Acute Regulation of Tyrosine Hydroxylase

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B.BiomedSci (Hons)

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

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ACKNOWLEDGEMENT OF COLLABORATION

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I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.

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Sarah Gordon

Acknowledgements

I would firstly like to express my sincere thanks to my supervisor Phil Dickson, who has given me enormous support throughout my PhD. Choosing the right supervisor is the single most important decision you need to make when doing a PhD; any achievement I have made throughout my PhD is testament to that fact. I could not have asked for a better supervisor, and am sincerely grateful for all your guidance throughout the years.

In addition I would like to thank my cosupervisor Peter Dunkley, who has provided insight and direction when I needed it most. You have been an important mentor to me throughout all of my studies.

I would like to thank all those who have contributed to the work presented in this thesis or have aided me throughout my PhD studies, especially Kelly Marquardt, Larisa Bobrovskaya, Ingo Lehmann, Julianne Webb and Jackie Shehadeh. Your help has been much appreciated.

I would like to thank my family and friends for providing much-needed support during my studies. Very very very special thanks go to Lisa (for always being up for a coffee, you've been a great side-kick!), Jackie, Kel and Gabby (for the much-needed distractions), Jess and Con (for listening to me whinge), Nick and Sis (for being beautiful), Mum and Dad (for the gene-pool, for pushing the right buttons, and for being wonderful), and especially Katie and Ames (for putting up with me).

Contents

Abstract

Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, is regulated acutely by a combination of phosphorylation of three key serine (Ser) residues (Ser19, Ser31 and Ser40), and feedback inhibition by the catecholamines. Phosphorylation of Ser40 directly increases TH activity by relieving feedback inhibition of the enzyme. The phosphorylation of Ser19 or Ser31 can potentiate the phosphorylation of Ser40 in a process known as hierarchical phosphorylation. The 2 major human TH isoforms, hTH1 and hTH2, are differentially regulated by hierarchical phosphorylation *in vitro*. In this study, the human neuroblastoma SH-SY5Y cell line has been transfected with hTH1 and hTH2, and it has been demonstrated that phosphorylation of Ser31 potentiates the phosphorylation of Ser40 in hTH1. Phosphorylation of the equivalent Ser31 residue in hTH2 was not detectable, and thus this enzyme is not subject to Ser31-mediated hierarchical phosphorylation of Ser40 *in situ*. This is the first study to demonstrate that hTH1 and hTH2 are differentially regulated *in situ*. In addition, we have examined the nature of feedback inhibition of TH by the catecholamines. In addition to the high affinity, non-dissociable dopamine binding that is relieved by Ser40 phosphorylation, we have identified a second low affinity, readily dissociable binding site which regulates TH activity both *in vitro* and *in situ* regardless of the phosphorylation state of the enzyme. This low affinity binding site responds to changes in cytosolic catecholamine levels *in situ* in order to regulate TH activity. This work has contributed to our understanding of the complex nature of the regulation of TH activity.

Abbreviations list

BH_4: tetrahydrobiopterin

CA: catecholamine

CaMKII: calcium/ calmodulin-dependent protein kinase II

COMT: catechol-O-methyltransferase

ERK: extracellular signal-regulated kinase

Fe(II): ferrous iron

Fe(III): ferric iron

hTH1-4: human tyrosine hydroxylase isoforms 1-4

K_D: dissociation constant

K_m: Michaelis constant

L-DOPA: L-dihydroxyphenylalanine

MAO: monoamine oxidase

OC charcoal: ovalbumin-coated charcoal

PKA: protein kinase A

ROS: reactive oxygen species

rTH: rat tyrosine hydroxylase

Ser: serine

TH: tyrosine hydroxylase

VMAT: vesicular monoamine transporter

V_{max}: maximal velocity

Publications

Lehmann I.T., Bobrovskaya L., **Gordon S.L.**, Dunkley P.R. and Dickson P.W. (2006) Differential regulation of the human tyrosine hydroxylase isoforms via hierarchical phosphorylation. J. Biol. Chem. **281**, 17644-17651.

Gordon S.L., Quinsey N.S., Dunkley P.R. and Dickson P.W. (2008) Tyrosine hydroxylase activity is regulated by two distinct dopamine-binding sites. J. Neurochem. **106**, 1614-1623.

Submitted manuscripts

Gordon S.L., Bobrovskaya L., Dunkley P.R. and Dickson P.W. (2008) Differential regulation of human tyrosine hydroxylase isoforms 1 and 2 *in situ*: isoform 2 is not phosphorylated at Ser35. J. Neurochem.

Gordon S.L., Webb J.K., Shehadeh J., Dunkley P.R. and Dickson P.W. (2008) The low affinity binding site on tyrosine hydroxylase: the role of the N-terminus and *in situ* regulation of enzyme activity. Neurochem. Res.

Conference Presentations

Gordon S.L. (2007). Tyrosine hydroxylase activity is regulated by two distinct dopamine-binding sites. Parkinson's Disease Satellite Meeting, Prince of Wales Medical Research Institute. **Oral presentation**.

Gordon S.L., Quinsey N.S., Dunkley P.R. and Dickson P.W. (2007) Tyrosine hydroxylase activity is regulated by two distinct dopamine-binding sites. International Brain Research Organisation World Congress, Melbourne. **Poster presentation**.

Gordon S.L., Quinsey N.S., Dunkley P.R. and Dickson P.W. (2008) Tyrosine hydroxylase activity is regulated by two distinct dopamine-binding sites *in situ*. Federation of European Neuroscience Society meeting, Geneva. **Poster presentation.**

Travel Grants

2008: IBRO WERC/CERC for attendance at Federation of European Neuroscience Society meeting.

Chapter 1: Introduction

1.1. The catecholamines

1.1.1. Functions of catecholamines

The catecholamines dopamine, noradrenaline and adrenaline are important neurotransmitters and hormones. Dopamine primarily acts as a neurotransmitter in the central nervous system (Bjorklund and Dunnett 2007), however it has also been suggested to have some paracrine and/or autocrine roles in the periphery (Goldstein et al. 1995). Noradrenaline and adrenaline are key neurotransmitters in the central nervous system (Moore and Bloom 1979). Peripherally, noradrenaline also has roles in neurotransmission in the sympathetic division of the autonomic nervous system, and both noradrenaline and adrenaline act as hormones when released from the adrenal medulla (Goldstein 2003; de Diego et al. 2008).

Due to the complexity of projections of central catecholaminergic neurons, the catecholamines have a diverse range of functions including (but not limited to) roles in the control of movement (Obeso et al. 2008), reward systems (Goodman 2008) and the fear response (Brunello et al. 2003), and have been implicated in the pathogenesis of a range of disorders including Parkinson's disease (Moore 2003), schizophrenia (Meisenzahl et al. 2007), Tourette's syndrome (Albin and Mink 2006), depression (Dunlop and Nemeroff 2007) and anxiety disorders (Brunello et al. 2003).

Peripherally, noradrenaline and adrenaline act upon α- and β-adrenoceptors to produce a wide range of physiological effects including vasoconstriction, an increase in the rate and force of cardiac muscle contraction, bronchodilation, pupil dilation and inhibition of gastrointestinal activity (Axelsson 1971; Yu and Koss 2003; Brodde et al. 2006; Johnson 2006). Through these actions, noradrenaline and adrenaline mediate the "alarm or stress response" or the "fight or flight response". There is also

a basal sympathetic tone present in the body contributing to the maintenance of homeostasis (Goldstein 2003; de Diego et al. 2008).

1.1.2. Intracellular catecholamines exist in two distinct environments

It is important to note that, although the catecholamines act as vital neurotransmitters and hormones, their accumulation within the cell may be detrimental. Intracellular catecholamines exist in two distinct environments: inside vesicles and free in the cytosol. Vesicular catecholamines are stabilised by their acidic environment, and interact with other vesicular components to decrease the osmotic pressure inside the vesicle (Travis and Wightman 1998). This interaction with the intravesicular storage matrix allows catecholamines to be highly concentrated (approximately 550mM) whilst remaining in a stable form.

Unlike vesicular catecholamines, cytosolic catecholamines are subject to enzymatic and auto-oxidation (Figure 1.1). Cytosolic catecholamines undergo oxidative deamination by monoamine oxidase (MAO) to produce reactive aldehydes, which are then further metabolised to form the more stable 3,4-dihydroxyphenylacetic acid (DOPAC, from dopamine) or 3,4-dihydroxyphenylglycol (DHPG, from noradrenaline and adrenaline) (Eisenhofer et al. 2004). Hydrogen peroxide is also produced during this reaction (Youdim and Bakhle 2006). Hydrogen peroxide may be eliminated enzymatically, or in the presence of iron it may participate in the Fenton reaction to produce the highly damaging hydroxyl radical (Jenner 2003). Catecholamines may also undergo auto-oxidation, and this pathway produces toxic catechol-quinones along with hydrogen peroxide (Stokes et al. 1999; Pattison et al. 2002). Catechol-quinones can covalently

modify proteins, lipids and DNA, suggesting that cytosolic catecholamines may be cytotoxic and/or genotoxic. The combined toxic effects of catechol-quinones and free radicals suggests that cytosolic catecholamines may contribute to oxidative stress within the cell (Jenner 2003). Oxidative stress has been implicated in the pathogenesis of Parkinson's disease. Chronic exposure to unregulated cytosolic dopamine has been demonstrated to be sufficient to result in neurodegeneration *in vivo* in mice (Chen et al. 2008).

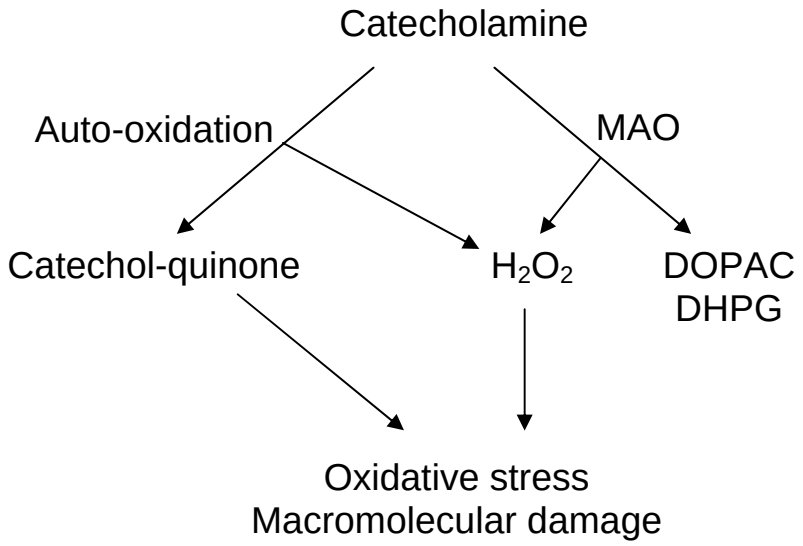


Figure 1.1: Oxidation of cytosolic catecholamines. Cytosolic catecholamines are subject to auto-oxidation, which produces toxic catechol-quinones and hydrogen peroxide (H_2O_2), both of which contribute to oxidative stress within the cell and may induce macromolecular damage. Enzymatic breakdown of catecholamines by monoamine oxidase (MAO) produces the stable metabolites DOPAC or DHPG, and also produces hydrogen peroxide. Modified from (Stokes et al. 1999).

1.1.3. Regulation of cytosolic catecholamines

The concentration of cytosolic catecholamines varies widely between different cell types. Cytosolic catecholamine levels in chromaffin cells were determined to be approximately 2-50μM (Mosharov et al. 2003). In PC12 cells and cultured midbrain dopaminergic neurons the cytosolic catecholamine content is <100nM (Mosharov et al. 2006). Cytosolic catecholamine levels are dependent on a range of cell-specific factors, including vesicular packaging, reuptake from the extracellular milieu, breakdown, and catecholamine biosynthesis (Figure 1.2).

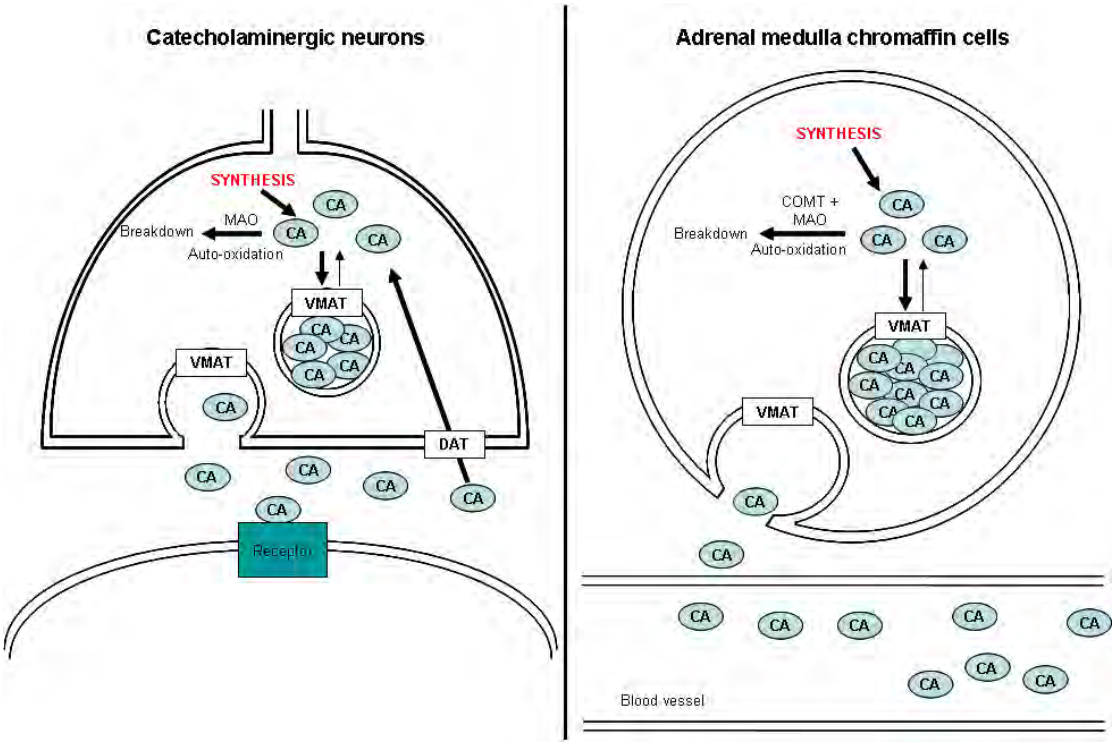


Figure 1.2: Regulation of cytosolic catecholamines. Cytosolic catecholamine (CA) levels are dependent on a range of factors, including packaging into vesicles via vesicular monoamine transporter (VMAT) activity, leakage from the vesicles, enzymatic breakdown due to monoamine oxidase (MAO) activity (and catechol-O-methyltransferase (COMT) activity in non-neuronal cells), breakdown due to auto-oxidation, and new synthesis of the catecholamines. Reuptake from the extracellular milieu by the dopamine or noradrenaline transporters (DAT) also contributes to cytosolic catecholamine levels in neuronal cells, however is unlikely to play a significant role in chromaffin cells.

Catecholamines are packaged into vesicles by the vesicular monoamine transporters 1 and 2 (VMAT1/2). VMAT1 is the primary transporter in neurons, while VMAT2 is responsible for packaging catecholamines in adrenal chromaffin cells (Henry et al. 1994). Vacuolar ATPase utilises ATP to actively pump H^+ into the vesicle. VMAT then exchanges these vesicular protons for cytoplasmic catecholamines by active transport. VMAT transports catecholamines into vesicles against the concentration gradient, resulting in a vesicular catecholamine concentration that is 5 orders of magnitude higher than cytosolic catecholamine levels.

Vesicular storage of catecholamines is not a static process, and a substantial amount of catecholamines leak from the vesicles (Parsons 2000; Eisenhofer et al. 2004). It has been suggested that under basal conditions the majority of catecholamines that are metabolised in the cell originate from leaked vesicular stores, rather than catecholamines that have been released via exocytosis events and taken back into the cell (Eisenhofer et al. 2004).

Reuptake of catecholamines from the extracellular milieu can contribute to cytosolic catecholamine levels in neurons. In neuronal cells catecholamines that have been released into the synaptic cleft are recycled back into the cell by the dopamine transporter or the noradrenaline transporter (Giros and Caron 1993; Mandela and Ordway 2006). Although monoamine transporters are present in adrenal chromaffin cells, it is unlikely that reuptake of catecholamines from the extracellular milieu significantly contributes to cytosolic catecholamine levels in these cells *in vivo* (Wakade et al. 1996). This results from two main factors: firstly there is a lower level of expression of the transporter in these cells (Cubells et al. 1995), but more importantly, the catecholamines very quickly diffuse into the bloodstream and

therefore have limited access to the reuptake mechanisms. This represents a major difference between the two cell types, with reuptake being a significant contributor to cytosolic catecholamine levels in neurons, while reuptake has a less important role in adrenal chromaffin cells.

Catecholamine breakdown is an important regulator of cytosolic catecholamine levels. As previously discussed, cytosolic catecholamines are subject to auto-oxidation and can be enzymatically degraded through the actions of MAO. In addition to MAO, chromaffin cells also contain catechol-O-methyltransferase (COMT), which breaks down noradrenaline and adrenaline into normetanephrine and metanephrine (Eisenhofer et al. 2004). MAO is present in neuronal cells in the outer membrane of mitochondria (Shih et al. 1999), whilst COMT is present in extraneuronal tissue in both soluble and membrane-bound forms which possess different affinities for the catecholamines (Eisenhofer et al. 2004).

The final main contributor to cytosolic catecholamine levels is the rate of synthesis of new catecholamines. Catecholamine synthesis must be sufficient to enable vesicular filling following catecholamine release via exocytosis, and to maintain sufficient basal catecholamine production to compensate for degradation. However, the rate of synthesis must also be regulated to ensure that catecholamines do not accumulate in the cytosol.

1.1.4. Catecholamine synthesis

The catecholamines are produced as part of a biosynthetic cascade (Figure 1.3). The first and rate-limiting step is the hydroxylation of L-tyrosine to form L-dihydroxyphenylalanine (L-DOPA), which is catalysed by the enzyme tyrosine

hydroxylase (TH; tyrosine 3-monooxygenase; E.C. 1.14.16.2) (Nagatsu et al. 1964). L-DOPA is converted to dopamine by the actions of aromatic amino acid decarboxylase. Dopamine is transported into vesicles where it is hydroxylated to form noradrenaline in cells containing dopamine-β-hydroxylase. In the final step of the biosynthetic cascade, noradrenaline is transported out of the vesicle and is converted to adrenaline by phenylethanolamine-N-methyl transferase in the cytosol (Kumer and Vrana 1996).

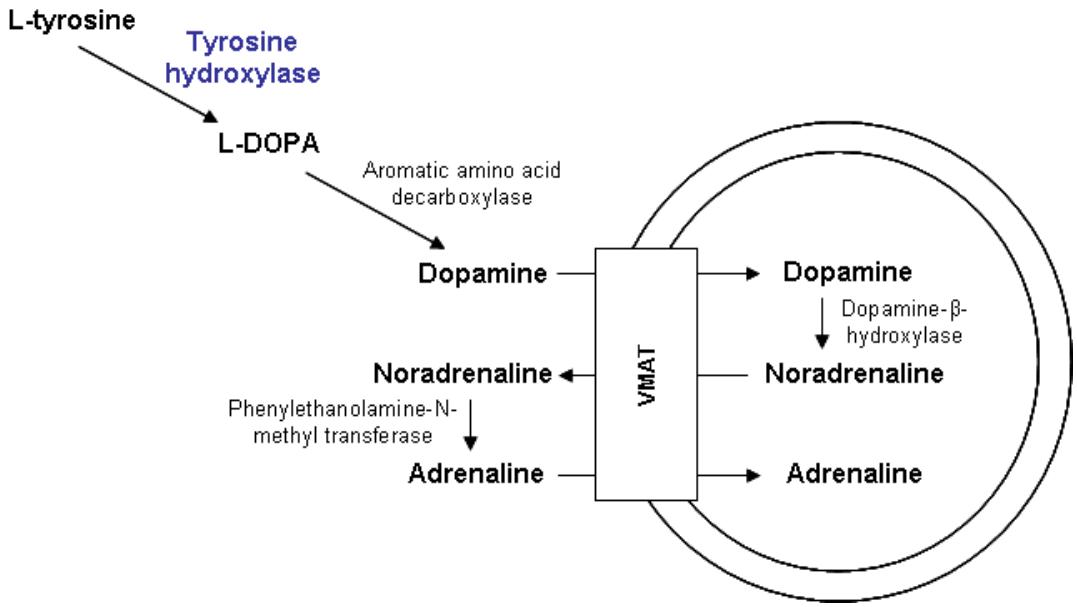


Figure 1.3: The catecholamine biosynthetic pathway. In the rate-limiting step of catecholamine biosynthesis, L-tyrosine is hydroxylated to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase. L-DOPA is converted to dopamine by aromatic amino acid decarboxylase. Dopamine is packaged into vesicles by vesicular monoamine transporter (VMAT) and can then be converted to noradrenaline in vesicles that contain dopamine-β-hydroxylase. Noradrenaline can be converted to adrenaline in the cytosol in cells that contain phenylethanolamine-N-methyl transferase. The catecholamines that are present in a particular cell are dependent on the enzymes that are expressed within that cell.

Secretion of catecholamines from the cell does not produce a concomitant decrease in intracellular catecholamine stores, indicating that catecholamine secretion and synthesis are linked (Zigmond et al. 1989). As the rate-limiting enzyme in catecholamine synthesis, TH is responsible for controlling the levels of catecholamines within the cell, and as such is subject to many different regulatory mechanisms. The focus of this thesis is the enzyme tyrosine hydroxylase, and specifically the ways in which the enzyme is regulated.

1.2. Tyrosine hydroxylase

TH belongs to the tetrahydrobiopterin-dependent amino acid hydroxylase family, which also includes tryptophan hydroxylase and phenylalanine hydroxylase. These enzymes exhibit substantial sequence homology around their C-terminal regions, and also share similar catalytic mechanisms (Fitzpatrick 1999). The enzymes are iron-containing mono-oxygenases, which utilise tetrahydrobiopterin (BH_4) and O_2 to hydroxylate their substrates.

The sites of TH synthesis reflect the important roles of the catecholamines as hormones and neurotransmitters. TH is present in the brain, adrenal gland and sympathetic nervous system of mammals, and is also found in the gut and retina (Dunkley et al. 2004). TH is highly evolutionarily conserved, with variants of the enzyme being identified in drosophila (Neckameyer and Quinn 1989), goldfish (Hornby and Piekut 1990), rat (Grima et al. 1985), bovine (Nagatsu et al. 1964; D'Mello et al. 1988) and primate (Lewis et al. 1994) species. There is significant structural and sequence homology between TH in different species (Nagatsu and Ichinose 1991).

1.2.1. Enzyme structure

TH can be divided into three discrete domains. The N-terminal third of the enzyme constitutes the regulatory domain (Daubner et al. 1993), while the extreme C-terminus contains the tetramerisation domain. The remaining portion of the enzyme forms the catalytic domain. The C-terminal two-thirds of the enzyme, constituting the catalytic domain and tetramerisation region, has been crystallised (Goodwill et al. 1997) (Figure 1.4).

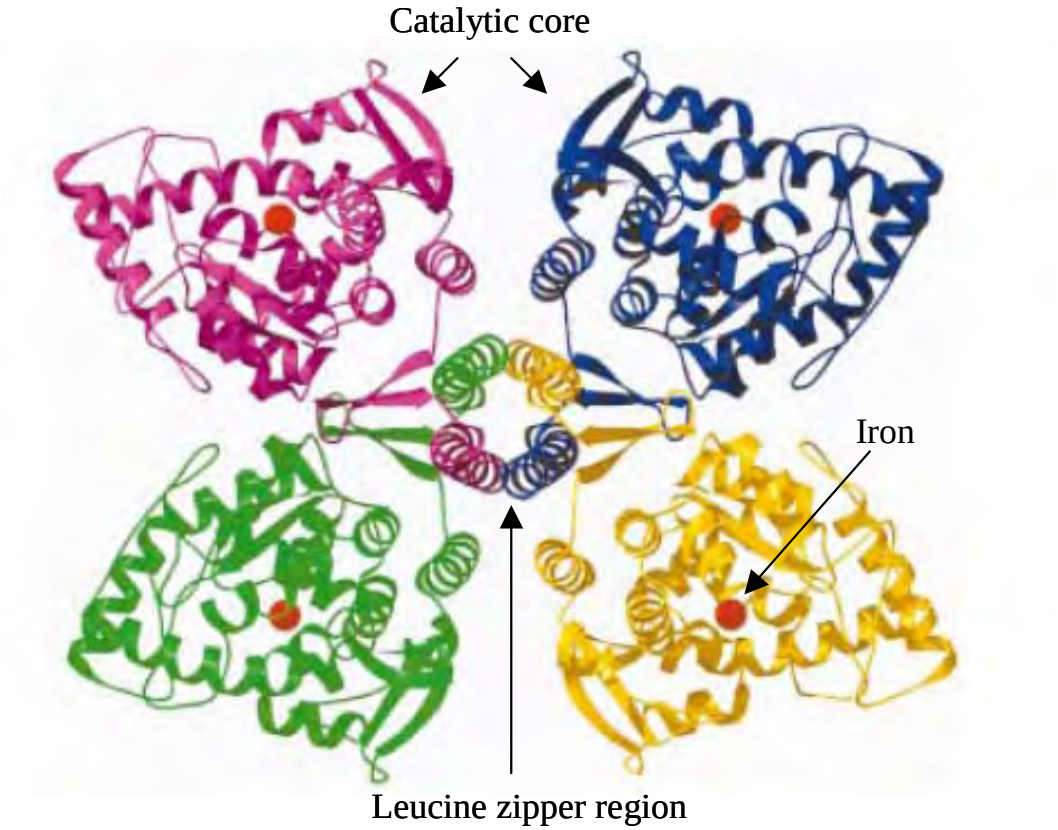


Figure 1.4: Crystal structure of the catalytic domains of the rat TH tetramer (taken from Goodwill et al. 1998; pdb 2TOH). Each TH monomer is represented by a different colour, and the iron atoms bound to the active site of each monomer are shown in red. The catalytic core, leucine zipper region and iron atoms are indicated.

TH is classically considered to exist as a homotetramer (Okuno and Fujisawa 1982), however the role of tetramerisation of TH is currently unknown. The C-terminus of TH contains a leucine-zipper region that is responsible for tetramerisation (Lohse and Fitzpatrick 1993; Vrana et al. 1994). Deletion or mutagenesis of this region can produce monomers or dimers of TH that are catalytically active (Lohse and Fitzpatrick 1993; Vrana et al. 1994). Crystal structures of TH suggest that the enzyme exists as a dimer of dimers, with the two dimers only interacting through the C-terminal tetramerisation domain (Goodwill et al. 1997).

The catalytic domain of TH contains the entire catalytic activity of the enzyme; that is, removal of the first N-terminal third of the enzyme does not affect TH activity (Daubner et al. 1993). TH requires ferrous iron (Fe(II)) for its activity, and utilises BH_4 and O_2 as co-substrates when hydroxylating tyrosine; as such, the binding regions for iron and all substrates exist in the C-terminal domain. TH binds one iron atom per subunit of TH enzyme (Dix et al. 1987; Haavik et al. 1991) which is situated at the active site of the enzyme (Goodwill et al. 1997). The crystal structure of TH with a pterin co-substrate bound to the active site has identified that the pterin co-substrate binds very closely to the iron atom, without coordinating with it (Goodwill et al. 1998). It has also been suggested that the bound pterin may contribute to the binding site for tyrosine (Goodwill et al. 1998).

A crystal structure of TH complete with the N-terminal regulatory domain is not available. The secondary structure of the first 60 amino acids of TH has been predicted to consist of two alpha helices connected by a turn (Nakashima et al. 2000). The regulatory domain of TH contains three key serine (Ser) residues (Ser19,

Ser31 and Ser40) that contribute to the modulation of TH activity. The regulatory role of phosphorylation of these residues will be discussed in later sections.

1.2.2. Enzyme function

In order for hydroxylation of tyrosine to occur, the substrates of the reaction must bind to TH in an ordered fashion, with BH_4 binding first, followed by molecular oxygen and then tyrosine (Fitzpatrick 1991). Molecular oxygen reacts with ferrous iron and BH_4 to produce the hydroxylating intermediate (Fe(IV)O) and 4a-hydroxypterin (Fitzpatrick 2003; Eser et al. 2007) (Figure 1.5). Fe(IV)O either breaks down non-productively or reacts with the aromatic side chain of tyrosine, hydroxylating it to form L-DOPA and leaving ferrous iron (Fitzpatrick 2003). 4a-hydroxypterin is dehydrated to form dihydrobiopterin (BH_2) and H_2O. BH_2 is recycled back to BH_4 by dihydropteridine reductase (Thony et al. 2000).

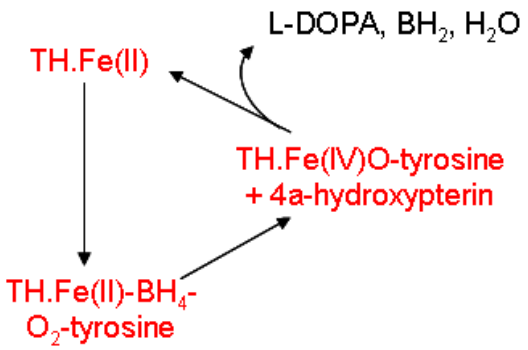


Figure 1.5: Tyrosine hydroxylation reaction. TH requires ferrous iron (Fe(II)) for activity. BH_4, O_2 and tyrosine bind to TH in an ordered fashion. The hydroxylating intermediate Fe(IV)O is formed along with 4a-hydroxypterin, and the reaction then proceeds with the hydroxylating intermediate either breaking down non-productively (not shown) or hydroxylating tyrosine to L-DOPA. 4a-hydroxypterin is dehydrated to produce dihydrobiopterin (BH_2) and H_2O.

TH requires ferrous iron for its activity, however the ferrous iron is readily oxidised by oxygen to produce ferric iron (Fe(III)) (Ramsey et al. 1996), which results in inactivation of TH. The inactive ferric iron is reduced back to the active ferrous form by BH_4 (Ramsey et al. 1996; Frantom et al. 2006).

1.2.3. Multiple human TH isoforms

TH is encoded by a single gene (Kumer and Vrana 1996), however multiple isoforms of the enzyme are present in higher order mammals. Subprimate species express only one TH isoform, while anthropoid species contain 2 isoforms that are generated by a single alternative splicing event (Haycock 2002). Humans express at least 4 TH isoforms (hTH1-4) produced by two alternative splicing events (Grima et al. 1987; Kaneda et al. 1987; Le Bourdelles et al. 1988). The presence of all four isoforms have been found in human brain (Lewis et al. 1993) and adrenal medulla (Haycock 1991). hTH1 and hTH2 are the major isoforms present in human brain and the adrenal gland (Coker et al. 1990; Haycock 1991; Lewis et al. 1993), while hTH3 and hTH4 mRNA comprise only 4 - 5% of total brain TH mRNA (Coker et al. 1990). It is unknown whether the different human isoforms are able to associate as heterotetramers.

The four TH isoforms differ only in their N-terminal domain, in the region immediately preceding the Ser31 residue in hTH1 (Figure 1.6). hTH1 shares significant homology with other mammalian TH (Nagatsu and Ichinose 1991). hTH2 has a 4 amino acid insert immediately N-terminal to the Ser31 residue, while hTH3 has an additional 27 amino acids in the same location. hTH4 contains both inserts,

with the 4 amino acid insert being N-terminal to the 27 amino acid insert (Grima et al. 1987; Kaneda et al. 1987).

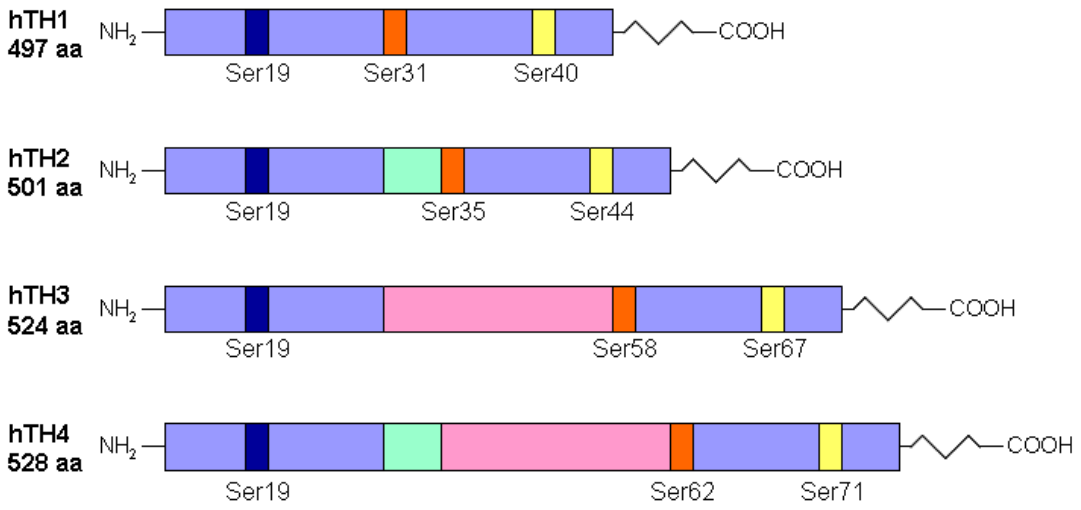


Figure 1.6: Schematic of the amino acid sequence of the four major human TH isoforms (hTH1-4). hTH1 has 497 amino acids, and shows substantial sequence homology to TH from other species. hTH2 has an additional 4 amino acids inserted N-terminal to the Ser31 residue of hTH1 (green region), hTH3 has an additional 27 amino acids (pink region), while hTH4 contains both inserts.

1.2.4. Other TH isoforms

The four major human TH isoforms have been well studied, and physiologically relevant levels of the four proteins have been identified in human tissue. In addition to the four well-recognised human TH isoforms, a number of other TH isoforms have been identified. In some cases only the mRNA for these isoforms has been detected in human tissue; it remains unknown whether the corresponding proteins are expressed *in vivo*. Some of these isoforms may play a role in disease states, although this remains controversial.

TH mRNA lacking exon 3 has been identified in the adrenal medulla at a level comparable to that of hTH3 and hTH4 (Dumas et al. 1996). There were much higher levels (11-34% of total TH mRNA) of this form of mRNA present in patients with progressive supranuclear palsy. Subsequent work performed by the same laboratory demonstrated that a TH protein isoform lacking exon 3 was expressed in human adrenal medulla, with a higher level of expression in a patient with progressive supranuclear palsy (Bodeau-Pean et al. 1999). The recombinant form of this protein was demonstrated to have decreased V_{max} and also decreased inhibition by dopamine compared to hTH3 (Bodeau-Pean et al. 1999). Exon 3 also contains the Ser31 and Ser40 residues, so this isoform of TH would not contain these important regulatory serine residues. This variant of TH has not been further investigated.

TH mRNAs lacking exon 8 alone, and also lacking both exons 8 and 9, have been identified in patients with neuroblastoma (Parareda et al. 2003). These are the only non-N-terminal splice variants of TH that have been identified. Recombinant hTH lacking exons 8 and 9 was shown to have no enzyme activity (Roma et al. 2007). It remains unknown whether a TH protein lacking exon 8 or exons 8 and 9 is expressed *in vivo*. A protein of correct size was identified in neuroblastoma tumours by western blotting utilising an anti-TH antibody (Roma et al. 2007), however this was not conclusively demonstrated to correspond to TH lacking exons 8 and 9. It remains possible that the lower molecular weight protein that was identified was a result of proteolysis of TH.

TH mRNA lacking exon 4 was identified in the adrenal of a normal patient during a screen for novel TH splice variants (Ohye et al. 2001). The skipping of exon 4 resulted in a premature stop codon at amino acid 147. No TH protein corresponding to this isoform has yet been identified.

1.3. Regulation of TH activity

As the rate-limiting enzyme in catecholamine biosynthesis, the activity of TH is directly responsible for the availability of catecholamine stores. Considering the high levels of catecholamine that may be released from the adrenal medulla following stimulation, and that secretion of catecholamines does not produce a concomitant decrease in intracellular catecholamine stores, TH activity must be able to increase rapidly when required.

TH is present in a variety of tissues which have very different levels of catecholamine release. With the diversity of conditions that TH must accommodate for, it is not surprising that the enzyme is subject to a wide variety of regulatory mechanisms. These can be loosely grouped into long-term and short-term regulation of the enzyme. The major long-term regulator of TH activity levels is the control of TH protein levels (Kumer and Vrana 1996). TH expression is under tight regulatory control, and although this forms an important regulatory mechanism, it will not be discussed in depth in this review.

TH is regulated acutely by a range of factors. Of these, the most extensively studied (and arguably the most important) contributors to regulation of enzyme activity are feedback inhibition of the enzyme by the catecholamines, and phosphorylation of three key Ser residues. The roles that each of these factors plays in the regulation of TH activity will be the focus of the remainder of the review.

1.3.1. Feedback inhibition

Feedback inhibition of TH by the catecholamines dopamine, noradrenaline and adrenaline has been recognised for decades. In fact, the three catecholamines were

identified as inhibitors of TH when the enzyme was first discovered (Nagatsu et al. 1964). Early studies theorised that end-product feedback inhibition was likely to be one of the most important regulators of TH activity *in vivo* (Spector et al. 1967). Despite this extensive history of research, the mechanism by which TH is inhibited by the end-products of catecholamine synthesis is not fully understood.

Feedback inhibition of TH is a well-recognised phenomenon. However, several factors have complicated research in the area, resulting in controversy regarding the mechanisms by which feedback inhibition occurs as well as the overall importance of feedback inhibition in the regulation of TH activity. Before exploring our current understanding of the mechanisms of TH feedback inhibition, an explanation of factors complicating research in the area will first be presented. Firstly, early studies performed on TH purified from a range of tissue sources were confounded by the fact that the isolated enzyme contained varying amounts of bound catecholamine (Andersson et al. 1988; Andersson et al. 1992). Thus, TH was already in a partially inhibited state when isolated from tissues. Secondly, it was shown using TH purified from tissues that enzyme activity was highest at approximately pH 5.0-6.0 (Nagatsu et al. 1964; Okuno and Fujisawa 1982, 1985), and this was considered the pH optimum of the enzyme. This is because the affinity of catecholamine binding to TH at acidic pH is significantly less than at physiological pH; the bound catecholamines which were inhibiting TH activity begin to dissociate from the enzyme at acidic pH (Fitzpatrick 1988; Haavik et al. 1990). This means that any studies performed at non-physiological pH do not reflect the true nature of the mechanism of dopamine inhibition of the enzyme. Thirdly, TH purified from tissues contains variable amounts of phosphate (Dunkley et al. 2004). As will be discussed in subsequent sections, the phosphorylation state of TH affects the binding of

catecholamines to the enzyme. The following discussion of feedback inhibition of TH will take into account these complicating factors.

