Discharging dopamine

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

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CHAPTER 2

Revisiting kinase-dependent regulation of tyrosine hydroxylase and hierarchical phosphorylation in vitro

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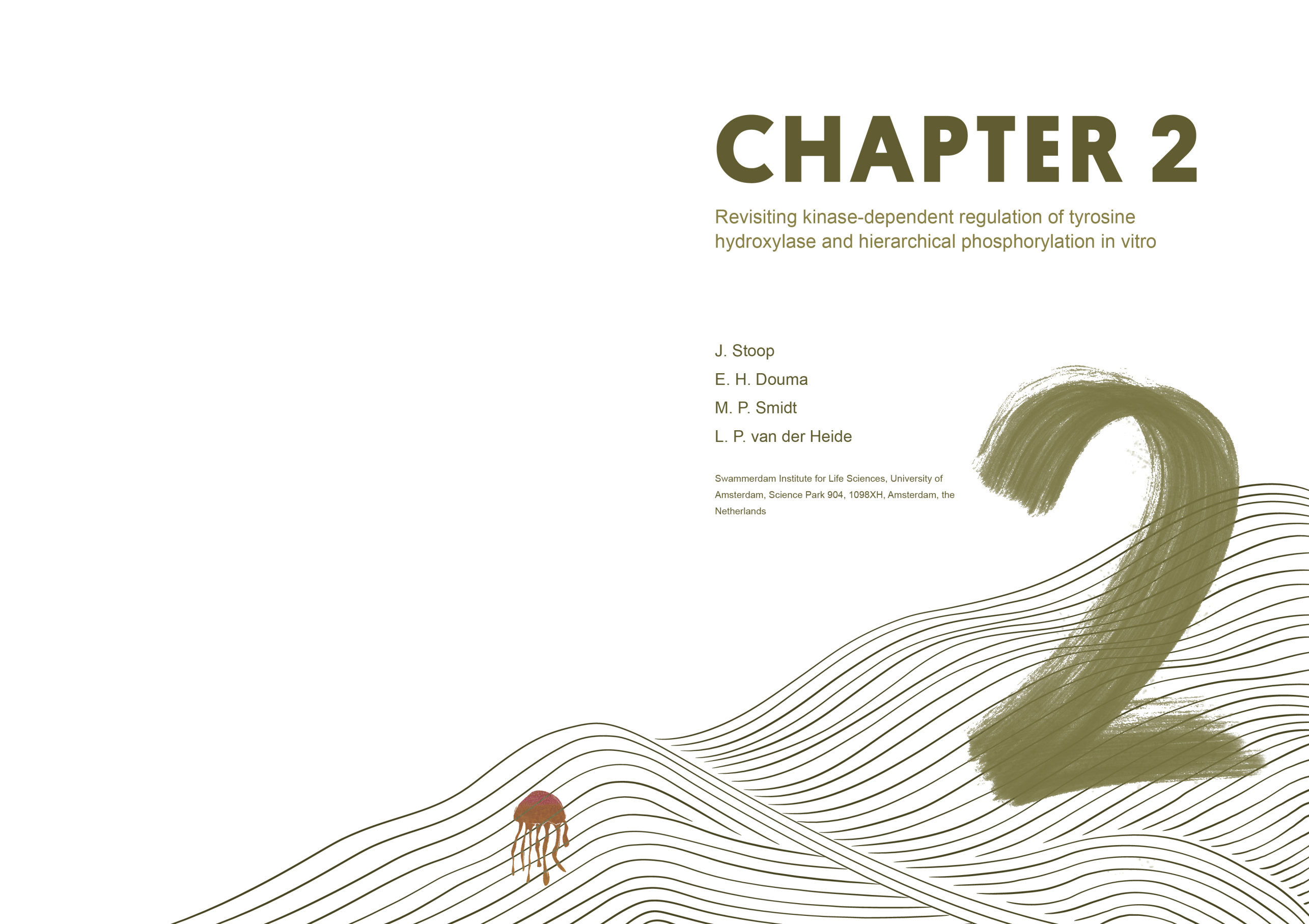


Abstract

The production of the neurotransmitter dopamine depends on the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme in this biosynthesis pathway. TH activity is regulated by the phosphorylation of serine (Ser) residues 19, 31, and 40 by a range of protein kinases. Previous studies in BACC and PC12 cells have suggested that phosphorylation of Ser40 by PKA is linked to an increase in the activity of TH, whereas Ser31 phosphorylation by ERK1/2 and CDK5 is linked to increased TH stability and subcellular localization. Additionally, Ser31 is suggested to facilitate subsequent phosphorylation of Ser40 in a manner called hierarchical phosphorylation. We revisited cAMP-dependent upstream signaling on Ser40 and Ser31 phosphorylation in dopaminergic MN9D cells and Th transfected Neuro2A cells. We confirm that cAMP-dependent signaling routes increase Ser40 phosphorylation levels that is mediated by PKA. Surprisingly, elevated cAMP signaling downregulated phospho-Ser31 levels presumably by inhibitory signaling between PKA and ERK1/2. Next, the postulated hierarchical phosphorylation hypothesis that proposes that Ser31 phosphorylation influences the extend of Ser40 phosphorylation is not supported in our model. Using phospho-mimetic and unphosphorylated mutants for Ser19, Ser31 and Ser40, we find no evidence to support this reported claim. Conversely, we show that Ser40 phosphorylation is required for Ser31 phosphorylation. Therefore, we suggest that Ser40 is crucial in controlling TH activity and the concomitant rate of dopamine synthesis.

Introduction

The catecholamine dopamine is involved in diverse brain functions, such as motor control, motivation, reward, and others [1]. Dysregulation has been linked to a number of neurological disorders, including Parkinson's disease, attention deficit/hyperactive/mood disorder, schizophrenia, addiction and gambling [2–11]. The enzyme tyrosine hydroxylase (TH) plays a central role in dopamine availability, as TH is the rate-limiting enzyme in its synthesis [12–15]. TH catalyzes the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylanine (L-DOPA), using the co-factors ferrous iron (Fe^{2+}), oxygen (O_2) and biopterin (BH4) [16,17]. Subsequently, the enzyme aromatic L-amino acid decarboxylase (AADC) converts L-DOPA into dopamine.

The 60 kDa TH protein consists of a C-terminal tetramerization domain, a central catalytic domain and an N-terminal regulatory domain [18–20]. Four TH proteins are able to oligomerize and form a functional tetrameric structure of 240 kDa [19]. The regulatory domain contains 3 conserved serine residues Ser19, Ser31 and Ser40 which all have a varying degree of influence on TH activity [21]. Additionally, Ser19 and Ser31 have been attributed diverse functions not directly related to enzymatic activity as will be further discussed below.

Ser19 phosphorylation is mediated by Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) and p38-regulated/activated protein kinase (PRAK) [22–26]. Phosphorylated Ser19 is found in a complex with 14-3-3 proteins that is believed to increase TH stability, as shown by decreased proteolysis upon trypsin treatment [27]. Additionally, this binding of 14-3-3 stimulates TH activity, presumably by preventing access of phosphatases to phosphorylated serine residues, which protects TH from being dephosphorylated [26,28,29].

Ser31 phosphorylation levels have been shown to be increased by KCL-induced depolarization in rat striatal slices. The depolarization induced increase in Ser31 phosphorylation was dependent on Ca^{2+} and extracellular signal-regulated kinases 1/2 (ERK1/2) [30–34]. Ser31 phosphorylation is linked to promote TH activity due to a decrease in Km value for the co-factor BH4 [24,33–35]. In addition to TH activity, Ser31 phosphorylation is linked to other processes such as stability, as cyclin-dependent kinase 5 (CDK5) induced the stabilization of the protein and reduced TH degradation after cycloheximide treatment [36]. Phosphorylation of Ser31 is suggested to be important in the somatodendritic compartments of dopaminergic cells, as higher phosphorylation levels are measured in these compartments, compared to terminal fields. The importance of phosphorylation was explained by a much greater dopamine turnover in these areas [37–40], and the phosphorylation of Ser31 is linked to subcellular localization along microtubules [24,33,34,41]. Finnigan *and colleagues* show that Ser31 phosphorylation by CDK5 or ERK1/2 targets TH to vesicles for transport along microtubules from the soma to the distal parts of neurites using vesicular monoamine transporter 2 (VMAT2) [41].

Ser40 is described to be phosphorylated by CAMKII, protein kinase- C (PKC) and A (PKA) [21,33,42–54]. Ser40 phosphorylation attenuates the end-product feedback inhibition of TH by catecholamines. This mechanism describes how catecholamines interfere with TH activity by binding almost irreversibly to the ferric iron in the catalytic domain of TH, thereby blocking binding of co-factor BH4 [12,15,20,51,54–64]. The result of this strict inhibitory control is that TH in dopaminergic neurons is present in a low activity state [33]. Ser40 phosphorylation is suggested to cause a conformational change due to electrostatic interactions, allowing the catecholamines to dissociate [65,66].

