antibody specific to phospho-Ser40 (Figure 7b) and, similarly, when Th-S31A or Th-S31D are introduced no phospho-Ser31 signal can be detected with the antibody specific to phospho-Ser31 (Figure 7f). Total tyrosine hydroxylase levels, independent of phosphorylation state, can still be detected in all conditions. Most interestingly, phospho-Ser31 levels are not detectable when Th-S40A is introduced, while basal levels are detectable but decreased with Th-S40D (Figure 7c; $p < 0.01$, $M = 0.53$). Since ERK1/2 is upstream of Th-Ser31 (Figure 6), we tested if Th-S40A could act as dominant negative on ERK1/2

FIGURE 6 MEK1/2 signaling affects tyrosine hydroxylase Ser31 protein phosphorylation in MN9D cells. (a) Schematic of the experimental setup to investigate how the inhibitors U0126 and PD98059 modulate phospho-Th (Ser31) levels by inhibiting MEK1/2 signaling, either with or without exposure to forskolin. MN9D cells were pre-incubated for 45 min in the presence or absence of either 10- μ M U0126 or 50- μ M PD98059, followed by 60 min addition of either vehicle or 10- μ M forskolin. (b) The effect of the presence or absence of either U0126 or PD98059 on the effect of forskolin on Th, phospho-Th (Ser31), phospho-Th (Ser40), Erk1/2, phospho-Erk1/2 (Thr202/Tyr20), and β -actin protein levels. (c) Quantitative analysis of phospho-Th (Ser31) levels corrected for total Th levels. (d) Quantitative analysis of total Th-corrected phospho-Th (Ser40) levels. (e) Quantitative analysis of phospho-Erk1/2 (Thr202/Tyr204) levels corrected for total Erk1/2 levels. $n = 4$ for each condition where each sample represents an independent cell culture preparation from the same passage number.