1.3.1.1. Competitive feedback inhibition versus BH_4

Early studies indicated that dopamine inhibition of TH was due to competitive inhibition of the bound catecholamine with the co-substrate BH_4 (Udenfriend et al. 1965; Ikeda et al. 1966; Waymire et al. 1972). In more recent studies using recombinant enzyme at physiological pH, it was demonstrated that addition of BH_4 could result in dissociation of dopamine from TH (Almas et al. 1992).

This mechanism of dopamine inhibition represents a kinetically-mediated, readily-reversible feedback inhibitory action which would provide classical homeostatic regulation of enzyme activity (Kumer and Vrana 1996). Altering intracellular levels of either BH_4 or catecholamines would change TH activity. This form of inhibition would require that dopamine be readily dissociated from the enzyme. It was shown that gel filtration of TH purified from tissue extracts resulted in a decrease in the Michaelis constant (K_m) for the co-substrate BH_4 (Ames et al. 1978; Okuno and Fujisawa 1982). This was suggested to be due to dissociation of bound catecholamines from the enzyme, as the addition of dopamine back into the mixture increased the K_m for BH_4 (Ames et al. 1978). Unfortunately the majority of these studies were performed at acidic pH on TH purified from tissues, so the validity and relevance of this mechanism of feedback inhibition remains unclear.

1.3.1.2. Non-competitive feedback inhibition: direct co-ordination of dopamine to the active-site iron

In direct conflict with data suggesting that the catecholamines bind to the enzyme in a readily-reversible manner is the fact that TH purified from tissues still retains dopamine bound to the enzyme. Presumably if catecholamines bind to TH in a readily-reversible manner then the catecholamines should all be dissociated from the enzyme during the purification process, however, half of the iron-containing TH isolated from bovine adrenal glands was demonstrated to be catecholamine-bound (Andersson et al. 1988). This suggests that catecholamines bind to TH in a much stronger manner than first thought.

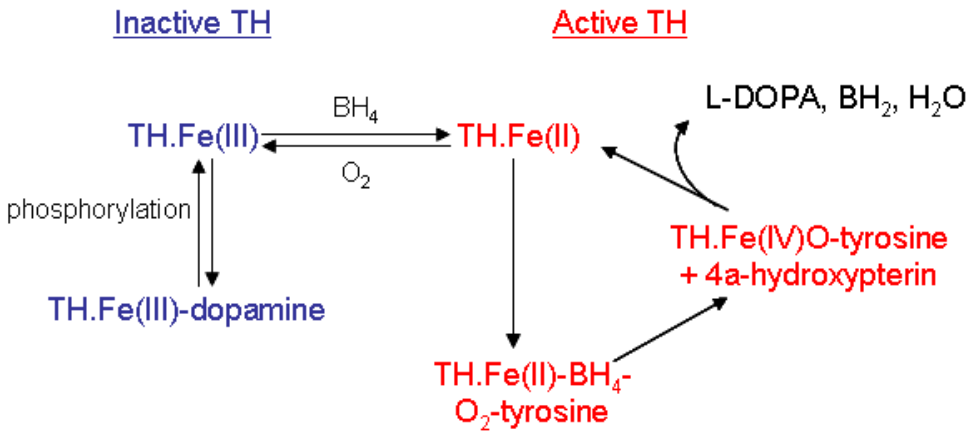


Figure 1.7: Schematic of activation states of TH. TH with iron in the ferrous (Fe(II)) state is active, and binds BH_4, O_2 and tyrosine in the hydroxylation reaction. The hydroxylating intermediate Fe(IV)O then hydroxylates tyrosine to L-DOPA, leaving the active site iron in the Fe(II) state. In the presence of O_2, Fe(II) undergoes oxidation to the ferric (Fe(III)) form, which inactivates TH. Fe(III) can be reduced to Fe(II) by BH_4. However, dopamine can covalently bind to Fe(III), trapping TH in the inactive state. Phosphorylation of Ser40 promotes the dissociation of dopamine from the enzyme.

It has been observed that TH purified from tissues exhibits a blue-green colour (Haavik et al. 1988). Raman resonance studies of the purified enzyme demonstrated

that this colour was produced by the direct coordination of catecholamines to the iron at the catalytic core of the enzyme (Andersson et al. 1988). The catecholamines bind to the ferric form of the iron (Fe(III)) in a bidentate fashion (Andersson et al. 1988). This traps the iron in the inactive ferric form, and as mentioned previously, it is the ferrous form of iron (Fe(II)) that is required for hydroxylation of tyrosine to occur (Figure 1.7).

1.3.1.3. Dopamine binding studies with recombinant TH

There are two conflicting sets of data provided by studies from TH purified from tissue extracts. Early data suggested that catecholamines bind to TH in a readily-reversible manner, however more recent data demonstrates that the catecholamines covalently bind to the iron in the catalytic core.

The rat TH enzyme was eventually expressed in a bacterial system which allowed the study of catecholamine-free, phosphate-free enzyme (Daubner et al. 1992; Ribeiro et al. 1992). Under these conditions it was found that the pH optimum of TH was approximately 7. It was then demonstrated that catecholamine binding to TH at physiological pH is non-dissociable (Daubner et al. 1992). Resonance Raman studies of the recombinant enzyme confirmed that the dopamine is indeed coordinated to ferric iron (Michaud-Soret et al. 1995). Crystallographic analyses of the related enzyme phenylalanine hydroxylase with bound catecholamine has added further support to that notion that the catecholamines bind directly to the active-site iron (Erlandsen et al. 1998).

The stoichiometry of dopamine binding to TH was determined to be 0.55 dopamine molecules/TH monomer (Daubner et al. 1992). The dissociation constant (K_D) for dopamine binding to TH has been determined to be in the very low nM

range (Ramsey and Fitzpatrick 1998; McCulloch et al. 2001; Sura et al. 2004). The 'off' rate for catecholamine binding to TH is extremely slow, and in one study the rate of dissociation of dopamine from the enzyme was essentially unmeasurable, even following 12h of incubation with a competitor (Ramsey and Fitzpatrick 1998; McCulloch et al. 2001; Sura et al. 2004). This effectively means that once catecholamines are bound they do not dissociate from the enzyme. Together, all this data suggests that dopamine binding to TH is not readily reversible.

1.3.1.4. Mechanism of inhibition

The mechanism by which dopamine inhibits TH activity seems to be a dual effect on both the maximal velocity (V_{max}) of the enzyme and the K_m for BH_4. However, there remains conflict regarding the degree of effect of dopamine on these two kinetic factors. Dopamine binding has been shown to decrease V_{max} to 5% of that of the wild-type enzyme, while also producing a small (2-fold) increase in the K_m for BH_4 (Daubner et al. 1992). This is in contrast to studies performed at pH 6 which suggested that dopamine primarily exerts its inhibitory actions by increasing the K_m for BH_4 (Ames et al. 1978; Ribeiro et al. 1992).

1.3.1.5. Role of the N-terminus in feedback inhibition

The N-terminal regulatory domain has also been suggested to contribute to the feedback inhibition of TH by the catecholamines. Deletion of the entire N-terminal regulatory domain substantially decreased the inhibition of TH by dopamine (Ribeiro et al. 1993). Further studies confined this effect to the first 38 N-terminal residues of TH (Ota et al. 1997; Nakashima et al. 1999).

Specifically two positively-charged residues, Arg37 and Arg38, have been demonstrated to be important for the stabilisation of dopamine binding to the enzyme (Nakashima et al. 2000). Mutation of these residues to electrically neutral Gly residues reduced the inhibition of TH by dopamine, suggesting an important role of the charged residues in dopamine inhibition. These residues lie in a putative turn surrounded by two alpha-helices, thereby forming two potentially important residues in determining the structure of the N-terminus of TH. This suggests an important role of the N-terminus in modulating dopamine binding to the enzyme.

1.3.1.6. Stabilisation of TH by dopamine

In addition to its inhibitory actions, dopamine binding to TH also acts to increase the stability of the enzyme. Incubation of TH with dopamine protects against time-dependent loss of enzyme activity (Okuno and Fujisawa 1991). In another study, binding of dopamine to TH increased the resistance of the enzyme to proteolysis, and also increased the thermal stability of TH (Martinez et al. 1996). It is theorised that there are two distinct pools of TH, a stable pool with low enzyme activity and a labile pool with high enzyme activity (Okuno and Fujisawa 1991).

1.3.1.7. Summary of feedback inhibition

There has been extensive research into the area of catecholamine-mediated inhibition of TH activity. Early work was complicated by several factors, however the availability of the recombinant enzyme clarified our understanding of feedback inhibition of TH activity by the catecholamines. The catecholamines bind covalently to the ferric form of the active site iron, trapping it in an inactive state. Once bound

in this way, the catecholamines are essentially unable to be dissociated from the enzyme.

It remains unclear whether the readily-reversible catecholamine inhibition that is competitive versus BH_4 (which was originally proposed to be the primary mechanism of feedback inhibition of TH) actually occurs, and indeed if it is physiologically relevant. The mechanism of catecholamine inhibition is also unclear. There are conflicting results regarding the magnitude of the effect of dopamine binding on V_{max} and the K_m for the co-substrate BH_4.

If dopamine is able to strongly inhibit enzyme activity by coordinating to the active site iron, and is unable to dissociate from the enzyme, then a mechanism for relief of this inhibition must exist in order for physiological levels of catecholamine synthesis to occur. As will be discussed, feedback inhibition of TH is relieved by phosphorylation of the Ser40 residue.

1.3.2. Phosphorylation

TH contains four serine (Ser) residues in its N-terminal domain which are known to be phosphorylated *in vivo*: Ser8, Ser19, Ser31 and Ser40. Ser8 is phosphorylated to a very low stoichiometry *in vivo*, has not been shown to effect TH activity, and no stimuli have been identified that can reproducibly increase the phosphorylation of the residue *in situ* (Dunkley et al. 2004); as such, it will not be discussed further in this review. Much of the focus in TH research has gone into determining the functional roles of phosphorylation of the remaining three Ser residues. A comprehensive review of the regulation of phosphorylation of TH and the consequences for enzyme activity and catecholamine biosynthesis has recently

been undertaken (Dunkley et al. 2004). Therefore, this discussion will primarily focus on the role of phosphorylation at each of the main Ser residues.

1.3.2.1. Phosphorylation of Ser40

Incubation of TH purified from tissues under conditions that promote the activation of protein kinase A (PKA) was shown to increase enzyme activity (Morgenroth et al. 1975). It was later demonstrated that PKA phosphorylates Ser40 (Campbell et al. 1986). Early studies were suggestive of a mechanism by which phosphorylation increased enzyme activity by relieving catecholamine inhibition of the enzyme (Ames et al. 1978), however this was only conclusively shown when the recombinant enzyme became available.

Phosphorylation of Ser40 in the non-catecholamine bound enzyme produces minimal effects on enzyme activity. Ser40 phosphorylation in non-catecholamine bound TH induces a small decrease in the K_m for BH_4, and does not affect V_{max} (Daubner et al. 1992; Ribeiro et al. 1992; McCulloch et al. 2001). However, phosphorylation of Ser40 in the catecholamine-bound enzyme substantially increases V_{max} by relieving catecholamine inhibition of the enzyme (Daubner et al. 1992; Ramsey and Fitzpatrick 1998).

Ser40 phosphorylation relieves feedback inhibition of TH by increasing the rate of dissociation of catecholamines from the enzyme (see Figure 1.6). As stated previously, the ‘off’ rate for catecholamine binding to TH is extremely slow (Haavik et al. 1990; Ramsey and Fitzpatrick 1998; McCulloch et al. 2001; Sura et al. 2004). Phosphorylation of Ser40 significantly increases the ‘off’ rate such that catecholamines are now able to dissociate from the enzyme; the result is an increase

in the K_D of catecholamine binding to the enzyme by 3 orders of magnitude (Ramsey and Fitzpatrick 1998; McCulloch et al. 2001; Sura et al. 2004). The increase in the off rate and thus the K_D of the enzyme not only results in dissociation of bound catecholamines, but also prevents catecholamines from the re-binding to the enzyme.

Phosphorylation of Ser40 increases the α-helical content of TH by approximately 10% (Martinez et al. 1996). In addition, it was demonstrated using gel filtration chromatography that TH phosphorylated at Ser40 adopts a more open conformation than the non-phosphorylated form (Bevilaqua et al. 2001). Phosphorylation of Ser40 has also been shown to increase the susceptibility of TH to tryptic digestions (McCulloch and Fitzpatrick 1999). This data together suggests that the enzyme substantially alters its structure upon phosphorylation of Ser40. The conformational change to the enzyme induced by Ser40 phosphorylation may contribute to the dissociation of the bound catecholamines.

In addition to this, two positively-charged residues close to Ser40, Arg37 and Arg38, have been demonstrated to be important for the stabilisation of dopamine binding to the enzyme (Nakashima et al. 2000). The introduction of a large negatively-charged phosphate group to the N-terminus of the enzyme at Ser40 may disrupt the interaction between these two residues and the dopamine molecule bound at the active site of the enzyme. This provides some reasoning for why dopamine binding to the active site of the enzyme would be affected by phosphorylation of an N-terminal residue.

While phosphorylation is primarily considered to be an acute regulator of protein activity, it has recently been demonstrated that phosphorylation of Ser40 is able to extend past this acute period for a more sustained period. Increases in Ser40 phosphorylation without an increase in TH protein levels has been shown to last for

at least 24 hours following the addition of a stimulus (Bobrovskaya et al. 2007a; Bobrovskaya et al. 2007b). Furthermore, sustained phosphorylation of Ser40 has been demonstrated to be due to different intracellular signalling pathways than those that are responsible for classical acute phosphorylation (Bobrovskaya et al. 2007a; Bobrovskaya et al. 2007b).

Thus, phosphorylation of Ser40 is responsible for directly increasing TH activity by relieving feedback inhibition of the enzyme. It achieves this by increasing the K_D of catecholamine binding, thereby promoting the dissociation of catecholamines from the enzyme. Phosphorylation of Ser40 is able to extend past the acute period for up to 24 hours in a phenomenon known as sustained phosphorylation. While the role of Ser40 phosphorylation in the regulation of enzyme activity is well-understood, the importance of phosphorylation of the Ser19 and Ser31 residues has only recently been ascertained.

1.3.2.2. Phosphorylation of Ser19

Phosphorylation of Ser19 has no direct effect on TH activity *in vitro* (Toska et al. 2002). There is evidence that Ser19-phosphorylated TH is able to associate with the 14-3-3 protein, and this results in a small increase in enzyme activity (Yamauchi and Fujisawa 1981; Yamauchi et al. 1981; Toska et al. 2002). Other studies have been unable to reproduce this effect (Haycock and Wakade 1992; Sutherland et al. 1993). It remains unknown whether this association actually occurs *in vivo,* and if it has any functional relevance.

Ser19 is phosphorylated *in situ* and *in vivo* to quite a high stoichiometry (Dunkley et al. 2004). A range of stimuli have been shown to promote Ser19

phosphorylation *in situ*, including depolarising stimuli and increases in intracellular Ca^{2+} (Dunkley et al. 2004). Thus, although Ser19 is clearly phosphorylated *in vivo*, and phosphorylation of this residue is known to be under regulatory control *in situ*, the functional consequences of Ser19 phosphorylation remained unclear.

While Ser19 phosphorylation does not directly increase TH activity, it does have indirect effects on enzyme activity. Phosphorylation of Ser19 results in the enzyme adopting a more open conformation (Bevilaqua et al. 2001), and increases the rate of phosphorylation of Ser40 by approximately 3-fold *in vitro* (Bevilaqua et al. 2001; Toska et al. 2002). This potentiation of Ser40 phosphorylation appears to occur in a hierarchical manner, as Ser40 phosphorylation does not affect the rate of Ser19 phosphorylation (Bevilaqua et al. 2001). Prior phosphorylation of Ser19 results in a 76% potentiation of Ser40 phosphorylation *in situ* (Bobrovskaya et al. 2004); this correlates to a 50% potentiation of TH activity when Ser19 is phosphorylated prior to Ser40 compared to TH phosphorylated at Ser40 alone (Bobrovskaya et al. 2004).

Therefore, although phosphorylation of Ser19 does not have a direct effect on TH activity, it is able to indirectly increase TH activity by increasing the rate of phosphorylation of Ser40. It is possible that this is the primary role of phosphorylation of Ser19.

1.3.2.3. Phosphorylation of Ser31

Phosphorylation of Ser31 alone has been demonstrated to induce a small (1.2-2 fold) increase in TH activity *in vitro* (Haycock et al. 1992; Sutherland et al. 1993). This effect seems to be primarily due to a small decrease in the K_m for the co-

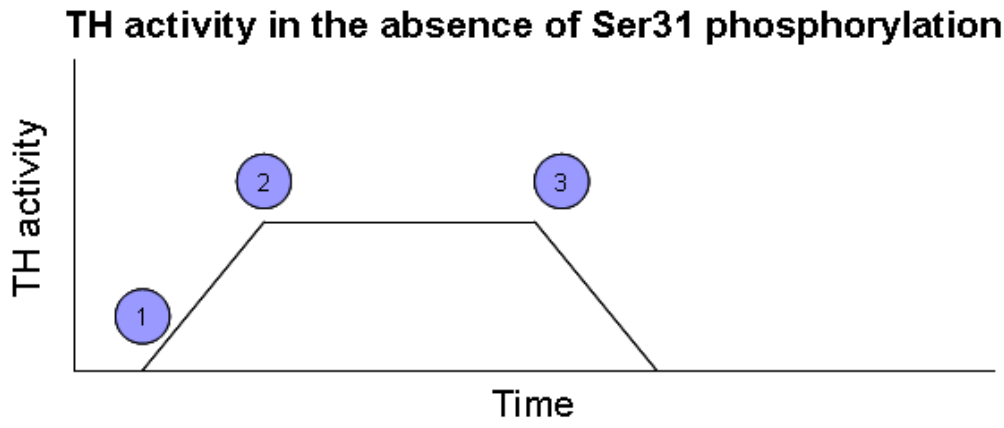
substrate BH_4 in the non-dopamine bound form of TH (Sutherland et al. 1993). In this respect phosphorylation of Ser31 and phosphorylation of Ser40 seem to have similar effects on the native (non-dopamine bound) form of TH. However, phosphorylation of Ser31 does not result in the dissociation of bound catecholamines from the enzyme (Haycock et al. 1992).

In addition to this small direct effect on TH activity, phosphorylation of Ser31 is able to potentiate the phosphorylation of Ser40 in a similar way to that described for Ser19. Prior phosphorylation of Ser31 increases the rate of Ser40 phosphorylation by 9-fold *in vitro* (Lehmann et al. 2006). Phosphorylation of Ser31 potentiates the phosphorylation of Ser40 by 2-fold *in situ*, and this equates to a 2-fold increase in TH activity compared to TH phosphorylated at Ser40 alone (Lehmann et al. 2006). Ser40 phosphorylation does not affect the rate of Ser31 phosphorylation, demonstrating that the effect of Ser31 phosphorylation is hierarchical in nature. The potentiation of Ser40 phosphorylation was found to exist only in the dopamine-free form of TH.

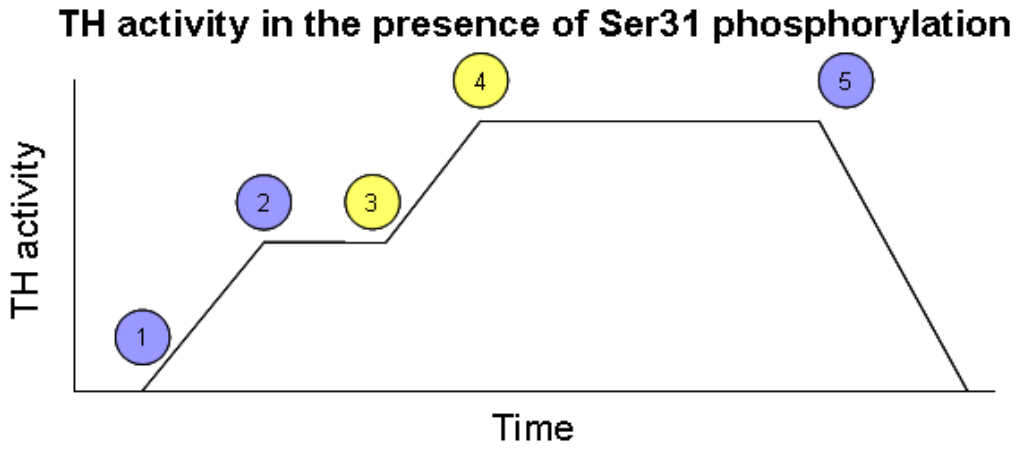
Phosphorylation of Ser31 *in situ* generally occurs more slowly than phosphorylation of Ser19 and Ser40 (Dunkley et al. 2004). In bovine adrenal chromaffin cells, phosphorylation of Ser19 and Ser40 can be achieved within 1-3 minutes, while phosphorylation of Ser31 generally takes approximately 5-10 minutes of stimulation. This would fit a model whereby phosphorylation of Ser31 would serve to prolong the effects of phosphorylation of Ser40 (Lehmann et al. 2006) (Figure 1.8). Following activation of Ser40 kinases, Ser40 would become phosphorylated within a number of minutes, resulting in the dissociation of catecholamines from the enzyme and an increase in enzyme activity. Following this, the activation of Ser40 phosphatases would result in dephosphorylation of Ser40, and

catecholamines would be free to re-bind to the enzyme and strongly inhibit TH activity. However, in the presence of Ser31 phosphorylation, re-phosphorylation of Ser40 on the catecholamine-free TH would be potentiated. This would lead to a pool of TH being in a prolonged state of activity.

Figure 1.8 (over page): Schematic of the effect of Ser31 phosphorylation on TH activity. In the absence of Ser31 phosphorylation, TH activity depends solely on the rate of phosphorylation of Ser40. Following an increase in intracellular Ca^{2+}, Ser40 kinases become active and phosphorylate Ser40 which increases TH activity due to the dissociation of bound catecholamines. TH activity plateaus when the rates of phosphorylation and dephosphorylation of Ser40 reaches equilibrium. TH activity is decreased when Ser40 kinase activity decreases (or Ser40 phosphatase activity increases), resulting in overall dephosphorylation of Ser40 and therefore re-binding of the catecholamines. Following an extended increase in intracellular Ca^{2+}, Ser31 kinases become active, and phosphorylation of Ser31 potentiates the phosphorylation of Ser40. This changes the equilibrium between phosphorylation and dephosphorylation of Ser40 to favour the phosphorylated state, resulting in an overall higher level of TH activity.



1. Ca^{2+} entry, Ser40 kinases active, Ser40 phosphorylation increases, dopamine dissociates, increased TH activity
2. Equilibrium phase (equilibrium of phosphorylation and dephosphorylation of Ser40)
3. Ser40 phosphorylation decreases, dopamine re-binds to TH, decreased TH activity



1. Ca^{2+} entry, Ser40 kinases active, Ser40 phosphorylation increases, dopamine dissociates, increased TH activity
2. Equilibrium phase (equilibrium of phosphorylation and dephosphorylation of Ser40)
3. Extended Ca^{2+} increase, Ser31 kinases active, potentiation of Ser40 phosphorylation, increased TH activity
4. Equilibrium phase, new equilibrium of phosphorylation and dephosphorylation of Ser40 with higher TH activity
5. Ser40 phosphorylation decreases, dopamine re-binds, decreased TH activity

1.3.2.4. Multiple isoforms of TH and hierarchical phosphorylation

Multiple TH isoforms exist in human brain and adrenal glands as a result of alternative splicing events. Of these, four isoforms have been well-characterised. Currently, it is unknown what causes or promotes the alternative splicing events that result in multiple TH isoforms. Also, it remains unknown whether alternative splicing of TH mRNA can be regulated.

hTH1 and hTH2 comprise the major TH isoforms, with hTH3 and hTH4 being expressed at a much lower level (Haycock 1991; Lewis et al. 1993). Immunocytochemistry studies have suggested that each of the four isoforms can be co-expressed and co-localise in some neuronal populations, however there is evidence for the selective targeting and/or expression of the isoforms in some neurons (Lewis et al. 1993). It remains unknown whether the isoforms are differentially expressed in different brain regions.

The catalytic domains of the four human TH isoforms are entirely conserved. This is supported by the finding that very little difference in specific activity exists between the recombinant isoforms (Haavik et al. 1991; Le Bourdelles et al. 1991; Sura et al. 2004). Each of the enzymes has been shown to be inhibited by catecholamines, and this is reversible by phosphorylation of Ser40 (Almas et al. 1992; Sura et al. 2004).

As the four human TH isoforms differ only in the amino acids immediately N-terminal to Ser31, it would be assumed that any major differences that exist between the isoforms occurs with regards to phosphorylation of this residue. This in fact has been found to be the case (Sutherland et al. 1993). Ser31 in hTH1 is phosphorylated by extracellular signal-regulated kinase (ERK) only to a stoichiometry of 0.5mol phosphate/mol TH subunit. ERK can phosphorylate hTH3 and hTH4 at their

corresponding Ser31 residues (Ser58 and Ser62 respectively) to a stoichiometry of 1mol phosphate/mol TH subunit. Conversely, the corresponding Ser31 residue in hTH2 (Ser35) cannot be phosphorylated by ERK *in vitro* (Sutherland et al. 1993; Lehmann et al. 2006). Thus, the different amino acid sequence immediately N-terminal to the corresponding Ser31 residue changes the ability of ERK to phosphorylate the enzyme.

The different amino acid sequence N-terminal to Ser35 in hTH2 has been theorised to alter the kinase specificity of the Ser residue, changing it from an ERK site to a target for calcium/ calmodulin-dependent protein kinase II (CaMKII) (Grima et al. 1987). Indeed, Mallet and colleagues have claimed that CaMKII can phosphorylate the residue *in vitro* (Le Bourdelles et al. 1991). However, this result was not able to be replicated (Lehmann et al. 2006). A kinase capable of phosphorylating Ser35 in hTH2 in a replicable manner is yet to be identified.

The different amino acid sequence N-terminal to the equivalent Ser31 residues in the four isoforms has important implications for the regulation of TH activity via hierarchical phosphorylation (Lehmann et al. 2006). Phosphorylation of Ser31 increases the rate of phosphorylation of Ser40 in hTH1. The absence of phosphorylation of Ser35 by ERK in hTH2 means that this effect is absent in hTH2 *in vitro*. Phosphorylation of the equivalent Ser31 residues in hTH3 and hTH4 (Ser58 and Ser62 respectively) does not increase the rate of phosphorylation of the relative Ser40 residues (Ser67 and Ser71) *in vitro*, suggesting that the 27 amino acid insert must interfere with Ser31-dependent hierarchical phosphorylation of Ser40.

The TH isoforms differ not only in the effect of the relative Ser31 residues on the phosphorylation of Ser40, but also in the effect of Ser19 on Ser40 phosphorylation. The prior phosphorylation of Ser19 in hTH1 and hTH2 increases

the rate of phosphorylation of the relative Ser40 residues (Ser40 and Ser44 respectively) *in vitro* (Lehmann et al. 2006). However, phosphorylation of Ser19 induces a greater potentiation of Ser44 phosphorylation in hTH2 than Ser40 phosphorylation in hTH1. This represents a relatively mild difference between the isoforms compared to the differential regulation of the enzymes by the phosphorylation, or lack thereof, of the relative Ser31 residues. It is unknown whether hTH1 and hTH2 are differentially regulated *in situ* or *in vivo*.

1.4. Summary and Aims

As the rate-limiting enzyme in catecholamine synthesis, TH is subject to a wide variety of regulatory controls. Many of these, such as feedback inhibition by the catecholamines, and phosphorylation of the three key Ser residues, offer several levels of control over enzyme activity. Despite extensive research into the mechanisms of regulation of TH activity, there remain several points of dispute.

The catecholamines are known to bind to TH with high affinity, and inhibit enzyme activity primarily by decreasing V_{max}. The effect of catecholamine binding on the K_m for BH_4 has been disputed. It was originally believed that catecholamines inhibit enzyme activity by competing with the co-substrate BH_4, however it has been shown that dopamine directly coordinates with the active-site ferric iron and may only be dissociated by phosphorylation of the Ser40 residue. The presence of competitive inhibition is yet to be confirmed, and the physiological relevance, if any, of this form of inhibition is unknown.

While the role of phosphorylation of Ser40 is well-recognised, the role of phosphorylation of the other two major Ser residues, Ser19 and Ser31, has only

recently been elucidated. Ser19 phosphorylation does not directly increase TH activity, while phosphorylation of Ser31 induces a small increase in TH activity. However, both sites are able to indirectly affect TH activity by potentiating the phosphorylation of Ser40 in a hierarchical manner; that is, prior phosphorylation of Ser19 or Ser31 increases the rate of phosphorylation of Ser40. This represents an important regulatory mechanism *in situ*. The lack of phosphorylation of the relative Ser31 residue in hTH2 (Ser35) represents a major difference between the two major human TH isoforms, hTH1 and hTH2. hTH1 is subject to hierarchical phosphorylation of Ser40 by prior Ser31 phosphorylation *in vitro*; this effect is absent in hTH2 due to the lack of phosphorylation of Ser35 *in vitro* (see Appendix).

This thesis will aim to further elucidate the mechanisms of regulation of TH activity, and will focus on the two major acute forms of regulation of TH activity, phosphorylation and feedback inhibition by the catecholamines (Figure 1.9). Phosphorylation of Ser31 contributes to the regulation of TH activity *in vitro*, and provides a mechanism by which the major human TH isoforms may be differentially regulated. This has been further investigated in Chapter 2, which has aimed to determine whether the differential regulation of the major human TH isoforms also occurs *in situ*. The mechanism of feedback inhibition of TH activity has long been a contentious issue. Chapter 3 provides a comprehensive investigation into the nature of dopamine binding to TH and the functional consequences of this binding on the regulation of TH activity *in vitro*. The physiological relevance of feedback inhibition of TH is further explored in Chapter 4, where the effect of altered cytosolic catecholamine levels on TH activity is examined *in situ*.

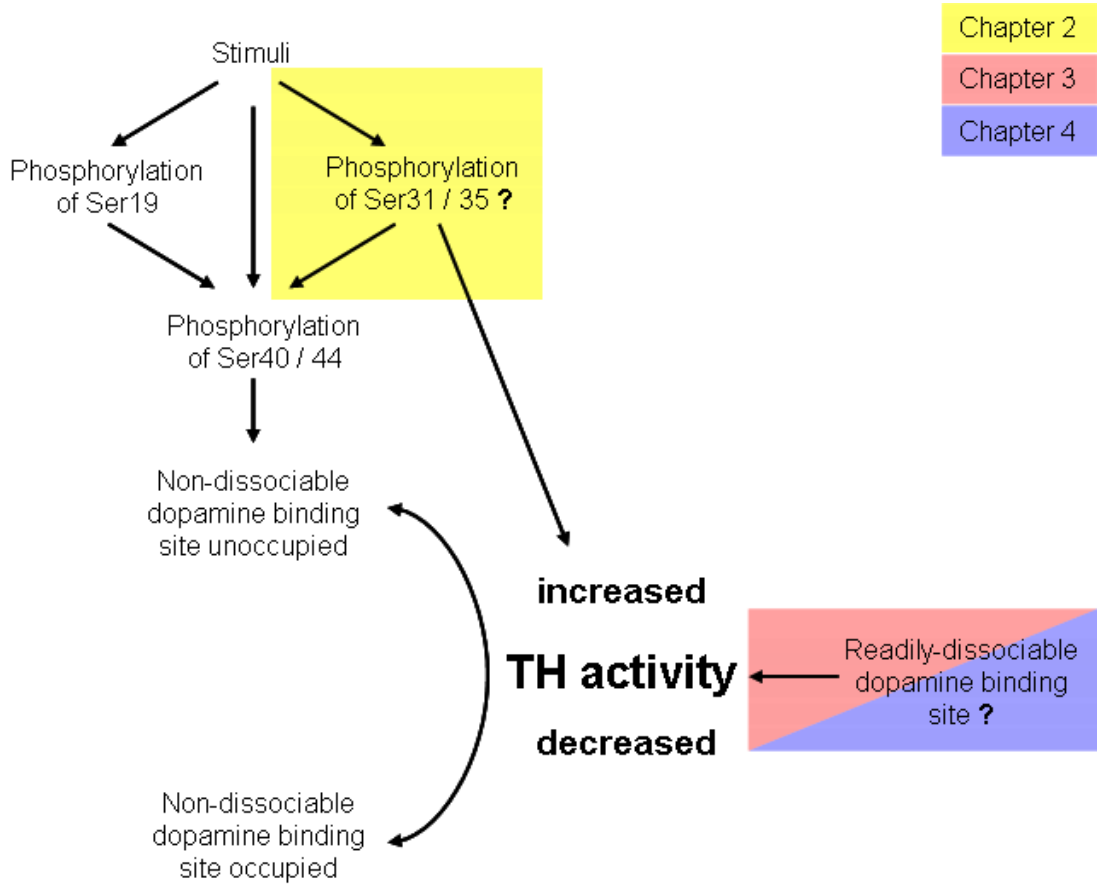


Figure 1.9: Current understanding of factors that influence TH activity. TH activity is regulated by a combination of feedback inhibition by the catecholamines and by phosphorylation of three key Ser residues. The phosphorylation of Ser19 and Ser31 can potentiate the phosphorylation of Ser40 in hTH1. It is unknown whether the phosphorylation of Ser35 can potentiate the phosphorylation of Ser44 in hTH2 due to the lack of Ser35 phosphorylation *in vitro*. Phosphorylation of Ser40 increases TH activity by relieving dopamine binding to the non-dissociable binding site. It is unknown whether dopamine can bind to TH in a readily-dissociable manner. Contentious issues are followed by a question mark, and it is these areas that will be further investigated in this thesis.

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Chapter 2: Differential regulation of human tyrosine hydroxylase isoforms 1 and 2 *in situ*: isoform 2 is not phosphorylated at Ser35

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This manuscript has been submitted to the Journal of Neurochemistry as an original article (19th December 2008).

The content of this manuscript has not been altered in any way except that footnotes, figures and associated figure legends have been placed in the text at appropriate positions. In addition, headings have been numbered to match style throughout thesis.

ACKNOWLEDGEMENT OF COLLABORATION

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers.

The work in the following chapter (Chapter 2) was submitted to the Journal of Neurochemistry in December 2008. I (Sarah Gordon) was the author of this manuscript, and the work embodied in this chapter was primarily performed by myself, with the exception of the following:

Figure 1: Characterisation of phospho-specific antibodies, parts A and B only, was performed by Dr Larisa Bobrovskaya.

The work embodied by these experiments is inextricably tied to the overall findings of the manuscript, and therefore this work will be discussed in the final chapter (Chapter 5) of this thesis.

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Sarah Gordon

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Associate Professor Phillip Dickson

2.1. Abstract

The major human tyrosine hydroxylase isoforms (hTH1 and 2) differ in their ability to be phosphorylated *in vitro*. hTH1 is phosphorylated at Ser31 by extracellular signal-regulated kinase (ERK). This kinase is not capable of phosphorylating hTH2 at Ser35 (the residue that corresponds to Ser31 in hTH1). We have stably transfected SH-SY5Y cells with hTH1 or hTH2 to determine if hTH2 can be phosphorylated at Ser35 *in situ*. Forskolin increased the phosphorylation of Ser40 in hTH1 and Ser44 in hTH2. Muscarine increased the phosphorylation of both Ser19 and Ser40/44 in both hTH1 and hTH2. EGF increased the phosphorylation of Ser31 in hTH1. Phosphorylation of Ser35 in hTH2 was not detected under any of the conditions tested. Inhibition of ERK by UO126 decreased the phosphorylation of Ser31 and this lead to a 50% decrease in the basal level of phosphorylation of Ser40 in hTH1. The basal level of Ser44 phosphorylation in hTH2 was not altered by treatment with UO126. Therefore phosphorylation of Ser31 contributes to the phosphorylation of Ser40 in hTH1 *in situ*, however this effect is absent in hTH2. This represents a major difference between the two human TH isoforms, and has implications for the regulation of TH activity *in vivo*.

2.2. Introduction

Tyrosine hydroxylase (TH)[1] [EC1.14.16.2], the rate-limiting enzyme in catecholamine biosynthesis (Nagatsu et al. 1964), is subject to a wide variety of regulatory mechanisms (Kumer and Vrana 1996). Long-term regulation of TH activity is primarily controlled by modulation of TH protein levels (Kumer and Vrana 1996). Acute regulation of TH activity occurs via two distinct forms of feedback inhibition by the catecholamines (Gordon et al. 2008) and also by phosphorylation of three key serine (Ser) residues: Ser19, Ser31 and Ser40 (Dunkley et al. 2004).

Phosphorylation of Ser40 directly increases TH activity by relieving the binding of catecholamines to the high affinity site on the enzyme (Daubner et al. 1992). Stimulation of Ser40 phosphorylation results in an increase in TH activity and catecholamine synthesis *in situ* and *in vivo* (Dunkley et al. 2004). While the role of phosphorylation of Ser40 is well-understood, the function of phosphorylation of Ser19 and Ser31 has only recently been elucidated.

Phosphorylation of Ser19 does not directly increase TH activity *in vitro* (Toska et al. 2002), however incubation of Ser19-phosphorylated TH with 14-3-3 protein is able to produce a small increase in enzyme activity (Yamauchi and Fujisawa 1981; Yamauchi et al. 1981). Phosphorylation of Ser31 induces a small (1.2-2-fold) increase in TH activity *in vitro*, primarily by decreasing the K_M for the cosubstrate tetrahydrobiopterin (BH_4) (Haycock et al. 1992; Sutherland et al. 1993). In addition to these small effects on TH activity, prior phosphorylation of Ser19 or Ser31 is able to increase the rate of phosphorylation of Ser40 by approximately 3-

[1] The abbreviations used are: TH, tyrosine hydroxylase; Ser, serine; hTH, human TH; BH_4, tetrahydrobiopterin; ERK, extracellular signal-regulated protein kinase; CaMKII, calcium/ calmodulin-dependent protein kinase II; DMEM, Dulbecco's Modified Eagle Medium.

fold and 9-fold respectively *in vitro* in a process known as hierarchical phosphorylation (Bevilaqua et al. 2001; Lehmann et al. 2006). Phosphorylation of Ser19 or Ser31 potentiates both the phosphorylation of Ser40 and the pSer40-induced increase in TH activity *in situ* (Bobrovskaya et al. 2004; Lehmann et al. 2006).