Besides the roles described for each individual phosphorylation site, prior phosphorylation of Ser19 and Ser31 has been suggested to increase the extent of Ser40 phosphorylation. This process is called hierarchical phosphorylation and has been explored in numerous studies [41,58,67,68]. Lehmann *and colleagues* (2006) were the first to report that in a dopamine-free experimental set-up phosphorylation of Ser31 and Ser19 produced a 9-fold increase in the rate of Ser40 phosphorylation in the presence of ERK1/2, while the phosphorylation of Ser40 had no effect on the rate of phosphorylation of either Ser31 or Ser19 [67]. Work from the same group found that downregulation of Ser31 phosphorylation by inhibiting the phosphorylation of ERK1/2 leads to a 50% decrease in basal levels of Ser40 phosphorylation [58]. Thus, Ser19 and Ser31 are proposed to indirectly stimulate TH catalytic activity by increasing the phosphorylation rate of Ser40 by its kinase [67].

Previous studies that investigated Th function have made use of a variety of cell types, such as rat pheochromocytoma (PC12) cells, bovine adrenal chromaffin cells (BACCs) and differentiated human SH-SY5Y cells [41,67,69–71]. In this study investigated signal transduction routes in the dopaminergic cell line MN9D. MN9D cells abundantly express dopamine neuron-specific markers, such as *Th*, *Aadc*, *Vmat2* and are capable of synthesizing and store dopamine [72–74]. Although they have a less efficient catecholamine reuptake system which is distinct from true dopaminergic neurons [72–75], we found MN9D cells as the best suitable dopaminergic cell line available to investigate Th regulation in a dopamine-rich environment [74].

Our results confirm that cAMP-dependent signaling induces Th phospho-Ser40 levels, presumably mediated by PKA. Surprisingly, this signaling route has revealed to downregulate Ser31 phosphorylation as well, which was not due to intrinsic changes within Th itself, but by upstream inhibitory signaling between PKA and Erk1/2. Finally, using phospho-mimetic or unphosphorylated mutants of *Th* we thoroughly tested if Th is subject to a form of hierarchical phosphorylation, in which one serine residue may affect the rate of phosphorylation of the other residue. Strikingly, we show that Ser40 phosphorylation is required for phosphorylation of Ser31, contradicting the existing hypothesis of hierarchical TH phosphorylation. Overall, our results suggest that cAMP-dependent phosphorylation of Ser40 is crucial for TH activity and subsequent regulation.

Materials and methods

Cell culture and transfection

Neuroblastoma 2A cells (Neuro2A, mouse neuroblastoma cells, ATCC) were cultured in 100 mm Petri dishes. Mouse dopaminergic MN9D cells [72,75], were grown in poly-d-lysine (PDL, P6407; Sigma-Aldrich) -coated 100 mm Petri dishes. Cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM, D6429; Sigma) supplemented with 2 mM l-glutamine (25030081; ThermoFisher Scientific), 1 unit/ml Penicillin/Streptomycin (15140163; ThermoFisher Scientific) and 10% (v/v) heat inactivated fetal bovine serum (HiFBS) MN9D: S181B; Biowest, Neuro2A: 10082147; ThermoFisher Scientific). Cells were incubated in a 37°C incubator with a humidified atmosphere of 95% air and 5% CO_2. Cells were not allowed to exceed 90% confluency before passaging and were not used for more than 30 passages.

For passaging, cultures were rinsed with phosphate buffered saline (PBS, 10010023; ThermoFisher Scientific) and incubated with 1 mL of 1x trypsin (15400054; ThermoFisher Scientific) in PBS for 5 minutes. For transient transfections of Neuro2A cells, cells were grown to ~75% confluency. A mixture of DNA plasmid and lipofectamine 2000 reagent (11668019; Invitrogen) was prepared according to manufacturer's instructions and incubated with the cells. After ~8 hours, the lipofectamine/DNA mix was replaced with regular growth medium and incubated for ~24 hours.

Plasmids

The DNA plasmids (pcDNA3.1 backbone; 6His N-terminal translational fusion; Genscript, Leiden) used encode either empty vector, mouse wild-type tyrosine hydroxylase or site-directed mutagenesis variants thereof. In these tyrosine hydroxylase mutants, the serine residues are replaced for either an alanine (A), to mimic an unphosphorylated state or aspartic acid (D), mimicking the phosphorylated state.

Chemical treatment and sample preparation

Before the experiments were performed the cells were serum-deprived for ~16 hours in DMEM (0% and 0.5% HiFBS for Neuro2A cells and MN9D cells, respectively). 0.5% HIFBS was included for MN9D cells to prevent detachment of cells. Experiments were performed in plates that were ~90% confluent. A table of the chemicals used in the experiments can be observed on the next page (Table 1). Controls were all treated with the appropriate amount of vehicle. After treatment, cells were washed with 1X PBS pH 7.4 and lysed in 100 or 200 µL, for 24 or 12 plates 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) supplemented with 50 mM dithiotretol (DTT; Merck Millipore). Samples were collected, sonicated for 3 min in a Bioruptor sonicator (Diagenode) at maximum potency, heated at 95°C for 5-10 min, and briefly spun down.

WES™ Protein detection

Evaluation of protein expression was performed using the Wes™ automated capillary western blot system (Protein Simple) according to manufactures instructions and under the default settings. Briefly, 1 part of prepared cell lysate samples were diluted in 9 parts of Milli-Q 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 (1:100, P40101; Pel-Freez); rabbit anti-phospho-tyrosine hydroxylase (Ser40) (2791S; CST); rabbit anti-phospho-tyrosine hydroxylase (Ser31) (13041S; CST); rabbit anti-β-actin (4907S; CST); rabbit anti-Creb (4820S; CST); rabbit anti-phospho-Creb (Ser133) (9198S; CST); rabbit anti-p44/42 MAPK (Erk1/2) (4695S; CST); rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (4370; CST). Samples from Supplemental Fig. 1 were probed with rabbit anti-phospho-tyrosine hydroxylase (Ser40) (P41301; Pel-Freez). All antibodies were diluted 1:50 in antibody diluent (042-203; ProteinSimple).

Table 1. Chemicals used for pharmacological treatment

Name	Concentration (μM)	Vendor	Category number
Forskolin	1-100	Cell Signaling Technology	3828S
Dibutyryl cAMP	500	Sigma-Aldrich	D0627
pCPT-cAMP	500	Sigma-Aldrich	C3912
8-Br-cAMP	500	Sigma-Aldrich	B7880
007-AM	10	ToCris	4853
H-89	30	Cayman Chemical	10010556
U0126	10	Cayman Chemical	70970
PD98059	50	Cayman Chemical	10006726
KN-62	10	Cayman Chemical	13318
KN-93			13319
SB203580			13067
SB202190			10010399
Olomoucine			10010240
Roscovitine			10009569
CHIR99021			13122
TWS119			10011251
LY294002			70920
Quercetin			6151-25-3

Table 2. Inhibition of protein kinases by commercially available protein kinase inhibitors (The first named kinase demonstrates the largest efficacy of kinase inhibition)

Inhibitor	Protein kinase targets	Reference
H-89	PKA, S6K1, MSK1	Lochner et al., 2006 [76], Bain et al., 2007 [77], Davies et al., 2000 [78]
PD98059	MAPKK	Cohen et al., 2002 [79], Rosen et al., 1994 [80], Davies et al., 2000 [78], Alessi et al., 1995 [81]
U0126	MEK2, MEK1, MKK1	Favata et al., 1998 [82], Bain et al., 2007 [77], Davies et al., 2000 [78]
KN-62	CAMKII, PRAK, GSK3	Davies et al., 2000 [78]
KN-93	CAMKII, CAMKI, CAMKIV	Gao et al., 2013 [83], Pellicena et al., 2014 [84]
SB203580	p38α MAPK, P38β MAPK, EGFR, JNK3	Gao et al., 2013 [83], Bain et al., 2007 [77], Davies et al., 2000 [78]
SB202190	p38α MAPK, P38β MAPK, CK1δ, JNK3	Gao et al., 2013 [83], Bain et al., 2007 [77], Davies et al., 2000 [78]
Olomoucine	CDK5, CDK1, CDK2, CDK7	Demange et al., 2013 [85]
Roscovitine	CDK5, CDK1, CDK2, ERK8, RSK1	Demange et al., 2013 [85], Bain et al., 2007 [77], Bain et al., 2003 [86]
CHIR99021	GSK3	Meijer et al., 2004 [87]
TWS119	GSK3	Daub et al., 2004 [88]
LY294002	PI3K, CK2, PLK1, PIM1, PIM3	Bain et al., 2007 [77], Davies et al., 2000 [78]
Quercetin	AMPK, PI3K, CK2, MAPKAPK, S6K1	Bain et al., 2007 [77], Davies et al., 2000 [78]

Western blotting
Samples were briefly spun down before loading. Proteins were loaded and separated on an SDS-PAGE gel (10–15% depending on molecular weight; 375 mM Tris-Cl pH 8.8, 0.1% APS, 0.1% SDS, 0.04% TEMED), followed by transfer to 0.2 µm nitrocellulose membrane. Subsequently, membranes were blocked in 5% ELK milk powder in TBS-T. Blots were incubated with the appropriate primary antibody in TBS-T overnight at 4 °C. Blots were incubated with rabbit anti-tyrosine hydroxylase (P40101; Pel-Freez; 1:1000) or mouse anti-β-actin (3700; CST; 1:5000). After incubation with the appropriate HRP-conjugated secondary antibody in TBS-T, the blots were exposed to enhanced chemiluminescence (ECL) reagents. ECL reagents contained 250 mM luminol (Fluka Analytical), 90 mM p-coumaric acid (Sigma-Aldrich), 1 M Tris-HCl pH 8.5 and 0.02% H_2O_2. Chemiluminescence was detected in an Odyssey FC Imaging System (LI-COR Biosciences). Densitometric analyses were performed using ImageStudio Lite software.