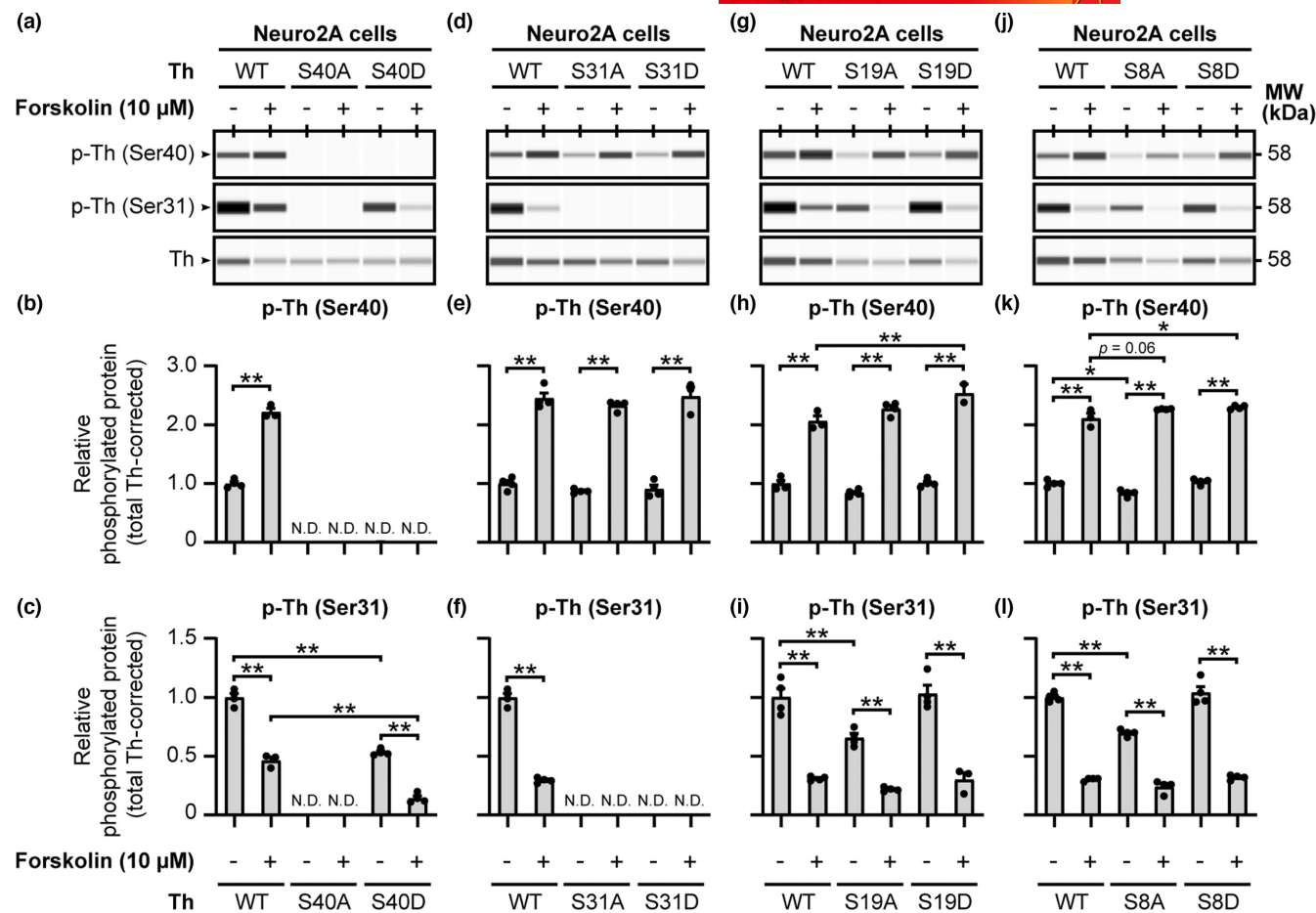


FIGURE 7 Phosphorylation of tyrosine hydroxylase at Ser40 is required for phosphorylation of Ser31 in Neuro2A cells. (a–l) The effect of mimicking absolute dephosphorylation or absolute phosphorylation states of serine residues Ser40 (a–c), Ser31 (d–f), Ser19 (g–i), or Ser8 (j–l) on Th, phospho-Th (Ser40), and phospho-Th (Ser31) protein levels, either in the presence or in the absence of 60-min exposure to 10-μM forskolin for 60 min. $n=4$ for each condition where each sample represents an independent cell culture preparation from the same passage number.

activity (Figure S1). However over-expressed Th-S40A did not have a significant effect on ERK1/2 phosphorylation as compared to Th-WT. Altogether, this indicates that Ser31 phosphorylation requires Ser40 phosphorylation and not the other way around per se. Additionally, both Th-S31A ($M=0.87$) and Th-S31D ($M=0.91$) had no effect on basal Ser40 phosphorylation (Figure 7e), indicating that the state of Ser31 phosphorylation is not affecting basal Ser40 phosphorylation.

The effects of forskolin (10 μM) on tyrosine hydroxylase phosphorylation in the Th-WT condition are comparable to the experiments that we demonstrated earlier (Figure 2), suggesting that the cAMP second messenger system is comparably involved in tyrosine hydroxylase phosphorylation for endogenous tyrosine hydroxylase in MN9D cells and when tyrosine hydroxylase is introduced in Neuro2A cells. Furthermore, forskolin significantly lowers the phospho-Ser31 levels of Th-S40D (Figure 7c; $p<0.01$, $M=0.53$ vs. 0.14) as well. When compared to Th-WT conditions, the basal levels of Ser31 phosphorylation are already lower for Th-S40D ($p<0.01$, $M=1.00$ vs. 0.53) and the exposure to forskolin leads to a further decrease in the levels of Ser31 phosphorylation ($p<0.01$, $M=0.46$ vs. 0.14).

Moreover, the forskolin-induced elevation in Ser40 phosphorylation levels are similar to Th-WT ($p<0.01$, $M=2.45$) for both Th-S31A (Figure 7e; $p>0.99$, $M=2.45$ vs. 2.33) and Th-S31D (Figure 7e; $p>0.99$, $M=2.45$ vs. 2.48).

To complement, we additionally examined—in a similar approach—the effects of the phosphorylation state of Ser19 (Figure 7g–i) and Ser8 (Figure 7j–l) on the phosphorylation states of Ser40 and Ser31. Although it is believed that the phosphorylation status of both Ser19 and Ser8 has no direct effect on tyrosine hydroxylase activity (Dunkley et al., 2004), we introduced tyrosine hydroxylase variants mimicking absolute unphosphorylated states of Ser19 or Ser8 (Th-S19A and Th-S8A, respectively) or absolute phosphorylation states of Ser19 or Ser8 (Th-S19D and Th-S8D, respectively) and investigated the influence on the phosphorylation of Ser40 and Ser31.