TH is encoded by a single gene. While most species express only a single isoform of TH (Haycock 2002), there are 4 human TH (hTH) isoforms (hTH1-4) which differ only in the number of amino acids N-terminal to the Ser31 residue in hTH1 (Grima et al. 1987; Kaneda et al. 1987). The sequence surrounding the other phosphorylation sites is identical in all TH isoforms. hTH1 is the smallest of the hTH enzymes, and is homologous to TH found in other species (Nagatsu and Ichinose 1991). hTH2 contains an additional 4 amino acids inserted immediately N-terminal to Ser31, hTH3 contains an additional 24 amino acids, while hTH4 contains both inserts (4+27). All four isoforms are expressed in human brain and adrenals (Haycock 1991; Lewis et al. 1993), with hTH1 and hTH2 being the two major isoforms, together comprising approximately 90% of TH in brain (Coker et al. 1990; Lewis et al. 1993).

hTH1 is phosphorylated at Ser31 *in vitro* by extracellular signal-regulated protein kinase (ERK) (Sutherland et al. 1993; Lehmann et al. 2006), however hTH2 cannot be phosphorylated at the equivalent Ser31 residue (Ser35) by ERK (Sutherland et al. 1993; Lehmann et al. 2006). The addition of four amino acids N-terminal to this residue in hTH2 was believed to change the kinase specificity of the site from an ERK site to a calcium/ calmodulin-dependent protein kinase II (CaMKII) site (Le Bourdelles et al. 1991). However, in other studies CaMKII was unable to phosphorylate the Ser35 residue in hTH2 *in vitro* (Lehmann et al. 2006). It is unknown whether hTH2 can be phosphorylated at Ser35 in intact systems.

Due to the important role that phosphorylation of Ser31 plays in regulating the rate of Ser40 phosphorylation, the absence of phosphorylation of Ser35 in hTH2 represents a major difference between the two isoforms (Lehmann et al. 2006). We have stably transfected the neuroblastoma cell line SH-SY5Y with hTH1 and hTH2 isoforms and stimulated the cells in an effort to determine whether hTH2 is able to be phosphorylated at Ser35 *in situ*.

2.3. Experimental procedures

2.3.1. Materials

pcDNA3.1, Lipofectamine 2000 and geneticin were from Invitrogen (Carlsbad, CA, USA). Wizard SV gel and PCR clean-up system and UO126 were from Promega (Madison, WI, USA). XL1-blue competent cells were from Stratagene (La Jolla, CA, USA). Quicklyse plasmid purification mini-prep kit and plasmid purification maxi-prep kit were from Qiagen (Hilden, Germany). SH-SY5Y cells were obtained from ATCC (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) was from GIBCO (Carlsbad, CA, USA). Foetal calf serum was from Bovogen (Essendon, Australia). Rat-tail collagen, forskolin, and muscarine chloride were from Sigma-Aldrich (St Louise, MO, USA). Epidermal growth factor was from Affinity BioReagents (Golden, CO, USA). L-[3,5-^{3}H]-tyrosine was from GE Healthcare (Buckinghamshire, England). Peptides were synthesised by Auspep (Parkville, Australia). Sulfolink immobilization kit and Aminolink plus immobilization kit were from Pierce (Rockford, IL, USA)

2.3.2. Preparation of pcDNA3.1 hTH1 and hTH2

pcDNA3.1 hTH1 was generously supplied by Ingo Lehmann. For pcDNA3.1 hTH2, hTH2 cDNA was amplified from pET3a hTH2 by PCR using primers constructed to encompass the BamHI and EcoRI sites (forward primer = CGTAGGA TCCATGCCCACCCCCGACGCAACC, reverse primer = CGTAGAATTCCTAGC CAATGGCACTCAGCGC). The PCR product was purified using the Wizard SV gel and PCR clean-up system according to the manufacturer's instructions. The purified PCR product and pcDNA3.1 were digested with BamHI and EcoRI, and then purified using the Wizard SV gel and PCR clean-up system. The purified hTH2 and pcDNA3.1 digests were then ligated together, and transformed into XL1-Blue competent cells. Transformed bacteria were selected using LB agar plates containing 100µg/mL ampicillin. Overnight cultures were inoculated from single colonies, and plasmids purified using the Quicklyse plasmid mini-prep kit according to the manufacturer's instructions. Insertion of hTH2 cDNA into pcDNA3.1 was confirmed by DNA sequencing.

Stocks of pcDNA hTH1 and pcDNA hTH2 were prepared using the plasmid purification maxi-prep kit according to the manufacturer's instructions, and were stored at -20ºC in DNAse/RNAse-free H_2O.

2.3.3. Cell culture

Transfected and wild-type SH-SY5Y cells were routinely maintained in 10% DMEM (DMEM supplemented with 10% foetal calf serum, 10mM Hepes and 2mM L-Glutamine) at 37ºC, 5% CO_2 in a humidified incubator. Cells were not allowed to exceed 90% confluency before passaging, and were not used above passage 22.

6-well and 12-well plates were coated with 10µg/mL rat-tail collagen in phosphate-buffered saline for 2h at 37ºC, and then washed with phosphate-buffered saline, prior to plating of cells. Cells were plated in collagen-coated plates (unless otherwise stated) at a density of 4-7x10^5 cells/well for 6-well plates, and 2-4x10^5 cells/well for 12-well plates, and were maintained in 10% DMEM until 80% confluent.

2.3.4. Stable transfection of SH-SY5Y cells

SH-SY5Y cells were plated in 12-well (non collagen-coated) plates at a density of 4x10^5 cells/well, and were incubated with 1.6µg pcDNA3.1 hTH1 or pcDNA3.1 hTH2 and 4µL Lipofectamine 2000 reagent for 24h, before media was aspirated and replaced with 10% DMEM supplemented with 0.06mg/mL geneticin (DMEM-G). Cells were maintained in DMEM-G; during this time, cell density was observed to decrease to <1% confluency, and then increase until 80% confluent. In wild-type (non-transfected) SH-SY5Y cells, incubation in DMEM-G resulted in 100% cell death. Cells were transferred to a 5mL flask, and were maintained in DMEM-G until cells were transferred to a 75mL flask, after which point hTH1 and hTH2 SH-SY5Y cells were routinely maintained in 10% DMEM. TH protein expression was analysed using western-blotting as described below.

2.3.5. TH activity assay

Cells were plated in 6-well plates. Cells were washed in serum-free media (DMEM supplemented with 10mM Hepes and 2mM L-Glutamine), and then pre-incubated in serum-free media at 37ºC, 5% CO_2 for 2h. Media was aspirated, cells were washed in phosphate-buffered saline, and 350µL of homogenisation buffer

(50mM Tris, pH 7.5, 1mM EGTA, 1mM EDTA, 1mM dithiothreitol, 80µM ammonium molybdate, 1x protease inhibitor cocktail, 1mM tetrasodium pyrophosphate, 5mM β-glycerophosphate and 1mM sodium vanadate) was added to each well. Wells were scraped, and cells were lysed by passing through a 26G needle. Samples were centrifuged at 18,000g for 15min at 4ºC, and the supernatants were collected and assayed for TH activity using a variation of the tritiated-water release assay (Reinhard et al. 1986) as described (Bobrovskaya et al. 2004). Briefly, TH activity assays were initiated by the addition of an equal volume of reaction mix (60mM potassium phosphate buffer, pH 7.4, 0.006% (v/v) 2-mercaptoethanol, 36µg/ml catalase, 24µM tyrosine, 4µCi L-[3,5-^{3}H]-tyrosine/mL, and 2mM BH_4). Background samples did not contain BH_4. Assays were performed at 30ºC for 9min. Reactions were linear under these conditions.

2.3.6. Treatment of SH-SY5Y cells

hTH1 SH-SY5Y and hTH2 SH-SY5Y cells were plated in 6- or 12-well plates. Cells were pre-incubated for 2h in serum-free media prior to cell treatment as described above. Cells were treated with 10mM forskolin, 10mM muscarine, 50ng/mL EGF or relative vehicle for control wells for 5 or 30min. The vehicle for muscarine and EGF treatments was H_2O, and the vehicle for forskolin was DMSO. Forskolin and DMSO were added to cells such that the final concentration of DMSO did not exceed 0.1%. For pre-incubation with UO126, cells were pre-incubated in serum-free DMEM for 1.5h and then were incubated with 10µM UO126 for 30min.

2.3.7. Preparation of phospho-specific antibodies and total TH antibody

To generate antibodies to detect TH phosphorylated at Ser40, Ser31, or Ser19, we synthesized phospho-peptides corresponding to residues 36–44 (GRRQpSLIED; pSer40TH), 27–35 (EAVTpSPRFI; pSer31TH) and 15–23 (RRAVpSEQDA; pSer19TH) of rat TH with the addition of a terminal cysteine residue as described (Cammarota et al. 2003). After purification by HPLC, an aliquot of each phospho-peptide was linked to diphtheria toxoid and used to immunize rabbits (for pSer19 and pSer31) and sheep (for pSer40). After the animals had been bled, serum was collected and antibodies were purified via phospho-peptide affinity chromatography. 2 mg of each phospho-peptide was coupled to 2mL of Sulfolink coupling gel using Sulfolink immobilization kit for peptides. Sera were purified according to the manufacturer's general protocol for affinity purification of protein (Pierce, Rockford, IL, USA). The specificity of the phospho-specific antibodies was assessed by immunoblotting using recombinant rat TH specifically phosphorylated at Ser40 (by protein kinase A), Ser31 (by ERK1), Ser19 (mutated form of rat TH (Ser40Ala) phosphorylated by CaMKII) and also non-phosphorylated recombinant rat TH as described (Lehmann et al. 2006). As shown in Fig. 1A, the phospho-specific antibodies recognised their respective phospho-rTH proteins in a concentration-dependent manner. The phospho-specific antibodies did not significantly cross-react with non-phosphorylated rTH, or with rTH phosphorylated at the alternative Ser residues. hTH1, hTH2 and hTH3/4 have different amino acid sequences immediately N-terminal to their relative Ser31 residues. The pSer31 antibody was therefore further assessed for the detection of the different human TH isoforms phosphorylated at the equivalent Ser31 residues (Fig. 1B). The pSer31 antibody recognised recombinant hTH1, hTH3 and hTH4 phosphorylated at the

equivalent Ser31 residues by ERK; hTH2 cannot be phosphorylated by ERK *in vitro* and thus was not analysed (Sutherland et al. 1993; Lehmann et al. 2006). To determine if the pSer31 antibody recognises the pSer35 hTH2 sequence, the pSer31 antibody was incubated for 1 hour at 25ºC with 500nM of pSer35 hTH2 peptide (VRGQpSPRFI), 500nM pSer31TH peptide or 500nM pSer40TH peptide. Both the pSer35 hTH2 peptide and the pSer31TH peptide were able to block the binding of the pSer31 antibody to the pSer31 TH immunoblot, while the pSer40TH peptide did not block binding (Fig. 1C). This data demonstrates that the pSer31 antibody is able to recognise hTH1, 3 and 4 phosphorylated at their equivalent Ser31 residues despite their different N-terminal amino acid sequences, and if hTH2 was phosphorylated at Ser35, then the pSer31 antibody could recognise it.

To generate antibodies to detect non-phosphorylated TH, rabbits were immunised with recombinant human TH protein lacking the first 156 residues to avoid any interaction of the antibody with the N-terminal regulatory domain, where phosphorylation could alter the binding of the antibody to the TH protein. Animals were bled and serum was collected. 2mg of recombinant rat TH was coupled to Aminolink coupling gel using Aminolink plus immobilization kit. Antibodies were purified according to the manufacturer's general protocol for affinity purification of protein (Pierce, Rockford, IL, USA).

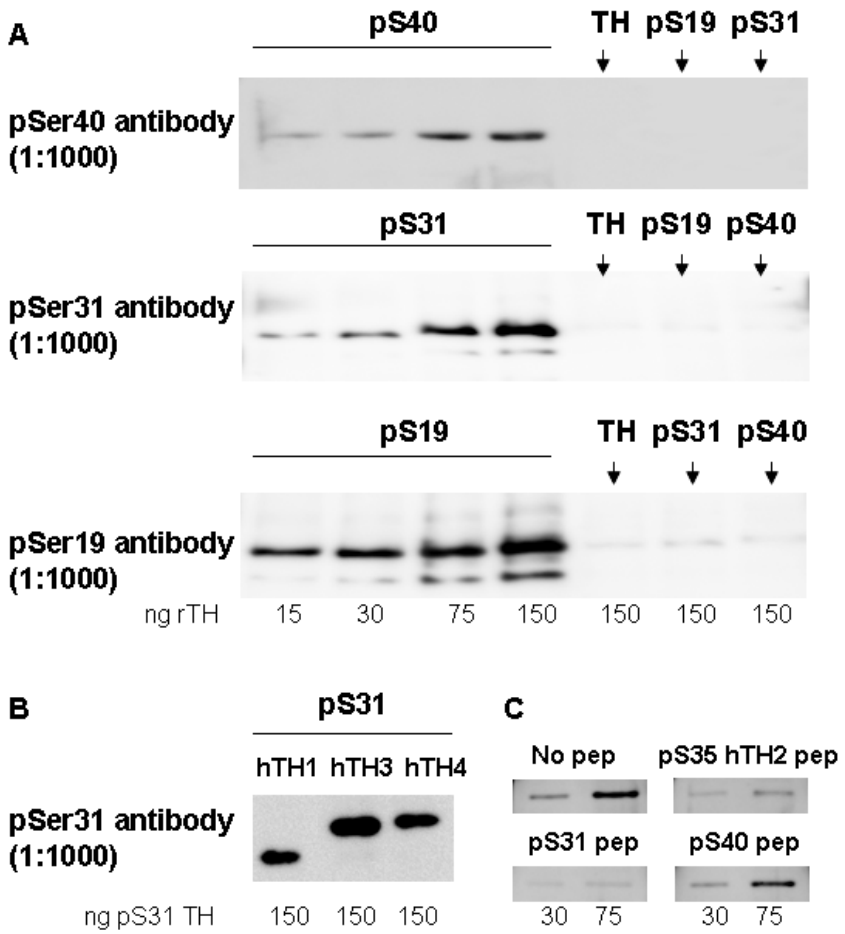

Fig. 1: Characterisation of phospho-specific antibodies. A) The specificity of phospho-specific antibodies was assessed by immunoblotting using different loadings of recombinant rat TH that was either non-phosphorylated (TH), or phosphorylated specifically at Ser40 (pS40), Ser31 (pS31) or Ser19 (Ser40Ala rTH phosphorylated by CaMKII, pS19). **B)** The ability of pSer31 antibody to detect human TH (hTH) isoforms 1, 3 and 4 phosphorylated by ERK (pS31 TH, 150ng loaded) was assessed by immunoblotting. **C)** pSer31 antibody was incubated without peptide (no pep), or with pSer35 hTH2, pSer31 TH or pSer40 TH peptides. The binding of the blocked antibody to pS31 TH (30 and 75ng loaded) was assessed by immunoblotting.

2.3.8. SDS-PAGE and western blotting

Following incubation of cells with various stimuli, media was aspirated and treatments were terminated by the addition of Laemlli sample buffer (50mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% DTT, 2mM EDTA). Samples were applied

to 10% SDS-polyacrylamide gels before being transferred to nitrocellulose as described (Jarvie and Dunkley 1995). Nitrocellulose membranes were then immunoblotted using phospho-specific or total TH antibodies as described previously (Cammarota et al. 2003). Analysis of site-specific TH phosphorylation was performed as previously described (Bobrovskaya et al. 2004). Immunoblots were visualized using the Image Reader LAS-3000 (Fujifilm) imaging system using ECL plus detection reagents. The density of total TH or phospho-specific TH bands was measured using MultiGauge V3.0 (Fujifilm).

2.3.9. Statistical Analyses

The data was expressed as a fold-increase of the mean of the control samples (control=1), and is presented as mean ± SEM for the stated number of experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's test for multiple comparisons or Student's unpaired *t*-tests for analysis of Ser31 phosphorylation.

2.4. Results

2.4.1. Stable transfection of hTH1 and hTH2 into SH-SY5Y cells

SH-SY5Y cells were stably transfected with hTH1 or hTH2 cDNA inserted in the pcDNA3.1 mammalian expression vector. The expression of hTH1 and hTH2 was confirmed by SDS-PAGE and western blotting (Fig. 2A). hTH1 and hTH2 can be distinguished as two different sized bands when the gels are run their entire length (>10cm). Similar separation of the two isoforms has previously been observed (Haycock 2002). hTH1 and hTH2 were expressed at similar levels in transfected SH-SY5Y cells. TH expression was not observed in wild-type cells. The expression of

hTH1 or hTH2 in SH-SY5Y cells did not change cellular morphology (Fig. 2, compare B, C and D).

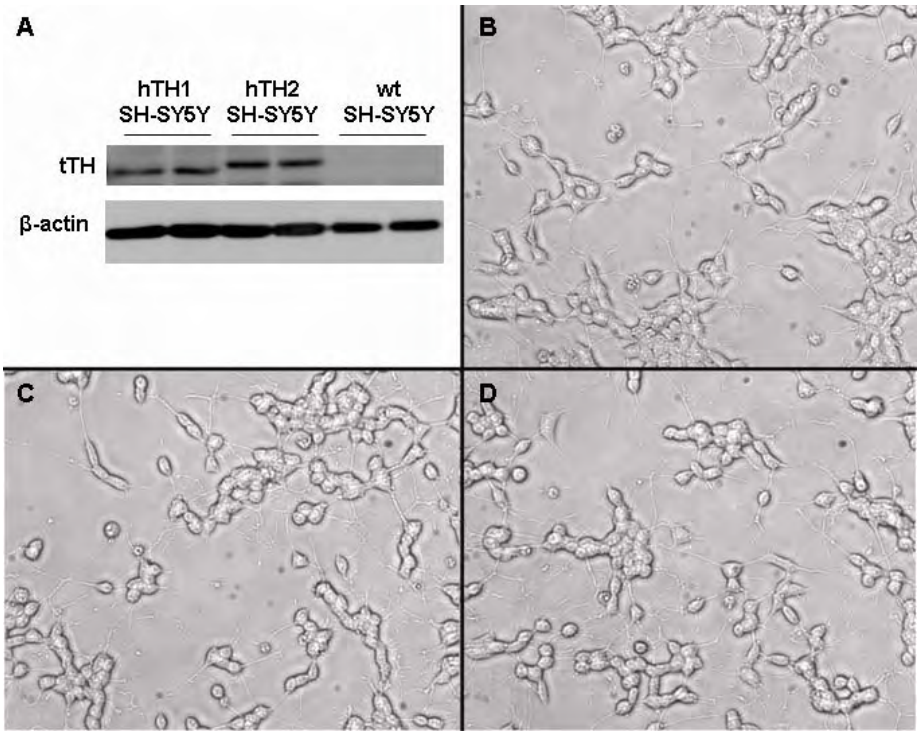

Fig. 2: Characterisation of stably-transfected SH-SH5Y cells. A) Wild-type (wt), hTH1 and hTH2 SH-SY5Y cells extracts were analysed for TH protein and β-actin content as described in methods. **B)** Wild-type, **C)** hTH1 and **D)** hTH2 SH-SY5Y cells were photographed using the Zeiss microscope to visualise cell morphology.

Basal TH activity in wild-type, hTH1 and hTH2 SH-SY5Y cells was assayed to determine whether the hTH expressed in the SH-SY5Y cells was active. TH activity in hTH1 and hTH2 cells was found to be significantly increased above the level of wild-type cells ($p<0.01$, Fig. 3). This concurs with data demonstrating that while catecholamines are undetectable in wild-type SH-SY5Y cells, the transfection

of cells with TH results in a significant increase in catecholamine levels[2]. Therefore the TH that was expressed in hTH1 and hTH2 SH-SY5Y cells was active.

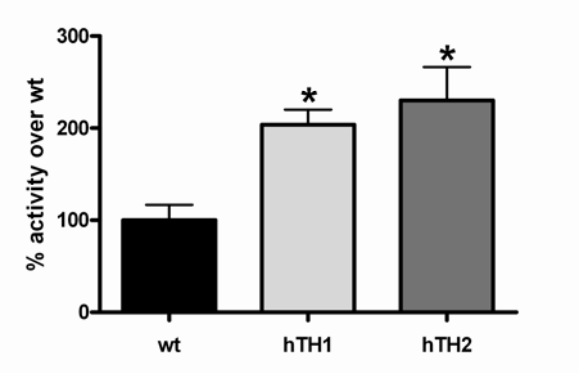

Fig. 3: hTH1 and hTH2 SH-SY5Y cells express active TH. Wild-type (wt), hTH1 and hTH2 SH-SY5Y cells were lysed and assayed for TH activity as described in methods section. Activity is expressed as a percentage of mean of wild-type activity (wt = 100%) + SEM (n=3). *p<0.01 vs wild-type

2.4.2. In situ phosphorylation of hTH1 and hTH2 at Ser19 and Ser40/44

The hTH1 and hTH2 SH-SY5Y cells were characterised with regards to the phosphorylation of the three main Ser residues in TH (Ser19, Ser31, Ser40 in hTH1; Ser19, Ser35 and Ser44 in hTH2). hTH1 and hTH2 SH-SY5Y cells were stimulated with 10µM forskolin for 5min, and assayed for phosphorylation and TH protein levels as described in the methods section. Stimulation with forskolin significantly increased the phosphorylation of Ser40/44 in both hTH1 and hTH2 cells ($p<0.01$, Fig. 4). There was no significant difference in the level of forskolin-induced phosphorylation of Ser40/44 between hTH1 and hTH2 cells. Ser31 phosphorylation levels were unchanged in hTH1, and phosphorylation of Ser35 was not detectable in hTH2 in either the control or stimulated cells (data not shown). Forskolin did not change Ser19 phosphorylation or total TH protein levels in either cell type.

[2] Franco, J. *et al*. 2008 Manuscript in submission

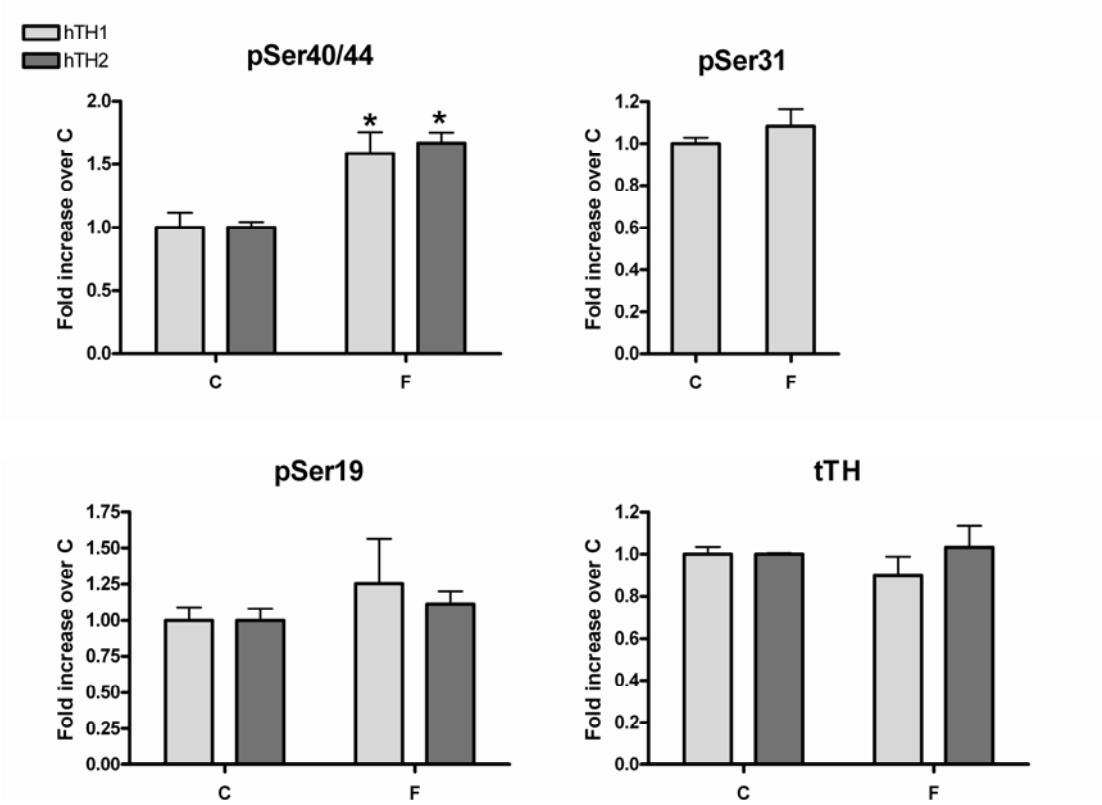

Fig. 4: Forskolin stimulation of hTH1 and hTH2 SH-SY5Y cells. SH-SY5Y cells were stimulated with 10µM foskolin (F) or the same volume of vehicle (C) for 5min. TH phosphorylation and TH protein levels were analysed and quantified as described in materials and methods. Data are presented as the average fold increase relative to mean of control levels + SEM (n=8 for C, n=5 for F). *p<0.01 vs corresponding control.

hTH1 and hTH2 SH-SY5Y cells were stimulated with 10µM muscarine for 5min. Muscarine was found to significantly increase the phosphorylation of Ser40/44 in both hTH1 and hTH2 cells ($p<0.001$, Fig. 5). Muscarine also induced a substantial increase in the phosphorylation of Ser19 in both hTH1 and hTH2 cells ($p<0.001$, Fig. 5). There was no significant difference in the level of muscarine-induced phosphorylation of Ser19 or Ser40/44 between hTH1 and hTH2 cells. There was no increase in the phosphorylation of Ser31 in hTH1 cells (Fig. 5), and again phosphorylation of Ser35 in hTH2 was not detectable in control or muscarine-

stimulated cells (see representative immunoblot, Fig. 5). There was no change in TH protein levels in either cell type.

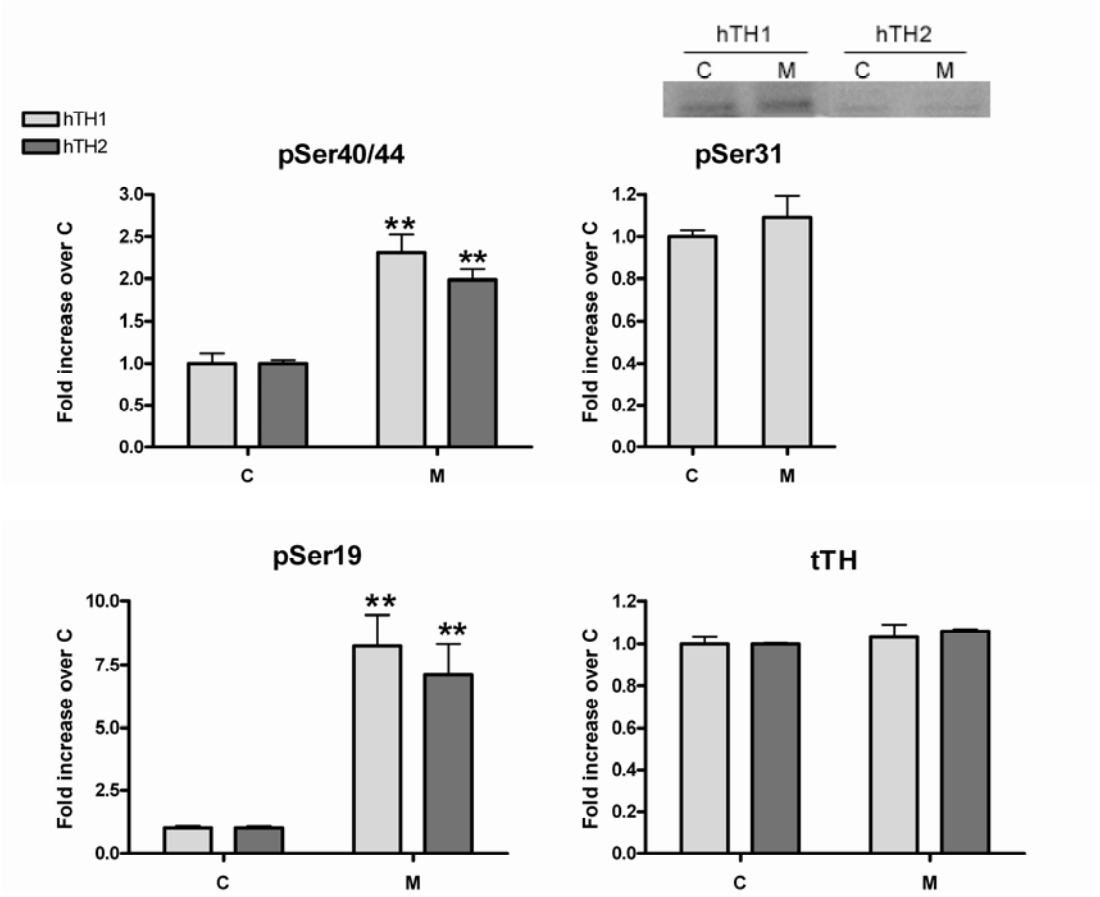

Fig. 5: Muscarine stimulation of hTH1 and hTH2 SH-SY5Y cells. SH-SY5Y cells were stimulated with 10µM muscarine (M) or the same volume of vehicle (C) for 5min. TH phosphorylation and TH protein levels were analysed and quantified as described in materials and methods. Representative pSer31 immunoblot is displayed in upper right quadrant. Data are presented as the average fold increase relative to mean of control levels + SEM (n=8 for C, n=5 for M). **p<0.001 vs corresponding control.

2.4.3. In situ phosphorylation of Ser31 in hTH1, but not Ser35 in hTH2

hTH1 and hTH2 cells were stimulated with 50ng/mL EGF for 30min. EGF significantly increased the phosphorylation of Ser31 in hTH1 ($p<0.05$, Fig. 6), however no phosphorylation of Ser35 in hTH2 was detected (see representative immunoblot, Fig. 6). Stimulation with EGF did not result in any changes in the

phosphorylation of Ser40 or Ser19, or TH protein levels. EGF increased the phosphorylation of ERK in both hTH1 and hTH2 cells (data not shown).

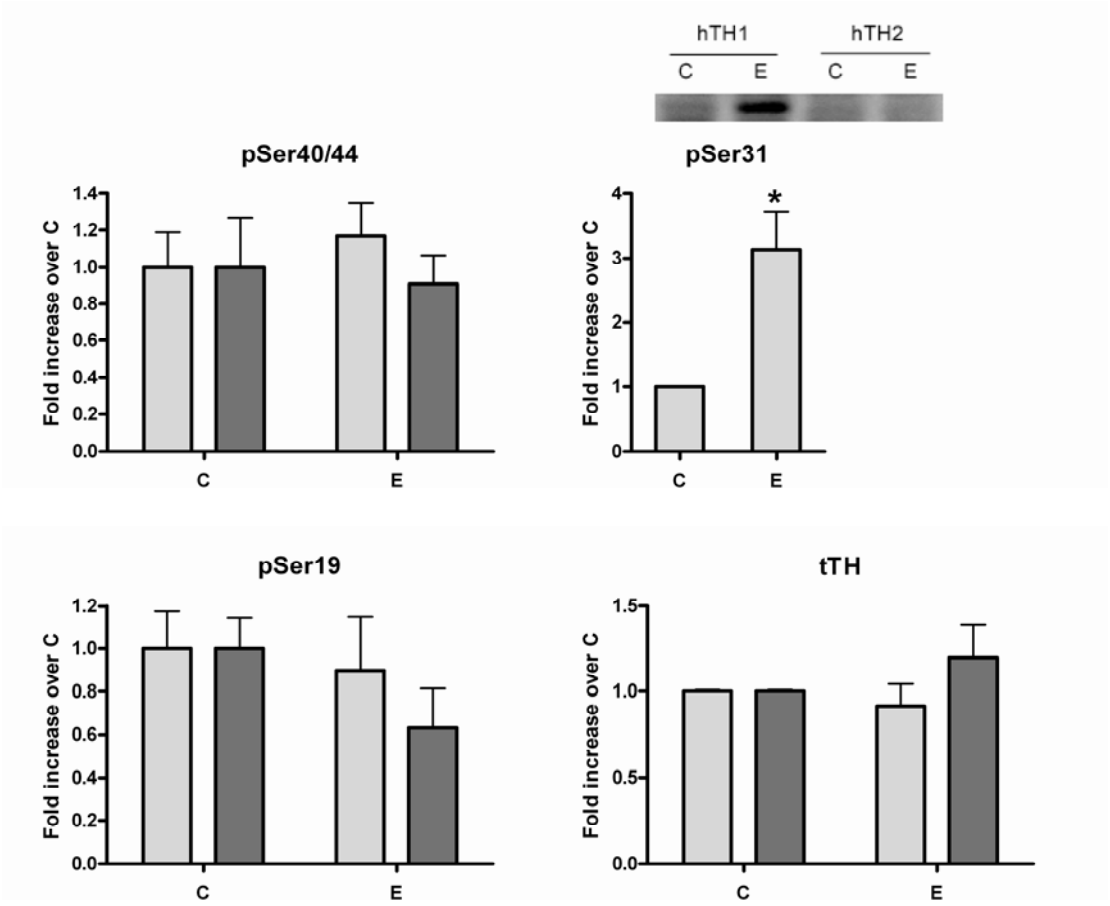

Fig. 6: EGF stimulation of hTH1 and hTH2 SH-SY5Y cells. SH-SY5Y cells were stimulated with 50ng/mL EGF (E) or the same volume of vehicle (C) for 30min. TH phosphorylation and TH protein levels were analysed and quantified as described in materials and methods. Representative pSer31 immunoblot is displayed in upper right quadrant. Data are presented as the average fold increase relative to mean of control levels + SEM (n=4). *$p<0.05$ vs corresponding control.

2.4.4. pSer31-dependent hierarchical phosphorylation contributes to basal pSer40 levels in hTH1, but not hTH2 cells

We could not detect any phosphorylation of Ser35 in hTH2 under basal conditions or using stimuli that increased the phosphorylation of Ser31 in hTH1. Phosphorylation of Ser31 can potentiate the phosphorylation of Ser40 *in vitro*

(Lehmann et al. 2006). We therefore decided to inhibit kinases that are known to contribute to Ser31 phosphorylation and determine the effect on Ser40/44 phosphorylation to see if phosphorylation of Ser31 was contributing to the phosphorylation of Ser40 in a hierarchical manner. hTH1 and hTH2 SH-SY5Y cells were incubated for 30min with 10µM UO126, which inhibits MEK, a kinase upstream of ERK1/2. UO126 significantly inhibited the phosphorylation of ERK in both hTH1 and hTH2 cells ($p<0.001$, Fig. 7). UO126 significantly inhibited basal Ser31 phosphorylation in hTH1 cells ($p<0.05$, Fig. 7); Ser35 phosphorylation in hTH2 cells was not detectable (data not shown). UO126 also significantly inhibited the basal phosphorylation of Ser40 in hTH1 ($p<0.05$), but did not inhibit the phosphorylation of Ser44 in hTH2 (Fig. 7). Ser19 phosphorylation levels and total TH levels were not significantly decreased following treatment with UO126. This data indicates that phosphorylation of Ser31 can contribute to Ser40 phosphorylation in hTH1, however this effect is absent in hTH2 cells due to the lack of Ser35 phosphorylation.

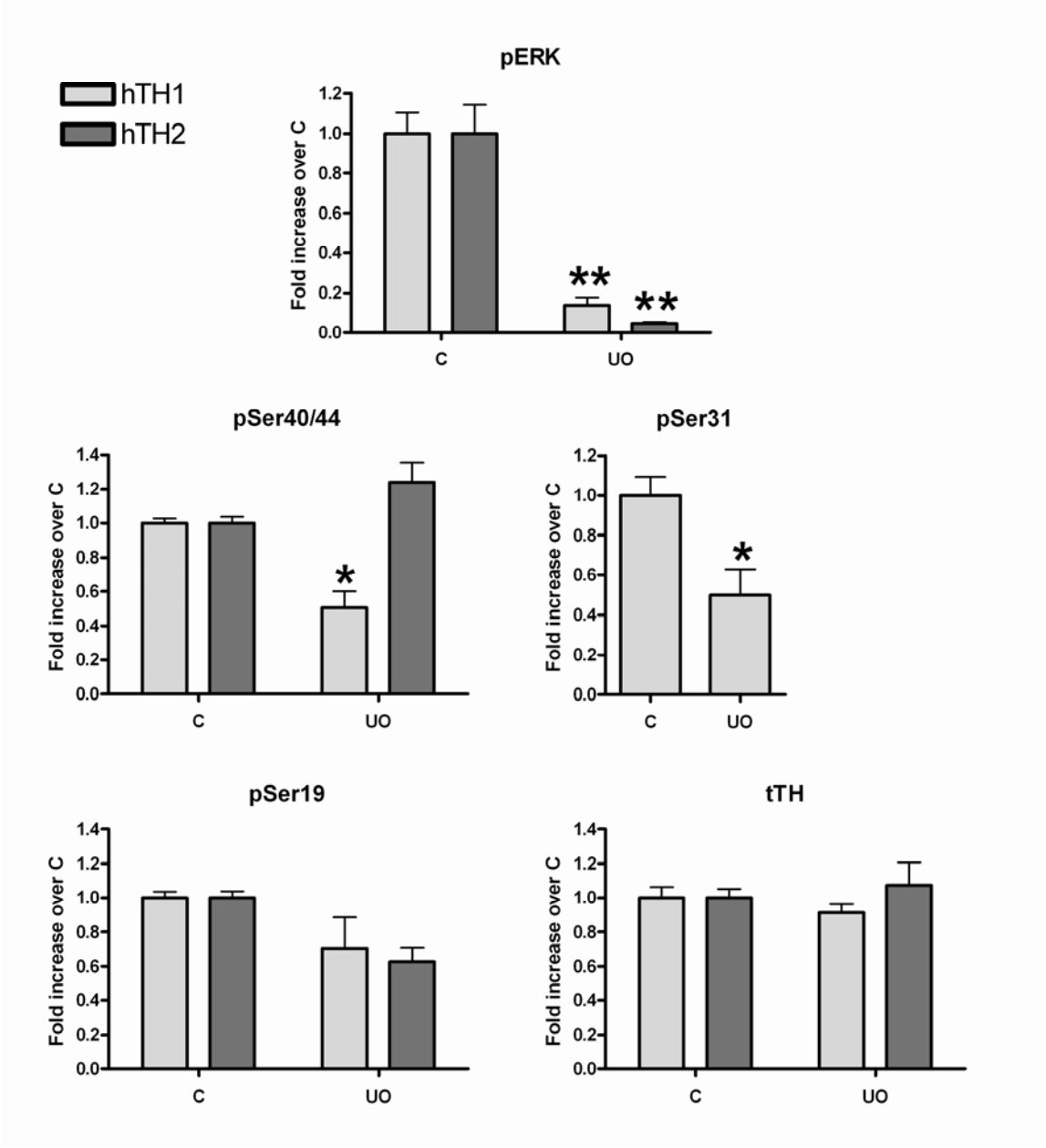

Fig. 7: UO126 treatment of hTH1 and hTH2 SH-SY5Y cells. SH-SY5Y cells were pre-incubated with 10µM UO126 (UO) or the same volume of vehicle (C) for 30min. TH phosphorylation and TH protein levels were analysed and quantified as described in materials and methods. Data are presented as the average fold increase relative to mean of control levels + SEM (n=5, n=6 for pERK). *$p<0.05$ vs corresponding control, **$p<0.001$ vs corresponding control.