Statistical analysis
The amount of phospho-protein is first corrected for the total amount of protein and normalized to the average of the control condition (vehicle). To determine statistical significance, one way analysis of variance (ANOVA) was performed, followed by Bonferroni's multiple comparisons post hoc test. The data are expressed as fold change compared to the control condition (control = 1) and presented as bar charts showing the mean ± SEM. Differences were considered significant at a p-value < 0.05. Asterisks indicate significance (n.s. $p > 0.05$, *$p < 0.05$ and **$p < 0.01$).

Results

Validation of phospho-specific Th antibodies
First, we tested the specificity and selectivity of the (phospho-specific) Th antibodies. We used Th transfected Neuro2A cells, a cell line that endogenously does not contain detectable Th protein levels (Fig. 1A). These Neuro2A cells were transfected with either pcDNA3.1 empty vector, wild-type Th (*Th-WT*) or Th mutants (*Th-S40A* and *Th-S31A* respectively).

The Th-specific antibody (P40101; Pel-Freez) successfully detected Th levels at peak values of 58 kDa, corresponding with its theoretical molecular weight (Fig. 1A-G). The Th Ser40 phospho-specific antibody (2791S; CST) successfully detected phosphorylated Th whereas it failed to detect phosphorylation in *Th-S40A,* demonstrating its specificity (Fig. 1B,C). Similarly, the antibody targeting Ser31 (13041S; CST) also showed high specificity for its respective residue (Fig. 1D,E). A presumed phospho-specific antibody targeting Ser40 phosphorylation (P41301; Pel-Freez) detected *Th-WT*, and also showed a peak for *Th-S40A* (100x zoomed in scale), demonstrating that this antibody is less specific for Ser40 Th phosphorylation and as such was not further used in this study (Supplemental Fig. 1A-C).

cAMP-dependent manipulation of Th phosphorylation in dopaminergic MN9D cells

Forskolin alters phosphorylation of Th in a dose-dependent matter
Forskolin rapidly increases cAMP levels via activation of adenylyl cyclases [89] and is known to induce Th phospho-Ser40 levels in PC12 cells and bovine adrenal cells [90–92]. To revisit regulation of Th phosphorylation in MN9D cells, we exposed these cells to forskolin and

monitored phospho-Ser40 levels with phospho-specific antibodies. Cells were exposed to either forskolin for 60 minutes at different concentrations (1, 3, 10, 30, or 100 µM; Fig. 2A-C, left panels) or to 10 µM forskolin at different time points (5, 15, 30, 60, or 120 minutes; Fig. 2A-C, right panels).

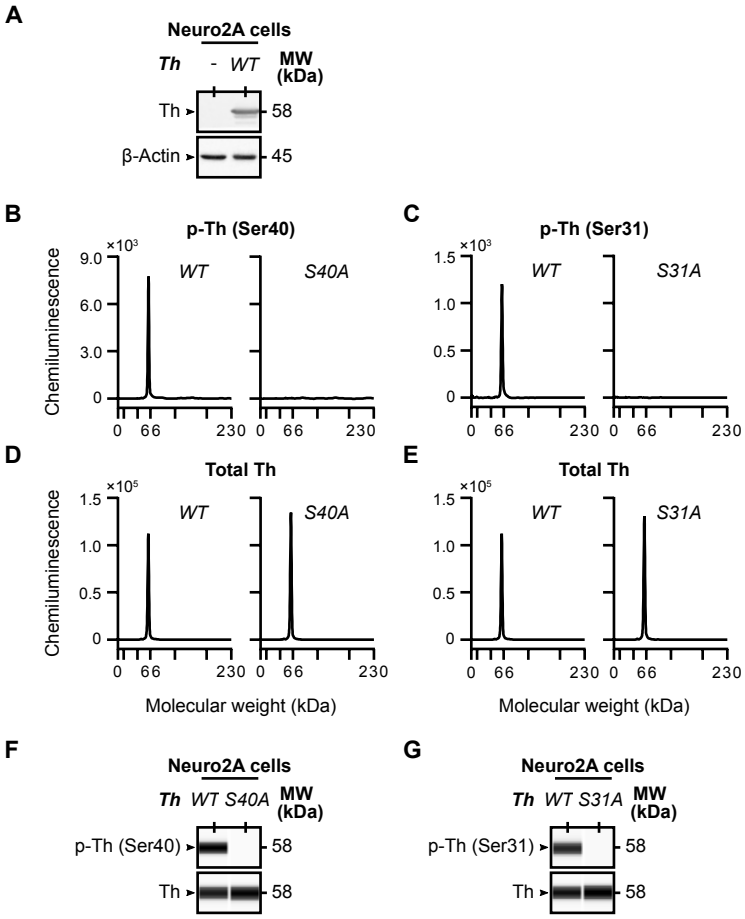


Figure 1: Validation of antibodies against phosphorylated Th forms

The specificity of the phosphorylated Th antibodies was validated using Neuro2A cells. Neuro2A cells were transfected with pcDNA3.1 empty vector, wild-type Th (*Th-WT*) or phospho-mimetic Th mutants. In these mutants, the serine residues at either position 40 (Ser40), position 31 (Ser31) or position 19 (Ser19) are replaced with an alanine (*Th- S40A*, *S31A* and *S19A,* respectively). **A |** Regular western blot images for protein levels of Th and β-actin. Neuro2A cells do not show detectable Th protein endogenously, however shows signal in the *Th-WT* condition. **B-E |** Electropherograms of chemiluminescence signals representing Th protein levels. Peak values of 58 kDa were found, which corresponds with its theoretical molecular weight. The antibody targeting (**B**) phospho-Ser40 shows signal in the *Th-WT* condition (left panel), but no signal for the *Th-S40A* mutant (right panel). The antibody targeting (**C**) phospho-Ser31 shows signal in the *Th-WT* condition (left panel), and no signal for the *Th-S31A* mutant (right panel). The antibody targeting (**D**,**E**) total Th shows signal for both the mutated and *WT-Th* variant. **F,G |** Images representing the upper electropherograms in a traditional western blot-like appearance.

In MN9D cells, forskolin increases Th phospho-Ser40 levels in a dose-dependent manner (Fig. 2B, left panel; $M > 2.79$). Increased phosphorylation of Ser40 could already be observed as early as 5 minutes after application (Fig. 2B, right panel; $p < 0.01$, $M > 4.24$). Surprisingly, phospho-Ser31 levels are decreased at all concentrations (Fig. 2C, left panel; $p < 0.01$, $M < 0.65$) and time-points tested (Fig. 2C, right panel; $p < 0.01$, $M < 0.40$). In conclusion, forskolin application leads to increases of phospho-Ser40 levels, while phospho-Ser31 levels are downregulated.

cAMP signaling affects Th protein phosphorylation in MN9D cells

Since the effects of forskolin on Th phosphorylation are suggested to be mediated via increases in intracellular cAMP, we subsequently tested several cell-permeable analogs of cAMP, to substantiate this hypothesis. We tested dibutyryl cAMP (N6,O2'-Dibutyryl cAMP salt), pCPT-cAMP (8-(4-Chlorophenylthio) cAMP salt) and 8-Br-cAMP (8-Bromo cAMP salt) at recommend effective concentrations [93,94]. These analogs differ in the potency of permeability and substrate affinity [95].