First, the effects of Ser19 phosphorylation on Ser40 and Ser31 phosphorylation. For Th-WT, Th-S19A, and Th-S19D, forskolin induced increases in Ser40 phosphorylation and decreases in Ser31 phosphorylation levels in all tyrosine hydroxylase variants. Moreover, basal levels of Ser40 phosphorylation were no different to



Th-WT for both Th-S19A (Figure 7h; $p=0.54$, $M=1.00$ vs. 0.84) and Th-S19D (Figure 7h; $p>0.99$, $M=1.00$ vs. 1.01). However, the forskolin-induced raise in Ser40 phosphorylation levels is relatively higher in Th-S19D as compared to Th-WT (Figure 7h; $p<0.01$, $M=2.53$ vs. 2.06), suggesting that a phosphorylated status of Ser19 boosts the effects of forskolin on Ser40 phosphorylation.

Regarding Ser31 phosphorylation, the levels of Ser31 phosphorylation after forskolin exposure are similar for Th-WT, Th-S19A, and Th-S19D conditions (Figure 7i). Interestingly, basal levels of Ser31 phosphorylation were lower for Th-S19A as compared to Th-WT (Figure 7i; $p<0.01$, $M=0.66$), while the basal levels of Ser31 phosphorylation of Th-S19D are similar to Th-WT. This suggests that Ser19 phosphorylation might support Ser31 phosphorylation.

Second, we attempted to investigate the effects of Ser8 phosphorylation status on Ser40 and Ser31 phosphorylation. Ser8 is not present in the human enzyme of tyrosine hydroxylase as the human (*Homo sapiens*) isoforms have a threonine at position 8 (Dunkley et al., 2004), while the rat (*Rattus norvegicus*) and mouse (*Mus musculus*) enzymes of tyrosine hydroxylase do incorporate a serine residue at position 8 (Ser8). Again, forskolin increased Ser40 phosphorylation and decreased Ser31 phosphorylation levels for all conditions. Interestingly, the basal levels of both Ser40 (Figure 7k; $p=0.02$, $M=0.84$ vs. 1.00) and Ser31 (Figure 7l; $p<0.01$, $M=0.70$ vs. 1.00) are lower for Th-S8A as compared to Th-WT, while levels of Th-WT and Th-S8D are similar. Furthermore, although not significant for Th-S8A, the forskolin-increased levels of Ser40 phosphorylation were higher in both Th-S8A (Figure 7k; $p=0.06$, $M=2.26$ vs. 2.11) and Th-S8D (Figure 7k; $p=0.01$, $M=2.30$ vs. 2.11) than in Th-WT. The phosphorylation status of Ser8 had no effect on the forskolin-induced effects on Ser31 phosphorylation.

In sum, mimicking total Ser40 dephosphorylation via Th-S40A leads to undetectable levels of Ser31 phosphorylation. This suggests, combined with the fact that both Th-S31A and Th-S31D had no effect on basal levels of Ser40 phosphorylation, that the state of Ser31 phosphorylation is dependent on the state of Ser40 phosphorylation, while Ser40 phosphorylation is independent of the state of Ser31 phosphorylation. This adds to our previous findings (Figure 6) that decreased levels of Ser31 phosphorylation had no consequences for Ser40 phosphorylation. The effects of Ser19 and Ser8 phosphorylation status on Ser40 and Ser31 phosphorylation remain more elusive. Measuring the activity of the enzyme under these conditions may provide additional insight into the roles of these phosphorylation sites.