2.5. Discussion

Phosphorylation of Ser31 plays an important role in the regulation of TH activity. Phosphorylation of Ser31 can directly increase TH activity, and also participates in the hierarchical phosphorylation of Ser40 *in vitro*. In this study we have demonstrated that while basal phosphorylation of Ser40 in hTH1 is potentiated by the phosphorylation of Ser31 *in situ*, hTH2 is not subject to the same regulatory mechanism. In addition, we have demonstrated using stimuli that induce a large increase in Ser31 phosphorylation in hTH1, and also under conditions that increase intracellular Ca^{2+} mobilisation, that there is no detectable phosphorylation of Ser35 in hTH2. This represents a fundamental difference between the regulatory mechanisms utilised by the major human TH isoforms.

To date, there have been no studies that have investigated the different phosphorylation profiles of hTH1 and hTH2 *in situ*. Active hTH1 and hTH2 were stably expressed in SH-SY5Y cells, a neuroblastoma cell line with a dopaminergic phenotype. Stimulation with forskolin induces a selective increase in the phosphorylation of Ser40/44 in hTH1 and hTH2 cells. Stimulation of hTH1 and hTH2 cells with muscarine results in an increase in the phosphorylation of both Ser19 and Ser40/44. This correlates with *in vitro* data demonstrating that the two major hTH isoforms are phosphorylated to an equal extent at Ser19 and Ser40/44 (Almas et al. 1992; Sutherland et al. 1993).

Stimulation of hTH1 cells with EGF resulted in a substantial increase in the phosphorylation of Ser31, which was not accompanied by an increase in Ser40 phosphorylation. It was expected that phosphorylation of Ser31 would potentiate the phosphorylation of Ser40. However in the transfected SH-SY5Y cells forskolin only induced a mild (1.6-fold) increase in the phosphorylation of Ser40, which is much

smaller than the approximately 4-fold forskolin-induced increase in Ser40 phosphorylation that was previously found in bovine adrenal chromaffin cells (Bobrovskaya et al. 2004; Lehmann et al. 2006). This may mean that phosphorylation of hTH1 and hTH2 in the transfected SH-SY5Y cells was already at a high level due to higher basal Ser40 kinase activity in these cells, and could explain why phosphorylation of Ser31 alone could not further increase Ser40 phosphorylation. In effect, we may have already exceeded the level of Ser40 phosphorylation that can be altered by hierarchical phosphorylation via Ser31. Therefore, to examine whether Ser31 phosphorylation was contributing to the phosphorylation of Ser40 *in situ*, we treated the cells with UO126 which significantly inhibited the phosphorylation of ERK in both hTH1 and hTH2 cells, and resulted in a 50% inhibition of Ser31 phosphorylation in hTH1. Importantly, there was a 50% inhibition of Ser40 phosphorylation in hTH1 that was not accompanied by a concomitant inhibition of Ser44 phosphorylation in hTH2. The inhibition of Ser40 phosphorylation in hTH1 cells must be due to an UO126-mediated inhibition of Ser31 phosphorylation; if UO126 was acting upstream of the Ser40 kinase pathway, then a decrease in phosphorylation of Ser40/44 would be present in both cell types. Therefore we have demonstrated that while hTH1 is regulated by Ser31-mediated hierarchical phosphorylation of Ser40 under basal conditions *in situ*, hTH2 is not subject to this form of regulation.

While EGF induced a substantial increase in the phosphorylation of Ser31 in hTH1, it did not increase the phosphorylation of Ser35 in hTH2. Activation of the muscarinic receptor results in the mobilisation of Ca^{2+} from intracellular stores and activation of Ca^{2+}-dependent kinases, such as CaMKII (Babb et al. 1996; Alberi et al. 2000). Muscarine potently increased the phosphorylation of Ser19 and Ser40/44 in

both hTH1 and hTH2, which is consistent with the activation of Ca^{2+}-dependent kinases, however stimulation with muscarine for 5min (Fig. 5) or 30min (data not shown) was not able to increase Ser31/35 phosphorylation in hTH1 or hTH2 cells. Therefore using two different stimuli that activate a variety of intracellular signalling pathways we have not detected Ser35 phosphorylation in hTH2 *in situ*.

This is the first study to demonstrate that hTH1 and hTH2 are differentially regulated *in situ*. The lack of phosphorylation of Ser35 in hTH2 *in situ* means that Ser35 is not able to facilitate the hierarchical phosphorylation of Ser44. In addition, while phosphorylation of Ser31 can directly increase hTH1 activity by decreasing the K_M for the cosubstrate BH_4, hTH2 would not be subject to this form of regulatory control. Haycock and colleagues have demonstrated that the human TH isoforms may be differentially distributed in different cell populations. For example, in dopaminergic cells of the substantia nigra, it was shown that although all four human TH isoforms were expressed in the cell body, hTH1 was selectively distributed along the axons and terminal fields of these neurons (Lewis et al. 1993). The selective targeting of the isoforms would provide a mechanism for the region-specific modulation of TH activity. hTH1 and hTH2 are subject to differential regulation, and therefore the rate of catecholamine synthesis that occurs a specific cellular region would depend on the relative amounts of the isoforms that are present in that region. Therefore the differential regulation of the major human TH isoforms may have important consequences for the control over catecholamine biosynthesis *in vivo*.

2.6. Acknowledgements

We would like to thank Dr. Ingo Lehmann for providing the pcDNA3.1 hTH1 vector, and Jacqueline Shehadeh for supplying the recombinant human TH

lacking the first 156 residues. We would like to thank Kelly Marquardt for assistance with tissue culture techniques. This work was supported by funding from the National Health and Medical Research Council (No. 455547).

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Chapter 3: Tyrosine hydroxylase activity is regulated by two distinct dopamine-binding sites

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This publication was accepted by the Journal of Neurochemistry (21st May 2008).

ACKNOWLEDGEMENT OF AUTHORSHIP

I hereby certify that the work embodied in this chapter contains a published paper/ scholarly work of which I am a joint author.

The work in the following chapter (Chapter 3) was submitted to, and accepted, by the Journal of Neurochemistry in May 2008. I (Sarah Gordon) was the author of this publication, and the work embodied in the publication was primarily performed by myself, with the exception of the following:

Figure 1: Measurement of dopamine binding to rat tyrosine hydroxylase, was performed by Associate Professor Phillip Dickson with assistance from Noelene Quinsey.

Figure 2: Analysis of dopamine binding to rat tyrosine hydroxylase (rTH) and pSer40 rTH, parts B, C, E and F only, was performed by Associate Professor Phillip Dickson with assistance from Noelene Quinsey.

Figure 3: Catecholamine binding to rat tyrosine hydroxylase (rTH) and pSer40 rTH, was performed by Associate Professor Phillip Dickson with assistance from Noelene Quinsey.

The work embodied by these experiments is inextricably tied to the overall findings of the paper, and therefore this work will be discussed in the final chapter (Chapter 5) of this thesis.

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Sarah Gordon

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Associate Professor Phillip Dickson

TYROSINE HYDROXYLASE ACTIVITY IS REGULATED BY TWO DISTINCT DOPAMINE BINDING SITES

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FOOTNOTES

[1] The abbreviations used are: TH, tyrosine hydroxylase; DOPA, dihydroxyphenylalanine; Ser, serine; BH_4, tetrahydrobiopterin; pSer40, Ser40-phosphorylated; PKA, protein kinase A; GSH, reduced L-glutathione; OC charcoal, ovalbumin-coated charcoal; rTH, rat TH; hTH, human TH.

Running Title: Two dopamine binding sites in tyrosine hydroxylase

ABSTRACT

Tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of the catecholamines dopamine, noradrenaline and adrenaline, is regulated acutely by feedback inhibition by the catecholamines and relief of this inhibition by phosphorylation of serine 40. Phosphorylation of serine 40 abolishes the binding of dopamine to a high affinity (K_D < 4nM) site on tyrosine hydroxylase, thereby increasing the activity of the enzyme. We have found that tyrosine hydroxylase also contains a second low affinity (K_D = 90nM) dopamine binding site, which is present in both the non-phosphorylated and the serine 40-phosphorylated forms of the enzyme. Binding of dopamine to the high affinity site decreases Vmax and increases the Km for the cofactor BH_4, while binding of dopamine to the low affinity site regulates tyrosine hydroxylase activity by increasing the Km for BH_4. Kinetic analysis indicates that both sites are present in each of the four human tyrosine hydroxylase isoforms. Dissociation of dopamine from the low affinity site increases tyrosine hydroxylase activity 12-fold for the non-phosphorylated enzyme and 9-fold for the serine 40-phosphorylated enzyme. The low affinity dopamine binding site has the potential to be the primary mechanism responsible for the regulation of catecholamine synthesis under most conditions.

Keywords: tyrosine hydroxylase, dopamine, catecholamines, phosphorylation, feedback inhibition.

INTRODUCTION

The enzyme tyrosine hydroxylase (TH)[1] [EC1.14.16.2] catalyses the rate-limiting step in the biosynthesis of the catecholamines dopamine, noradrenaline and adrenaline, hydroxylating L-tyrosine to form dihydroxyphenylalanine (DOPA) (Nagatsu et al. 1964). Catecholamine secretion does not produce a concomitant decrease in intracellular catecholamine stores (Wakade et al. 1988), indicating that the secretion and synthesis of catecholamines are closely linked. Efficient and precise control of catecholamine availability is achieved through the regulation of TH activity.

Long-term regulation of TH activity is primarily controlled by TH protein synthesis (Kumer and Vrana 1996). Acute regulation of TH activity is modulated by phosphorylation of three key serine (Ser) residues on TH, Ser19, Ser31 and Ser40 (Dunkley et al. 2004). Phosphorylation of Ser19 does not directly increase TH activity (Dunkley et al. 2004), but prior phosphorylation of Ser19 does increase the rate of phosphorylation of Ser40 (Bevilaqua et al. 2001; Bobrovskaya et al. 2004). Similarly, phosphorylation of Ser31 only has a small effect on TH activity directly, producing a maximum 2 fold increase in activity *in vitro* (Haycock et al. 1992; Sutherland et al. 1993), but prior phosphorylation of Ser31 also increases the rate of Ser40 phosphorylation (Lehmann et al. 2006).

Phosphorylation of Ser40 directly increases TH activity by relieving end-product feedback inhibition by the catecholamines (Le Bourdelles et al. 1991). The catecholamines interact with the catalytic iron at the active site of TH (Almas et al. 1992), and decrease enzyme activity primarily by decreasing Vmax (Daubner et al. 1992). Catecholamines bind with high affinity to TH, with a K_D of < 4 nM (McCulloch et al. 2001; Sura et al. 2004), reconfiguring the enzyme to a strongly inhibited, but more stable conformation (Okuno and Fujisawa 1991). Phosphorylation of Ser40 substantially decreases the affinity of the catecholamines for the enzyme, increasing the K_D for dopamine to 78-208 nM (McCulloch et al. 2001; Sura et al. 2004), thereby relieving feedback inhibition and increasing enzyme activity. Regulation of TH activity by phosphorylation of Ser40 is able to extend past the acute period and can last for up to 48 hours (Bobrovskaya et al. 2007a; Bobrovskaya et al. 2007b).

Previous data is suggestive of a second type of inhibitory effect of the catecholamines. Early reports suggested that feedback inhibition occurs through competitive binding, whereby catecholamines compete with the cofactor tetrahydrobiopterin (BH_4) for binding to the enzyme (Zigmond et al. 1989). This mechanism would provide acute feedback control of TH that may be overcome by altering relative concentrations of BH_4 and catecholamine (Kumer and Vrana 1996). The nature of this form of inhibition, and the role that it plays in TH regulation was unclear.

We have investigated the nature of dopamine binding to TH. We confirmed the presence of the high affinity dopamine binding site, but have found that TH also contains a second low affinity dopamine binding site, which is present in both the non-phosphorylated and the Ser40-phosphorylated (pSer40) forms of the enzyme. Both sites are able to regulate TH activity. The low affinity dopamine binding site has the potential to be the primary mechanism responsible for the regulation of catecholamine synthesis under most conditions.

EXPERIMENTAL PROCEDURES

Materials - BH_4 was obtained from Dr. B. Shircks laboratory (Jona, Switzerland). Protein kinase A (PKA), dopamine hydrochloride, bovine catalase, reduced L-glutathione (GSH), and chicken egg albumin (ovalbumin) were all obtained from Sigma-Aldrich (St Louis, USA). Activated charcoal was obtained from BDH Biochemicals (Poole, England). L-[3,5-^{3}H]-tyrosine and [2,5,6-^{3}H]-dopamine were obtained from GE Healthcare (Buckinghamshire, England). Optiphase 'HiSafe' scintillation cocktail was from Wallac (Turku, Finland).

Expression of recombinant TH – rat TH (rTH) was expressed in *E. coli* according to previously described methods (Bevilaqua et al. 2001). Human TH isoforms 1-4 (hTH1-4) were expressed in *E. coli* according to previously described methods (Lehmann et al. 2006). TH was eluted in 300mM NaCl, 50mM Tris, pH 7.4, at a final concentration ranging from 15-30μM, and was stored in 15% glycerol at -20°C until required. No exogenous iron is added to the TH protein preparation.

Phosphorylation of Ser40 - Phosphorylation of TH at Ser40 was performed by incubating 0.8 - 6µM TH in the presence of 50mM Tris, pH 7.4, 20mM $MgCl_2$, 500µM ATP and 14U PKA for 20 min at 30˚C. Under these conditions TH is maximally phosphorylated at Ser40. The pSer40 TH was then used in place of TH in the TH activity assay solution (see below).

Preparation of ovalbumin-coated charcoal (OC charcoal) - This procedure is a development of a method originally used to measure binding of thyroid hormone to prealbumin (Munro et al. 1989). Charcoal was activated by the addition of 1M HCl, and then neutralised by sequentially washing with H_2O, 0.5M HEPES, 50mM HEPES, and 50mM potassium phosphate buffer. The final 1% w/v charcoal solution was stored at 4°C in 50mM potassium phosphate buffer, pH 7.4. When required, the charcoal solution was spun at 18000 x g for 10 minutes and the pellet resuspended in buffer A (5mM GSH, 10mg/ml ovalbumin, 0.06mg/ml catalase, 100mM potassium phosphate, pH 7.4) to a final concentration of 2% w/v charcoal. The charcoal solution was incubated at 25˚C with shaking for 1 hour, and spun at 18000 x g for 10 minutes. The supernatant was discarded and replaced with fresh buffer A. The 2% OC charcoal slurry was then incubated at 25˚C with shaking until required (no longer than 8 hours). The OC charcoal was used to bind free dopamine in the TH activity assay solution.

Measurement of catecholamine binding to TH - The binding of catecholamines to TH was routinely investigated by the sedimentation velocity meniscus depletion method of Howlett *et al.* (Howlett et al. 1978) using a Beckman Benchtop TL-100 ultracentrifuge. Solutions (100µl) containing 70-200nM rTH or 560-600nM pSer40 rTH and [^{3}H]-dopamine in 50mM potassium phosphate, pH 7.4, 5mM GSH, 50nM EDTA, 10nM catalase and 5mg/ml ovalbumin were centrifuged in a TLA100 rotor at 386000 x g for 1 h at 20^{O}C to deplete the meniscus of TH and bound ligand. In this procedure TH was sedimented to the point where it was depleted from the top 20% of the solution but still remained free in solution. The TH does not precipitate under these conditions. After centrifugation the concentration of free ligand at the meniscus was determined by scintillation counting of 20µl samples removed from the meniscus. The measured values were corrected for sedimentation of free ligand, determined by centrifuging solutions containing radiolabelled ligand but no TH. Generally 6-8 % of the ligand was depleted from the meniscus

under these conditions. The measured values were also corrected for radiolabelled molecules that could not bind TH. This was done by centrifuging solutions containing radiolabelled ligand in the presence of excess TH. Further controls are outlined in the Supplemental Data. Data was analysed by standard procedures to obtain the relationship between the molar binding function R (moles of ligand bound/mol of TH subunit) and the concentration of free ligand.

To confirm results obtained by the sedimentation velocity meniscus depletion method, microcalorimetry was performed using a Microcal MCS isothermal titration microcalorimeter. 6µM TH or pSer40 TH in 50mM potassium phosphate pH 7.4, 5mM GSH was maintained at 26^{O}C in a 1.36ml cell. The cell was titrated with 10 aliquots (10µl each) of 100µM dopamine in the same buffer. The data was analysed using the Origin software provided with the instrument. The data was corrected for the heat produced by dilution of the ligand.

Determination of TH activity - Dopamine was solubilised in 5mM HCl to prevent oxidation of the dopamine. The TH activity assay solution (suitable amount of TH or pSer40 TH, 5mM GSH and 100mM potassium phosphate, pH 7.4 in the presence of 20µM dopamine for dopamine-bound TH or an equal volume of 5mM HCl for non-dopamine bound TH) was incubated at 25˚C for 10 min, and then assayed for TH activity. Final concentrations of TH present in the TH activity assay solution ranged from 0.05 to 5µM to ensure that sufficient activity for dopamine-bound TH was obtained and that substrate conversion did not exceed 10%.

For OC charcoal-treated TH, the TH activity assay solution was prepared as described above but with the addition of 10mg/ml ovalbumin and 0.06mg/ml catalase. The TH activity assay solution was incubated with an equal volume of OC charcoal for 15 min at 25˚C, and spun at 18000 x g at 4˚C for 10 min. The supernatant was collected and assayed for TH activity.

TH activity was determined using the tritiated water release assay of Reinhard *et al.* (Reinhard et al. 1986). The activity of the TH in the activity assay solution was analysed in the presence of 24µM tyrosine (with 4µCi L-[3,5-^{3}H]-tyrosine/mL), 60mM potassium phosphate buffer, pH 7.4, 0.006% 2-mercaptoethanol, and 36µg/ml catalase. 20µM dopamine or an equal volume of 5mM HCl was added to

the final assay mixture, and each reaction was initiated by the addition of 1-2000 μM BH_4. Control samples did not contain BH_4. Conditions were chosen such that substrate conversion did not exceed 10%. Assays were performed at 30˚C for a period not exceeding 10 min. The TH activity assay was linear under these conditions.

RESULTS AND DISCUSSION

Dopamine binding to TH - It is currently accepted that dopamine binds to TH with high affinity, and that this binding is only relieved by phosphorylation of Ser40 (McCulloch et al. 2001; Sura et al. 2004). Older data suggests a different type of inhibitory interaction between dopamine and TH (Zigmond et al. 1989), but this has not been investigated in depth in recombinant systems. To further investigate the nature of dopamine feedback inhibition of TH, dopamine binding to rTH was analysed using the sedimentation velocity meniscus depletion method as described in 'Experimental Procedures'. The saturation curve for dopamine binding to TH did not appear to fit a single site model as the binding of dopamine to rTH increased rapidly at low dopamine concentrations, but continued to increase slowly at higher concentrations of dopamine (Fig. 1A). Therefore the data was transformed using the method of Scatchard (Scatchard 1949) (Fig. 1B). The non-linear nature of the Scatchard plot suggested that two dopamine binding sites exist in rTH, each displaying different affinities for dopamine. The saturation curve data was therefore fitted to a two-site model. The K_D for the high affinity site was 4 ± 1nM and for the low affinity site was 90 ± 34nM. The overall stoichiometry of dopamine binding was found to approach 1 mol dopamine/mol TH subunit (Fig. 1A).

Previous work has shown that TH contains a single high affinity dopamine binding site (Ramsey and Fitzpatrick 2000; McCulloch et al. 2001; Sura et al. 2004), with binding parameters similar to that established for the high affinity site in our experiments. The rate of dissociation from the high affinity site is essentially unmeasurable (Sura et al. 2004). However, data obtained from the saturation curve demonstrates that two distinct dopamine binding sites exist in TH. Assuming that the properties of the high affinity site were similar to that previously described in literature, we examined the properties of

dopamine binding to rTH in a competition experiment. [^{3}H]-dopamine was allowed to equilibrate with rTH and then 2mM dopamine was added to the mixture. The amount of [^{3}H]-dopamine bound to TH was then determined. The addition of excess dopamine results in the dissociation of approximately half of the [^{3}H]-dopamine from rTH (Fig. 2A). This suggests that rTH contains a high affinity dopamine binding site from which there is negligible dissociation of dopamine and a low affinity dopamine binding site from which dopamine readily dissociates.

In order to investigate the binding of dopamine to the high affinity, non-dissociating site directly, rTH was incubated with increasing concentrations of [^{3}H]-dopamine and was then incubated with 2mM dopamine to dissociate [^{3}H]-dopamine from the low affinity site. The stoichiometry of dopamine binding to the high affinity site was determined to be approximately half of that measured in the absence of excess dopamine (Fig. 2B, compare with Fig. 1A). Scatchard analysis of the data produced a linear curve (Fig. 2C), and the K_D was determined to be <10nM, which approximates that determined for the high affinity site in the two-site model of dopamine binding. This is also consistent with the K_D previously determined for dopamine binding to TH (McCulloch et al. 2001; Sura et al. 2004).

Phosphorylation of Ser40 relieves feedback inhibition of TH by dopamine by increasing the K_D for dopamine by at least 23-fold compared to that for the non-phosphorylated enzyme (McCulloch et al. 2001; Sura et al. 2004). We wished to determine the nature of dopamine binding to pSer40 rTH with regard to our new two-site dopamine binding model. We analysed the binding of dopamine to pSer40 rTH in the same way as that for non-phosphorylated rTH. pSer40 rTH was incubated with [^{3}H]-dopamine and allowed to reach equilibrium before being incubated with 2mM dopamine. Addition of excess dopamine resulted in the dissociation of 97% of [^{3}H]-dopamine from pSer40 rTH (Fig. 2D), suggesting that pSer40 rTH does not contain the high affinity dopamine binding site. The stoichiometry of dopamine binding to pSer40 rTH was determined to be approximately 1 mol dopamine/mol TH subunit (Fig. 2E), which is the same as that determined for non-phosphorylated TH (see Fig. 1A). The K_D for dopamine binding was 59 ± 3nM (Fig. 2E) which is similar to that determined for the low affinity dopamine binding site in rTH. The K_D is also similar to that previously measured for dopamine binding to pSer40 TH (Sura et al. 2004).

Scatchard transformation of the data produced a linear curve (Fig. 2F), indicating that pSer40 rTH does not contain binding sites with different affinities for dopamine.

The overall stoichiometry of dopamine binding to rTH and pSer40 rTH is essentially the same even though rTH contains two distinct dopamine binding sites while pSer40 rTH contains only the low affinity dopamine binding site. This result was confirmed using isothermal titration microcalorimetry (data not shown). The stoichiometry of dopamine binding to rTH using this method was 0.84 ± 0.01 mol dopamine/mol TH subunit and that to pSer40 rTH was 0.79 ± 0.03 mol dopamine/mol TH subunit.

The crystal structure of the catalytic core and tetramerisation region suggests that TH exists as a dimer of dimers, with the two dimers only interacting through the leucine zipper region (Goodwill et al. 1997; Goodwill et al. 1998). Dopamine binds to TH such that the total stoichiometry for dopamine binding to TH is approximately 1 mol dopamine/mol TH subunit, irrespective of whether TH is phosphorylated or not. Taken together, this suggests that a model for our data could be that there is one high affinity site and one low affinity site for each dimer of non-phosphorylated TH. pSer40 TH also contains two dopamine binding sites per dimer, but they are both low affinity sites.

In many TH expressing cells the primary catecholamine will be either noradrenaline or adrenaline. In order to determine whether these two catecholamines bind to TH in the same way as dopamine, competition experiments were performed. [^{3}H]-dopamine was mixed with increasing concentrations of dopamine, noradrenaline and adrenaline and then incubated with rTH and the amount of [^{3}H]-dopamine bound was determined. All three catecholamines can completely inhibit the binding of [^{3}H]-dopamine to rTH (Fig. 3A). All three catecholamines therefore appear to bind to both sites in rTH. When catecholamine binding to pSer40 rTH was determined it was again found that the three catecholamines could completely inhibit [^{3}H]-dopamine binding (Fig. 3B). Dopamine has a slightly higher affinity for rTH than noradrenaline and adrenaline (Fig. 3A), however similar levels of each of the catecholamines were required to inhibit the binding of [^{3}H]-dopamine to pSer40 rTH (Fig. 3B), suggesting that the affinity of binding of each of the catecholamines for the low affinity site of TH is very similar.

The effect of dopamine binding on the kinetic constants of rTH and pSer40 rTH - The effect of dopamine binding on the kinetic constants of rTH was determined. Substrate inhibition was found to occur at high concentrations of BH_4, which is consistent with previous studies (Mann and Gordon 1979). Kinetic analyses were performed using BH_4 concentrations not exhibiting cofactor inhibition.

The kinetic constants of rTH and pSer40 rTH in the absence or presence of dopamine were determined using increasing concentrations of BH_4. Vmax was high for rTH (19 ± 4 µM DOPA/min/µM TH subunit, Fig. 4A) and this decreased substantially in the presence of dopamine (0.28 ± 0.03µM DOPA/min/µM TH subunit, Fig. 4B). Vmax for pSer40 rTH was high (16 ± 1µM DOPA/min/µM TH subunit, Fig. 4C) and remained high in the presence of dopamine (15 ± 4 µM DOPA/min/µM TH subunit, Fig. 4D).

The Km for BH_4 was low for rTH (10.48 ± 0.18µM, Fig. 4A) and dramatically increased in the presence of dopamine (776 ± 122µM, Fig. 4B). Similarly, pSer40 rTH also had a low Km for BH_4 (5.78 ± 0.73 µM, Fig. 4C) which substantially increased in the presence of dopamine (294 ± 73 µM, Fig. 4D).

Eadie-Hofstee transformations of the data were performed to determine the relative contributions that each dopamine binding site makes to the overall effect of dopamine on the kinetic constants for TH. The slope of the curve indicates the Km for BH_4. These plots were linear (Fig. 4, see inset graphs), so although there are two dopamine binding sites, the data suggests that the Km for BH_4 for each cofactor binding site is similar.

Dopamine binding to TH decreases Vmax and increases the Km for BH_4. Dopamine binding to pSer40 rTH has no major effect on Vmax but results in a large increase in the Km for BH_4. In order to determine how dopamine binding to the high and low affinity sites affected the kinetic constants of TH, it was necessary to investigate the effect of the two sites individually. To do this we developed a procedure in which we utilised ovalbumin-coated charcoal (OC charcoal), which binds approximately 99% of the free dopamine in the assay mixture (see Supplemental Data for OC charcoal method validation). TH was incubated with dopamine to saturate both sites and was then incubated with OC charcoal. The binding of free dopamine to the OC charcoal would induce dissociation of dopamine from the low affinity site but

not the high affinity site. Thus, TH could be studied with dopamine bound only to the high affinity site. In order to determine the effect of the low affinity site, dopamine was added back into the TH activity assay solution prior to analysis of activity.

The data in Fig. 5A (squares) shows the BH_4 concentration curve for TH with dopamine bound only to the high affinity site. As expected, Vmax is low (see Table S1 in Supplemental Data for detailed summary of kinetic constants), however the curve did not seem to exhibit regular rectangular hyperbolic shape. There is an initial rapid increase in TH activity at low BH_4 concentrations, but the activity continued to steadily increase at higher BH_4 concentrations. The data was subjected to an Eadie-Hofstee transformation, which produced a non-linear curve (Fig. 5B, squares). As the slope of the curve corresponds to the Km for BH_4, this suggests that there are two distinct Kms for BH_4 in TH when dopamine is bound only to the high affinity site. The first site had a low Km for BH_4 (8 ± 2 μM), while the second site had a high Km for BH_4 (252 ± 46 μM). This result was replicated using gel filtration to dissociate dopamine from the low affinity site in place of the OC charcoal (see Supplemental Data). One explanation for these results would be that dopamine binding to the high affinity site inhibits the binding of BH_4 to one of the BH_4 binding sites on the dimer, thereby increasing the Km for BH_4, but does not affect the binding of BH_4 to the other site on the dimer, which will still have a low Km. This would suggest that the high affinity dopamine binding site regulates TH activity by decreasing Vmax and increasing the Km for BH_4 at the corresponding BH_4 binding site.

The addition of dopamine to the OC charcoal-treated TH should reverse the effect of the OC charcoal as dopamine will now be bound to both the high and low affinity sites. Vmax remained low when the supernatant mixture was incubated with additional dopamine (Fig. 5A, circles and see Table S1 in Supplemental Data). Eadie-Hofstee transformation of the data produced a single curve (Fig. 5B, circles). The low Km site was no longer evident, and there was a single Km for BH_4 of 528 ± 113 μM, which is similar to that for dopamine-bound TH that had not been treated with OC charcoal (see Fig. 4B). This indicates that the removal of dopamine from the low affinity site is readily reversible. With dopamine bound to both the high and low affinity sites on TH, it is likely that dopamine will inhibit the

binding of BH_4 to both BH_4 sites on the dimer, thereby resulting in a high Km for BH_4. The binding of dopamine to the low affinity site therefore exerts the majority of its effect by increasing the Km for BH_4.

Ser40-phosphorylated rTH was incubated with dopamine and following incubation with OC charcoal was assayed in the presence or absence of additional dopamine. In the absence of additional dopamine, pSer40 rTH had a high Vmax (Fig. 5D, squares) and Eadie-Hofstee transformation of the data revealed a single low Km for BH_4 of 28 ± 3 μM (Fig. 5E, squares). These results are similar to those obtained for non-dopamine-bound pSer40 rTH (see Fig. 4D), suggesting that the binding of dopamine to pSer40 rTH is readily reversible. When pSer40 rTH was incubated in the presence of additional dopamine the Vmax remained high (Fig. 5D, circles). However, the single Km for BH_4 increased to 258 ± 4 μM (Fig. 5E, circles) which is consistent with dopamine binding to the low affinity site inhibiting the binding of BH_4 to the enzyme. This further demonstrates that the low affinity dopamine binding site exerts its effects by increasing the Km for BH_4.

Effect of the low affinity site on TH activity - The suggested physiological concentration range of BH_4 is 1-100μM (Abou-Donia and Viveros 1981; Levine et al. 1981; Nagatsu 1983). We wanted to determine the effect of the low affinity site on TH activity over this concentration range. Therefore, rTH (Fig. 5C) and pSer40 rTH (Fig. 5F) were incubated with dopamine, treated with OC charcoal to dissociate dopamine from the low affinity site, and then assayed in the presence or absence of additional dopamine over 1-100μM BH_4. This assay provides an estimate of the maximal effect that the site will have on TH activity in a physiological setting.

Dissociation of dopamine from the low affinity site of rTH by treatment with OC charcoal results in a 12-fold increase in activity over the physiological concentration range of BH_4 (Fig. 5C). Similarly, dissociation of dopamine from the low affinity sites of pSer40 rTH results in a 9-fold increase in activity (Fig. 5F).

Two distinct dopamine binding sites exist in each of the 4 major human TH isoforms - There are 4 major human TH isoforms (hTH1-4). hTH1 most closely resembles TH found in other mammalian species, such as rat TH and bovine TH, while hTH2-4 contain an additional 4, 27 and 31 (4 + 27) amino

acids inserted N-terminal to the Ser31 residue in the hTH1 isoform (Grima et al. 1987; Le Bourdelles et al. 1988). hTH1 and hTH2 are the most prominent forms found in human tissue (Haycock 1991, 2002).

The activity assays performed for rTH were repeated for hTH1-4 and kinetic constants for the enzymes under various conditions were determined (see Table S1 in Supplemental Data). These were found to be similar to those determined for rTH.

hTH1-4 and pSer40 hTH1-4 were incubated with dopamine, treated with OC charcoal, and then assayed in the absence or presence of additional dopamine. Eadie-Hofstee plots of OC charcoal-treated hTH1-4 assayed in the absence of additional dopamine were non-linear (data not shown). This is a similar result to that obtained for rTH (see Fig. 5B). The kinetic constants of hTH1-4 and pSer40 hTH1-4 were in a similar range to that determined for rTH and pSer40 rTH (see Table S1 in Supplemental Data). Thus, kinetic analyses suggested that each of the four hTH isoforms has two distinct dopamine binding sites. The data obtained in the OC charcoal experiments was plotted over the physiological BH_4 concentration range to determine the effect of the low affinity site on TH activity (Fig. 6). Dissociation of dopamine from the low affinity site is able to increase TH activity in a similar way to that demonstrated for rTH (refer to Fig. 5).

Regulatory role of dopamine on TH activity and cytosolic catecholamine levels

TH activity is regulated by feedback inhibition by the catecholamines dopamine, noradrenaline and adrenaline. Previous work has suggested that dopamine binds with high affinity to TH, and is only dissociated from the enzyme by phosphorylation of Ser40 (Daubner et al. 1992). The work shown here has demonstrated that a second type of dopamine binding site exists in TH, namely a low affinity binding site from which dopamine is readily-dissociable.

The low affinity dopamine binding site has previously been recognised in pSer40 TH (McCulloch et al. 2001; Sura et al. 2004), but this is the first study to demonstrate that the site is also present in non-phosphorylated TH and to elucidate the functional consequences of dopamine binding to the site in both non-phosphorylated and pSer40 TH. The low affinity dopamine binding site is able to regulate TH

activity in both the non-phosphorylated and pSer40 forms of the enzyme, with dissociation of dopamine from the site increasing activity 12- and 9-fold respectively.

Under basal conditions, only a small proportion of TH is phosphorylated. For example, in rat brain, basal phosphorylation stoichiometry of Ser40 is 0.02-0.05 mol pSer40 TH/mol total TH (Salvatore et al. 2000). This suggests that under these conditions the majority of TH would contain both the high affinity and low affinity dopamine binding sites. The cytosolic concentration of catecholamine in PC12 cells has been determined to be <100nM (Mosharov et al. 2006). The K_D for dopamine binding to the high affinity site of TH has been determined to be in the very low nM range ((McCulloch et al. 2001; Sura et al. 2004) and this paper) and the off rate for dopamine binding was essentially unmeasurable (Sura et al. 2004). Therefore, under basal conditions, the high affinity dopamine binding site would be almost fully saturated.

The low affinity dopamine binding site has the potential to provide a major form of regulation of TH activity under most conditions by responding to cytosolic catecholamine levels (see Fig. 7). Under basal conditions, the high affinity site is occupied, and regulation of catecholamine synthesis rests solely with the low affinity dopamine binding site. The K_D for the low affinity site as determined in our studies is close to cytosolic catecholamine levels, so the low affinity site would respond to the cytosolic catecholamine levels and regulate TH activity accordingly. When low levels of catecholamine release occurs, cytosolic dopamine levels will decrease as vesicular filling occurs. This would result in dissociation of dopamine from the low affinity site and cause an increase in TH activity to replenish catecholamine stores (Fig. 7 panel A). Conversely, if cytosolic catecholamine levels increase dopamine will bind to the low affinity site to inhibit TH activity.

Following high levels of release of catecholamines from the cell, TH activity must be increased in order to facilitate vesicular filling (Wakade 1988; Wakade et al. 1988). Phosphorylation of Ser40 abolishes the high affinity dopamine binding site, increasing TH activity to provide higher levels of catecholamine synthesis (Dunkley et al. 2004). This switches catecholamine synthesis to a high throughput system, with higher levels of catecholamine synthesis to match the required rate of vesicular

packaging. Once vesicles become filled, there may be a lag period before TH is dephosphorylated at Ser40 and enzyme activity is reduced by dopamine re-binding to the high affinity site. The low affinity binding sites on pSer40 TH would be capable of responding to increased cytosolic concentrations of catecholamines to decrease TH activity and therefore prevent accumulation of cytosolic catecholamines during this time. (See Fig. 7 panel B). Conversely, if cytosolic catecholamine levels decrease the catecholamines will be released from the low affinity site to increase TH activity even for the pSer40 form of the enzyme.

The low affinity dopamine binding site would act to maintain cytosolic catecholamine levels within a narrow range irrespective of the phosphorylation status of TH, uptake of catecholamines into vesicles, degradation of catecholamines or reuptake from extracellular pools. This would have two major consequences. Firstly, the site may act as the primary regulator of TH activity under most conditions, maintaining the required rate of catecholamine synthesis to meet low level needs. Dissociation of dopamine from the low affinity site has the capacity to increase TH activity 9-12 fold regardless of the phosphorylation status of the enzyme. Phosphorylation of Ser40 would only become necessary when high levels of catecholamine synthesis are required.

The low affinity dopamine binding site may also have a protective role within the cell by preventing the accumulation of catecholamines in the cytosol. Cytosolic catecholamines are subject to auto- or enzymatic oxidation, which potentially places cells under oxidative stress and is thought to contribute to dopaminergic cell degeneration (Jenner 2003). Accumulation of cytosolic catecholamines has been hypothesised to contribute to the pathogenesis of Parkisonson's disease (Barzilai et al. 2001). The low affinity binding site may respond automatically to changes in cytosolic catecholamines concentrations to regulate TH activity. This occurs regardless of the phosphorylation state of the enzyme. Therefore, the low affinity dopamine binding site may have a protective function by modulating TH activity to prevent the harmful accumulation of cytosolic catecholamines.

ACKNOWLEDGEMENTS

We would like to thank Ingo Lehmann for help with the expression of the human TH isoforms. We would like to thank John Rostas for reading the manuscript. This work was supported by grants from the National Health and Medical Research Council (No. 455547), the Australian Research Council (No. DP0343355) and the Hunter Medical Research Institute from a donation from the Lions Club of Adamstown (Newcastle).