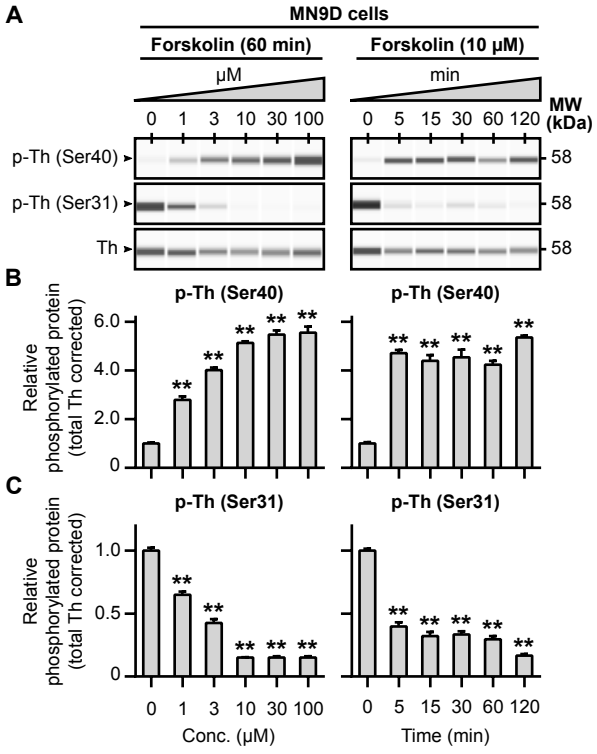


Figure 2: Forskolin increases Th phospho-Ser40 and decreases phospho-Ser31 levels in MN9D cells

The effect of forskolin on Th phosphorylation was investigated in MN9D cells. MN9D cells were exposed to different concentrations of forskolin for 60 minutes (left panels) or for different time-points at 10 µM (right panels). **A |** The effect of forskolin on Th, phospho-Th (Ser40), and phospho-Th (Ser31) levels. Quantitative analysis of (**B**) phospho-Ser40 and (**C**) phospho-Ser31 levels shows that forskolin increases Ser40 and downregulates Ser31 phosphorylation levels in MN9D cells, both dose- and time- dependently. Data are mean ± SEM, *$p < 0.05$ and **$p < 0.01$ (One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test), $n = 4$.

First, we incubated MN9D cells with 500 µM dibutyryl cAMP at different time points (5, 15, 30, 60, or 120 minutes; Fig. 3A-C). Similar to forskolin, dibutyryl cAMP induced potent increases in phospho-Ser40 levels (Fig. 3B; $p < 0.01$, $M > 2.06$) and decreases in phopsho-Ser31 levels (Fig. 3C; $p < 0.01$, $M < 1.06$). Regarding Ser31 phosphorylation, post-hoc analysis only showed significant decreases after at least 30 minutes of incubation with dibutyryl cAMP ($p < 0.03$, $M < 0.675$).

Incubation with other analogs dibutyryl cAMP, pCPT-cAMP or 8-Br-cAMP for 30 or 60 minutes demonstrated increases phospho-Ser40 levels and decreases in phospho-Ser31 levels (Fig. 3D-F). Dibutyryl cAMP ($M > 11.5$) showed the most robust increase for Ser40 phosphorylation, while 8-Br-cAMP ($M > 4.12$) and pCPT-cAMP ($M > 4.26$) were comparable. The robust increase in phospho-Ser40 by dibutyryl cAMP was not matched by an equally robust downregulation of Ser31 as all three analogs performed similarly ($M < 0.6$).

Besides activating PKA, cAMP can activate exchange protein activated by cyclic AMP (Epac) [96,97]. Therefore, we investigated the effect of 007-AM, a cAMP analog that specifically activates Epac, but not PKA at 10 µM for 30 or 60 minutes on Th phosphorylation (Fig. 3G-I). 007-AM also showed significant increases in phospho-Ser40 levels (Fig. 3H; $M > 2.95$). Th Ser31 phosphorylation levels were also downregulated by 007-AM (Fig. 3I; $M < 0.40$). Please note that, β-actin protein levels are reduced in 007-AM treated cells. This could have been due to cells detaching during the treatment with this inhibitor.

Protein kinase signaling upstream of Th phosphorylation in dopaminergic cells

Various kinases have been described to be upstream of Ser40 and Ser31 phosphorylation. Since forskolin/cAMP/PKA signaling is a well described pathway upstream of Ser40, we tested if we could block phosphorylation of Ser40 with H-89, a specific PKA inhibitor (Fig. 4A) [76–78].

Cells were exposed to 30 µM H-89 or vehicle, prior to forskolin treatment. H-89 treatment indeed attenuated Ser40 phosphorylation in response to forskolin treatment (Fig. 4B,C; $p < 0.01$, $M_{H\text{-}89} = 1.86$ vs $M_{H\text{-}89+Forskolin} = 2.93$). Unexpectedly, we observed that H-89 also affected phospho-Ser31 levels, as it decreased upon addition of H-89 and decreased even further after forskolin treatment (Fig. 4B,D; $p < 0.01$, $M_{H\text{-}89} = 0.34$ vs $M_{H\text{-}89+Forskolin} = 0.20$). The effectiveness of H-89 treatment was confirmed by measuring the level of Creb phosphorylation on Ser133, a well described PKA substrate [98,99]. Indeed, H-89 reduced phospho-Creb levels dramatically (Fig. 4E,F; $p < 0.01$, $M_{Forskolin} = 29.16$ vs $M_{H\text{-}89+Forskolin} = 3.49$), indicating that the inhibitor is effective. As ERK1/2 and CDK5 are the major protein kinases responsible for the phosphorylation of Ser31 [24,33,100–104], we speculated that the reduction of Ser31 phosphorylation may be the result of crosstalk between the cAMP and Erk1/2 pathway [42,105–108]. H-89 significantly lowers phospho-Erk1/2 levels (Fig. 4E,G; $p < 0.01$, $M_{vehicle} = 1.00$ vs $M_{H\text{-}89} = 0.43$), while forskolin also downregulates the phosphorylation of Erk1/2 (Fig. 4E,G; $p < 0.01$, $M_{vehicle} = 1.00$ vs $M_{Forskolin} = 0.30$). Additionally, there is a significant difference in forskolin-induced effects on phospho-Erk1/2 levels after pre-incubation with H-89 (Fig. 4E,G; $p < 0.05$, $M_{H\text{-}89} = 0.43$ vs $M_{H\text{-}89+Forskolin} = 0.15$).

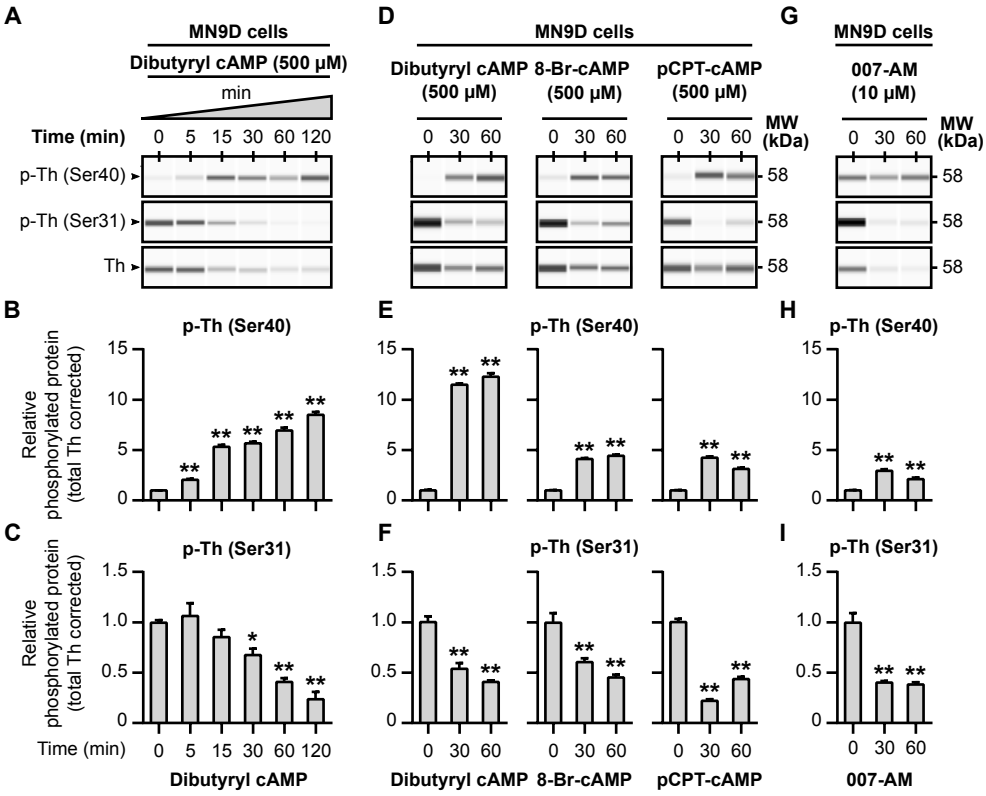


Figure 3: cAMP analogs increase Th phospho-Ser40 and decrease phospho-Ser31 levels in MN9D cells

cAMP-mediated signaling was investigated on Th phosphorylation in MN9D cells, using cAMP analogs. **A-C |** Time-response experiments for the effects of 500 µM cAMP analog dibutyryl cAMP on Th, phospho-Ser40, phospho-Ser31, and β-actin levels in MN9D cells. Quantifications of **(B)** phospho-Ser40 and **(C)** phospho-Ser31 shows that dibutyryl cAMP time-dependently increases Ser40 and downregulates Ser31 phosphorylation levels. **D-F |** The effect of 30- and 60-minutes exposure of 500 µM cAMP analogs dibutyryl cAMP (left panel), 8-Br-cAMP (middle panels), pCPT-cAMP (right panels) on Th, phospho-Ser40, phospho-Ser31, and β-actin levels. **G-I |** The effect of 30- and 60-minutes exposure of 10 µM Epac activation with 007-AM on Th, phospho-Ser40, phospho-Ser31, and β-actin levels. Quantitative analysis of **(E,H)** phospho-Ser40 and **(F,I)** phospho-Ser31 shows that all three cAMP analogs and the specific Epac activator 007-AM increase Ser40 and decrease Ser31 phosphorylation levels. Data are mean ± SEM, $*p < 0.05$ and $**p < 0.01$ (One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test), n ≥ 4.