4 | DISCUSSION

The phosphorylation state of tyrosine hydroxylase plays a cardinal role in regulating tyrosine hydroxylase activity. As such, phosphorylation is of major importance in the signaling cascades eventually leading to the endogenous production of dopamine. In the present study, we focused on the mechanisms regulating tyrosine

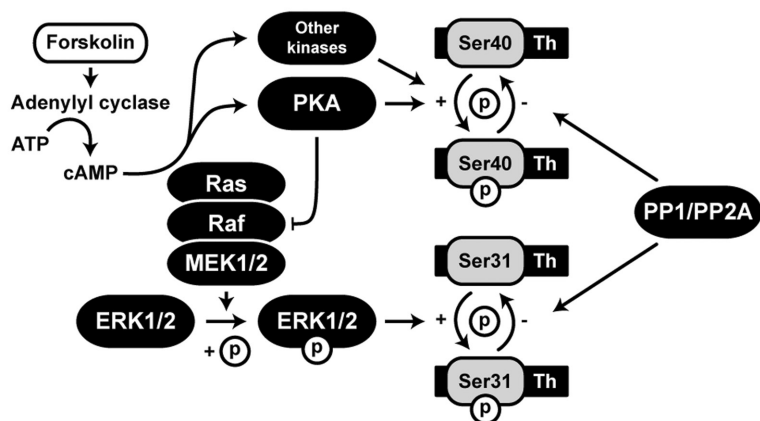
hydroxylase phosphorylation and confirmed basic principles as described in previous studies. However, in contradiction to previously described findings, our results suggest that Ser40 is of major importance for the regulation for tyrosine hydroxylase as it needs to be phosphorylated first before Ser31 can be phosphorylated.

PKA and ERK are the most prominent kinases to mediate the phosphorylation of Ser40 and Ser31, respectively (Dunkley et al., 2004; Dunkley & Dickson, 2019). We approximated the influence of these kinases on tyrosine hydroxylase phosphorylation using a subset of kinase inhibitors. Indeed, we found that PKA is involved in the phosphorylation of Ser40 and ERK in the phosphorylation of Ser31. There is, however, cross-talk between cAMP-dependent kinase signaling and ERK phosphorylation, affecting the state of Ser31 phosphorylation. Phosphorylation of ERK is linked to the phosphorylation of Ser31, and, presumably, the phosphorylation of ERK is down-regulated via decreased phosphorylation of mitogen-activated protein kinase kinase (MEK) via PKA (Dumaz & Marais, 2005; Dunkley & Dickson, 2019; Haycock, 1993; Impey et al., 1998; Stork & Schmitt, 2002). MEK and ERK are part of the Ras/Raf/MEK/ERK signaling cascade (Chang et al., 2003). PKA can phosphorylate three serine residues within c-Raf that independently block c-Raf activation by Ras (Dhillon et al., 2002; Dumaz & Marais, 2003; Sidovar et al., 2000). The phosphorylation of c-Raf at Ser43 directly interferes with activation by Ras through steric hindrance, while phosphorylation of Ser233 and Ser259 recruit 14-3-3 proteins that block the activation of c-Raf by Ras indirectly (Dumaz & Marais, 2003; Light et al., 2002; Wu et al., 1993). Thus, via this inhibition of Raf activity, PKA activation can down-regulate ERK phosphorylation and tyrosine hydroxylase Ser31 phosphorylation (Figure 8a). In our in vitro model, ERK1/2 activity accounts for a substantial amount of Ser31 phosphorylation, but it can not be excluded that CDK5 also partly contributes to Ser31 phosphorylation as complete inhibition of ERK1/2 does not prevent some residual Ser31 phosphorylation.

Besides this cross-talk on kinase activity level, some suggest that the phosphorylation of Ser40 and Ser31 are interconnected. It is proposed that a function of phosphorylation of Ser31 phosphorylation is to promote phosphorylation of Ser40, and the phosphorylation of Ser40 happens in a hierarchical way (Gordon et al., 2009; Lehmann et al., 2006): Ser31 phosphorylation allows Ser40 to be phosphorylated. However, we found no such interdependency. Instead, we found evidence that Ser31 phosphorylation is dependent on Ser40 phosphorylation. If we introduce a tyrosine hydroxylase variant that mimics Ser40 in a totally unphosphorylated state, we could not detect Ser31 phosphorylation levels. On the contrary, if we introduce a tyrosine hydroxylase variant that mimics total unphosphorylated Ser31, we were still able to detect and manipulate Ser40 phosphorylation. In addition, both the tyrosine hydroxylase variants that mimics total unphosphorylated Ser31 and the variant that mimics total Ser31 phosphorylation had no effect on basal Ser40 phosphorylation levels. Moreover, down-regulation of Ser31 phosphorylation via ERK inhibition had no effect on basal Ser40 phosphorylation levels, a setup others provided as proof for the hierarchical phosphorylation of tyrosine hydroxylase (Gordon et al., 2009). Therefore, we