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FIGURE LEGENDS

Figure 1: Measurement of dopamine binding to rTH A) rTH was incubated with different concentrations of [^{3}H]-dopamine for 30 min at 20^{O}C. Assay of the amount of [^{3}H]-dopamine bound was performed using the sedimentation velocity meniscus depletion method. The final concentrations of TH used were 70 and 120nM. The data was fitted to a two-site model, and the solid line represents the best fit of the data. High affinity site: K_D = 4 ± 1nM, R_{max} = 0.36 ± 0.06 mol dopamine/mol TH subunit. Low affinity site: K_D = 90 ± 34nM, R_{max} = 0.58 ± 0.01 mol dopamine/mol TH subunit. **B)** Scatchard transformation of (A). R is the molar binding function (mol ligand bound/mol TH subunit). Results shown are representative.

Figure 2: Analysis of dopamine binding to rTH and pSer40 rTH. **A)** 200nM rTH was incubated with [^{3}H]-dopamine for 30 min at 20^{O}C and then further incubated in the absence (-) or presence (+) of 2mM dopamine. The amount of [^{3}H]-dopamine bound was determined using the sedimentation velocity meniscus depletion method. Bars indicated are standard error, n=3. **B)** 200nM rTH was incubated with different concentrations of [^{3}H]-dopamine for 30 min at 20^{O}C after which 2mM dopamine was added before assay of the amount of [^{3}H]-dopamine bound using the sedimentation velocity meniscus depletion method. The data was fitted to a single-site model, and the solid line represents the best fit of the data. K_D = 8.3 ± 0.6nM, R_{max} = 0.41 ± 0.01 mol dopamine/mol TH subunit. Results shown are representative. **C)** Scatchard transformation of (B). **D)** 560nM pSer40 rTH was incubated with [^{3}H]-dopamine for 30 min at 20^{O}C and then further incubated in the absence (-) or presence (+) of 2mM dopamine. The amount of [^{3}H]-dopamine bound was determined using the sedimentation velocity meniscus depletion method. Bars indicated are standard error, n=3. **E)** 600nM pSer40 rTH was incubated with different concentrations of [^{3}H]-dopamine for 30 min at 20^{O}C. Assay of the amount of [^{3}H]-dopamine bound was performed using the sedimentation velocity meniscus depletion method. The data was fitted to a single-site model, and the

solid line represents the best fit of the data. K_D = 59 ± 3nM, R_{max} = 0.91 ± 0.01 mol dopamine/mol TH subunit. Results shown are representative. **F)** Scatchard transformation of (E). R is the molar binding function (mol ligand bound/mol TH subunit).

Figure 3: Catecholamine binding to rTH and pSer40 rTH. 70nM rTH **(A)** and 600 nM pSer40 rTH **(B)** were incubated with [^{3}H]-dopamine and increasing concentrations of ● dopamine, ■ noradrenaline and ▲ adrenaline. The amount of [^{3}H]-dopamine bound was determined using the sedimentation velocity meniscus depletion method. 100% was defined as the amount of [^{3}H]-dopamine bound when only [^{3}H]-dopamine was added. Results shown are representative.

Figure 4: Effect of dopamine on Km for BH_4 and Vmax. TH activity was assayed over differing concentrations of BH_4 to determine enzyme kinetics. Kinetic analyses were performed using BH_4 concentrations not exhibiting cofactor inhibition. Lines plotted are rectangular hyperbolic regression lines calculated to show enzyme kinetics in the absence of cofactor inhibition. Inset graphs are Eadie-Hofstee transformations of original concentration curves. **A)** rTH. **B)** rTH was incubated with 20µM dopamine prior to assay. **C)** pSer40 rTH. **D)** pSer40 rTH was incubated in the presence of 20µM dopamine prior to assay. The final concentrations of rTH and pSer40 rTH were: A) 0.05µM, B) 5µM, C) 0.05µM D) 0.05µM. Results shown are single experiments. Similar results were obtained in ≥3 experiments. V is µM DOPA/min/µM TH subunit, S is BH_4 concentration (µM).

Figure 5: Effect of two dopamine binding sites on rTH and pSer40 rTH activity. Kinetic analyses were performed using BH_4 concentrations not exhibiting cofactor inhibition. Lines plotted are rectangular hyperbolic regression lines calculated to show enzyme kinetics in the absence of cofactor inhibition. **A)** rTH was incubated with dopamine and then incubated with OC charcoal. Assays were performed in the absence (■) or presence (●) of additional dopamine to determine the effect of dopamine binding to the high affinity and low affinity binding sites respectively. **B)** Eadie-Hofstee transformation of A. **C)** rTH

was assayed as in (A) over the physiological concentration range of BH_4. Bars indicated are standard error, n=3. **D)** rTH was phosphorylated at Ser40 and incubated with dopamine. This mixture was then incubated with OC charcoal. Assays were performed in the absence (■) or presence (●) of additional dopamine. **E)** Eadie-Hofstee transformation of D. **F)** pSer40 rTH was assayed as in (D) over the physiological concentration range of BH_4. n=3, bars indicated are standard error. The final concentration of rTH (A-C) was 2µM and pSer40 rTH (D-F) was 0.06µM. A,B,D,E: Results shown are single experiments. Similar results were obtained in ≥3 experiments. V is µM DOPA/min/µM TH subunit, S is BH_4 concentration (µM).

Figure 6: Effect of dopamine binding on hTH1-4 and pSer40 hTH1-4 activity. Kinetic analyses were performed using BH_4 concentrations not exhibiting cofactor inhibition. Lines plotted are rectangular hyperbolic regression lines calculated to show enzyme kinetics in the absence of cofactor inhibition. Results shown have been plotted over the physiological concentration range of BH_4. Left column: hTH was incubated with dopamine and then incubated with OC charcoal. Assays were performed in the absence (■) or presence (●) of additional dopamine to determine the effect of dopamine binding to the high affinity and low affinity binding sites respectively. Right column: hTH was phosphorylated at Ser40 and incubated with dopamine. This mixture was then incubated with OC charcoal. Assays were performed in the absence (■) or presence (●) of additional dopamine. The final concentration of hTH was 2-4µM and pSer40 hTH was 0.05-0.2 µM. Results shown are representative.

Figure 7: Role of the low affinity site in regulating TH activity and cytosolic dopamine concentrations. Dopamine (D) is contained in two compartments within the cell. Dopamine is synthesised in the cytosol, and is then packaged into vesicles. **A)** Non-phosphorylated TH contains a low affinity (L) and a high affinity dopamine binding site. Dopamine does not dissociate from the high affinity site (D_H). The low affinity site is able to respond to cytosolic catecholamine concentrations, ensuring sufficient catecholamine production for vesicular filling under conditions of low-level

catecholamine release. **B)** Under conditions of high levels of catecholamine release, TH activity must be increased to ensure sufficient vesicular filling. When high levels of catecholamine synthesis are required, TH is phosphorylated at Ser40 (P), which abolishes the high affinity dopamine binding site, increasing TH activity. pSer40 TH still contains the low affinity dopamine binding site, which is able to respond to cytosolic concentrations of catecholamine to prevent catecholamines from accumulating in the cytosol.

Figure 1

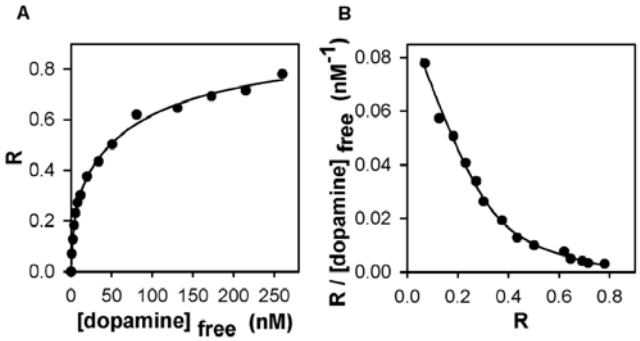

A
B
R
[dopamine] free (nM)
R / [dopamine] free (nM-1)
R
0.0
0.2
0.4
0.6
0.8
0
50
100
150
200
250
0.00
0.02
0.04
0.06
0.08

Figure 2

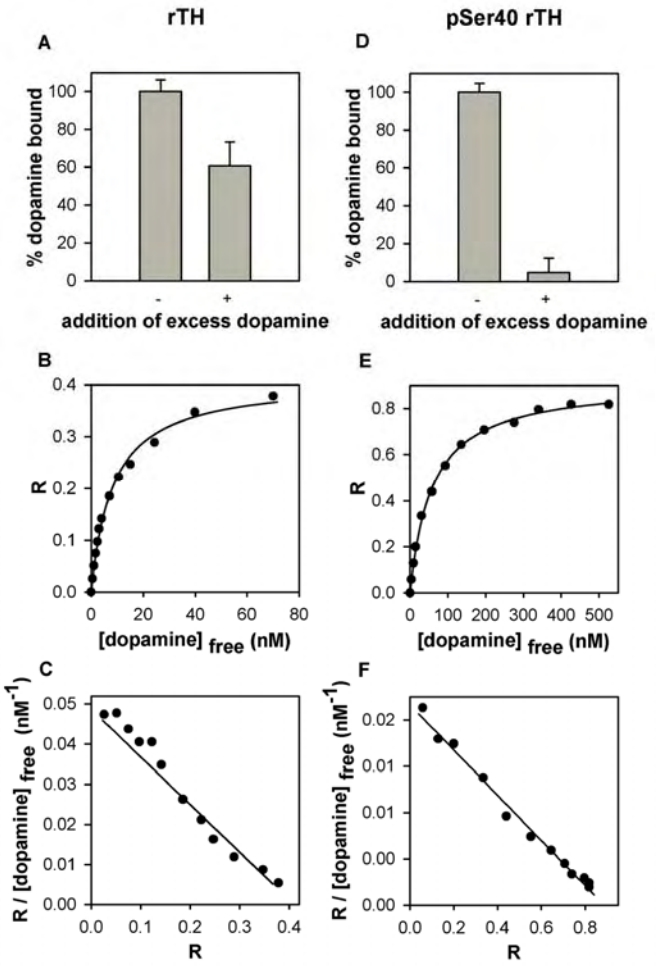

rTH
pSer40 rTH
A
D
% dopamine bound
addition of excess dopamine
B
E
R
[dopamine] free (nM)
C
F
R / [dopamine] free (nM-1)
R

Figure 3

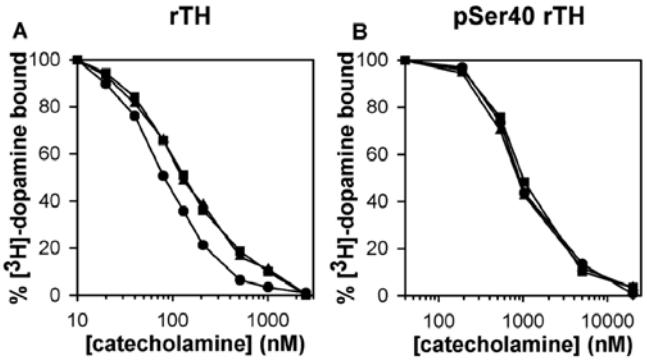

A
rTH
% [3H]-dopamine bound
100
80
60
40
20
0
10
100
1000
[catecholamine] (nM)
B
pSer40 rTH
% [3H]-dopamine bound
100
80
60
40
20
0
100
1000
10000
[catecholamine] (nM)

Figure 4

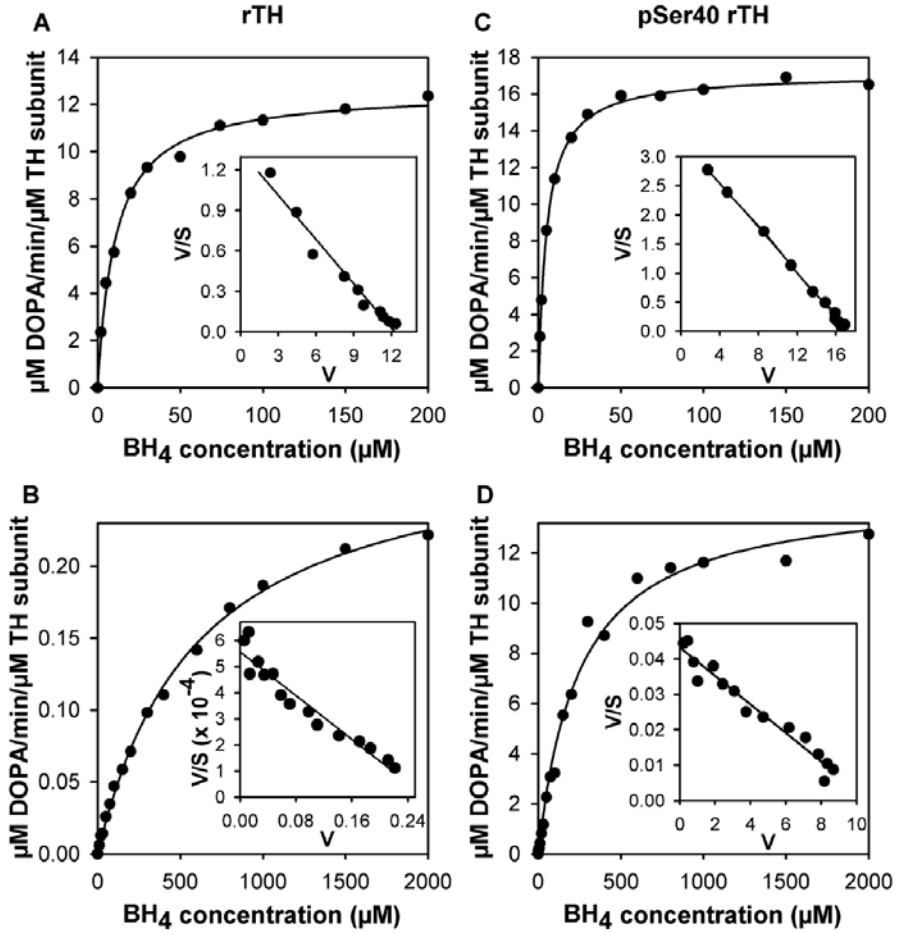

A
rTH
C
pSer40 rTH
B
D
µM DOPA/min/µM TH subunit
BH4 concentration (µM)
V/S
V
V/S (x 10-4)

Figure 5

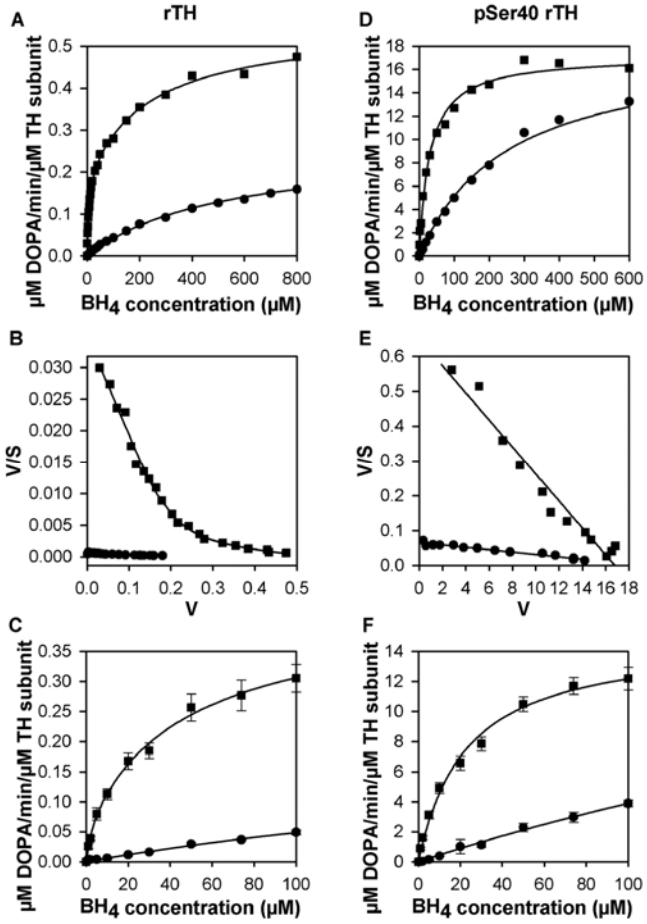

A
rTH
μM DOPA/min/μM TH subunit
BH4 concentration (μM)
D
pSer40 rTH
μM DOPA/min/μM TH subunit
BH4 concentration (μM)
B
V/S
V
E
V/S
V
C
μM DOPA/min/μM TH subunit
BH4 concentration (μM)
F
μM DOPA/min/μM TH subunit
BH4 concentration (μM)

Figure 6

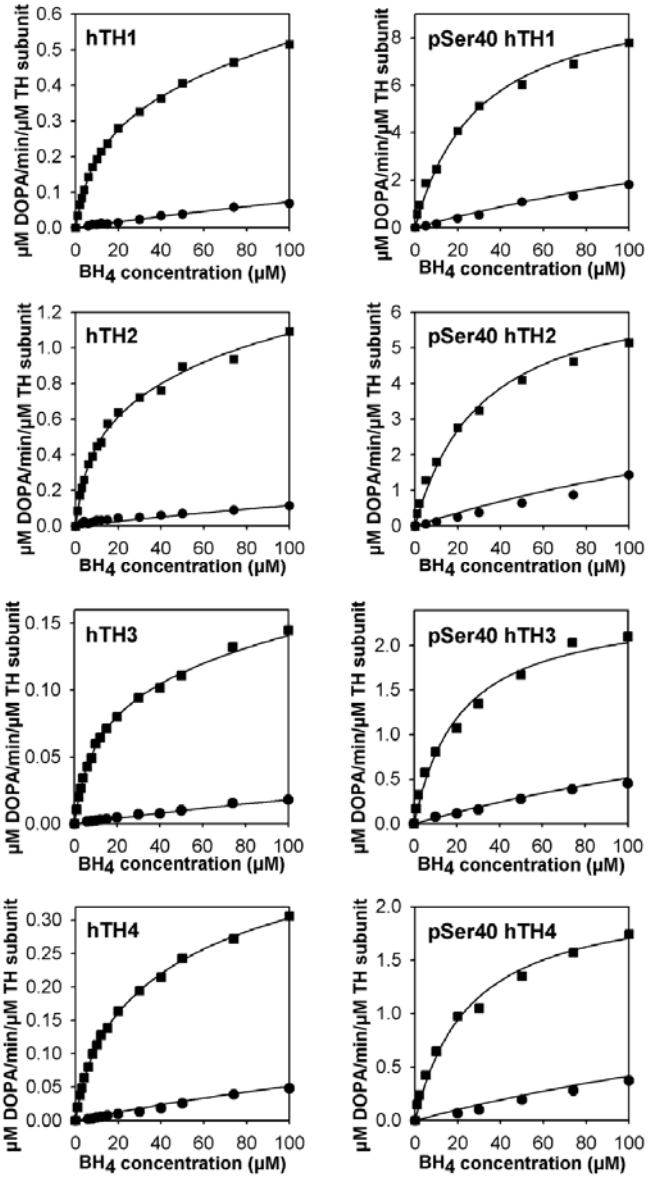
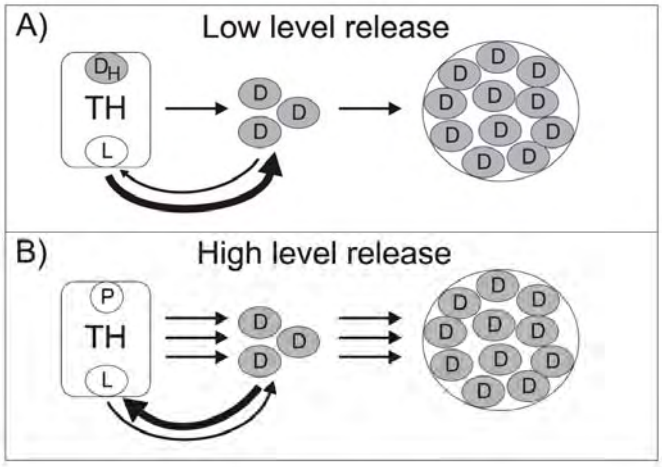

hTH1
pSer40 hTH1
hTH2
pSer40 hTH2
hTH3
pSer40 hTH3
hTH4
pSer40 hTH4
µM DOPA/min/µM TH subunit
BH4 concentration (µM)

Figure 7



SUPPLEMENTAL DATA

Controls for sedimentation velocity meniscus depletion method

The proportion of the radiolabelled ligand that could not bind TH was 7-9% when radiolabelled ligand was less than 6 weeks old, after which the proportion of radiolabelled ligand which could not bind TH increased. Consequently the [^{3}H]-dopamine was used within 6 weeks of the analysis date. Both TH and the catecholamines are relatively unstable and therefore it was important to determine that they remained stable over the period of the experiment. Under the conditions developed for use in the binding experiments it was found that TH had a half time of inactivation of 5 h at the minimum concentrations used in these experiments (70 nM subunit). At higher TH concentrations the enzyme was more stable. There was no significant difference in the stability of TH and pSer40 TH. It has been shown that high concentrations of GSH can protect the catecholamines from oxidation at neutral pH (Boomsma et al. 1991). It was found that essentially 100% of dopamine was recovered after 4 h incubation at 20^{O}C in 50mM potassium phosphate, pH 7.4, 5mM GSH, 50nM EDTA, 10nM catalase and 5mg/ml ovalbumin as determined by HPLC and electrochemical detection (Livett 1987).

OC charcoal method validation

To determine whether OC charcoal affects TH activity, a time course of activity was performed on rTH treated with OC charcoal or buffer A alone. As shown in Fig. S1A, rTH activity is unchanged following OC charcoal treatment, and the activity remains linear over the same period of time as rTH treated with buffer A alone. The dopamine-binding capability of OC charcoal was determined by incubating 20µM dopamine containing 1µCi [2,5,6-^{3}H]-dopamine, 5mM reduced glutathione, 100mM potassium phosphate buffer, pH 7.4 with an equal volume of OC charcoal or buffer A alone at 25˚C for 15 min. The mixture was spun at 18000 x g for 10 minutes at 4˚C, and 100µl supernatant measured for radioactivity. OC charcoal removes approximately 99% of dopamine from the assay mixture (Fig. S1B).

Results obtained with the OC charcoal incubation step could be replicated using gel filtration. A Superdex 75 column (dimensions 11cm x 0.9cm) was equilibrated with 50mM Tris, 100mM NaCl, pH 7.4. The TH assay solution was prepared in the presence of dopamine as described in Experimental Procedures, and applied to the column. Chromatography was carried out at 25°C, and fractions containing the highest concentration of protein as determined by the method of Bradford (Bradford 1976) were collected and assayed for TH activity in the absence of additional dopamine. Vmax for the enzyme was low (Fig. S1C) and Eadie-Hofstee transformation of the data revealed a non-linear curve and two distinct Kms for BH_4 (Fig. S1D) similar to that determined for rTH treated with OC charcoal (Fig. 5A and B, squares).

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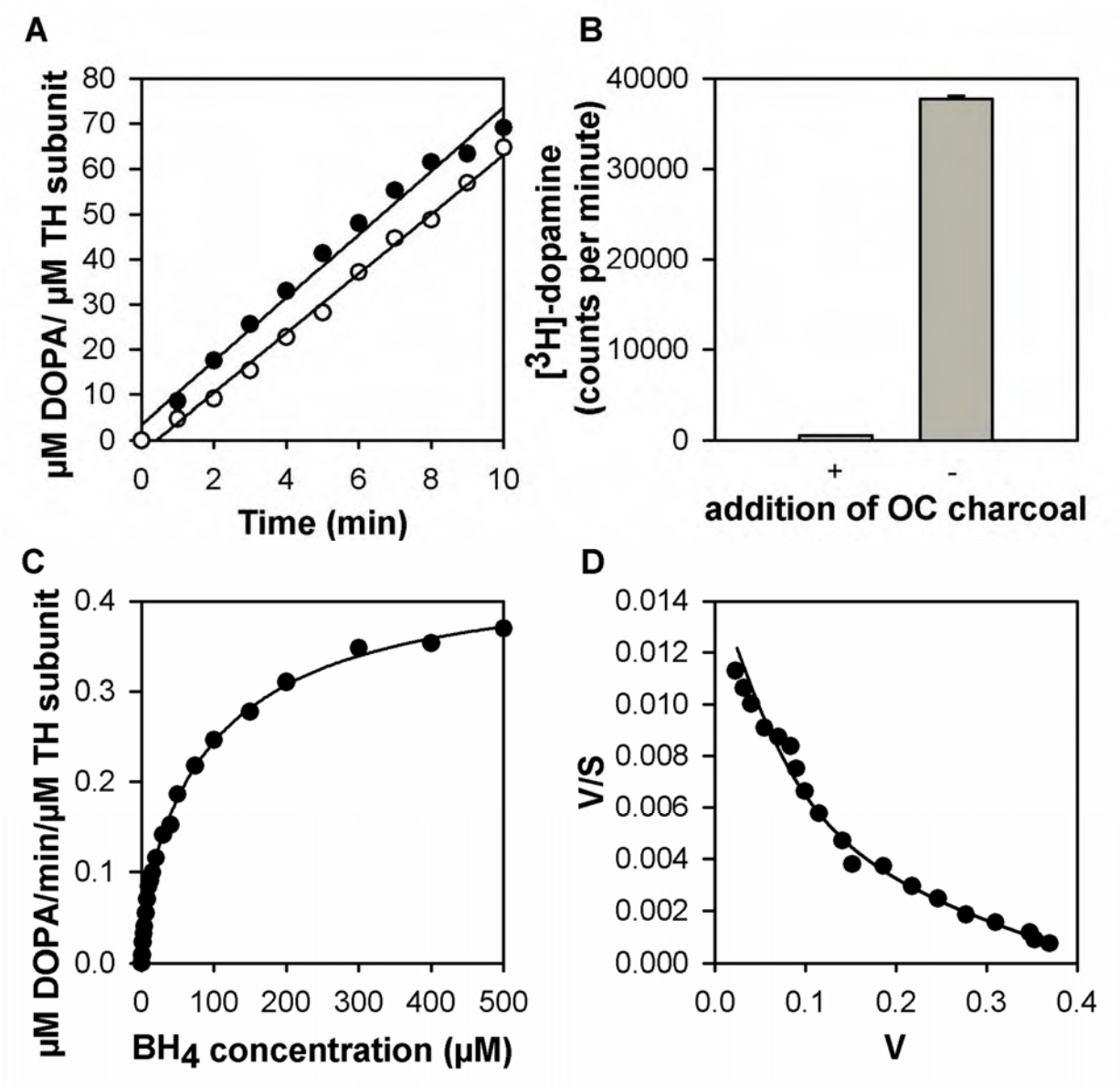

Figure S1: OC charcoal method validation. A) rTH was incubated with OC charcoal (●) or buffer (○) and the supernatant mixture assayed for TH activity. **B)** OC charcoal (+) or buffer (-) mixture was incubated with dopamine spiked with [^{3}H]-dopamine, and the supernatant assayed for [^{3}H]-dopamine. Bars indicated are standard error, n = 3. **C)** rTH was incubated with dopamine and then subjected to gel filtration. TH activity was assayed in the absence of additional dopamine. **D)** Eadie-Hofstee transformation of C. Results displayed are single experiments except where noted.

Table S1

TH: Km for BH_4 (µM)

	rTH	SE	hTH1	hTH2	hTH3	hTH4
No DA	10.48	0.18	7.5	6.46	7.4	5.21
+ DA	776	122	565	590	500	517
+ DA + OC + DA	528	113	584	337	516	373
+ DA + OC - DA Site 1	8.5	2.21	9	6.8	8.6	6.57
+ DA + OC - DA Site 2	252	46	239	162	195	73

TH: Vmax (µM DOPA/min/µM TH subunit)

	rTH	SE	hTH1	hTH2	hTH3	hTH4
No DA	19.00	4.15	5.66	9.67	5.1	5.87
+ DA	0.28	0.03	0.68	0.2	0.12	0.18
+ DA + OC + DA	0.28	0.08	0.5	0.1	0.11	0.23
+ DA + OC - DA Site 1	0.12	0.05	0.31	0.3	0.09	0.12
+ DA + OC - DA Site 2	0.23	0.07	0.82	0.4	0.16	0.33

pSer40 TH: Km for BH_4 (µM)

	pS40 rTH	SE	pS40 hTH1	pS40 hTH2	pS40 hTH3	pS40 hTH4
No DA	5.78	0.73	7.17	8.09	6.56	5.83
+ DA	294	73	517	332	251	510
+ DA + OC + DA	258	4	320	276	317	325
+ DA + OC - DA	27.61	2.86	30.35	31	24	25

pSer40 TH: Vmax (µM DOPA/min/µM TH subunit)

	pS40 rTH	SE	pS40 hTH1	pS40 hTH2	pS40 hTH3	pS40 hTH4
No DA	16.24	1.35	12.10	11.51	6.88	9.81
+ DA	15.09	3.59	12.14	8.98	5.09	13.36
+ DA + OC + DA	18.03	0.13	7.94	5.46	2.15	1.76
+ DA + OC - DA	20	2.67	10.15	6.89	2.56	2.11

Table S1: Kinetic constants for rTH and human TH isoforms. rTH and hTH were assayed in the presence (+ DA) or absence (no DA) of dopamine and the data plotted against rectangular hyperbolic regression curves to obtain kinetic constants. rTH and hTH were incubated with dopamine, treated with OC charcoal, and assayed in the presence (+ DA + OC + DA) or absence (+ DA + OC – DA) of additional dopamine. Data was plotted against single or two-site models to obtain kinetic constants. Experiments and analyses were then repeated

for rTH and hTH that had first been phosphorylated at Ser40. Results are average of ≥ 3 experiments for rTH, and single experiments for hTH isoforms.

Chapter 4: The low affinity dopamine binding site on tyrosine hydroxylase: the role of the N-terminus and *in situ* regulation of enzyme activity

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This manuscript has been submitted to Neurochemical Research as an original article (16th December 2008).

The content of this manuscript has not been altered in any way except that footnotes, figures and associated figure legends have been placed in the text at appropriate positions. In addition, headings have been numbered to match style throughout thesis.

ACKNOWLEDGEMENT OF COLLABORATION

I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers.

The work in the following chapter (Chapter 4) was submitted to Neurochemical Research in December 2008. I (Sarah Gordon) was the author of this manuscript, and the work embodied in this chapter was performed by myself, with the exception of the following:

Preparation and expression of the 157-498 hTH mutant (utilised in Figures 1 and 2) was performed by Jacqueline Shehadeh.

Figure 1: Analysis of dopamine binding to hTH1 and 157-498 hTH, was performed by Julianne Webb under the supervision of Associate Professor Phillip Dickson and myself.

Figure 2: Analysis of the functional effect of dopamine binding to hTH1 and 157-498 hTH, was performed by Julianne Webb under the supervision of Associate Professor Phillip Dickson and myself.

The work embodied by the experiments performed by Julianne Webb and Jacqueline Shehadeh will NOT be discussed in depth in the final chapter (Chapter 5) of this thesis, however the results obtained from the *in situ* experiments (performed by myself) will be discussed in Chapter 5.

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Sarah Gordon

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Associate Professor Phillip Dickson

4.1. Abstract

Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, is inhibited *in vitro* by catecholamines binding to two distinct sites on the enzyme. The N-terminal regulatory domain of TH contributes to dopamine binding to the high affinity site of the enzyme. We prepared an N-terminal deletion mutant of TH to examine the role of the N-terminal domain in dopamine binding to the low affinity site. Deletion of the N-terminus of TH removes the high affinity dopamine binding site, but does not affect dopamine binding to the low affinity site. The role of the low affinity site *in situ* was examined by incubating PC12 cells with L-DOPA to increase the cytosolic catecholamine concentration. This resulted in an inhibition of TH activity *in situ* under both basal conditions and conditions that promoted the phosphorylation of Ser40. Therefore the low affinity site is active *in situ* regardless of the phosphorylation status of Ser40.

4.2. Introduction

Tyrosine hydroxylase (TH; EC 1.14.16.2) is the rate-limiting enzyme in catecholamine biosynthesis [1] and as such is subject to a wide variety of regulatory mechanisms [2]. Long-term regulation of TH activity is primarily controlled by modulation of TH protein levels [2]. Acute regulation of TH activity occurs via phosphorylation of three key serine (Ser) residues (Ser19, Ser31 and Ser40) [3], and also by two distinct forms of feedback inhibition by the catecholamines [4].

Catecholamines bind to two distinct sites on TH. Catecholamines can bind to a high affinity, non-dissociable site through direct coordination of the active site iron [5], and this results in the inhibition of TH activity by decreasing Vmax and increasing the K_M for the obligate cosubstrate tetrahydrobiopterin (BH_4) [4; 6]. The interaction between dopamine and iron suggests that the high affinity dopamine binding site is likely to be situated at the active site of the enzyme. Dopamine binding to the high affinity site can only be reversed by phosphorylation of Ser40 [6; 7]. This indicates that the N-terminal regulatory domain plays a role in the binding of dopamine to the high affinity site.

Catecholamines also bind to a low affinity, readily dissociable site which inhibits TH activity by increasing the K_M for BH_4 [4]. Dopamine binding to the low affinity site is not regulated by phosphorylation of the enzyme *in vitro*. The location of the low affinity site on the enzyme is currently unknown. The K_D of the low affinity site is approximately 60-90nM, which approximates the concentration of cytosolic catecholamines in PC12 cells and cultured midbrain dopaminergic neurons [8]. Therefore, it is possible that the low affinity dopamine binding site is able to regulate TH activity *in situ* by responding to cytosolic catecholamine levels.

In this study, we have investigated the role of the N-terminal regulatory domain on the binding of dopamine to the low affinity site of TH. In addition we have used PC12 cells to analyse the role of the low affinity site in the control of TH activity *in situ*. This is the first study to investigate the physiological relevance of the low affinity binding site *in situ*.

4.3. Experimental procedure

4.3.1. Materials

pET-14b was from Novagen (Madison, USA). Quicklyse plasmid purification mini-prep kit was from Qiagen (Hilden, Germany). Vent DNA polymerase was from New England Biolabs (Ipswich, USA). Wizard SV gel and PCR clean-up system was from Promega (Madison, USA). Chelating sepharose fast flow beads, L-[3,5-^{3}H]-tyrosine and [2,5,6-^{3}H]-dopamine were obtained from GE Healthcare (Buckinghamshire, England). Activated charcoal was obtained from BDH Biochemicals (Poole, England). BH_4 was obtained from Dr. B. Shircks laboratory (Jona, Switzerland). Dopamine hydrochloride, chicken egg albumin (ovalbumin), L-dihydroxyphenylalanine (L-DOPA), forskolin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St Louis, USA). PC12 cells were from ATCC (Manassas, USA). Dulbecco's Modified Eagle Medium (DMEM) and horse serum were obtained from GIBCO (Carlsbad, USA). Fetal calf serum was from Bovogen (Essendon, Australia). ^{14}C-tyrosine was from American Radiolabelled Chemicals (St Louis, USA).

4.3.2. Methods

4.3.2.1. Preparation of TH

The 157-498 hTH mutant was generated by amplifying the required region from pET3a hTH1 by PCR using Vent DNA polymerase. The primers used included the Nde1 and BamHI sites embedded within the sequence (forward: GGGAATTCCATATGAGCCCCGCGGGGCCCAAGGTC, reverse: GTACGGATC CCTAGCCAATGGCACTCAGCGC). The PCR product was purified using the Wizard SV gel and PCR clean-up system according to the manufacturer's instructions. The purified PCR product and pET-14b were digested with NdeI and BamHI, and then purified using the Wizard SV gel and PCR clean-up system. The purified 157-498 hTH and pET-14b digests were then ligated together, and transformed into DH5α. Transformed bacteria were selected using LB agar plates containing 100µg/mL ampicillin. Overnight cultures were inoculated from single colonies, and plasmids purified using the Quicklyse plasmid mini-prep kit according to the manufacturer's instructions. Insertion of the correct 157-498 hTH cDNA into pET-14b was confirmed by DNA sequencing. The plasmid was transformed into competent BL21 DE3 *E. coli* and utilised for subsequent expression of the 157-498 hTH protein.

hTH1 was expressed and purified as previously described [9]. 157-498 hTH was expressed according to previously described methods [10], except that the bacterial pellets were resuspended in 20mM $NaPO_4$, 500mM NaCl, pH 7.4. 157-498 hTH was purified using chelating sepharose fast flow beads charged with 100mM $NiSO_4$. The columns were washed with 20mM imadazole, 20mM $NaPO_4$, 500mM NaCl, pH 7.4, and then bound TH was eluted with 300mM imadazole, 20mM $NaPO_4$, 500mM NaCl, pH 7.4. The eluted 157-498 hTH was dialysed overnight in

20mM $NaPO_4$, 150mM NaCl, pH 7.4. The dialysed protein was stored at -20°C in 15% glycerol until required.

4.3.2.2. Measurement of catecholamine binding to TH

The binding of catecholamines to TH was investigated by the sedimentation velocity meniscus depletion method of Howlett *et al.* [11] using a Beckman Benchtop TL-100 ultracentrifuge as described by Gordon *et al.* [4].

4.3.2.3. Measurement of in vitro TH activity

Ovalbumin-coated charcoal (OC charcoal) was prepared as described Gordon *et al.* [4]. For non-dopamine-bound TH, the TH activity assay solution (suitable amount of TH, 10mg/ml ovalbumin, 0.06mg/ml catalase, 5mM GSH and 100mM potassium phosphate, pH 7.4) was incubated at 25˚C for 10 min. For dopamine-bound TH, the TH activity assay solution was incubated at 25˚C for 10 min in the presence of 20µM dopamine, and was then incubated with an equal volume of OC charcoal at 25˚C for 15 min and centrifuged at 18000 x g at 4˚C for 10 min. The supernatant was collected and assayed for TH activity in the presence or absence of additional dopamine using the tritiated water release assay of Reinhard *et al.* [12] at 20 or 2000µM BH_4.

4.3.2.4. Cell culture

PC12 cells were routinely maintained in DMEM supplemented with 10% horse serum, 5% fetal calf serum, 10mM Hepes and 2mM L-Glutamine at 37ºC, 5% CO_2 in a humidified incubator. 24-well tissue culture plates were coated with 10µg/mL collagen for 2 hours at 37ºC prior to addition of cells. PC12 cells were

plated on 24-well plates at a density of $0.1x10^6$ cells/well. The cells were left to grow for 2-3 days until wells were approximately 80% confluent.