The role of Erk1/2 upstream of Ser31 phosphorylation was further investigated with the MEK1/2 inhibitors U0126 and PD98059 (Fig. 5A), both small molecule kinase inhibitors of the MAPK pathway. Both U0126 or PD98059 resulted in a downregulation of Ser31 phosphorylation (Fig. 5B,C; $p < 0.01$, $M_{U0126} = 0.44$, $M_{PD98059} = 0.47$), without affecting Ser40 phosphorylation (Fig. 5B,D). Moreover, forskolin decreases the phosphorylation of Ser31 even further in combination with these two inhibitors (Fig. 5B,C; $p < 0.01$, $M_{U0126+Forskolin} = 0.14$, $M_{PD98059+Forskolin} = 0.13$). The effects of the inhibitors and forskolin on phospho-Ser31 levels matched the effects of these inhibitors on phosphorylation of Erk1/2 substantiating the potential role of the Erk1/2 pathway upstream of Ser31 phosphorylation.

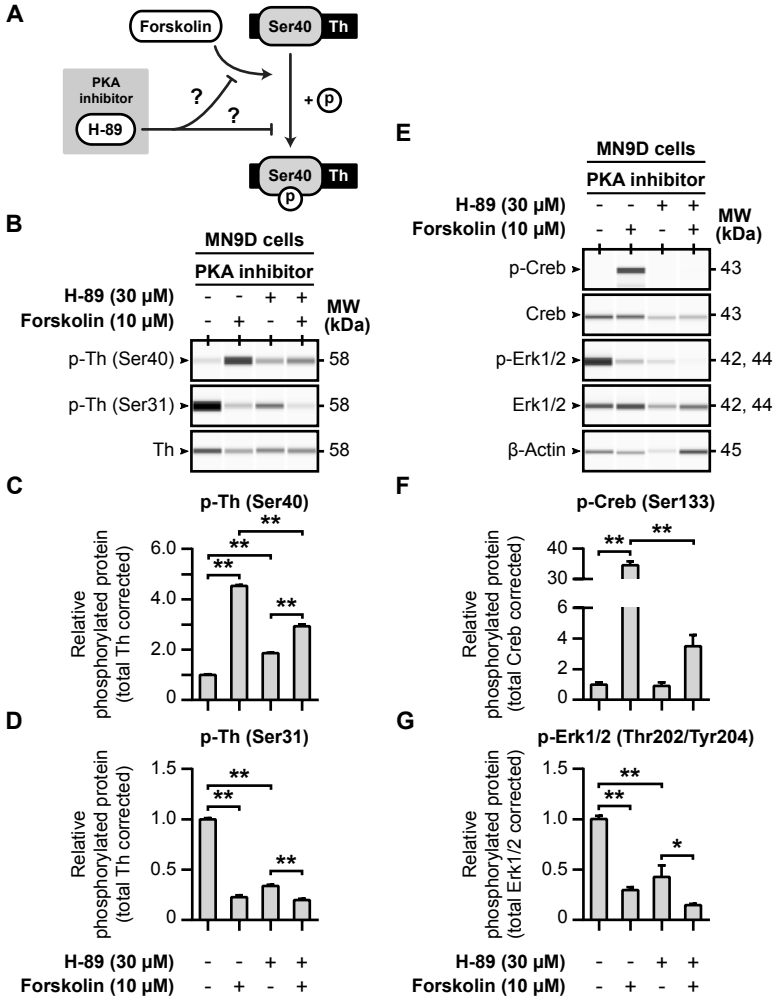


Figure 4: Inhibition of PKA signaling attenuates Th phospho-Ser40 and phospho-Ser31 levels in MN9D cells

The role of PKA-mediated signaling on Th phosphorylation was investigated using the PKA inhibitor H-89. **A |** Schematic of the experimental setup to investigate how H-89 modulates Ser40 phosphorylation levels, either with or without exposure to forskolin. MN9D cells were preincubated for 45 minutes in the presence or absence of 30 µM H-89, followed by 60 minutes exposure to either vehicle or 10 µM forskolin. **B-D |** The effect of the presence or absence of H-89 on the effect of forskolin on Th, phospho-Ser40 and phospho-Ser31 levels. Quantitative analysis showed that **(C)** phospho-Ser40 levels are higher when incubated with H-89. With or without H-89, forskolin increased Ser40 phosphorylation levels and the effect of forskolin is downregulated when pre-incubated with H-89. Quantifications demonstrate that **(D)** phospho-Ser31 levels are decreased when incubated with H-89. With or without H-89, forskolin decreases phospho-Ser31 levels and no effect of H-89 on phospho-Ser31 was observed when exposed to forskolin. **E-G |** The effect of the presence or absence of H-89 on the effect of forskolin on Creb, phospho-Creb (Ser133), Erk1/2, phospho-Erk1/2 (Thr202/Tyr20), and β-actin levels. Data analysis demonstrated **(F)** no differences in phospho-Creb (Ser133) levels when incubated with H-89. Forskolin significantly increased phospho-Creb (Ser133) levels and when incubated with H-89, this effect is downregulated and not significant. Quantitative analysis showed that **(G)** H-89 incubation decreases phospho-Erk1/2 (Thr202/Tyr204) levels. With or without H-89, forskolin decreases phospho-Erk1/2 (Thr202/Tyr204) levels, with no difference in phosphorylation levels after forskolin exposure due to H-89 incubation. Data are mean ± SEM, $*p < 0.05$ and $**p < 0.01$ (One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test), $n = 4$.

Exploring additional upstream Th protein kinase signaling routes

After thorough investigation into the effects of PKA and Erk1/2 signaling on Th phosphorylation (Fig. 4 and Fig. 5), we tested several additional kinase inhibitors on Th phosphorylation in our in vitro set-up. Table 3 below represents fold changes (corrected for total Th levels) of inhibitor treated MN9D cells on Th Ser40 and Ser31 phosphorylated protein levels compared with vehicle. Significance was determined with a two-sided unpaired student's t-test. MN9D cells were incubated with 10 µM compound or vehicle for 105 minutes.

Table 3. Protein kinase inhibitors on Th phospho-Ser40 and phospho-Ser31 levels (fold change; n = 4)

Inhibitor	Kinase	p-Ser40	p-value	p-Ser31	p-value
KN-62	CAMKII	0.96	0.367	0.98	0.704
KN-93	CAMKII	0.84	0.113	0.70	0.100
SB203580	P38MAPK	1.59	0.000	1.84	0.000
SB202190	P38MAPK	1.37	0.018	1.44	0.003
Olomoucine	CDK5	0.98	0.807	0.85	0.064
Roscovitine	CDK5	2.13	0.000	0.34	0.000
CHIR99021	GSK3	1.33	0.005	0.86	0.044
TWS119	GSK3	1.21	0.090	1.08	0.155
LY294002	PI3K	1.51	0.264	0.60	0.022
Quercetin	PI3K	1.17	0.000	0.53	0.000

Interestingly, CAMKII inhibition using the inhibitors KN-62 or KN-93 did not affect phosphorylation levels of Ser40 or Ser31, although prior studies demonstrated that Ser40 can be phosphorylated by CAMKII in vitro [48]. Second, results demonstrate that inhibition of p38 mitogen-activated protein kinases (p38MAPK) by SB203580 or SB202190 significantly increases Th phospho-Ser40 and phospho-Ser31 in MN9D cells. In literature, p38-stimulated protein kinase is shown to phosphorylate Th Ser19 in situ [21,109], however this was linked to protect Ser19 and Ser40 from dephosphorylation by phosphatases [26,28,29]. Third, the CDK5 inhibitor Roscovitine, but not Olomoucine increased the phosphorylation of Ser40 and downregulated Ser31 phosphorylation in MN9D cells. In literature, Th can be phosphorylated at Ser31 by CDK5 in vitro [21,30,36], which corresponds to inhibition by Roscovitine. However, the structurally dissimilar Olomoucine fails to do so, suggesting a Roscovitine effect not related to CDK5. The GSK3 inhibitors CHIR99021 and TWS119 also led to inconsistent results as CHIR99021 but not TWS119 was able to increase Th phospho-Ser40 levels and downregulate Ser31 phosphorylation. Next, PI3K inhibitors LY294002 and Quercetin significantly downregulated Ser31 phosphorylation in MN9D cells, which has not been described before.

In summary, PKA inhibition demonstrated that cAMP-dependent Ser40 phosphorylation is attenuated, which indicates that PKA is upstream of Th Ser40 phosphorylation. The cAMP-dependent downregulation of Th Ser31 and Erk1/2 phosphorylation is suggested to be mediated by a PKA-MEK1/2 inhibitory crosstalk mechanism, which places Erk1/2 upstream of Ser31 phosphorylation.