(a) Regulation of tyrosine hydroxylase Ser40 and Ser31 phosphorylation



(b) Hierarchical tyrosine hydroxylase phosphorylation

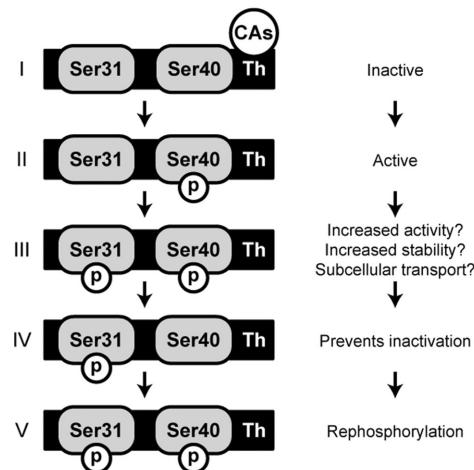


FIGURE 8 Hierarchical phosphorylation of tyrosine hydroxylase is manipulated through PKA and MEK1/2 cross-talk in MN9D cells. (a) Hypothesized intra-cellular signal-transduction pathways for the (de)phosphorylation of tyrosine hydroxylase (Th) at Ser40 and Ser31. Alterations in cyclic nucleotide levels can lead to changes in protein kinase activity, resulting in changes in the phosphorylation of tyrosine hydroxylase. Protein phosphatases are able to dephosphorylate tyrosine hydroxylase. This figure demonstrates the complexity of tyrosine hydroxylase protein phosphorylation with a variety of key players. (b) Hypothesized model of hierarchical phosphorylation of tyrosine hydroxylase. In the fully inactive form of tyrosine hydroxylase, there is no phosphorylation of tyrosine hydroxylase at Ser31 or Ser40 and, importantly, catecholamine binding to the protein inhibits the enzyme from its catalytic activity (I). Tyrosine hydroxylase phosphorylation at Ser40 lifts this feedback inhibition by catecholamines, which are competitive with the enzyme's cofactor tetrahydrobiopterin (BH₄). Thereby Ser40 phosphorylation promotes the initiation of tyrosine hydroxylase activity (II). Subsequently, Ser40 can be dephosphorylated or Ser31 can be phosphorylated additionally. The phosphorylation of tyrosine hydroxylase at Ser31, tyrosine hydroxylase is required to be phosphorylated at Ser40 (III). If both serine residues are phosphorylated, Ser31 phosphorylation might promote the protein to be in a state of increased activity and/or stability (Halloran & Vulliet, 1994; Moy & Tsai, 2004), or support subcellular transport (Jorge-Finnigan et al., 2017). However, when Ser40 is subsequently dephosphorylated, the phosphorylation of Ser31 presumably supports sustained tyrosine hydroxylase activity by impeding catecholaminergic feedback inhibition (IV). Moreover, by preventing or delaying this feedback inhibition mechanism, Ser31 phosphorylation might additionally promote more rapid rephosphorylation of Ser40 (V). This would mean that Ser31 phosphorylation has a supportive role, while Ser40 phosphorylation is the essential mechanism in the activation of tyrosine hydroxylase catalytic activity.

found no evidence for the hierarchical phosphorylation of Ser40, and our results are conflicting with previous explanations (Gordon et al., 2009; Lehmann et al., 2006).

That our data are not in accordance with previous data is partly the result from the different approaches that are used. To examine the effects of Ser31 phosphorylation on Ser40 phosphorylation, we used, as described above, a more direct approach and investigated tyrosine hydroxylase variants that mimic opposing phosphorylation states. On the other hand, the main evidence advocating that tyrosine hydroxylase is hierarchically phosphorylated results from a more correlative and kinase-dependent approach where the selective increases (Lehmann et al., 2006) or decreases (Gordon et al., 2009) in Ser31 phosphorylation are linked to effects on Ser40 phosphorylation levels.