4.3.2.5. L-DOPA treatment

L-DOPA stocks were made up in H_2O and then filter-sterilised prior to addition to cells. Concentrated filter-sterilised L-DOPA was added directly to media to a final concentration of 20µM. Cells were incubated in L-DOPA for 24 hours.

4.3.2.6. Measurement of in situ TH activity

TH activity assays were performed as described [13]. Following incubation of cells with L-DOPA, the media was aspirated and the cells washed with hepes-buffered saline (HBS; 150mM NaCl, 5.5mM D-glucose, 3.8mM K_2HPO_4, 1mM $MgSO_4$, 1.5mM $CaCl_2$, 15mM Hepes, pH 7.4). Following the addition of 18µM tyrosine in HBS (spiked with approximately 1µCi ^{14}C-tyrosine per well) to the cells, the wells were immediately covered with an acrylic tube capped with a rubber stopper that was fitted with a plastic container containing 300µL 1M NaOH. For the phosphorylation assay, 10µM forskolin was added along with the tyrosine to the cells. Cells were maintained for 10 minutes at 37ºC, and then reactions were terminated by the injection of 250µL 10% TCA through the rubber stopper and onto the cells. The apparatus was left for 24 hours at room temperature to allow the NaOH to absorb the $^{14}CO_2$ produced by catecholamine synthesis. Following this, a 250µL aliquot of the NaOH was added to 3mL scintillation fluid and vortexed, and then ^{14}C levels were measured by scintillation counter.

4.3.2.7. SDS-PAGE and western blotting

Cells were incubated with L-DOPA as described above. Cells were washed with HBS, and then incubated with 18µM tyrosine in HBS for 10 minutes. For the phosphorylation assay, 10µM forskolin was added along with the tyrosine to the cells. Following aspiration of the HBS, reactions were terminated through the addition of SDS buffer (50mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% DTT, 2mM EDTA). Samples were applied to 10% SDS-polyacrylamide gels before being transferred to nitrocellulose as described [14]. Nitrocellulose membranes were then immunoblotted using phospho-Ser40-specific or total TH antibodies as described previously [15]. Analysis of site-specific TH phosphorylation was performed as previously described [16]. Immunoblots were visualized using the Image Reader LAS-3000 (Fujifilm) imaging system using ECL plus detection reagents. The density of total TH or phospho-Ser40 TH bands was measured using MultiGauge (Fujifilm).

4.3.2.8. Cell viability assay

Following incubation of cells with L-DOPA, media was aspirated from cells and cells were incubated with 300µL of 5mg/mL MTT for 30 minutes. The MTT solution was aspirated from the cells, and the cells were then solubilised in 500µL DMSO for 10 minutes. 200µL of the solubilised cell solutions were transferred to a microplate, and absorbance was measured at 560nm.

4.3.2.9. Statistical Analyses

Data is expressed where appropriate as a percentage of the mean of the control samples (control=100%), and is presented as mean ± SEM for the stated number of experiments. Statistical significance was assessed using Student's

unpaired *t*-tests or one-way ANOVA followed by Tukey's test for multiple comparisons.

4.4. Results

4.4.1. Role of the N-terminus in dopamine binding to TH

Dopamine binds in a non-dissociable manner to the high affinity site in TH [7; 17], however the binding of dopamine to the low affinity site is readily reversible [4]. These differences can be utilised to investigate the presence of the two different sites in TH [4]. Dopamine binding to hTH1 and the N-terminal deletion mutant 157-498 hTH was assessed by allowing [^{3}H]-dopamine to equilibrate with TH and then adding excess (2mM) dopamine. The amount of [^{3}H]-dopamine bound to TH was then determined. The addition of excess dopamine results in the dissociation of approximately half of the [^{3}H]-dopamine from hTH1 (Figure 1A), which correlates with data demonstrating that TH contains a high affinity dopamine binding site from which there is negligible dissociation of dopamine and a low affinity dopamine binding site from which dopamine readily dissociates [4]. When 157-498 hTH was analysed it was found that dopamine could readily bind to this deletion mutant, indicating that it still has a functional dopamine binding site(s). However, the addition of excess dopamine results in the dissociation of 87% of the [^{3}H]-dopamine from 157-498 hTH (Figure 1B). This is significantly different to the effect determined in hTH1 ($p<0.01$). This suggests that the N-terminal deletion mutant does not contain the non-dissociable high affinity dopamine binding site.

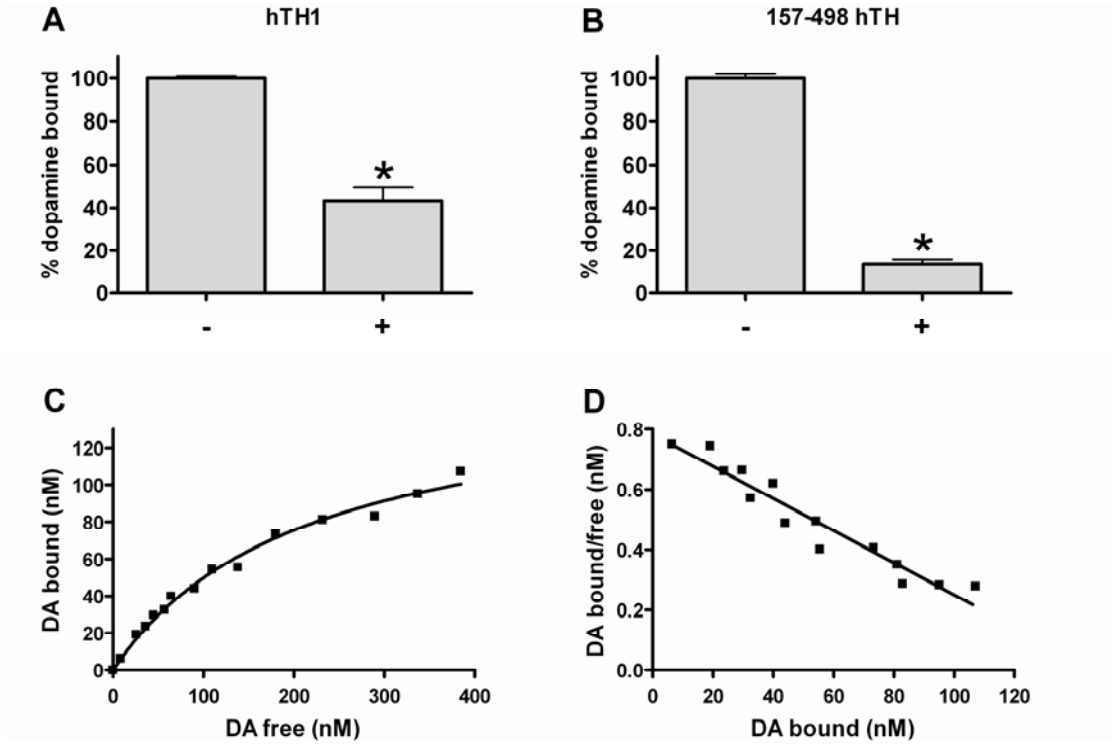

Figure 1: Analysis of dopamine binding to hTH1 and 157-498 hTH. **A)** hTH1 or **B)** 157-498 hTH was incubated with [^{3}H]-dopamine for 30 min at 20^{O}C and then further incubated in the absence (-) or presence (+) of 2mM dopamine. The amount of [^{3}H]-dopamine bound was determined using the sedimentation velocity meniscus depletion method. The data is presented as mean ± SEM, n=3, * $p<0.001$ vs (-). **C)** 157-498 hTH was incubated with different concentrations of [^{3}H]-dopamine for 30 min at 20^{O}C. Assay of the amount of [^{3}H]-dopamine bound was performed using the sedimentation velocity meniscus depletion method. The data was fitted to a single-site model, and the solid line represents the best fit of the data. K_D = 215 ± 25nM. Results shown are representative. **D)** Scatchard transformation of (C).

The binding of dopamine to 157-498 hTH was further analysed using the sedimentation velocity meniscus depletion method to obtain a saturation curve for dopamine binding (Figure 1C). The K_D for dopamine binding to 157-498 hTH was determined to be 215 ± 25nM, which approximates the previously determined K_D for dopamine binding to the low affinity site [4; 7; 17]. The saturation curve data was transformed according to the method of Scatchard [18]. In contrast to the results with

full-length TH which showed a non-linear Scatchard plot due to the presence of both the high and low affinity sites on the enzyme [4], the linear nature of the Scatchard transformation indicates that 157-498 hTH does not contain binding sites with different affinities for dopamine (Figure 1D). This correlates with the competition experiment in Figure 1B which demonstrates that 157-498 hTH lacks the high affinity dopamine site.

4.4.2. Effect of the low affinity site on *in vitro* TH activity

In order to investigate the functional effects of dopamine binding to the high and low affinity sites on TH activity separately, the OC charcoal procedure was used as described in the methods. TH was first incubated with dopamine and then treated with OC charcoal (DA + OC). The OC charcoal binds the free dopamine in the assay mix and results in the dissociation of dopamine from the low affinity site alone [4]. This procedure allows us to assess the effect of dopamine binding to the high affinity site on TH activity. Subsequent addition of dopamine back into the assay mixture saturates the low affinity site and allows us to assess the effect of dopamine binding to the low affinity site on TH activity (DA + OC + DA). We have previously demonstrated that the inhibition of TH activity by dopamine binding to the low affinity site occurs solely due to an increase in the K_M of the cosubstrate BH_4 [4]. Therefore the experiments were performed at both sub-saturating (20µM) and saturating (2000µM) BH_4 concentrations in order to assess the functionality of the low affinity binding site.

The results in Figure 2 show that for full-length hTH1, dopamine binding to the high affinity site alone (DA + OC) inhibits TH activity to approximately 15% of control (non-dopamine bound) levels at 20µM BH_4 (Figure 2A). There is a similar

level of high affinity site-mediated inhibition of TH activity (24% of control) at 2000μM BH_4, which reflects the dual effect of the high affinity site on both the Vmax of the enzyme and the K_M for BH_4 [4; 6]. The subsequent binding of dopamine to the low affinity site on hTH1 (DA + OC + DA) results in a further inhibition of TH activity (3% of control) at 20μM BH_4 (Figure 2A). However, at 2000μM BH_4 the further addition of dopamine does not significantly inhibit TH activity compared to TH with dopamine bound to the high affinity site alone; this reflects the effect of the low affinity site on the K_M for BH_4, and thus the ability of high concentrations of BH_4 to reverse the low affinity binding site-mediated inhibition of hTH1.

The effect of dopamine binding to the N-terminal deletion mutant was then examined. 157-498 hTH was incubated with dopamine and then treated with OC charcoal, thereby resulting in the dissociation of dopamine from the low affinity site. There was no significant decrease in TH activity at 20μM or 2000μM BH_4 following OC charcoal treatment (DA + OC) (Figure 2B). This concurs with the dopamine binding data which suggests that 157-498 hTH contains no high affinity site. The effect of dopamine binding to the low affinity site was determined by the addition of dopamine back into the assay mixture following the OC charcoal treatment. Dopamine binding to the low affinity site of 157-498 hTH (DA + OC + DA) resulted in an inhibition of TH activity to 9% of control levels at 20μM BH_4. Similar to the effect found in hTH1, this inhibition of 157-598 hTH was completely reversed by the addition of 2000μM BH_4. This data is consistent with 157-498 hTH containing only a low affinity dopamine binding site whose effects are mediated solely by an effect on the K_M for BH_4. This suggests that the N-terminal domain of TH is not required for a functional low affinity binding site.

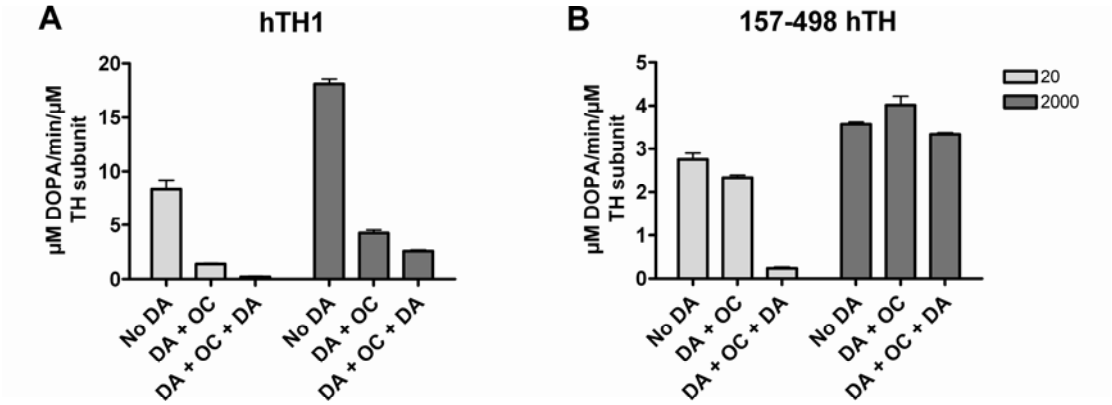

Figure 2: Analysis of the functional effect of dopamine binding to hTH1 and 157-498 hTH. **A)** hTH1 or **B)** 157-498 hTH was incubated in the absence of dopamine (no DA), in the presence of dopamine and then further incubated with OC charcoal (+ DA + OC), or as with +DA + OC followed by the further addition of dopamine (+DA + OC + DA). TH activity was then determined at 20µM or 2000µM BH_4. The data is presented as mean ± SEM, n=3.

4.4.3. Functional effects of the low affinity site on *in situ* TH activity

The role of the low affinity site in controlling TH activity in intact cells was examined. The low affinity binding site should be sensitive to the concentration of cytosolic catecholamines. Incubation of PC12 cells with L-DOPA significantly increases cytosolic catecholamine levels [8; 19]. *In situ* catecholamine synthesis was determined using ^{14}C-tyrosine. PC12 cells were incubated with 20µM L-DOPA for 24 hours to increase cytosolic catecholamine levels, and the cells were then assayed for *in situ* TH activity as described in the methods section. Addition of L-DOPA to the cells significantly inhibited *in situ* TH activity ($p<0.001$) to approximately 25% of control levels (Figure 3a). To determine the effect of L-DOPA on the *in situ* activity of TH phosphorylated at Ser40, PC12 cells were stimulated with forskolin, which is known to selectively increase the phosphorylation of TH at Ser40 [20; 21]. Forskolin significantly increased TH activity by 1.8-fold ($p<0.05$ vs control, Figure

3b). This increase in activity was blocked in cells that were incubated in 20µM L-DOPA ($p<0.05$ vs F, Figure 3b).

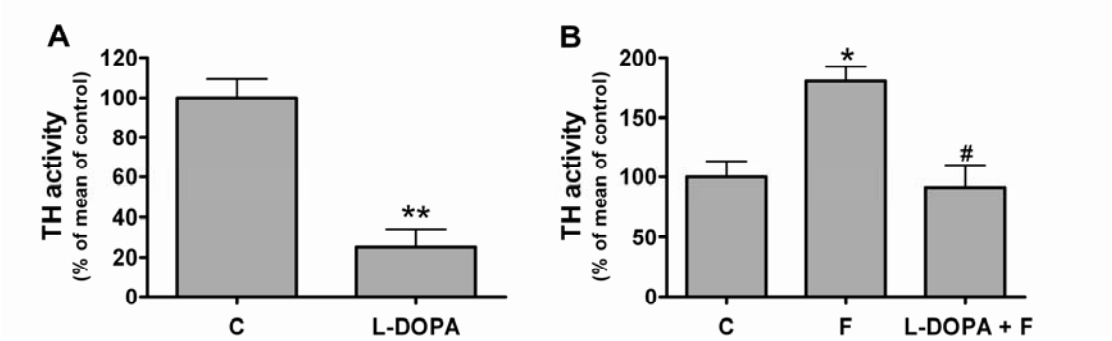

Figure 3: Effect of L-DOPA on *in situ* TH activity. **A)** PC12 cells were incubated with 20µM L-DOPA for 24 hours, and then assayed for *in situ* TH activity as described in methods. Control cells (C) were incubated in the absence of L-DOPA. **B)** PC12 cells were incubated in 20µM L-DOPA for 24 hours, and then assayed for *in situ* TH activity in the absence or presence of forskolin (F) as described in methods. Control cells (C) were incubated in the absence of L-DOPA. The data is presented as percentage of mean of control levels ± SEM, n = 5. ** $p<0.001$ vs control, * $p<0.05$ vs control, $^{\#}$ $p<0.05$ vs F.

Phosphorylation of Ser40 serves to increase TH activity by causing the dissociation of dopamine from the high affinity site of TH. Therefore, L-DOPA may decrease *in situ* TH activity by promoting dephosphorylation of the enzyme or by inhibiting phosphorylation. To determine if this was the case, PC12 cells were incubated with L-DOPA for 24 hours, and cell extracts were subjected to western blotting. No change in Ser40 phosphorylation levels (Figure 4a) or total TH protein levels (Figure 4b) were identified when cells were treated with L-DOPA. Treatment with forskolin significantly increased the phosphorylation of Ser40 ($p<0.01$, Figure 4a) without increasing total TH protein levels (Figure 4b). Prior incubation of PC12 cells with L-DOPA had no effect on the phosphorylation of Ser40 in forskolin-treated cells. There have also been conflicting results regarding the toxicity of L-DOPA on cultured cells [22-25]. PC12 cells were incubated in 20µM L-DOPA for

24 hours, and were then assayed for cell viability as described in the methods section. Incubation with L-DOPA had no effect on cell viability (Figure 4c).

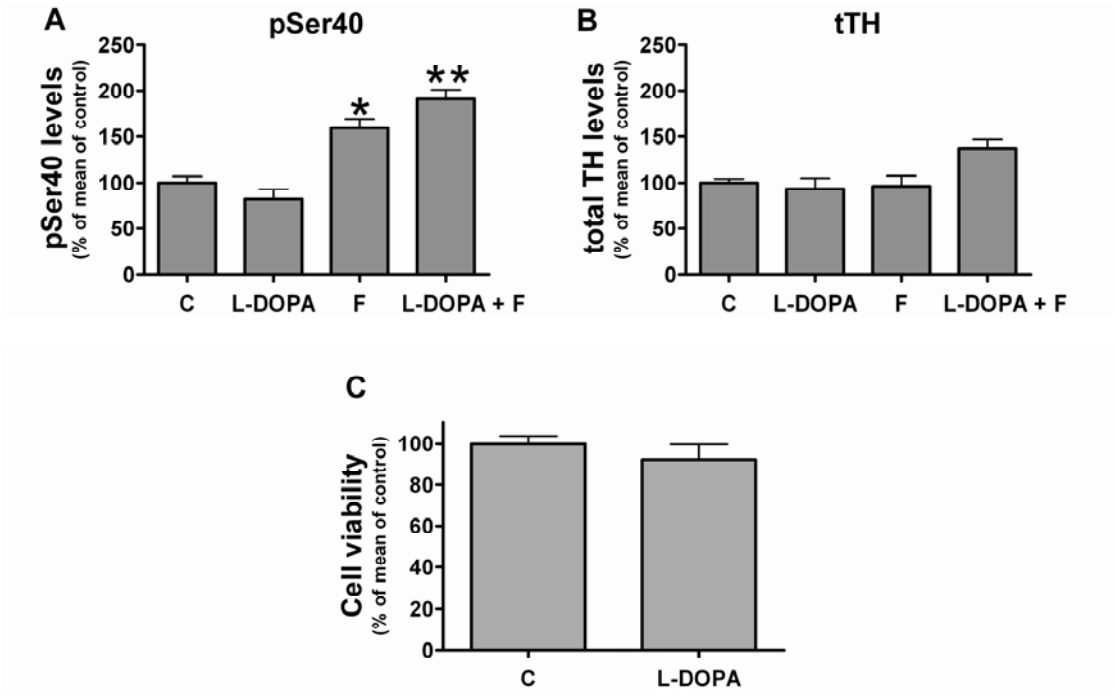

Figure 4: Effect of L-DOPA on phosphorylation of TH. **A)** and **B)** PC12 cells were incubated with 20µM L-DOPA for 24 hours, and then treated with or without forskolin (F) for 10 minutes. Control cells (C) were incubated in the absence of L-DOPA. Cell extracts were subjected to SDS-PAGE and immunoblotting using anti-pSer40 antibody (A) or anti-total TH protein antibody (B). **C)** PC12 cells were incubated for 24 hours with 20µM L-DOPA, and then assayed for cell viability. Control cells (C) were incubated in the absence of L-DOPA. The data is presented as percentage of mean of control levels ± SEM, n = 6 for A and B, n = 3 for C. * $p<0.01$, ** $p<0.001$ vs control.

4.5. Discussion

We have recently demonstrated that TH contains a low affinity, readily-dissociable dopamine binding site [4]. Unlike the high affinity dopamine binding site, the low affinity site is not regulated by phosphorylation of Ser40 *in vitro*. In order to further determine the role of the N-terminus in the regulation of dopamine

binding to the low affinity site, we constructed a mutant lacking the first 156 amino acids of TH which comprise the N-terminal regulatory domain of TH.

The N-terminal deletion mutant was shown to lack the high affinity dopamine binding site. This correlates with data demonstrating that the N-terminus of TH is involved in stabilising the binding of dopamine to the high affinity site [26-28]. However, dopamine was still able to bind to the low affinity site of the N-terminal deletion mutant with a K_D which approximates that found in the full-length phosphorylated and non-phosphorylated TH [4; 7; 17]. This suggests that the N-terminal regulatory domain is not involved in the binding of dopamine to the low affinity site. Dopamine binding to the low affinity site inhibits TH activity by increasing the K_M for BH_4, and is reversible by high concentrations of BH_4 [4]. We have demonstrated that the functionality of dopamine binding to the low affinity binding site is not regulated by the N-terminal regulatory domain *in vitro*.

The non-dissociable nature of the high affinity site suggests that dopamine binding to this site is not regulated by cytosolic catecholamine levels. However, catecholamines may readily dissociate from the low affinity site, and the K_D of dopamine binding to this site is in a range similar to that of cytosolic dopamine levels [4; 8]. This suggests that TH may respond to perturbations in cytosolic catecholamine levels via the low affinity site in order to regulate catecholamine synthesis. The data presented in this paper demonstrates that in intact cells under both basal and stimulatory conditions, increases in cytosolic catecholamine levels results in an inhibition of TH activity. This effect on *in situ* TH activity was not due to decreases in Ser40 phosphorylation levels, total TH protein levels or cell viability. Therefore, this inhibition of *in situ* TH activity is likely to be due to feedback inhibition of the enzyme by the catecholamines binding to the low affinity site.

This is the first study to demonstrate that TH activity may be regulated by the low affinity dopamine binding site *in situ* in a manner that is independent of phosphorylation. This suggests that the low affinity dopamine-binding site on TH is physiologically important. By inhibiting TH activity and therefore catecholamine synthesis in the presence of high levels of cytosolic catecholamines, the low affinity dopamine-binding site would act to maintain an equilibrium of cytosolic catecholamines.

Cytosolic catecholamines are subject to both auto-oxidation and enzymatic oxidation to produce potentially toxic products, including hydrogen peroxide and dopamine quinone [29; 30]. Oxidation of the catecholamines, especially dopamine, has been implicated in the pathogenesis of Parkinson's disease [31]. Therefore, by responding to cytosolic catecholamines, the low affinity binding site may act to prevent the harmful accumulation of cytosolic catecholamines.

4.6. Acknowledgements

This work was supported by a grant from the National Health and Medical Research Council (No. 455547).

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Chapter 5: Conclusions and future directions

5.1. Overview of regulation of TH

The regulation of TH activity is a complex process. As the rate-limiting enzyme in catecholamine biosynthesis, TH is subject to a wide variety of regulatory mechanisms. Feedback inhibition of TH by the catecholamines and phosphorylation of three key Ser residues are primary acute regulators of enzyme activity. There is important interplay between these regulatory processes. This thesis has aimed to further elucidate the mechanisms by which TH is regulated, and has primarily focused on investigating the differential regulation of the human TH isoforms, and the mechanisms by which feedback inhibition occurs (Figure 5.1).

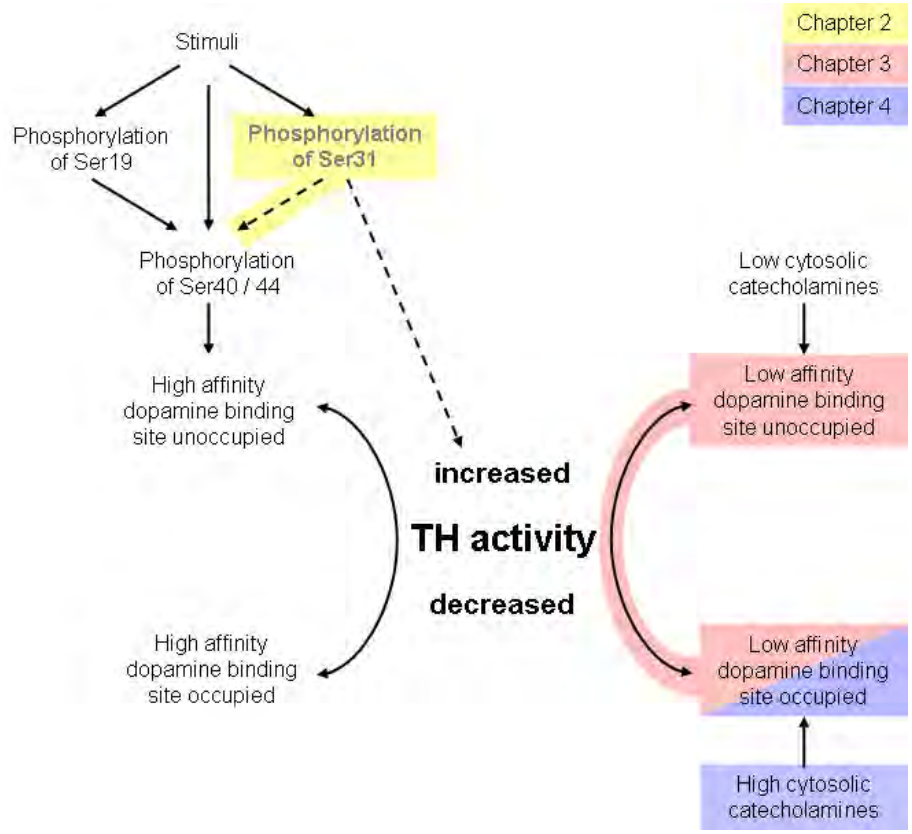


Figure 5.1: Regulation of TH activity by phosphorylation and feedback inhibition by the catecholamines. Full arrows indicate regulatory mechanisms that apply to both hTH1 and hTH2, dotted arrows indicate regulatory mechanisms that apply only to hTH1. Phosphorylation of Ser31 (grey writing) only occurs for hTH1. Coloured boxes indicate new findings obtained from this thesis.

5.2. Differential regulation of human TH isoforms *in situ*

Catecholamine binding to TH at the high affinity dopamine binding site exerts a strong inhibitory effect on the enzyme (Daubner et al. 1992). This binding is relieved by phosphorylation of Ser40 (Ramsey and Fitzpatrick 1998). Phosphorylation of the other two major Ser residues, Ser19 and Ser31, facilitates the phosphorylation of Ser40 in a hierarchical manner *in vitro* and *in situ* (Bevilaqua et al. 2001; Bobrovskaya et al. 2004; Lehmann et al. 2006). Phosphorylation of Ser31 also has a small direct effect on TH activity *in situ* (Salvatore et al. 2001).

There are 4 well-characterised human TH isoforms. Of these, hTH1 and hTH2 are the most highly expressed in brain and the adrenal medulla (Haycock 1991; Lewis et al. 1993). The major human TH isoforms have been demonstrated to display similar catalytic activities (Haavik et al. 1991; Le Bourdelles et al. 1991; Sura et al. 2004), and are regulated by catecholamine binding in similar ways (Sura et al. 2004; Gordon et al. 2008). The only major difference between the isoforms lies in their regulation via phosphorylation of their relative Ser31 residues (Table 5.1). While hTH1 can be phosphorylated at Ser31 *in vitro*, no kinase has been identified that is capable of reproducibly phosphorylating the equivalent Ser31 residue (Ser35) in hTH2. As a result of this, hTH1 and hTH2 have been demonstrated to be differentially regulated by hierarchical phosphorylation *in vitro* (Lehmann et al. 2006). Phosphorylation of Ser31 in hTH1 potentiates the phosphorylation of Ser40 *in vitro*. This effect is absent in hTH2 due to the lack of Ser35 phosphorylation (Lehmann et al. 2006).

We wished to determine whether the major hTH isoforms are also differentially regulated *in situ*. Data presented in Chapter 2 has demonstrated that phosphorylation of Ser40 in hTH1 is potentiated by the phosphorylation of Ser31

under basal conditions *in situ*. Furthermore, it has been shown that phosphorylation of Ser35 in hTH2 is not detectable *in situ*, meaning that this site is not able to participate in the hierarchical phosphorylation of hTH2 at the equivalent Ser40 residue (Ser44) *in situ*. This demonstrates that hTH1 and hTH2 are functionally different *in situ* (Table 5.1). This has potential implications for the regulation of TH activity *in vivo*.

	hTH1	**hTH2**
Phosphorylated at Ser31/35?	**Yes** (*in vitro*[1,2] and *in situ*[3])	**No** (*in vitro*[1,2] and *in situ*[3])
Hierarchical phosphorylation of Ser40/44 by Ser31/35?	**Yes** (*in vitro*[2] and *in situ*[3])	**No** (*in vitro*[2] and *in situ*[3])
Phosphorylated at Ser19?	Yes (*in vitro*[1,2,4] and *in situ*[3])	Yes (*in vitro*[1,2] and *in situ*[3])
Hierarchical phosphorylation of Ser40/44 by Ser19?	Yes (*in vitro*[2,4])	Yes (*in vitro*[2])

Table 5.1: Differential regulation of human TH isoforms *in vitro* and *in situ*. References: 1: (Sutherland et al. 1993); 2: (Lehmann et al. 2006); 3: Gordon et al. 2008, chapter 2 of this thesis; 4: (Toska et al. 2002).

5.3. Physiological implications of differential regulation of the human TH isoforms.

The expression of multiple isoforms of TH is evolutionarily conserved in higher order mammals. Anthropoids express two isoforms of TH, which correlate with hTH1 and hTH2, while only humans express the additional isoforms hTH3 and hTH4 (Lewis et al. 1994; Haycock 2002). This suggests that the presence of multiple TH isoforms must have some physiological relevance. The finding that the two major

hTH isoforms are differentially regulated *in situ* provides evidence for why multiple TH isoforms may be present in higher order mammals. This may allow for region-specific modulation of TH activity and catecholamine synthesis in different cell populations based on the differential distribution and/or expression of the isoforms.

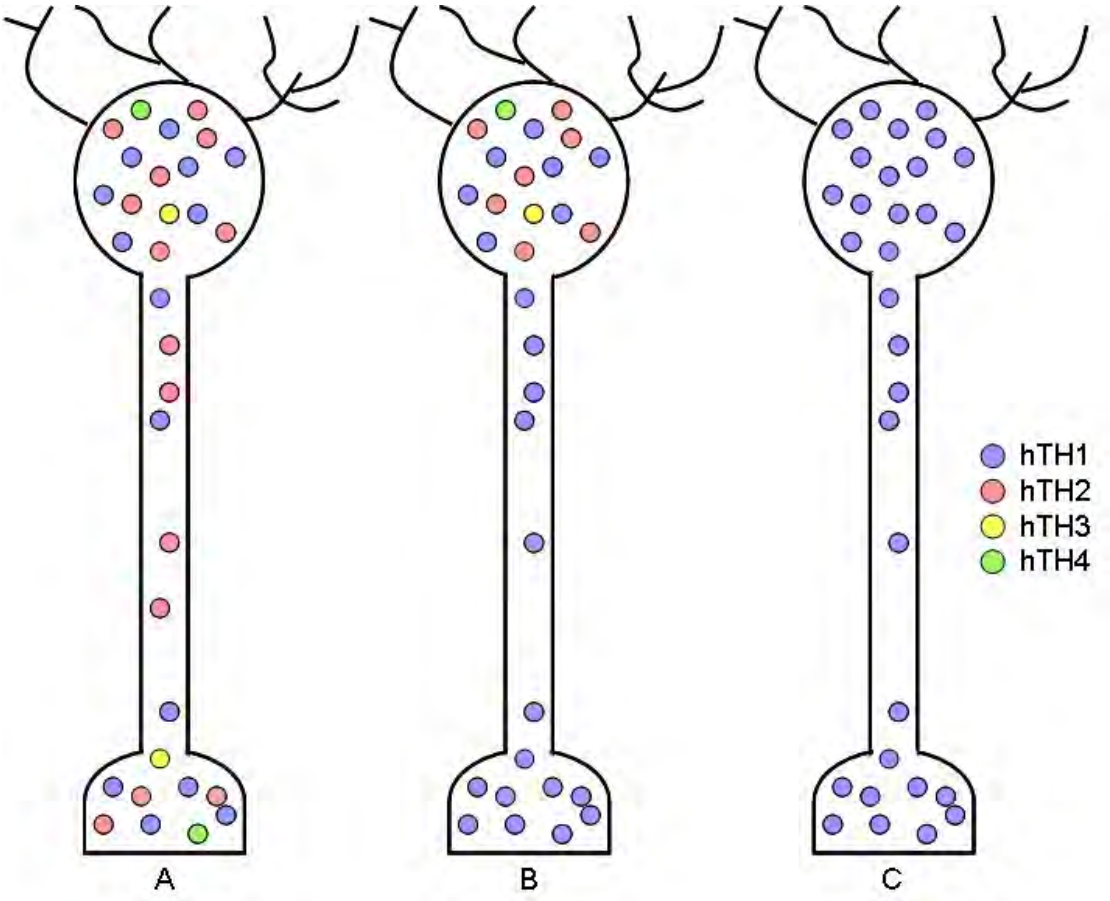


Figure 5.2: Models for cellular distribution of human TH isoforms. A) Stochastic distribution of hTH1-4. hTH1 and hTH2 are the major human TH isoforms, and hTH3 and hTH4 are expressed to a lower extent. The isoforms are distributed within the cell based solely upon their expression levels. **B)** Differential intracellular distribution of hTH isoforms. The isoforms are targeted to different intracellular regions, with hTH1 being selectively distributed in axons and terminal fields. **C)** Differential expression of hTH isoforms. The expression of the isoforms differs between cell types. Illustrated here is a cell selectively expressing hTH1.

There are three possible scenarios for the distribution and localisation of multiple TH isoforms in cells (Figure 5.2). Firstly, the distribution of the isoforms

within the cell may be a stochastic process; that is, the distribution of the four isoforms depends solely on the relative amounts of each isoform expressed within that cell (Figure 5.2 A). hTH1 and hTH2 are known to be the major human TH isoforms, with hTH3 and hTH4 being expressed at a much lower level (Haycock 1991; Lewis et al. 1993). In immunolabelling experiments using isoform-specific antibodies it was demonstrated that at least in some neurons hTH2, hTH3 and hTH4 are co-expressed and co-localise with hTH1 in the one cell (Lewis et al. 1993). It is therefore likely that at least in some cell populations the four human TH isoforms are distributed according to the model presented in Figure 5.2 A.

In the second model for the distribution of the isoforms, hTH1-4 may be differentially transported to specific regions of the cell (Figure 5.2 B). This would require that the different amino acid sequences in the N-terminal domain of TH be involved not only in the differential regulation of the isoforms as demonstrated in Chapter 2, but also be involved in differential protein trafficking. Immunocytochemical studies have suggested that in some neurons the human TH isoforms may be differentially distributed (Lewis et al. 1993). All four TH isoforms are expressed in the cell bodies of dopaminergic neurons of the substantia nigra, with hTH1 and hTH2 being the two major isoforms. However, hTH1 was found to be selectively expressed in the axons and terminal fields of the nigrostriatal projections. This data could reflect different affinities of the isoform-specific antibodies that were utilised, and therefore requires further study for confirmation. However, should this data be shown to be correct, it does suggest that mechanisms exist that provide for differential transport of the human TH isoforms to different neuronal compartments.

The sequestering of TH isoforms into different intracellular compartments as shown in Figure 5.2 B would allow for region-specific modulation of TH activity.

For example, ERK has been shown to be enriched in the synaptosomal fraction of neuronal extracts (Ortiz et al. 1995). Targeting hTH1 to the terminal fields of neurons may result in colocalisation of hTH1 with ERK. This would result in a higher level of TH activity due to the ability of ERK-mediated phosphorylation of Ser31 to potentiate the phosphorylation of Ser40. This would serve to concentrate TH activity at areas where it is required, for example in the terminal fields of dopaminergic neurons of the nigrostriatal tract. In effect, the differential distribution of the isoforms would provide for region-specific regulation of catecholamine synthesis which would be dependent on the isoform that predominates in that cellular region.

It is also tempting to suggest that differential expression of the isoforms between different cell groups could contribute to the regulation of catecholamine biosynthesis. Haycock and colleagues have demonstrated using isoform-specific antibodies that anti-hTH1 immunoreactivity alone was evident in some neurons of the rostral linear nucleus; no evidence of hTH2, 3 or 4 expression was found in these cells (Lewis et al. 1993). Again, these results may simply reflect the different affinities or stabilities of the isoform-specific antibodies that were used, however this is the first study to provide some evidence for a cell type-specific expression of the TH isoforms as modelled in Figure 5.2 C. Cell type-specific expression of the different TH isoforms remains an attractive but unconfirmed concept that would provide for differential catecholamine biosynthesis based on individual cellular requirements for catecholamine production. A comprehensive investigation into the inter- and intra-cellular distribution of the human TH isoforms has not yet been undertaken.

5.4. Regulation of TH activity *in vitro* and *in situ* by the low affinity binding site

Regulation of TH activity by feedback inhibition is a well-recognised phenomenon. There are two contrasting theories regarding the regulation of TH activity by the catecholamines, one suggesting that catecholamines bind to TH in a manner that is competitive with BH_4, and the other suggesting that catecholamines bind to TH in a non-dissociable manner by coordinating directly with ferric iron (Kumer and Vrana 1996).

Work presented here (Chapter 3) has shown that TH contains two distinct dopamine binding sites, one that binds dopamine with high affinity in a non-dissociable manner, and one that binds dopamine with low affinity in a readily-dissociable manner (Figure 5.1). Dopamine binding to the high affinity site is relieved by phosphorylation of Ser40, however dopamine binding to the low affinity site acts independently of phosphorylation. Both sites utilise different mechanisms to regulate TH activity *in vitro*. The low affinity dopamine binding site is able to regulate TH activity for both the non-phosphorylated and Ser40-phosphorylated forms of the enzyme *in vitro*, and does this primarily by increasing the K_m for BH_4.