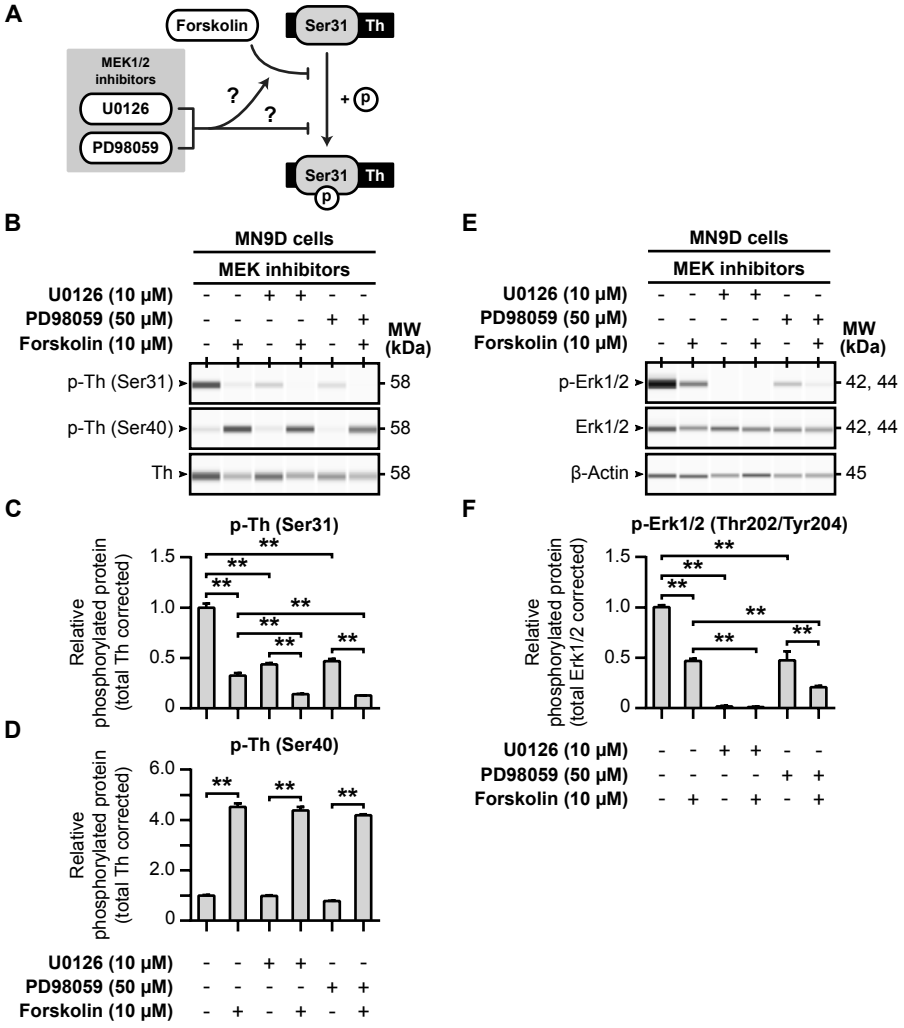


Figure 5: Inhibition of MAPK signaling attenuates Th phospho-Ser31 and phospho-Erk1/2 levels in MN9D cells

MAPK-mediated signaling was explored on the phosphorylation of Th and Erk1/2 using the MEK1/2 inhibitors U0126 and PD98059. **A |** Schematic of the experimental setup to investigate how the inhibitors U0126 and PD98059 modulate phospho-Ser31 levels by inhibiting MEK1/2 signaling, either with or without exposure to forskolin. MN9D cells were preincubated for 45 minutes in the presence or absence of either 10 μM U0126 or 50 μM PD98059, followed by 60 minutes exposure to either vehicle or 10 μM forskolin. **B-D |** The effect of either U0126, PD98059 and/or forskolin on Th, phospho-Ser31 and phospho-Ser40 levels. Quantitative analysis of **(C)** phospho-Ser31 levels. With or without U0126 or PD98059, forskolin downregulates phospho-Ser40 levels compared to vehicle conditions. In both the presence or absence of forskolin, however, phospho-Ser31 levels are lower when incubated with U0126 or PD98059. Quantifications of **(D)** phospho-Ser40 levels. With or without U0126 or PD98059, forskolin increases phospho-Ser40 levels. No differences in phospho-Ser40 levels are found between vehicle or either U0126 or PD98059. **E,F |** The effect of either U0126, PD98059 and/or forskolin on Erk1/2, phospho-Erk1/2 (Thr202/Tyr20), and β-actin levels. Quantitative analysis shows that **(F)** both U0126 and PD98059 downregulate phospho-Erk1/2 (Thr202/Tyr204). Data are mean ± SEM, *p < 0.05 and **p < 0.01 (One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test), n = 4.

Hierarchical phosphorylation: Ser40 phosphorylation is required for Ser31 phosphorylation

Our results regarding application of forskolin and cAMP analogs show that upon the increase in Ser40 phosphorylation Ser31 is decreased, whereas the state of Ser31 did not influence the levels of Ser40 phosphorylation (Fig. 2, Fig. 3 and Fig. 5, respectively). This is contrary to the postulated hierarchical phosphorylation hypothesis as this hypothesis postulates that an increase in Ser31 phosphorylation should result in an increase in Ser40 phosphorylation [67]. These findings do not suggest that this hypothesis is true as our results demonstrate that MEK1/2 inhibitors reduced the levels of phospho-Ser31 without affecting Ser40 phosphorylation (Fig. 5). In order to elucidate this possible interaction between Ser 8, 19, 31 and 40 phosphorylation events we generated mutant TH forms where these residues are either changed to an alanine (unphosphorylated form) or an aspartate (phospho-mimetic).

First, we show the effects of introducing Ser08 mutants or Ser19 mutants (Fig. 6A-C or Fig. 6D-F, respectively) on relative phosphorylation levels of Ser31 and Ser40 in transfected Neuro2A cells. *Th-S08A* had a significant downregulating effect on basal Ser40 phosphorylation (Fig. 6A,B; $p < 0.05$, $M = 0.84$) and whereas *Th-S08D* had no significant effect on basal Ser40 phosphorylation ($p > 0.05$, $M = 1.03$). Interestingly, in *Th-S08D* transfected cells, the effect of forskolin on Ser40 is modestly higher compared to *Th-WT* (Fig. 6A,B; $p < 0.05$, $M_{Th\text{-}S08D} = 2.30$ vs $M_{Th\text{-}WT} = 2.11$). Also, *Th-S08A* showed lower basal Ser31 phosphorylation levels compared to *Th-WT* (Fig. 6A,C; $p < 0.01$, $M = 0.70$). *Th-S19D* transfected cells affected the phosphorylation of Ser40 by forskolin, as forskolin treated *Th-S19D* transfected cells showed a modest increase in Ser40 phosphorylation compared to *Th-WT* (Fig. 6D,E; $p < 0.01$, $M_{Th\text{-}S19D} = 2.53$ vs $M_{Th\text{-}WT} = 2.06$). No differences were observed between *Th-S19A* mutant and *Th-WT* when exposed to forskolin, however *Th-S19A* had a significant downregulating effect on basal Ser31 phosphorylation (Fig. 6D,F; $p < 0.01$, $M = 0.66$), whereas *Th-S19D* did not.

Second, we demonstrated the effects of introducing Ser31 mutants or Ser40 mutants (Fig. 7A-C or Fig. 7D-F, respectively) on relative phosphorylation levels of Ser31 and Ser40 in transfected Neuro2A cells. *Th-S40D* had a significant effect on basal Ser31 phosphorylation (Fig. 7D,F; $p < 0.01$, $M = 0.53$) and on the effect of forskolin on Ser31 phosphorylation (Fig. 7D,F; $p < 0.01$, $M = 0.46$ vs. 0.14) compared with *Th-WT*. Both *Th-S31A* ($M = 1.18$) and *Th-S31D* ($M = 1.08$) had no significant effect on basal Ser40 phosphorylation (Fig. 7A,B), indicating that the state of Ser31 phosphorylation does not affect Ser40 phosphorylation. Most interestingly, phospho-Ser31 levels are not detectable in the *Th-S40A* mutant, while basal levels are detectable but decreased in the *Th-S40D* mutant (Fig. 7D,F; $p < 0.01$, $M = 0.53$). This suggests that Ser31 phosphorylation is dependent on the Ser40 phosphorylation state.

In summary, it has been suggested that the phosphorylated state of Ser08 facilitates the phosphorylation of Ser31 and Ser40, whereas the phosphorylated state of Ser19 facilitates Ser31 phosphorylation. Most interestingly, Ser40 phosphorylation is required for Ser31 phosphorylation while it is independent of the state of Ser31 phosphorylation.