Lehmann et al. (2006) used either angiotensin II in bovine adrenal chromaffin cells (BACCs) or purified ERK in a mixture with purified tyrosine hydroxylase to selectively increase Ser31 phosphorylation levels. Both settings resulted in an increase in Ser31 phosphorylation levels, while both angiotensin II and purified ERK had no direct effect on Ser40 phosphorylation levels. If tyrosine hydroxylase was exposed to forskolin or active PKA subsequently to the pre-treatment

with angiotensin II or ERK, respectively, Ser40 phosphorylation levels are potentiated as compared to the increases by forskolin and PKA alone. The authors attribute the potentiated increases in Ser40 phosphorylation to the increases in Ser31 phosphorylation. The link between increased Ser31 phosphorylation levels and potentiated Ser40 phosphorylation levels was only found in a physiological improbable dopamine-free situation. Moreover, forskolin only significantly affects Ser40 phosphorylation in the BACCs, while in our data, forskolin additionally down-regulates Ser31 phosphorylation levels in both Neuro2A and MN9D cells. In addition, in the study by Gordon et al. (2009), Ser31 phosphorylation levels were down-regulated in SH-SY5Y cells by the inhibition of ERK phosphorylation via U0126 and this led to an additional decrease in basal Ser40 phosphorylation. We used a similar setup in MN9D cells and demonstrated that U0126 only led to a decrease in Ser31 phosphorylation while Ser40 levels were unchanged.

Thus, in these studies, they use a kinase-dependent approach where the effects on Ser31 phosphorylation are linked to the eventual effects on Ser40 phosphorylation. However, our data suggest that cross-talk between cAMP, PKA, and ERK signaling in MN9D cells causes the observed effects. The interaction between these



different signaling pathways is very complex and depending on the specific cell type and context, it can lead to opposing effects on ERK (Dumaz & Marais, 2005). Therefore, we explored the effects of the phosphorylation state of Ser31 on Ser40 phosphorylation, and vice versa, in a more direct way via the tyrosine hydroxylase variants that mimic a specific phosphorylation state. We found no direct proof that the state of Ser31 phosphorylation alters basal Ser40 phosphorylation or can potentiate forskolin-induced changes in Ser40 phosphorylation.

Nonetheless, this does not mean that the proposed theory of Lehmann et al. (2006) on tyrosine hydroxylase phosphorylation is untrue. They proposed that Ser31 phosphorylation provides a mechanism for sustained activation of tyrosine hydroxylase by the facilitation of Ser40 rephosphorylation before dopamine binds to tyrosine hydroxylase and inactivates the enzyme (Lehmann et al., 2006). The phosphorylation of Ser40 is pivotal in the activation of tyrosine hydroxylase as it releases the catecholamine feedback inhibition on tyrosine hydroxylase. In fact, it is because of the strict inhibitory control of catecholamines that the majority of tyrosine hydroxylase endures a low activity state, with low Ser40 phosphorylation levels (Salvatore et al., 2000, 2001). Ser31 phosphorylation indeed provides a mechanism for sustained tyrosine hydroxylase activity, but subsequently to Ser40 phosphorylation. The phosphorylation of Ser31 might reduce the possibility of catecholamines to bind to tyrosine hydroxylase, and thereby to deactivate tyrosine hydroxylase. Thus, if Ser40 is dephosphorylated after the prior phosphorylation of both Ser40 and Ser31, then the Ser31 phosphorylation plausibly increases the rate of rephosphorylation of Ser40 before the catecholamines bind to tyrosine hydroxylase and inactivate the protein. This can provide a mechanism of sustained tyrosine hydroxylase activity in high demanding situations, for example, upon depolarization. Cell stimulation by depolarization activates ERK in a Ca^{2+} -dependent manner with an increase in Ser31 phosphorylation as result (Frodin et al., 1995; Kansy et al., 2004; Lindgren et al., 2002; Mitchell et al., 1990; Pavlović-Šurjančev et al., 1992; Rosenblum et al., 2000; Salvatore et al., 2001). Presumptively, in such high demanding situations, Ser31 phosphorylation provides a mechanism of sustained tyrosine hydroxylase activity upon prior activation of tyrosine hydroxylase via Ser40 phosphorylation (Figure 8b) emphasizing the complexity of the *in vivo* situation as compared to the *in vitro* models.