This work is extended in Chapter 4, which provides data demonstrating that the low affinity binding site is able to modulate TH activity *in situ* in response to alterations in cytosolic catecholamine levels. The low affinity site is active *in situ* under both basal conditions and conditions where TH is phosphorylated at Ser40.

The finding that there is a low affinity site on TH that is active both *in vitro* and *in situ*, and acts independently of phosphorylation of Ser40, has important implications for the regulation of TH activity in different body systems.

5.5. Cell type-specific regulation of TH

TH is predominantly expressed in two distinct cell types: endocrine cells and neurons. In chromaffin cells of the adrenal medulla, noradrenaline and adrenaline are released into the bloodstream in an endocrine manner. In dopaminergic, noradrenergic and adrenergic neurons of the CNS, and in post-ganglionic sympathetic neurons of the autonomic nervous system, the catecholamines are released locally and act as neurotransmitters. These cells are different in several ways with regards to the regulation of catecholamine availability.

The two cell types contain different amounts of catecholamines. Although the concentration of catecholamines inside the vesicles is relatively similar, the size and number of vesicles differs between cell types. Adrenal chromaffin cells contain many large vesicles, whilst neuronal cells contain fewer small or medium vesicles (Teschemacher 2005; Garcia et al. 2006). This enables chromaffin cells to release relatively large quantities of catecholamines into the bloodstream, and neuronal cells to release smaller amounts of the catecholamines into the synaptic cleft.

In neuronal cells, the dopamine and noradrenaline transporters are responsible for recycling catecholamines back into the neuron via reuptake from the synaptic cleft (Gether et al. 2006). When released from the adrenal gland, the catecholamines are quickly taken up into the bloodstream and transported around the body (Teschemacher 2005), thereby limiting the access of the transporters in chromaffin cells to free circulating catecholamines. This represents a second major difference between the cell types regarding mechanisms influencing catecholamine availability.

Despite the differences in catecholamine levels and reuptake mechanisms between these cells types, the same biosynthetic machinery is responsible for catecholamine production in both cases and therefore must be capable of responding

appropriately in these very different environments. As such, TH activity differs greatly between these two cell types. In neuronal cells, TH activity when measured at physiological pH is very low (Okuno and Fujisawa 1985). TH activity measured from adrenal extracts is approximately 15-30-fold higher than that measured in brain extracts (Okuno and Fujisawa 1985), demonstrating the TH has a comparatively higher specific activity in the adrenal medulla than in the brain.

This difference in the specific activity of TH is representative of system requirements. In the adrenal gland, the higher level of catecholamine release and the relative lack of reuptake of catecholamines from the periphery would necessitate a much higher level of catecholamine synthesis. In neuronal cells the lower level of catecholamine release coupled with the recycling of available catecholamines would require a lower level of TH activity. Therefore, it is likely that different regulatory mechanisms predominate in each of these cells types to provide for the large difference in the required rate of catecholamine synthesis and therefore specific activity of TH.

5.5.1. Regulation of TH activity in adrenal chromaffin cells

Catecholamine secretion from the adrenal gland is achieved by the release of acetylcholine from the splanchnic nerve. Acetylcholine binds to nicotinic receptors on chromaffin cells and causes membrane depolarisation, thereby resulting in an increase in intracellular Ca^{2+} (de Diego et al. 2008). Increases in intracellular Ca^{2+} results in exocytosis of catecholamine-containing vesicles from the adrenal gland (de Diego et al. 2008). Depolarisation-induced increases in intracellular Ca^{2+} also causes

the activation of kinase pathways that result in increased phosphorylation of all three major Ser residues on TH (Salvatore et al. 2001).

The basal release of catecholamines from the adrenal gland is involved in the maintenance of sympathetic tone in the body (de Diego et al. 2008). Increases in the rate of splanchnic nerve firing results in the mobilisation of catecholamine stores, thereby causing the mass activation of adrenergic receptors and producing the hallmark peripheral effects that characterise the “fight or flight” response. Secretion of catecholamines from chromaffin cells does not result in a concomitant decrease in intracellular catecholamine stores (Wakade et al. 1988). This suggests that TH activity must be rapidly increased following catecholamine release. Regardless of the degree of catecholamine secretion, the increase in TH activity required to compensate for exocytosis is likely to be a phosphorylation-mediated event.

There is a higher level of phosphorylation of all three major Ser residues of TH isolated from rat adrenal medulla compared to that in rat forebrain (Saraf et al. 2007). As shown in Figure 5.2, while similar amounts of TH protein have been loaded from the adrenal medulla and forebrain extracts, the level of phosphorylation of Ser19, 31 and 40 is much higher in the adrenal gland. Phosphorylation of Ser40 directly increases TH activity by increasing the rate of dissociation of catecholamines from the high affinity dopamine binding site (Daubner et al. 1992; Ramsey and Fitzpatrick 1998). Therefore the higher level of phosphorylation of Ser40 in the adrenal medulla is likely to be directly responsible for the comparatively higher specific activity of the enzyme in that tissue.

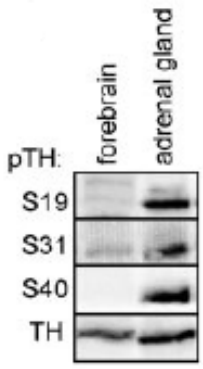
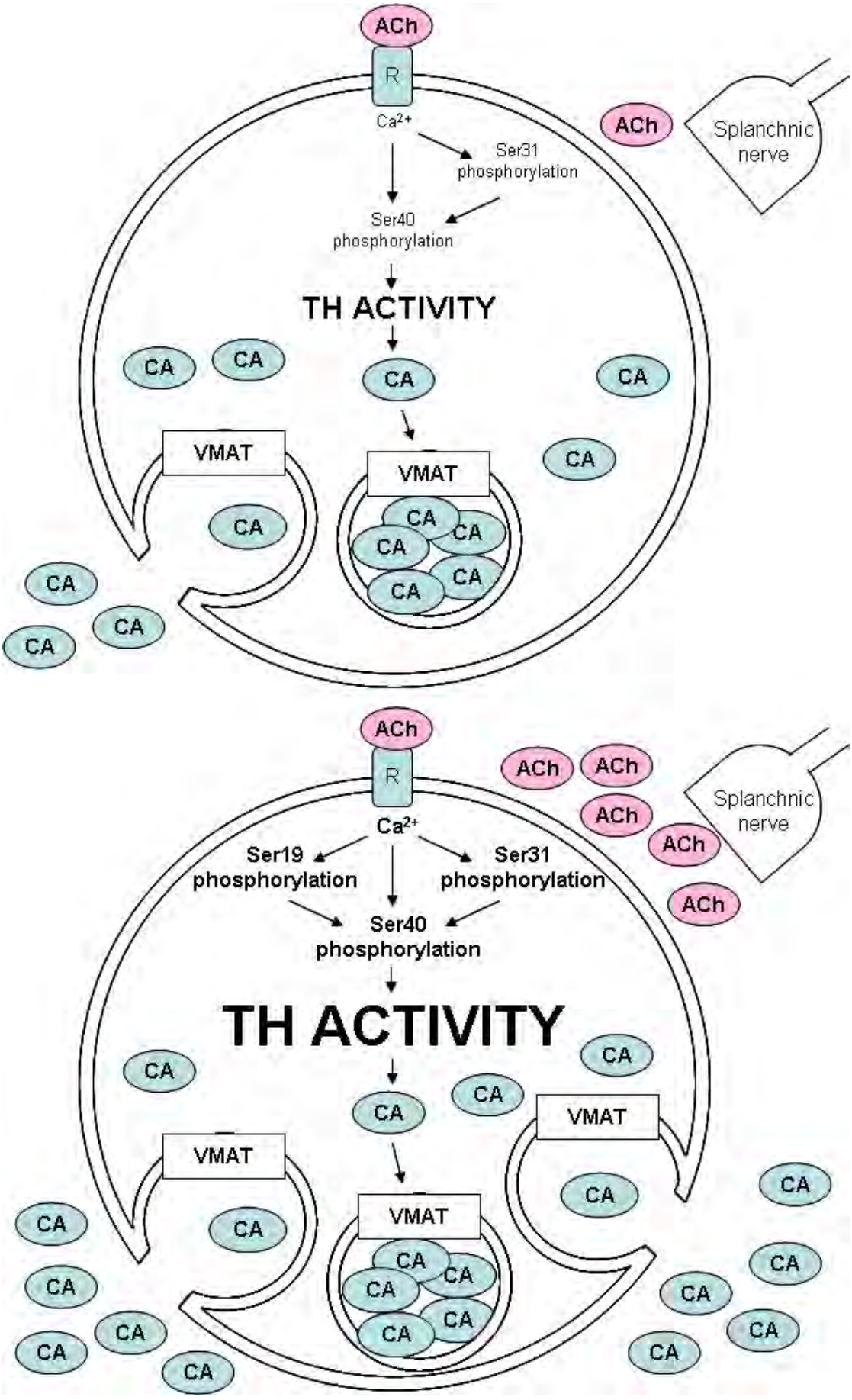


Figure 5.2: Phosphorylation of TH in different tissue extracts. Taken from (Saraf et al. 2007).

There is a high level of phosphorylation of both Ser19 and Ser31 in the adrenal gland (Figure 5.2) (Saraf et al. 2007). Work presented in this thesis (Chapter 2) has demonstrated that Ser31 phosphorylation contributes to the basal phosphorylation of Ser40 *in situ*. In addition, it has been demonstrated in bovine adrenal chromaffin cells that increases in the phosphorylation of Ser19 and Ser31 potentiates the stimulus-induced increase in Ser40 phosphorylation and hence TH activity (Bobrovskaya et al. 2004; Lehmann et al. 2006). This suggests that hierarchical phosphorylation of Ser40 by phosphorylation of Ser19 and/or Ser31 plays an important role in the regulation of TH activity in the adrenal gland under conditions of both low-level and high-level catecholamine release (Figure 5.3).

Figure 5.3 (next page): Regulation of TH activity in adrenal chromaffin cells under basal (top) and stimulated (bottom) conditions. Release of acetylcholine (ACh) from the splanchnic nerve results in an increase in intracellular Ca^{2+}. Increases in intracellular Ca^{2+} will result in exocytosis of catecholamines (CA). TH activity must be increased following catecholamine release to re-fill vesicles. Phosphorylation of Ser19, 31 and 40 contribute to this required increase in TH activity.


ACh
R
Ca2+
Ser31 phosphorylation
Ser40 phosphorylation
TH ACTIVITY
Splanchnic nerve
CA
VMAT
ACh
Ser19 phosphorylation

In mouse adrenal chromaffin cells, the cytosolic concentration of catecholamines is approximately 2-50μM (Mosharov et al. 2003), which exceeds the K_D of the low affinity, readily-dissociable dopamine binding site and essentially negates any role that this site may play in the regulation of TH activity in the adrenal gland. It is therefore likely that regulation of TH activity in the adrenal gland is achieved primarily by phosphorylation of the three major Ser residues.

5.5.2. Regulation of TH activity in neuronal cells

Midbrain dopaminergic neurons of the substantia nigra and ventral tegmental area normally fire in a tonic fashion at relatively low frequency, with intermittent burst firing or phasic neurotransmission (Heien and Wightman 2006). These two broad firing patterns play differing roles in dopaminergic neurotransmission (Heien and Wightman 2006; Marinelli et al. 2006). Tonic firing maintains a baseline of dopaminergic activity, essentially providing a pacemaker activity of dopaminergic neurons. Noradrenergic neurons of the locus coeruleus also display pacemaker firing patterns (Alreja and Aghajanian 1991). Phasic or burst firing of dopaminergic neurons results from a rapid increase in neuron firing rate, and can be altered by a number of factors (Marinelli et al. 2006). Peripheral sympathetic neurons also primarily exhibit bursting patterns of activity (McAllen and Malpas 1997). These two forms of firing activity differ in the amount of catecholamine that are released from the cell; as a result, they have different requirements for catecholamine synthesis and hence TH activity.

5.5.2.1. Regulation of TH activity in neuronal cells: tonic firing

Tonic firing of dopaminergic neurons results in the establishment of an equilibrium of dopamine release and reuptake, where exocytosis of vesicular dopamine stores is matched by recycling of dopamine from the extracellular milieu by the dopamine transporter (Heien and Wightman 2006). This maintains a relatively constant extracellular dopamine concentration (Figure 5.4). Due to the establishment of this equilibrium during tonic firing, new dopamine synthesis would only need to accommodate for breakdown of dopamine (Figure 5.5).

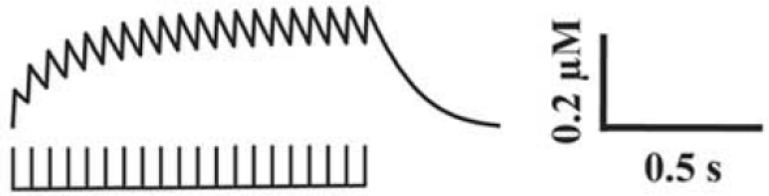
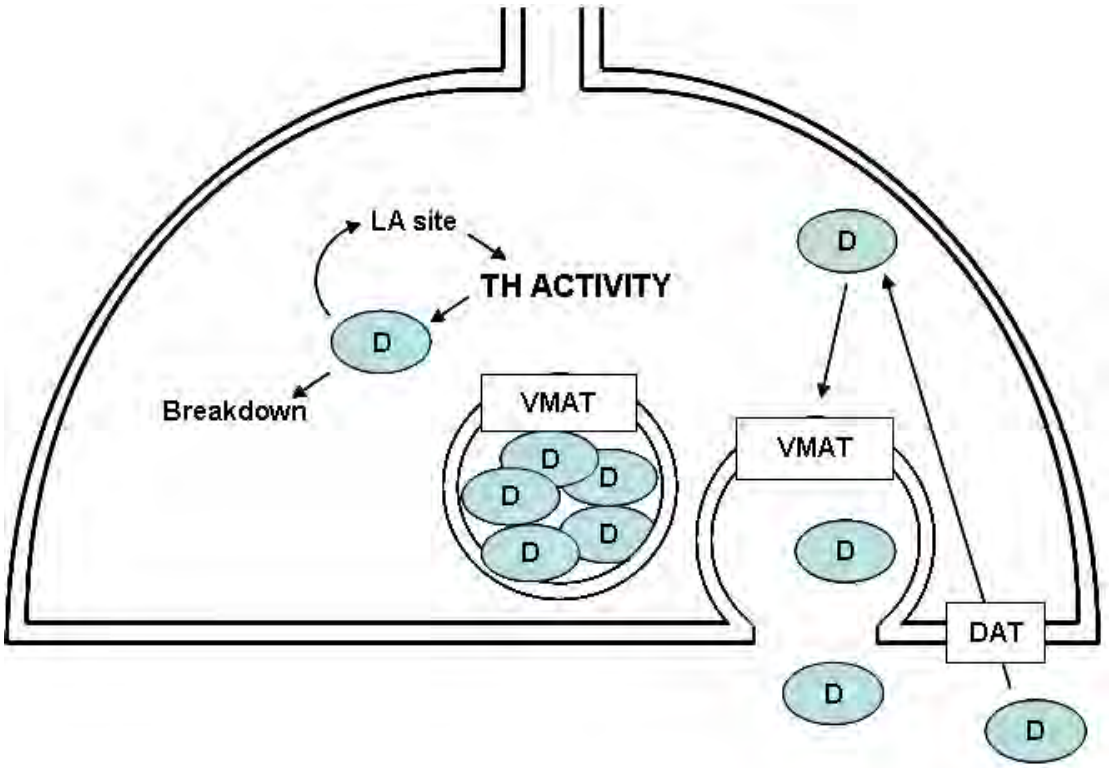
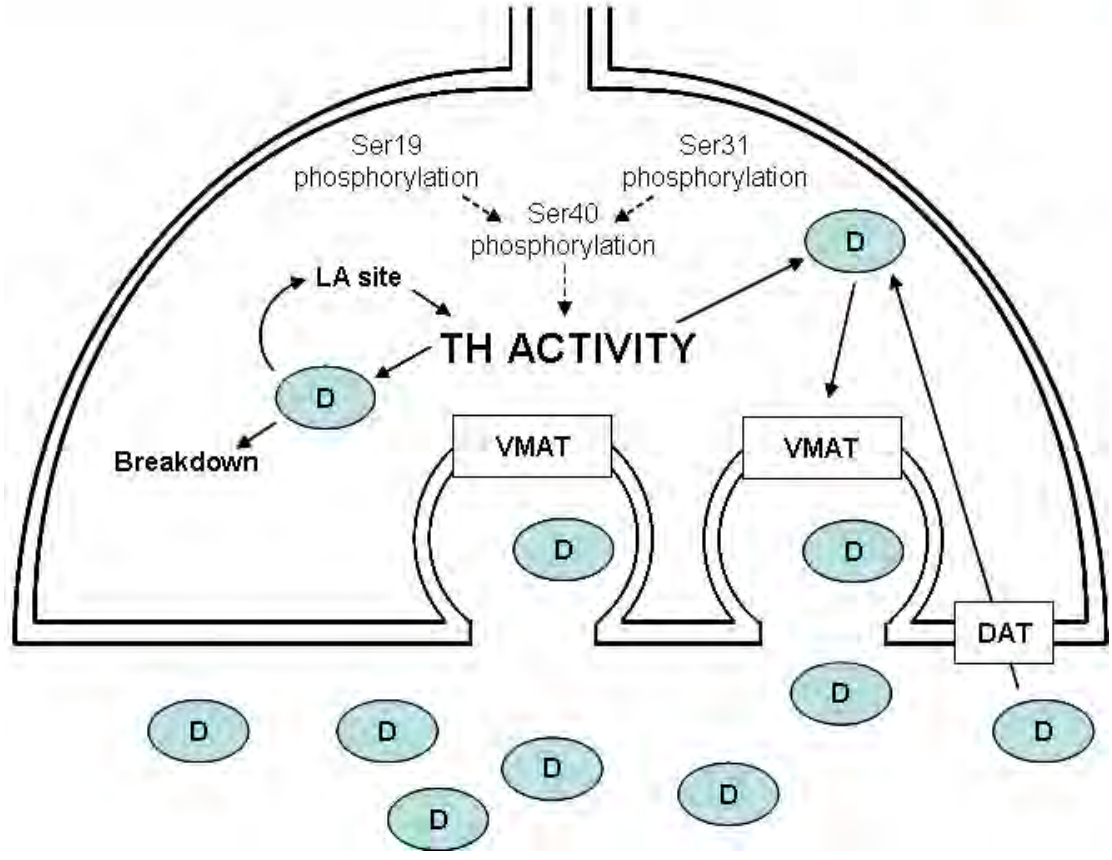


Figure 5.4: Extracellular dopamine concentrations during tonic firing. Vertical bars represent electrical stimulation pulses (350 µA, 2 ms duration) delivered at 20 Hz to the ventral tegmental area to mimic tonic firing. Simulation of the extracellular dopamine concentration in the striatum is shown above vertical bars. The actual measured dopamine concentration displays substantial similarity to simulated concentration (not shown). Taken from (Heien and Wightman 2006).

Figure 5.5 (next page): Regulation of TH activity in dopaminergic neurons during tonic (top) and phasic (bottom) firing. During tonic firing, reuptake of dopamine (D) by the dopamine transporter (DAT) matches the rate of exocytosis, and therefore TH activity only needs to accommodate for the rate of dopamine breakdown. TH is phosphorylated at Ser40 to a low stoichiometry in dopaminergic neurons. The cytosolic catecholamine concentration approximates the K_D of the low affinity site (LA site), and thus this site would be capable of regulating TH activity in dopaminergic neurons. During phasic firing, reuptake of dopamine is no longer able to accommodate for the rate of exocytosis. An increase in TH activity would be required under these conditions. It is possible that phosphorylation of Ser19, 31 and 40 may be increased under these conditions, and may contribute to the required increase in TH activity (broken arrows). The low affinity binding site would still be active under these conditions.


LA site
TH ACTIVITY
D
Breakdown
VMAT
D
D
D
D
D
VMAT
D
DAT
D
D


Ser19
phosphorylation
Ser31
phosphorylation
Ser40
phosphorylation
LA site
TH ACTIVITY
D
Breakdown
VMAT
VMAT
D
D
DAT
D
D
D
D
D
D

Under basal conditions in rat dopaminergic neurons, TH is phosphorylated at Ser40 to a low stoichiometry, with <5% of TH subunits being phosphorylated at this residue (Salvatore et al. 2000). Therefore, the majority of TH will be in a form where it contains both the high affinity and low affinity dopamine binding sites. Dopamine essentially does not dissociate from the high affinity site, and therefore the majority of TH under basal conditions in neurons will have dopamine covalently bound to the high affinity binding site and inhibiting TH activity.

Under these conditions TH will still be able to respond to any changes in cytosolic dopamine concentrations through dopamine binding to the low affinity, readily-dissociable binding site (Figure 5.5). The K_D of the low affinity dopamine binding site approximates the concentration of cytosolic catecholamines in midbrain dopaminergic neurons (Mosharov et al. 2006). The binding of dopamine to the low affinity site would therefore establish a basal rate of dopamine synthesis that is dependent on cytosolic dopamine levels. When cytosolic dopamine levels decrease, dopamine will dissociate from the low affinity site and TH activity will increase. Conversely, if cytosolic dopamine levels increase, dopamine will bind to the low affinity site to dampen TH activity and allow clearance of the cytosolic dopamine. Thus, the low affinity dopamine binding site would act to maintain an equilibrium of cytosolic dopamine.

The low affinity dopamine binding site acts independently of phosphorylation, and therefore it is not reliant upon intracellular signalling events. Instead, it acts as an automatic sensor of cytosolic catecholamine concentrations. This is a classical homeostatic form of feedback inhibition. As tonic firing forms the most common form of dopaminergic firing (Heien and Wightman 2006), the low

affinity dopamine binding site is likely to be the dominant regulator of TH activity in dopaminergic neurons.

5.5.2.2. Regulation of TH activity in neuronal cells: phasic firing

Phasic firing of dopaminergic neurons is characterised by a high frequency of spiking events, which results in an increase in the amount of dopamine released at the dopaminergic terminals (Marinelli et al. 2006). During phasic firing of neurons, dopamine release exceeds dopamine reuptake, such that dopamine continues to accumulate in the extracellular space (Heien and Wightman 2006) (Figure 5.6). This would necessitate a higher rate of dopamine synthesis compared to tonic firing, as the increased amount of dopamine release relative to reuptake would result in an overall depletion of intracellular stores.

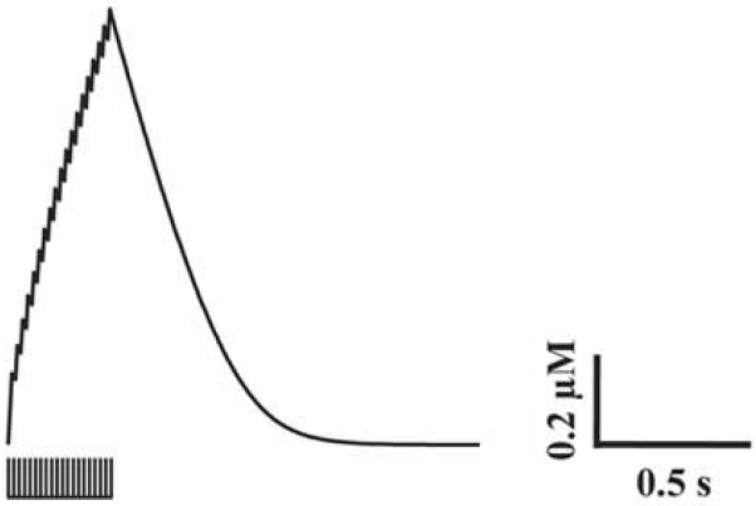


Figure 5.6: Extracellular dopamine concentration during phasic firing. Vertical bars represent electrical stimulation pulses (350 µA, 2 ms duration) delivered at 60 Hz to the ventral tegmental area to mimic phasic firing. Simulation of the extracellular dopamine concentration in the striatum is shown above vertical bars. The actual measured dopamine concentration displays substantial similarity to simulated concentration (not shown). Taken from (Heien and Wightman 2006).

Under these conditions, the dopamine dissociating from the low affinity binding site may not be able to fully accommodate for the required increase in rate of dopamine synthesis. Thus, phosphorylation of Ser40, and the relief of catecholamine inhibition from the high affinity dopamine binding site may be required to ensure sufficient catecholamine biosynthesis (Figure 5.5).

Similar to the link between catecholamine release and synthesis in the adrenal gland, it is possible that factors that alter phasic catecholamine release can also alter TH phosphorylation. Cocaine administration, which results in a decrease in dopaminergic activity (Marinelli et al. 2006), also decreases the phosphorylation of Ser19, 31 and 40 in the nucleus accumbens and caudate, and there is a concomitant decrease in TH activity in these brain regions (Jedynak et al. 2002). Similarly, quinpirole, a D2 receptor agonist, decreases dopaminergic firing (Marinelli et al. 2006), and also specifically decreased the phosphorylation of TH at Ser40 in rat striatal slices as well as producing a concomitant decrease in TH activity (Lindgren et al. 2001). Conversely, D2 receptor antagonists increase the phosphorylation of TH at Ser19, 31 and 40 (Salvatore et al. 2000).

Hierarchical phosphorylation of Ser40 via phosphorylation of Ser19 and Ser31 may contribute to the regulation of TH activity in dopaminergic cells during phasic firing. Cocaine and D2 receptor antagonists affect the phosphorylation of all three major Ser residues. This implies that a single stimulus could increase the phosphorylation of all three major Ser residues, thereby allowing the potentiation of Ser40 phosphorylation by the phosphorylation of Ser19 and/or Ser31. As previously discussed, this effect would also be dependent on which hTH isoforms predominate in specific neurons. Neurons containing high levels of hTH1 would be expected to

have higher levels of TH activity due to the effect of Ser31 phosphorylation on the potentiation of Ser40 phosphorylation.

Even following phosphorylation of Ser40, the low affinity dopamine binding site still retains the ability to regulate TH activity. This was demonstrated in PC12 cells, where the Ser40 phosphorylation-induced increase in TH activity was completely inhibited by preincubating the cells with high concentrations of L-DOPA. The cytosolic catecholamine content of PC12 cells is similar to that of midbrain dopaminergic neurons (Mosharov et al. 2006). Therefore, it is likely that in dopaminergic neurons, under conditions where Ser40 is phosphorylated and dopamine is no longer bound to the high affinity site, the low affinity site is still able to regulate TH activity by responding to cytosolic dopamine levels.

5.6. Physiological implications of the low affinity binding site on oxidative stress and neurodegenerative disease

Parkinson's disease is likely to be a multifactorial disorder without a single causative agent. In sporadic Parkinson's disease it is likely that several factors combine over an individual's lifetime to produce the neurodegeneration that is a hallmark of the disease (Moore et al. 2005; Olanow 2007). Oxidative stress has been implicated as one as the most likely causative agents contributing to disease pathogenesis (Jenner and Olanow 1996; Jenner 2003; Barnham et al. 2004). Oxidative stress results from the overproduction of reactive oxygen species (ROS) which can then oxidatively modify proteins, lipids and nucleic acids (Barnham et al. 2004). The oxidation of these macromolecules can alter their functionality, or

produce further toxic products. Furthermore, the generation of ROS can lead to dysregulation of intracellular Ca^{2+} and thereby initiate apoptosis cascades.

It is likely that a number of different pathways contribute to the accumulation of ROS in Parkinson's disease. Cytosolic dopamine has been implicated as a contributor to oxidative stress within dopaminergic neurons. Dopamine can undergo enzymatic breakdown and auto-oxidation, both of which may result in the production of ROS (Stokes et al. 1999; Jenner 2003; Youdim and Bakhle 2006). Auto-oxidation of dopamine also produces toxic dopamine-quinone (Stokes et al. 1999). Furthermore, dopamine itself has been suggested to contribute to ROS production by coordinating with intracellular Fe^{3+}, thereby promoting the Fenton reaction (Barnham et al. 2004).

The accumulation of cytosolic catecholamines has been hypothesised to contribute to the pathogenesis of Parkinson's disease (Barzilai et al. 2001). Recently it was demonstrated using transgenic mice engineered to overexpress the dopamine transporter in striatal neurons that chronic exposure to increased cytosolic dopamine levels *in vivo* is sufficient to induce neurodegeneration (Chen et al. 2008).

A major finding of this thesis has been that TH contains a low affinity dopamine binding site. The K_D of this low affinity, readily-dissociable site approximates the cytosolic concentration of catecholamines in PC12 cells and midbrain dopaminergic neurons (Mosharov et al. 2006). This suggests that the low affinity dopamine binding site regulates TH activity in neuronal cells by responding to changes in cytosolic catecholamine levels. This theory is supported by data presented in Chapter 4 which demonstrates that incubation of PC12 cells with L-DOPA inhibits TH activity through a mechanism that most likely involves catecholamines binding to the low affinity site.

The low affinity dopamine binding site is not only active under basal conditions, but can also regulate the activity of the Ser40-phosphorylated form of the enzyme both *in vitro* and *in situ*. Thus, regardless of the phosphorylation state of TH, the low affinity dopamine binding site is able to respond to cytosolic catecholamine levels to regulate TH activity accordingly.

Therefore the low affinity dopamine binding site would act to maintain an equilibrium of cytosolic catecholamines in neurons. This would not only provide for sufficient basal TH activity in neuronal cells, but also prevent the harmful accumulation of cytosolic catecholamines. This suggests that the low affinity dopamine binding site may have a neuroprotective role *in vivo*.

5.7. Future directions

5.7.1. Further elucidation of the role of multiple TH isoforms

Work presented here has demonstrated that stimuli that increase Ser31 phosphorylation in hTH1 *in situ* do not increase the phosphorylation of hTH2 at Ser35, and that this results in hTH1 and hTH2 being differentially regulated by hierarchical phosphorylation *in situ*. The differential regulation of hTH1 and hTH2 *in vitro* and *in situ* suggest that the level of expression of the different human TH isoforms could have implications for the tissue-specific level of TH activity and catecholamine synthesis.

A systematic investigation of the relative abundance and cellular distribution of each of the human TH isoforms in different brain regions, and also in the adrenal medulla, has not yet been undertaken. Our laboratory and associates are currently

undertaking such an analysis of the intra- and intercellular distribution of the different human TH isoforms by western blotting and immunohistochemistry.

5.7.2. Further elucidation of the role of hierarchical phosphorylation in the regulation of TH activity

Hierarchical phosphorylation of Ser40 in hTH1 via phosphorylation of Ser31 contributes to the basal level of phosphorylation of Ser40 *in situ*. This effect is absent in hTH2 *in situ*. Less is known about the *in situ* actions of Ser19 phosphorylation in potentiating the phosphorylation of the equivalent Ser40 residues in the different human TH isoforms. These studies may prove difficult, as a kinase capable of selectively phosphorylating Ser19 has not yet been identified; all Ser19 kinases identified to date also phosphorylate Ser40 to some degree (Dunkley et al. 2004). Therefore, stimulation or inhibition of kinases involved in Ser19 phosphorylation *in situ* would be likely to also affect Ser40 phosphorylation. To overcome this issue, Ser19Ala mutants of the human TH isoforms could be utilised to investigate whether Ser19 phosphorylation has a differential effect on Ser40/44 phosphorylation in hTH1 and hTH2 *in situ*.

5.7.3. Further elucidation of the role of the low affinity site in the regulation of TH activity

In order to further elucidate the role of the low affinity site in the regulation of TH activity, the location of the low affinity site must be determined. Dopamine binding to the low affinity site acts to increase the K_m for the co-substrate BH_4 without altering V_{max}. The mechanism of action of dopamine binding to the low

affinity site suggests that dopamine could be binding to the active site of the molecule. Mutagenesis of TH was performed to remove the regulatory domain of TH, and in these studies the low affinity, but not the high affinity, dopamine binding site was retained (Chapter 4). This suggested that the N-terminal regulatory domain is important in stabilising dopamine binding to the high affinity binding site, and also that the low affinity site is contained within the catalytic core of the enzyme. Mutagenesis of active site residues has suggested that the low affinity and high affinity dopamine binding sites are indeed contained within the active site of the enzyme[1]. The exact residues involved in the binding of dopamine to TH have not yet been defined, however Tyr371 is likely to play a pivotal role in feedback inhibition of TH activity[1]. The equivalent residue in phenylalanine hydroxylase has also been implicated in facilitating the binding to dopamine to the active-site iron (Erlandsen et al. 1998).

Once the active site residues involved in dopamine binding to the high and/or low affinity sites of TH have been elucidated, site-directed mutagenesis of these sites may allow the production of TH lacking either the high or low affinity binding sites. Transfection of these mutants into SH-SY5Y cells would allow the elucidation of the importance of the roles of these binding sites in regulating TH activity, and in maintaining an equilibrium of cytosolic catecholamines.

[1] G. Briggs, S. Gordon, P. Dunkley and P. Dickson, unpublished data, 2008.

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Appendix

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 26, pp. 17644–17651, June 30, 2006

Differential Regulation of the Human Tyrosine Hydroxylase Isoforms via Hierarchical Phosphorylation*

Received for publication, November 14, 2005, and in revised form, April 18, 2006 Published, JBC Papers in Press, April 27, 2006, DOI 10.1074/jbc.M512194200

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Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of the catecholamines dopamine, noradrenaline, and adrenaline. In response to short term stimuli TH activity is primarily controlled by phosphorylation of serine 40. We have previously shown that phosphorylation of serine 19 in TH can indirectly activate TH via a hierarchical mechanism by increasing the rate of phosphorylation of serine 40. Here we show that phosphorylation of serine 31 in rat TH increases the rate of serine 40 phosphorylation 9-fold *in vitro*. Phosphorylation of serine 31 in intact bovine chromaffin cells potentiated the forskolin-induced increase in serine 40 phosphorylation and TH activity more than 2-fold. Humans are unique in that they contain four TH isoforms but to date no significant differences have been shown in the regulation of these isoforms. Phosphorylation of the human TH isoform 1 at serine 31 by extracellular signal-regulated protein kinase (ERK) also produced a 9-fold increase in the rate of phosphorylation of serine 40, whereas little effect was seen in the TH isoforms 3 and 4. ERK did not phosphorylate human TH isoform 2. The effect of serine 19 phosphorylation on serine 40 (44 in TH2) phosphorylation is stronger in TH2 than in TH1. Thus hierarchical phosphorylation provides a mechanism whereby the two major human TH isoforms (1 and 2) can be differentially regulated with only isoform 1 responding to the ERK pathway, whereas isoform 2 is more sensitive to calcium-mediated events.

Tyrosine hydroxylase (TH)[2] [EC1.14.16.2] is the rate-limiting enzyme in the biosynthesis of the catecholamines dopamine, noradrenaline, and adrenaline (1). Short term regulation of TH is accomplished by dynamic changes in the phosphorylation state of the enzyme (2, 3). Although four serine residues have been shown to be phosphorylated in TH, only three of these serine residues (Ser^{19}, Ser^{31}, and Ser^{40}) are regulated *in vivo* (4). The most important mechanism of TH activation is phosphorylation of Ser^{40}, which decreases the feedback inhibition by the catecholamines (5–7). Phosphorylation of dopamine bound TH at Ser^{40} by protein kinase A (PKA) can activate TH by up to 20-fold (5). The direct effect of Ser^{19} and Ser^{31} phosphorylation on TH activation is much more modest. Phosphorylation of Ser^{19} by calcium calmodulin-dependent protein kinase (CaMKII) will only increase TH activity in the presence of the 14-3-3 protein (8–10), and this results only in a 2-fold increase in the activity. The phosphorylation of Ser^{31} by extracellular signal-regulated protein kinase (ERK) produces less than a 2-fold increase in TH activity, primarily by decreasing the affinity of the cofactor tetrahydrobiopterin (BH_4) (11–13). Phosphorylation of Ser^{31} in TH has also been shown to increase the stability of TH (14).

Humans have four TH protein isoforms, whereas anthropoids have two, and other mammalian species only have one (15). TH is encoded by a single gene, and the multiple isoforms are because of multiple mRNAs generated by alternative splicing of the single gene (16–18). The human TH1 (hTH1) variant is like the subunits in all other species. The other human TH isoforms hTH2, hTH3, and hTH4 have inserts that lead to the expression of proteins containing 4, 27, and 31 (4 + 27) amino acids inserted immediately N-terminal to Ser^{31} in the hTH1 isoform. The hTH1 and hTH2 isoforms are the most prominent forms in human tissue samples and human cell lines (15, 19, 20). Analysis of recombinant forms of the four human isoforms indicate that their steady state kinetic parameters are comparable among the non-phosphorylated forms and among the phosphorylated forms (12, 21–23). All four human TH isoforms showed the same dopamine binding characteristics as rat TH (rTH) (23).

As there is little direct effect of Ser^{19} and Ser^{31} phosphorylation on TH activity we have been exploring the possibility that phosphorylation of these sites may indirectly effect TH activation by hierarchical phosphorylation (3). That is, phosphorylation of Ser^{19} and Ser^{31} could alter the rate of Ser^{40} phosphorylation and therefore TH activation. We have shown that phosphorylation of Ser^{19} increases the rate of phosphorylation of Ser^{40} in TH 3-fold, whereas Ser^{40} phosphorylation has no effect on the rate of Ser^{19} phosphorylation (24). Therefore the phosphorylation of Ser^{19} increases the rate of phosphorylation of Ser^{40} in a hierarchical manner. This result has been confirmed both qualitatively and quantitatively by others using a different methodology (10). We have further shown that Ser^{19} phosphorylation can potentiate Ser^{40} phosphorylation and TH activation in intact cells (25).