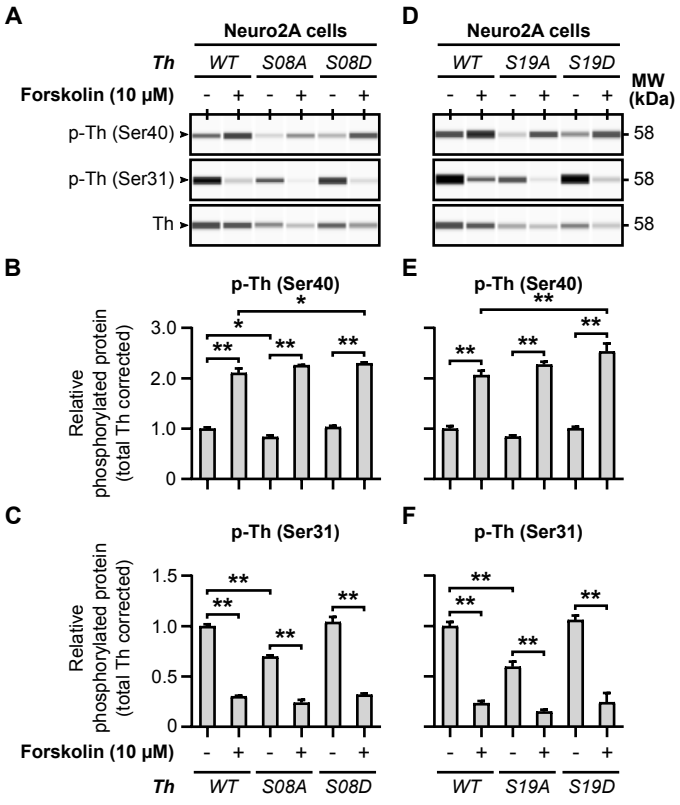


Figure 6: Ser08 or Ser19 mutagenesis affects the phosphorylation state of Ser40 and Ser31 in transfected Neuro2A cells

The effect of Th Ser08 and Ser19 unphosphorylated and phospho-mimetic mutagenesis on Th phosphorylation was tested in Neuro2A cells. Cells were transfected with plasmid DNA encoding either wild-type Th (*Th-WT*) or Th phospho-mimetic mutants. We used site-directed mutagenesis of Th Ser08 and Ser19, to an alanine (*Th-S08A* and *Th-S19A*, respectively) or aspartic acid (*Th-S08D* and *Th-S19D*, respectively). To manipulate phosphorylation, cells were exposed to either 10 µM forskolin or vehicle for 60 minutes. **A-F |** The effect of phospho-mimetic mutants of (**A-C**) Ser08 and (**D-F**) Ser19 on Th, phospho-Ser40 and phospho-Ser31 levels, either in the presence or absence forskolin. Quantitative analysis shows that compared with *Th-WT*, **(B)** phospho-Ser40 levels are downregulated in *Th-S08A* and the effect of forskolin on Ser40 phosphorylation is increased in *Th-S08D*. **(C)** *Th-S08A* downregulated basal Ser31 phosphorylation. **(E)** Phosho-Ser40 levels are higher in *Th-S19D* in forskolin treated cells. **(F)** Phospho-Ser31 levels are decreased in *Th-S19A* transfected cells. Data are mean ± SEM, **p* < 0.05 and ***p* < 0.01 (One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test), *n* = 4.

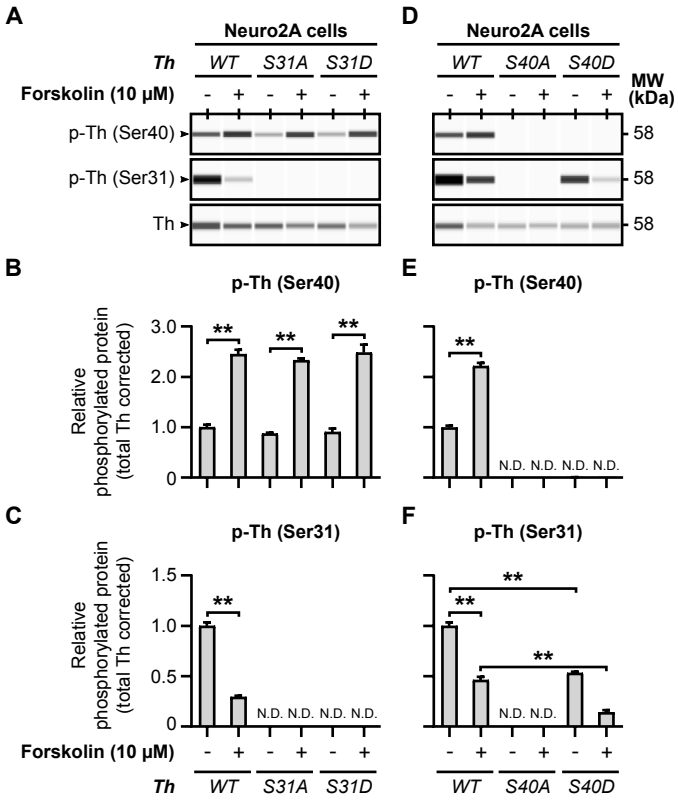


Figure 7: Phosphorylation of Th at Ser40 is required for Ser31 phosphorylation in transfected Neuro2A cells

The effect of Th Ser31 and Ser40 unphosphorylated and phospho-mimetic mutagenesis on Th phosphorylation was tested in Neuro2A cells. Cells were transfected with plasmid DNA encoding either wild-type Th (*Th-WT*) or Th phospho-mimetic mutants. We used site-directed mutagenesis of Th Ser31 and Ser40, to an alanine (*Th-S31A* and *Th-S40A*, respectively) or aspartic acid (*Th-S31D* and *Th-S40D*, respectively). To manipulate phosphorylation, cells were exposed to either 10 µM forskolin or vehicle for 60 minutes. **A-F |** The effect of phospho-mimetic mutants of (**A-D**) Ser31 and (**E-H**) Ser40 on Th, phospho-Ser40 and phospho-Ser31 levels, either in the presence or absence forskolin. Quantitative analysis shows that compared with *Th-WT*, **(B)** comparable phospho-Ser40 levels are detectable in either *Th-S31A*, or *Th-S31D*. **(C,E)** When *Th-S31A*, *Th-S31D*, *Th-S40A* and *Th-S40D* are introduced, no phosphorylation signal can be detected (N.D., not detected) for the specific phospho-antibody, whereas total Th levels can still be detected in all conditions. **(F)** Contrarily, in *Th-S40A* samples, no signal can be detected (N.D.) for phospho-Ser31, this while in *Th-S40D*, phospho-Ser31 protein is detected. *Th-S40D* downregulated basal and forskolin induced phospho-Ser31 levels. Data are mean ± SEM, $*p < 0.05$ and $**p < 0.01$ (One-way analysis of variance (ANOVA) with Bonferroni multiple comparison post hoc test), $n = 4$.

Discussion

In this chapter our objective was to revisit Th regulation in MN9D cells and to investigate a possible hierarchical phosphorylation mechanism involving Ser08, Ser19, Ser31 and Ser40 in Th transfected Neuro2a cells as a complementarity neuronal cell model. We demonstrated that cAMP signaling induced phosphorylation of Th at Ser40 in MN9D cells. Surprisingly, Ser31 and Erk1/2 phosphorylation was downregulated by elevated cAMP signaling, presumably by crosstalk inhibition of Erk1/2 by cAMP-mediated transduction routes. Assessment of hierarchical phosphorylation of Th using mutants that mimic phosphorylated or unphosphorylated states of serine residues demonstrate that Ser40 phosphorylation is required for Ser31 phosphorylation, whereas this is not the other way around. These results indicate that Ser40 could be considered the rate-limiting step in Th activity and function.

Previous studies have shown the importance of cAMP-dependent signaling on Th phosphorylation in vivo and phosphorylation of Th Ser40 by PKA in vitro [21,33,42,43,49–51,54]. Besides activating PKA, cAMP can also activate Epac. The role of Epac in Th regulation has not been addressed before. We demonstrated that Epac activation increases phosphorylation of Th Ser40, which suggests that Epac signals upstream of Ser40 as well. We suggest that Epac mediates down-stream signaling by activating RAP proteins that alter protein kinase signaling such as CAMKII and PKB [96,97]. CAMKII in turn is confirmed to phosphorylate Th Ser40 in situ [48]. Also, there are suggestions of an inhibitory crosstalk between PKB with PKA, which can lead to alterations of serine phosphorylation [110,111].

In this chapter, we demonstrated that Th Ser31 was downregulated in response to forskolin or cAMP analogs. ERK1/2, the member of the RAS, RAF, MEK, ERK pathway, is the main kinase linked to the phosphorylation of Ser31 [24,30,33,34,36,42,67]. We suggest that the phosphorylation of Erk1/2 is downregulated through increased activity of PKA, which has been described in various cell lines [42,105–108]. Indeed, enhanced cAMP signaling, and inhibition of Erk1/2 downregulate Erk1/2 and Th Ser31 phosphorylation in MN9D cells (Fig. 8A). The phosphorylation of Ser31 could subsequently be mediated by the upstream kinase CDK5. Although it is unclear what the exact mechanism behind cAMP-mediated ERK1/2 inhibition is in our model, previous studies have suggested that PKA is able to phosphorylate 3 serine residues within c-RAF that independently block c-RAF activation by RAS [112–114]. Phosphorylation of c-RAF at Ser43 directly interferes c-RAF activation by RAS through steric hindrance, and phosphorylation of c-RAF at Ser233 and Ser259 recruits 14-3-3 proteins that block the activation of c-RAF by RAS [114–116]. Therefore, PKA activation leads to inhibition of c-RAF that is upstream in a complex of MEK1/2 and ERK1/2 [117,118].