In contrast to the function and regulation of Ser31 phosphorylation, the function and regulation of Ser40 phosphorylation is well-described and conclusive. Our data support that Ser40 phosphorylation is up-regulated by the increased levels of cAMP (Harris et al., 1974; Joh et al., 1978; Lovenberg et al., 1975; Morgenroth et al., 1975; Roskoski & Roskoski, 1987), either directly via cAMP analogues or indirectly via the forskolin-induced activation of cAMP producing adenyl cyclases. The main roles of Ser40 phosphorylation are to lift the strong inhibitory feedback by bound catecholamines and to increase the affinity of tyrosine hydroxylase to its cofactor tetrahydrobiopterin (BH_4). Thereby, Ser40 phosphorylation initiates the activation of tyrosine hydroxylase. The eventual

hydroxylation reaction further requires the ferric iron of tyrosine hydroxylase to be oxidized to its ferrous form by BH_4 . Tyrosine hydroxylase contains a mononuclear non-heme iron atom, and its ferrous form unites the cofactors BH_4 and oxygen in the catalytic subunit before L-tyrosine binds. Subsequent to Ser40 phosphorylation, the ferrous iron therefore further facilitates enzymatic activity (Chow et al., 2009; Fitzpatrick, 1999; Tekin et al., 2014). Although the whole process initiating the catalysis of L-tyrosine to L-DOPA is more complex than only Ser40 phosphorylation, Ser40 phosphorylation is believed to be the most important regulatory mechanism of tyrosine hydroxylase activity as it initiates the whole activation process by releasing the strong feedback inhibition by catecholamines that are almost irreversibly bound to the ferric iron (Di Giovanni et al., 2012; Kumer & Vrana, 1996).

In a recent paper, Bueno-Carrasco et al. (2022) found evidence for the structural mechanism for tyrosine hydroxylase feedback inhibition by dopamine and described the counteracting role of Ser40 phosphorylation in this process (Bueno-Carrasco et al., 2022). The authors used cryo-EM to determine the structure of tyrosine hydroxylase with and without dopamine. Interestingly, they discovered that the flexible N-terminal of tyrosine hydroxylase includes an α -helix. This α -helix is structurally fixed in the active site of the enzyme upon binding of dopamine to tyrosine hydroxylase and supports the tight binding of dopamine to the active site (Bueno-Carrasco et al., 2022). Ser40 is located in the beginning of the α -helix (residues 39–58) and its phosphorylation forces the α -helix to egress from the active center of tyrosine hydroxylase, thereby releasing the structural fixation of dopamine to the active site and increasing the dissociation rate of dopamine from tyrosine hydroxylase (Ramsey & Fitzpatrick, 2000). Accordingly, Ser40 phosphorylation is the first and foremost essential mechanism in the activation of tyrosine hydroxylase catalytic activity as it initiates the release of catecholaminergic feedback inhibition.

As such, tyrosine hydroxylase Ser40 phosphorylation plays a major role in the regulation of presynaptic dopamine neurotransmission (Levitt et al., 1965; Nagatsu & Nagatsu, 2016; Udenfriend, 1966). Therefore, the modulation of tyrosine hydroxylase activity via the phosphorylation of Ser40 can provide a great therapeutic target for diseases in which dopamine neurotransmission is affected, like Parkinson's disease.

AUTHOR CONTRIBUTIONS

JS, EHD, and MvdV conducted the experimental work under the supervision of LPvdH. JS and EHD wrote the paper under the supervision of LPvdH and MPS. JS, EHD, MPS, and LPvdH interpreted the data and were involved in refining experiments. LPvdH conceptualized the original idea and expanded on this in close collaboration with MPS.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing non-financial interests but the following competing financial interests: MPS and LPvdH are founders, CEO, and shareholders of Macrobian Biotech BV.

DATA AVAILABILITY STATEMENT

Data are available upon request.

ORCID

Jesse Stoop  <https://orcid.org/0000-0002-3047-2987>

Erik H. Douma  <https://orcid.org/0000-0001-9565-9972>

Lars P. van der Heide  <https://orcid.org/0000-0003-1779-425X>

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