Since the protein kinase inhibitors targeting PKA or ERK1/2 were unable to completely block phosphorylation of either Ser31 or Ser40 we addressed some potential other kinases. Accordingly, we tested a variety of protein kinase inhibitors on serine phosphorylation of Ser31 and Ser40. We demonstrate that p38MAPK inhibitors (SB203580 & SB202190) increase phospho-Ser40 and phospho-Ser31 levels in MN9D cells. In literature however, Th Ser19 is known to be phosphorylated in situ by a p38-stimulated protein kinase [21,109], with no evidence to phosphorylate Th at Ser40 or Ser31. This unknown upregulation of both serine residues is possibly due to crosstalk interactions of p38MAPK with PP2A, as p38MAPK is known to decrease the activity of PP2A, a known serine phosphatase of Th [21,119,120]. Also, we show

that PI3K inhibition increased phospho-Ser40 and downregulated phospho-Ser31 levels. The upregulation of Ser40 phosphorylation by PI3K inhibitors is suggested to be caused by blocking PI3K-PKB inhibitory crosstalk with PKA [110,111,121,122]. Additionally, PKB can phosphorylate B-Raf and C-Raf in a similar manner as PKA and therefore reduces MAPK signaling activity [123,124]. GSK3 and CDK5 signaling on Th phosphorylation remains still unclear, as the effects are not comparable between the inhibitor pairs.

Our results demonstrate that through cAMP signaling, phospho-Ser40 levels are increased, whereas phospho-Ser31 and phospho-Erk1/2 levels are decreased. Additionally, MEK1/2 inhibitors reduced the levels of phospho-Ser31 without affecting Ser40 phosphorylation. Thus, our results conflict with previous explanations which have stated that Ser19 and Ser31 positively affect Ser40 phosphorylation by its upstream kinase [58,67]. In our study, this hierarchical interdependence was further explored using Th phospho-mimetic mutants resembling phosphorylated or unphosphorylated protein. Strikingly, we found evidence that Ser31 phosphorylation depends on the phosphorylation of Ser40 as transfected cells with the unphosphorylated Ser40A mutant demonstrated a complete absence of Ser31 phosphorylation. On the contrary, the phospho-mimetic or unphosphorylated Ser31 mutants, did not affect the level of Ser40 phosphorylation. Moreover, downregulation of Ser31 phosphorylation via inhibition of Erk1/2 signaling had no effect on basal Ser40 phosphorylation levels, indicating that the described hypothesis of Ser31-dependent hierarchical phosphorylation does not apply to our system.

It should be noted that the use of phospho-mimetic or unphosphorylated Th mutants may not always mimic the actual phosphorylation state as there is variation in chemical features and kinetic properties. McCulloch *and colleagues* suggest that the serine hydroxyl contributes to the stabilization of the catecholamine-inhibited enzyme, thereby suggesting that *Th-S40A* does not provide an accurate mimic of the unphosphorylated enzyme as the serine hydroxyl is lost in the alanine mutant [125]. This interaction is clearly disrupted by phosphorylation of Ser40. However, other researchers found *Th-S40A* to be suitable mimic as it was demonstrated that activation of Th requires Th to be phosphorylated at serine 40, as it failed to activate Th using the *S40A* mutant [51]. Additionally, Nakashima *and colleagues* showed that using the *S40A* mutant, dopamine and 3,4-Dihydroxyphenylacetic acid (DOPAC) was still accumulated in mouse AtT-20 pituitary gland cells, proving that the Th mutant is still enzymatically active for hydrolyzation of L-Tyrosine [126].

Nevertheless, we describe that Ser40 being phosphorylated is required for Ser31 phosphorylation, shown in Fig. 8B. Considering this hierarchal mode of action, Ser40 phosphorylation is the rate-limiting step in Th activity and facilitates the functionality of the other serine phosphorylation sites. Altogether, Th Ser40 phosphorylation plays a major role in the regulation of presynaptic dopamine neurotransmission [12,15,50]. Therefore, the modulation of TH activity via the phosphorylation of Ser40, the rate-limiting site may provide great therapeutical relevance.

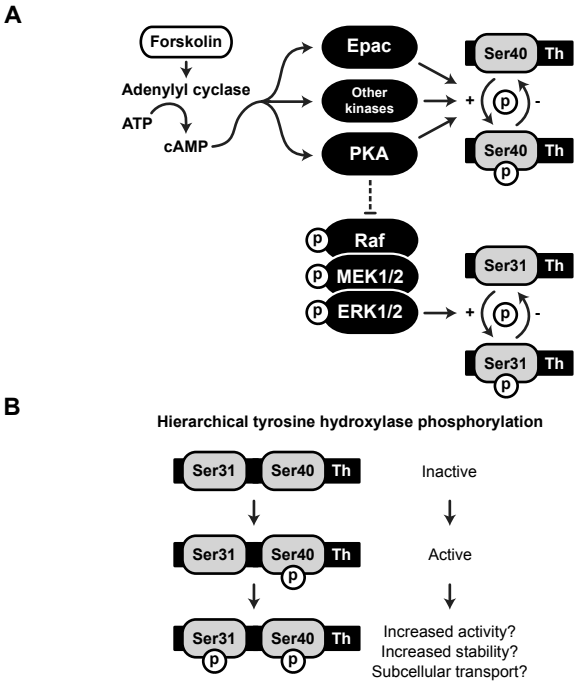
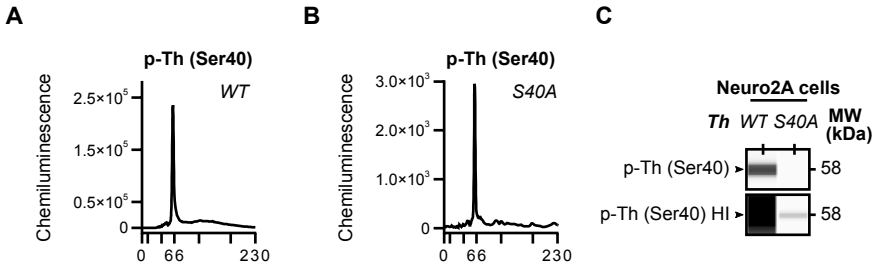


Figure 8: Hierarchical phosphorylation of Th is manipulated through PKA and MEK1/2 crosstalk in MN9D cells

A | Hypothesized intracellular signal-transduction pathways for the phosphorylation of Th at Ser40 and Ser31. Alterations in cAMP levels can lead to changes in protein kinase or Epac activity, resulting in the phosphorylation of Th Ser40 and Ser31. We suggest that increased PKA activity increases phospho-Ser40 and downregulates phospho-ERK1/2 (Thr202/Tyr204), which in turn leads to downregulated phosphorylation levels of Th Ser31. **B |** Hypothesized model of hierarchical phosphorylation of Th. In the fully inactive form of Th, Th is not phosphorylated, and binding of catecholamines inhibits the enzyme from its catalytic activity. To become active, Th may be first phosphorylated at Ser40 to lift this feedback inhibition by catecholamines. Ser40 phosphorylation enables the ability of Th to be additionally phosphorylated at Ser31 by its kinase. If both serine residues are phosphorylated, Ser31 phosphorylation might promote the protein to be in a state of increased activity and/or stability and mediate subcellular transport. Thus, Ser40 phosphorylation is required to increase activity and facilitate other functions of Th. *Abbreviations: ATP, Adenosine triphosphate; cAMP, Cyclic adenosine monophosphate; ERK1/2, Extracellular Signal-Regulated Kinase 1/2; MEK1/2, MAP (Mitogen-Activated Protein) Kinase/ERK Kinase 1/2; PKA, Protein kinase A; Th, Tyrosine hydroxylase.*

Supplemental



Supplemental Figure 1: Validation of a presumed phospho-specific antibody targeting phosphorylated Th Ser40 (Pel-Freez)

A,B | Electropherograms of chemiluminescence signals representing Th protein levels. These Neuro2A cells were transfected with plasmid DNA encoding either wild-type *Th* (*Th-WT*) or *Th-S40A* mutant. Peak values of 58 kDa were found for Th protein, which corresponds with its theoretical molecular weight. The antibody targeting Th phosphorylated at Ser40 residue (Pel-Freez) shows chemiluminescence signal in the (**A**) *Th-WT* condition, and protein signal for the (**B**) *Th-S40A* mutant. **C |** Images representing the electropherograms of (**A**) and (**B**) in a traditional western blot-like overview, showing phospho-Ser40, and high exposure (HI) of phospho-Ser40. Chemiluminescence signal in the *Th-S40A* mutant determined that this antibody is not specific for Ser40 Th phosphorylation and therefore, this antibody is not used further in this study.

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