In this report we have investigated whether Ser^{31} phosphorylation could also alter the rate of Ser^{40} phosphorylation. The results in this report show that Ser^{31} phosphorylation has a major effect on the rate of Ser^{40} phosphorylation and TH activation in intact cells and *in vitro*. These results also provide, for the first time, a mechanism by which the four human TH isoforms can be differentially regulated by hierarchical phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Protease Inhibitor Mixture was from Roche Applied Science. Angiotensin II (AngII), HEPES, phenol red, EGTA, EDTA, and PKA were obtained from Sigma. BH_4 was obtained from Dr. B. Schirck's laboratories, Jona, Switzerland. Forskolin was obtained from Biomol. SDS-PAGE reagents were from Bio-Rad Laboratories. Molecular

* This work was supported by grants from the Australian Research Council (number DP0343355), the Research Management Committee (University of Newcastle), the National Health and Medical Research Council (number 351118), and the Hunter Medical Research Institute from a donation from the Lions Club of Adamstown (Newcastle). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: TH, tyrosine hydroxylase; AngII, angiotensin II; BACCs, bovine adrenal chromaffin cells; BH_4, tetrahydrobiopterin; CaMKII, calcium/calmodulin-dependent protein kinase; ERK, extracellular signal-regulated protein kinase; hTH1, hTH2, hTH3, and hTH4, human TH isoforms 1–4; rTH, rat TH; PKA, protein kinase A; MOPS, 4-morpholinepropanesulfonic acid.

weight PAGE standards, nitrocellulose membrane (Hybond ECL), ECL plus kit, anti-rabbit immunoglobulin (horseradish peroxidase-linked whole antibody from donkey), and anti-mouse immunoglobulin (horseradish peroxidase-linked whole antibody from sheep) were obtained from Amersham Biosciences. The hTH2 Ser^{35} phosphopeptide ($GQS^{P}PR$) was synthesized by AUSPEP (Australia). Tissue culture reagents were from Sigma and were of analytical or tissue culture grade. Forskolin was dissolved in dimethyl sulfoxide before use. Appropriate solvent controls were performed for every condition.

Expression and Purification of TH and ERK and Purification of CaMKII—rTH was expressed in *Escherichia coli* and purified according to previously described procedures (5) with modifications as described (24). The recombinant rTH is not phosphorylated and does not contain bound catecholamine. The TH mutants were generated using the QuikChange site directed mutagenesis kit (Stratagene). The hTH2 and hTH4 isoforms were generated from the hTH1 and hTH3 isoforms, respectively, using the conditions described (23). The introduction of the mutation and the absence of other mutations were confirmed by DNA sequencing. The S19A mutations were further confirmed by the absence of Ser^{19} phosphorylation using the $Ser(P)^{19}$-specific antibody after phosphorylation with CaMKII. ERK2 was expressed from the plasmid NPT7–5His-ERK2-R4F and purified essentially as described (26) except that elution from the nickel-charged chelating-Superose column was with an imidazole gradient (0–400 mM). CaMKII was purified from whole brain by calmodulin-Sepharose chromatography (27). All of the recombinant DNA experiments were carried out in accordance with the guidelines of the Office of the Gene Technology Regulator, Australia.

Determination of TH Activity and the Incorporation of Radioactivity into TH—The phosphorylation reactions for TH were performed in 50 mM Tris-HCl, pH 7.5, 100 μM ATP, 12.5 mM $MgCl_2$ except for CaMKII, which were performed at 1 mM ATP. To obtain dopamine-bound TH, the TH was initially incubated in a buffer containing 75 mM MOPS, pH 7.2, 75 mM GSH, 15 μM dopamine for 20 min at 20 °C. Phosphorylation reactions were performed in the same buffer but with the addition of 100 μM ATP and 12.5 mM $MgCl_2$. TH activity was determined using the tritiated water release assay of Reinhard *et al.* (28). After dopamine binding and phosphorylation of TH, the TH was purified over a heparin-Sepharose column. The TH activity was then measured in a buffer containing 25 μM tyrosine, 60 mM potassium phosphate, pH 7.2, 0.006% β-mercaptoethanol, 36 μg/ml catalase, 500 μM BH_4 at 25 °C for 8 min. For the analysis of incorporation of radioactivity into TH, [γ-^{32}P]ATP was included in the phosphorylation reactions. The phosphorylation reactions were stopped by the addition of an equal volume of 4% SDS, 2 mM EDTA, 1% dithiothreitol, 50 mM Tris, pH 6.8. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The TH bands were detected by staining with Coomassie Blue-R250, the gel section containing TH was cut out, and the incorporated radioactivity was measured in a scintillation counter (Wallac 1410, Pharmacia).

TH Site Analysis—After TH was phosphorylated in the presence of [γ-^{32}P]ATP as described above the samples were applied to sodium dodecyl sulfate polyacrylamide gels and then transferred to nitrocellulose as described by Jarvie and Dunkley (29). The nitrocellulose filter was then subject to Ponceau staining. The TH band was cut out of the nitrocellulose, and the TH was eluted from the nitrocellulose by trypsin digestion. The HPLC analysis of the resultant phosphopeptides was performed as described previously (30). In the CaMKII rate experiments TH was precipitated by 10% trichloroacetic acid, washed twice with 10% trichloroacetic acid, and then once with acetone; the TH was then digested with trypsin and then directly injected onto the HPLC column.

Measurement of Site-specific TH Phosphorylation, TH Activity, Ser^{40} Kinase Activity and the Stoichiometry of Ser^{31} Phosphorylation in Bovine Adrenal Chromaffin Cell Extracts—Bovine adrenal chromaffin cells (BACCs) were prepared as described (31) with modifications (32). BACCs were plated and treated as described (25) except that cells were initially treated with 100 nM AngII for 30 min rather than anisomycin. Site-specific TH phosphorylation, TH activity, and Ser^{40} kinase activity in BACC extracts were measured as described (25). For measurement of the stoichiometry of Ser^{31} phosphorylation in BACCs, recombinant rat TH was maximally phosphorylated at Ser^{31} by incubation with ERK and was used as a calibration standard. A stoichiometry of 0.45 was assumed for the ERK-phosphorylated TH, as this was the average stoichiometry determined in a number of experiments. The stoichiometry of Ser^{31} phosphorylation in untreated BACCs was determined as described for Ser^{19} and Ser^{40} phosphorylation (25).

Statistical Analysis—For multiple comparisons statistical significance was assessed by the Tukey's test for multiple comparisons, protected by a one-way analysis of variance. Student's *t* test was used for pairwise comparisons.

RESULTS

The Effect of Ser^{31} Phosphorylation on the Forskolin-induced Increase in Ser^{40} Phosphorylation and TH Activity in Intact Cells—To examine the effect of Ser^{31} phosphorylation on Ser^{40} phosphorylation in intact cells we needed to establish conditions in which BACCs could be stimulated to independently increase the level of Ser^{31} or Ser^{40} phosphorylation without increasing Ser^{19} phosphorylation. The data in Fig. 1*A* show that when BACCs were incubated with AngII for 30 min, the level of Ser^{31} phosphorylation increased 3–4-fold with respect to control, whereas Ser^{40} phosphorylation and Ser^{19} phosphorylation did not change compared with the control ($p > 0.05$). An incubation of BACCs with forskolin for 4 min increased only Ser^{40} phosphorylation. The stoichiometry of phosphorylation of Ser^{31} was measured as described under "Experimental Procedures." A value for Ser^{31} stoichiometry in untreated chromaffin cells of 0.04 ± 0.003 (mean ± S.E., $n = 8$) was obtained. This indicates that the stoichiometry of Ser^{31} phosphorylation after AngII treatment would be between 0.12 and 0.16.

When the BACCs were treated first with AngII and then forskolin, the change in the levels of phosphorylation of Ser^{19} and Ser^{31} were the same as when the cells were individually treated with AngII or forskolin. This suggests that the effects of AngII and forskolin on BACCs are independent of each other. In contrast to this result, the increase in the level of Ser^{40} phosphorylation in cells treated with AngII plus forskolin was almost 2-fold greater than the increase in Ser^{40} phosphorylation in cells treated with forskolin alone. This difference was significant ($p < 0.001$). The effect of AngII and forskolin on TH activity is also shown in Fig. 1*A*. The treatment of cells with AngII produced no significant increase in TH activity when compared with the activity in untreated cells ($p > 0.05$). The addition of forskolin increased TH activity in BACCs ~2-fold. In cells treated with forskolin plus AngII, the increase of TH activity over control was 2.2-fold greater than that in cells treated with forskolin alone. This difference was statistically significant ($p < 0.001$).

The data shown above suggest that phosphorylation of Ser^{31} mediated by AngII can potentiate the forskolin-induced increase in Ser^{40} phosphorylation. If this were true then AngII should not increase the Ser^{40} phosphorylating activity in forskolin-treated cells. The level of TH Ser^{40} kinase activity in extracts of cells was determined as described under "Experimental Procedures" using exogenous TH as a substrate. The cells were treated with AngII, forskolin, or both forskolin and

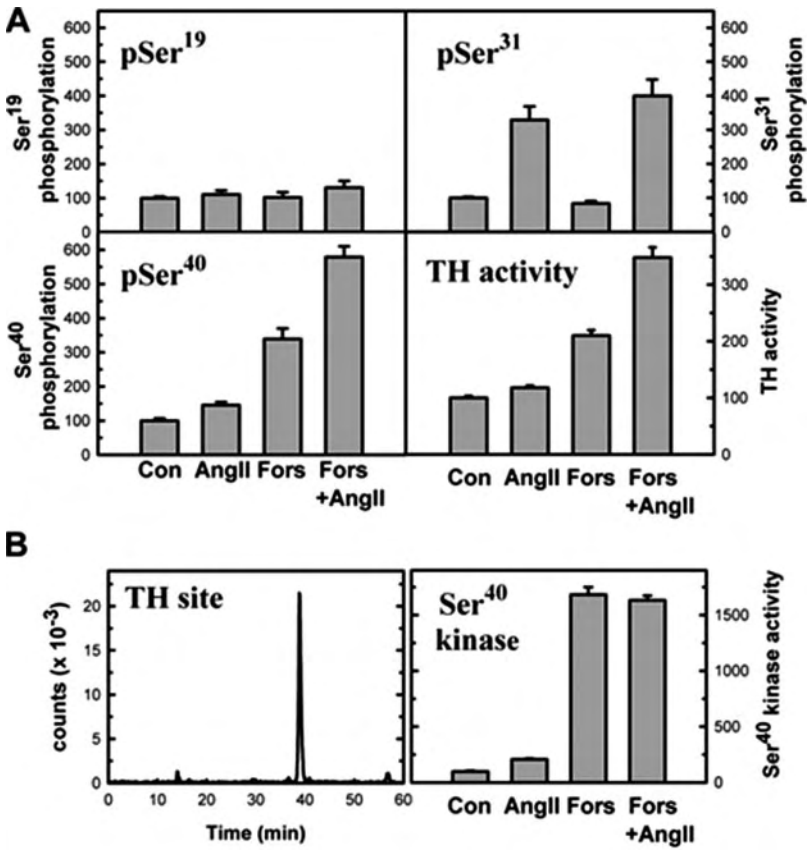

FIGURE 1. **Effect of Ser^{31} phosphorylation on the forskolin-induced increase in Ser^{40} phosphorylation and TH activation in BACCs.** *A*, BACCs were incubated with or without 100 nM AngII at 37 °C for 30 min. The cells were then incubated with or without 1 μM forskolin for a further 4 min at 37 °C. The cells were then processed, and the level of phosphorylation in TH of Ser^{19} (*pSer^{19}*), Ser^{31} (*pSer^{31}*), Ser^{40} (*pSer^{40}*) or TH activity was determined as described under "Experimental Procedures." *Con*, control basal; *Fors*, forskolin. Results are presented as the percent of the mean of the control ± S.E. For each condition the number of individual experiments was between 6 and 10. Statistical analysis: Ser^{19}, all conditions not significantly different from control ($p > 0.05$); Ser^{31}, Con *versus* Fors and AngII *versus* Fors+AngII were not significant ($p > 0.05$), all other comparisons were significant ($p < 0.001$); Ser^{40}, Con *versus* AngII was not significant ($p > 0.05$), all other comparisons were significant ($p < 0.001$); TH activity, Con *versus* AngII was not significant ($p > 0.05$), all other comparisons were significant ($p < 0.001$). *B*, TH site. Cells treated with AngII and forskolin as described above were processed, and the Ser^{40} kinase assay was run as described under "Experimental Procedures," except that [γ-^{32}P]ATP was also included in the assay, and HPLC analysis of the radiolabeled tryptic peptides was performed as described under "Experimental Procedures." The elution profile is shown. In Ser^{40} kinase, cells were treated as described above and then processed, and the level of TH Ser^{40} kinase activity was determined as described under "Experimental Procedures." The results are presented as the percent of the mean of the control ± S.E. For each condition the number of individual experiments was four. Statistical analysis, Con *versus* AngII and Fors *versus* Fors+AngII were not significant ($p > 0.05$), all other comparisons were significant ($p < 0.001$).

AngII. The data in Fig. 1*B* show the results from a phosphopeptide analysis of TH under the conditions used for the Ser^{40} kinase assay after treatment with AngII and forskolin. The results show a single radioactive peak with an elution position similar to that obtained for the Ser(P)^{40} tryptic peptide in our system (33). This indicates that essentially only the Ser^{40} is phosphorylated. The very low stoichiometry of Ser^{19} and Ser^{31} under these conditions would mean that we would not expect any potentiation of exogenous TH Ser^{40} phosphorylation because of Ser^{19} or Ser^{31} phosphorylation in the Ser^{40} kinase assay and so would be measuring the true Ser^{40} kinase activity. The results in Fig. 1*B* show that treatment of cells with forskolin produced ~17-fold increase in Ser^{40} kinase activity in the cell extracts. When extracts of cells treated with AngII and forskolin were analyzed for Ser^{40} kinase activity, the activity was not significantly different from that in extracts of cells treated with forskolin alone ($p > 0.05$). Therefore the phosphorylation of Ser^{31} mediated by AngII must be potentiating the forskolin-induced increase in TH Ser^{40} phosphorylation.

Stoichiometry and Site Analysis of the Phosphorylation of rTH by ERK and PKA—The data shown above indicated that phosphorylation of Ser^{31} could increase the rate of phosphorylation of Ser^{40} and activation of TH in intact cells. To understand the mechanism of this we examined the effect of Ser^{31} phosphorylation on Ser^{40} phosphorylation and TH activation *in vitro*. In these experiments ERK was used to phosphorylate Ser^{31}. To phosphorylate Ser^{31} in rTH, recombinant ERK was expressed in *E. coli* and purified as described under "Experimental Procedures." The specificity of ERK phosphorylation of rTH was determined. rTH was maximally phosphorylated with ERK. The ERK-phosphorylated rTH was then subjected to tryptic digestion, and the resulting labeled phosphopeptides were analyzed using HPLC as described under "Experimental Procedures." The results in Fig. 2, *panel E* show a major peak eluting at ~20 min and three minor peaks. Taking into account the elution position of the Ser(P)^{40} peptide (PKA-phosphorylated rTH; see Fig. 2, *panel P*) the elution position of the major peak was consistent with that previously determined for the Ser(P)^{31} peptide from rat TH (11). Essentially identical results were obtained with the ERK phosphorylation of dopamine-bound rTH (Fig. 2 *panel DE*). Previous results have shown that ERK could also phosphorylate Ser^{8} in rTH but only at a low level (11). More recent results suggested that ERK could phosphorylate Ser^{8} at a rate ~9-fold less than Ser^{31} (34), so it was possible that under the conditions used to maximally phosphorylate Ser^{31} there may have been significant phosphorylation of Ser^{8}. The elution position of the smallest of the three minor peaks was consistent with that previously defined to be the elution position of the Ser(P)^{8} peptide in rTH (11). To confirm this, ERK was used to phosphorylate a S8A mutant of rTH. As can be seen in Fig. 2, *panel E* (S8A), the middle peak of the three minor peaks disappears in the S8A mutant, confirming that this peak is in fact the Ser(P)^{8} peptide. The very low level of Ser^{8} phosphorylation suggested that it was unlikely to have any significant impact on Ser^{40} phosphorylation and so wild-type rTH was used in all subsequent experiments. The nature of the two other minor peaks is unclear. Once rTH was maximally phosphorylated by ERK, the subsequent addition of PKA only resulted in phosphorylation of Ser^{40} in dopamine-free (Fig. 2 *panel EP*) or dopamine-bound (Fig. 2, *panel DEP*) TH.

Analysis of the Effect of Ser^{31} Phosphorylation on Ser^{40} Phosphorylation and TH Activity in rTH—We first determined whether the effect of Ser^{31} phosphorylation on TH in intact cells was because of Ser^{31} phosphorylation increasing the ability of PKA to phosphorylate Ser^{40} and activate dopamine-bound rTH. The results in Fig. 3*A* show that addition of dopamine inhibited rTH activity ~40-fold (*D*). Phosphorylation of Ser^{31} by ERK did not significantly activate dopamine-bound rTH (Fig. 3*A*, *DE*). The addition of very high levels of PKA was able to fully reactivate the dopamine-bound rTH (*DP150*). The addition of a lower con-

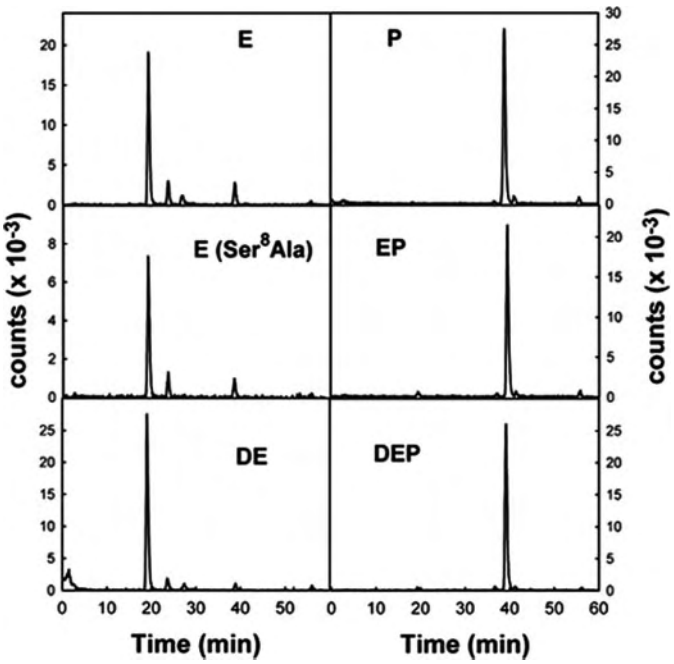

FIGURE 2. **Site analysis of ERK and PKA phosphorylation of dopamine-free rTH and dopamine-bound rTH.** rTH and Ser[8]Ala rTH were phosphorylated by ERK and/or PKA and subject to site analysis as described under "Experimental Procedures." The elution profiles of the ^{32}P-labeled peptides are shown. *E*, rTH (4 μM) was phosphorylated in a buffer containing [γ-^{32}P]ATP by ERK (0.8 μM) for 20 min at 30 °C. *E* (*Ser[8]Ala*), S8A rTH (2 μM) was phosphorylated in a buffer containing [γ-^{32}P]ATP by ERK (0.8 μM) for 20 min at 30 °C. *DE*, dopamine-bound rTH (4 μM) was phosphorylated in a buffer containing [γ-^{32}P]ATP by ERK (0.8 μM) for 20 min at 30 °C. *P*, rTH (4 μM) was phosphorylated in a buffer containing [γ-^{32}P]ATP by 5 units PKA for 10 min at 30 °C. *EP*, rTH (4 μM) was phosphorylated in a buffer containing unlabeled ATP by ERK (0.8 μM) for 20 min at 30 °C. [γ-^{32}P]ATP was then added to the reaction mix followed by 10 units PKA and the reaction incubated for a further 5 min. *DEP*, dopamine-bound rTH (4 μM) was phosphorylated in a buffer containing unlabeled ATP by ERK (0.8 μM) for 20 min at 30 °C. [γ-^{32}P]ATP was then added to the reaction mixture followed by 400 units of PKA, and the reaction mixture was incubated for a further 10 min.

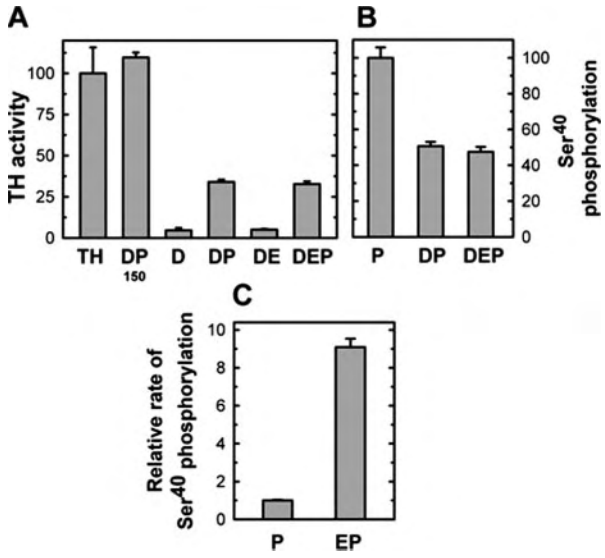

FIGURE 3. **Analysis of the effect of Ser^{31} phosphorylation on Ser^{40} phosphorylation and TH activity in rTH.** *A*, rTH activity was determined *in vitro* as described under "Experimental Procedures" after various treatments. *TH*, not treated; *DP150*, dopamine-bound rTH phosphorylated with 150 units of PKA for 20 min; *D*, dopamine-bound TH; *DP*, dopamine-bound rTH phosphorylated with 20 units of PKA for 20 min; *DE*, dopamine-bound rTH maximally phosphorylated with ERK; *DEP*, dopamine-bound rTH maximally phosphorylated with ERK and then phosphorylated with 20 units of PKA for 20 min. rTH activity is shown relative to untreated rTH, which was defined as 100%. For each condition the number of individual experiments was three. Statistical analysis, DEP was not significantly different from DP ($p > 0.05$). *B*, Ser^{40} phosphorylation of rTH was determined using Western blotting as described under "Experimental Procedures" after various treatments. *P*, dopamine-free rTH phosphorylated with 20 units of PKA for 20 min; *DP*, dopamine-bound rTH phosphorylated with 20 units of PKA for 20 min; *DEP*, dopamine-bound rTH maximally phosphorylated with ERK and then phosphorylated with 20 units of PKA for 20 min. Ser^{40} phosphorylation is shown the relative to dopamine-free rTH phosphorylated with PKA, which was defined as 100%. For each condition the number of individual experiments was six. Statistical analysis, DEP was not significantly different from DP ($p > 0.05$). *C*, dopamine-free rTH was incubated with ERK (*EP*) or without ERK (*P*) in the presence of unlabeled ATP for 25 min, [γ-^{32}P]ATP was then added, and the incorporation of radiolabel into rTH was measured as described under "Experimental Procedures" for 3.5 min after addition of PKA. Initial rates were determined by linear regression analysis. For each condition three rate experiments were performed. The results are presented relative to the mean rate of phosphorylation of rTH by PKA (*P*), which was assigned a value of one. Statistical analysis, EP was significantly different from P ($p < 0.001$).

centration of PKA was able to partially reactivate rTH (Fig. 3*A*, *DP*). Prior phosphorylation of Ser^{31} by ERK had no effect on the ability of this lower PKA concentration to reactivate rTH (*DEP* is not significantly different from *DP*). The effect of Ser^{31} phosphorylation on Ser^{40} phosphorylation in dopamine-bound TH was examined directly (Fig. 3*B*). Again, prior phosphorylation of Ser^{31} did not alter the PKA-mediated increase in the phosphorylation of Ser^{40} in dopamine-bound TH (*DEP* is not significantly different from *DP*). The results show that phosphorylation of Ser^{31} could not increase the rate of phosphorylation of Ser^{40} nor increase the activation of dopamine-bound rTH by PKA.

We therefore determined whether the potentiation of TH activation by Ser^{31} phosphorylation in intact cells could be because of Ser^{31} phosphorylation increasing the ability of PKA to phosphorylate Ser^{40} in dopamine-free TH. As there is only a very small effect on rTH activity by Ser^{40} phosphorylation in dopamine-free TH and as Ser^{31} phosphorylation produces only a small change in rTH activity, we examined Ser^{40} phosphorylation directly. The effect of Ser^{31} phosphorylation on the initial rate of Ser^{40} phosphorylation by PKA in dopamine-free rTH was determined, and the results are presented in Fig. 3*C*. The data show that the rate of Ser^{40} phosphorylation by PKA for Ser^{31} phosphorylated rTH (Fig. 3*C*, *EP*) is 9-fold greater than that for rTH not phosphorylated at Ser^{31} (*P*). In contrast, prior phosphorylation of Ser^{40} did not alter the rate of phosphorylation of Ser^{31} by ERK (not shown), indicating that the effect of the phosphorylation of Ser^{31} is hierarchical.

Stoichiometry and Site Analysis of the Phosphorylation of the Human TH Isoforms by ERK and CaMKII—The sequence comparison of the human TH isoforms is shown in Fig. 4*A*. It can be seen that the human TH isoforms differ in sequence only around the Ser^{31} site in hTH1. This suggested that the human TH isoforms may differ with respect to the effect of hierarchical phosphorylation via Ser^{31} (or its equivalent) phosphorylation. The four human isoforms were expressed and purified as described under "Experimental Procedures." The phosphorylation of the isoforms by ERK was examined. hTH1 was phosphorylated to a maximum stoichiometry of 0.5, whereas hTH3 and hTH4 could be phosphorylated to a maximum stoichiometry of 1.0, but there was very little phosphorylation of hTH2 by ERK (not shown). These results are consistent with that determined previously (12). The site analysis of hTH1 phosphorylated by ERK and CaMKII is shown in Fig. 4*B*. The relative positions of the peaks found for the Ser^{31}, Ser^{19}, and Ser^{40} tryptic phosphopeptides are consistent with those determined previously (12). When hTH1 was phosphorylated by ERK, there was also a small peak that eluted at 20 min. This peak was also found when hTH2 was phosphorylated by ERK, which would suggest that this peak is because of the phosphorylation of a protein in the ERK preparation rather than hTH1. hTH2 contains an additional four amino acids inserted N-terminal to Ser^{31} in hTH1. There is no information in the literature on the elution position of the hTH2 tryptic Ser^{35} phosphopeptide (GQS^{P}PR). The hTH2 tryptic Ser^{35} phosphopeptide was therefore synthesized, and as can be seen in Fig. 4*B* this peptide elutes very early under the conditions used. As expected, when hTH2 was phosphorylated by ERK the major

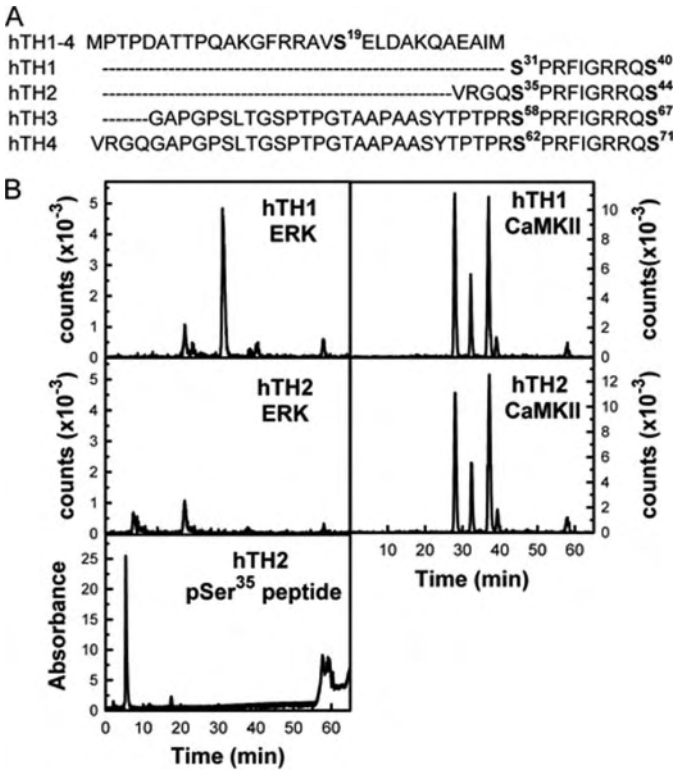

FIGURE 4. **Site analysis of hTH1 and hTH2 after phosphorylation by ERK or CaMKII.** *A*, sequence comparison of human TH isoforms. Sequences of the human TH isoforms are identical except for sequences inserted between amino acid 30 and 31 in hTH1. The sequence of hTH1 around that region is shown together with the inserted sequences in hTH2, hTH3, and hTH4. Figure is adapted from Ref. 1. *B*, hTH1 and hTH2 were phosphorylated by ERK or CaMKII in the presence of [γ-^{32}P]ATP and subject to site analysis as described under "Experimental Procedures." The elution profiles of the ^{32}P-labeled peptides are shown. hTH2 Ser(P)^{35} (*pSer^{35}*) peptide, the synthesized GQS^{P}PR peptide run on the same HPLC column.

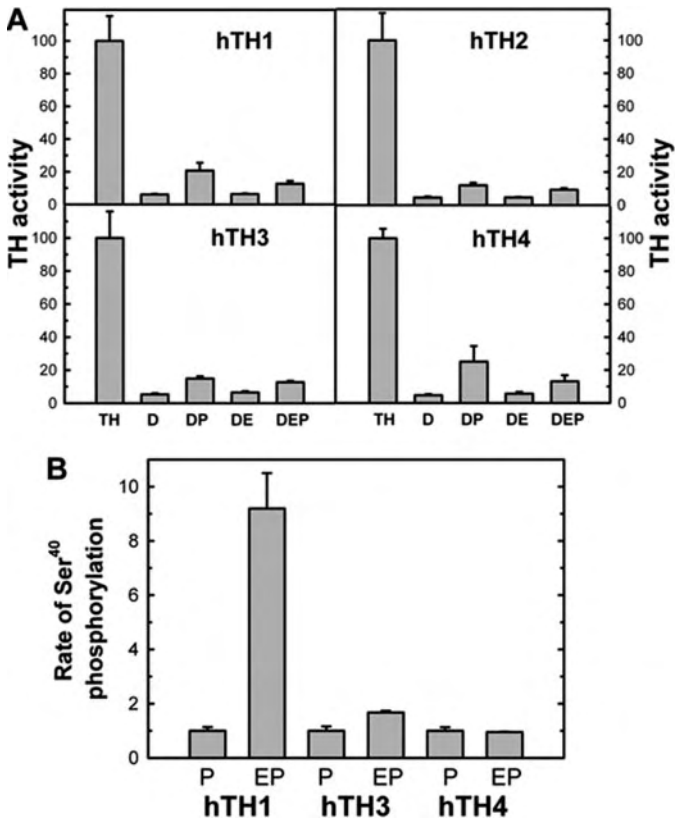

FIGURE 5. **Effect of ERK phosphorylation on the PKA-mediated increase in TH activity and Ser^{40} (or its equivalent) phosphorylation in the human TH isoforms.** *A*, TH activity of the four human isoforms in the presence of dopamine was determined *in vitro* after various treatments as described under "Experimental Procedures." The conditions and nomenclature are the same as in Fig. 3. TH activity is shown relative to untreated TH, which was defined as 100%. For each condition the number of individual experiments was three. Statistical analysis, for each isoform the value for DP was not significantly different from DEP ($p > 0.05$). *B*, the effect of ERK phosphorylation on the rate of PKA phosphorylation of the dopamine-free hTH1, hTH3, and hTH4 isoforms was determined as described under "Experimental Procedures." The conditions and nomenclature are as described in Fig. 3. The results are presented relative to P, which was assigned a value of one. For each condition the number of individual experiments was four. Statistical analysis, for hTH1 and hTH3 the value for P was significantly different from EP ($p < 0.01$), whereas for hTH4 the value of P was not significantly different from EP ($p > 0.05$).

peak found in hTH1 was no longer found (Fig. 4*B*, *hTH2 ERK*). Rather, in addition to the same small peak found in hTH1 that eluted at around 20 min, there was an additional minor peak that eluted very early. This early eluting peak was not found when hTH1 was phosphorylated by ERK, which suggests that it is derived from the phosphorylation of hTH2. This "peak" appeared to be two to three unresolved peaks. These peaks consistently eluted at least 2 min later than the synthesized hTH2 tryptic Ser^{35} phosphopeptide and so this suggests that ERK cannot phosphorylate hTH2 at Ser^{35} but may be phosphorylating another site(s) at very low levels. When hTH2 was phosphorylated by CaMKII (Fig. 4*B*, *hTH2 CaMKII*), the result was essentially the same as that for hTH1 phosphorylated by CaMKII (Fig. 4*B*, *hTH1 CaMKII*). This was surprising as it has been claimed that the Ser^{35} site in hTH2 can be phosphorylated by CaMKII (22). We could find no evidence for the phosphorylation of an hTH2 tryptic Ser^{35} phosphopeptide by CaMKII with mobility similar to that of the synthesized Ser^{35} phosphopeptide. This result is consistent with previous work where attempts to demonstrate the phosphorylation of hTH2 Ser^{35} in intact human neuroblastoma cells (prelabeled with $^{32}P_i$, treated with veratridine, forskolin, or phorbol ester, immunoprecipitated with isoform-specific antibodies, trypsinized, and analyzed by HPLC with radiochemical detection) indicated, rather, that hTH2 Ser^{35} was essentially not phosphorylated under any of the conditions.[3]

Effect of ERK Phosphorylation on the Human TH Isoforms—We examined the effect of ERK phosphorylation on the rate of activation of the dopamine-bound human TH isoforms by PKA. The data is shown in Fig. 5*A*. All four isoforms showed similar levels of inhibition by dopamine. Addition of low concentrations of PKA produced partial reactivation of each isoform (Fig. 5, *DP*). In all four isoforms, prior phosphorylation by ERK (Fig. 5, *DEP*) had no effect on the PKA-mediated activation as, in each case, DEP was not significantly different from DP.

We next investigated whether Ser^{31} phosphorylation could potentiate Ser^{40} phosphorylation in dopamine-free human TH isoforms. The results in Fig. 5*B* show that the effect seen in hTH1 was essentially the same as that for rTH in that Ser^{31} phosphorylation by ERK increased the rate of Ser^{40} phosphorylation by PKA ~9-fold. In contrast, phosphorylation of Ser^{58} in hTH3 by ERK increased the rate of Ser^{67} phosphorylation by PKA only ~1.7-fold. In the hTH4 isoform, Ser^{62} phosphorylation by ERK did not alter the rate of Ser^{71} phosphorylation by PKA. The Ser^{35} residue in hTH2 could not be significantly phosphorylated by ERK or CaMKII and so for this isoform of TH the equivalent potentiation could not occur.

Determination of the Role of Ser^{19} Phosphorylation in the Phosphorylation of Ser^{40} in hTH1 and Ser^{44} in hTH2—When the phosphorylation of hTH1 and hTH2 was examined by site analysis we con-

[3] J. W. Haycock, unpublished observations.

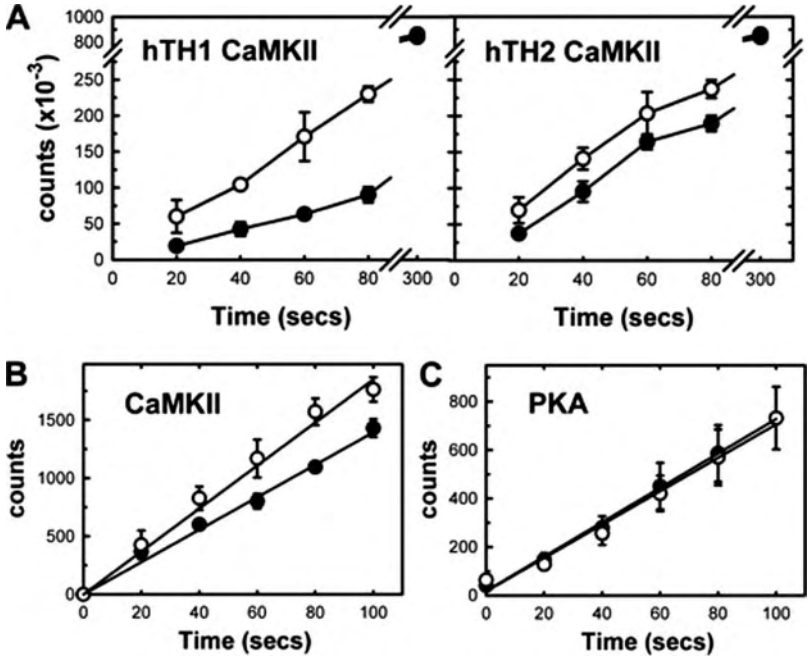

FIGURE 6. **Effect of Ser^{19} phosphorylation on the phosphorylation of Ser^{40} in hTH1 and the phosphorylation of Ser^{44} in hTH2.** *A*, hTH1 and hTH2 were phosphorylated by CaMKII in the presence of [γ-^{32}P]ATP for the times shown up to 80 s. 6-Fold more CaMKII was then added, and the sample was incubated for a further 3.5 min to obtain maximal phosphorylation of each TH isoform. The incorporation of radioactivity into each site was measured as described under "Experimental Procedures." The data is combined from three independent experiments. *Open circles*, Ser^{19}; *closed circles*, Ser^{40} (hTH1) or Ser^{44}(hTH2). *B*, S19A hTH1 and S19A hTH2 were phosphorylated by CaMKII in the presence of [γ-^{32}P]ATP and the incorporation of radioactivity into TH measured as described under "Experimental Procedures." The data are combined from three independent experiments. *Closed circles*, hTH1; *open circles*, hTH2. *C*, hTH1 and hTH2 were phosphorylated by PKA in the presence of [γ-^{32}P]ATP and the incorporation of radioactivity into TH measured as described under "Experimental Procedures." The data are combined from three independent experiments. *Closed circles*, hTH1; *open circles*, hTH2.

sistently found that when hTH1 and hTH2 were phosphorylated by CaMKII, the ratio of Ser^{44} to Ser^{19} phosphorylation in hTH2 was higher than the ratio of Ser^{40} to Ser^{19} phosphorylation in hTH1 (see Fig. 4*B*). This raised the possibility that the 4-amino-acid insert in hTH2 was either directly altering the rate of phosphorylation of Ser^{44} or altering the effect of Ser^{19} phosphorylation on the rate of Ser^{44} phosphorylation. We therefore examined the rate of phosphorylation of Ser^{19}, Ser^{40}, and Ser^{44} in the two isoforms by CaMKII. This was done by quantitating the radioactivity incorporated into Ser^{19}, Ser^{40}, and Ser^{44} by site-analysis as described in methods. The results in Fig. 6*A* show that when hTH1 and hTH2 are maximally phosphorylated by addition of high levels of CaMKII and incubation for 300 s, the incorporation of radioactivity into Ser^{19}, Ser^{40}, and Ser^{44} was, as expected, the same. As the amount of radioactivity incorporated into hTH1 and hTH2 was the same, the data were directly comparable. The results show that the rate of phosphorylation of Ser^{19} was the same in hTH1 and hTH2. In contrast the rate of phosphorylation of Ser^{44} in hTH2 was increased by 150% when compared with the rate of phosphorylation of Ser^{40} in hTH1. To examine whether this was because of the effect of Ser^{19} phosphorylation or because of the 4-amino-acid insert in hTH2 directly effecting CaMKII phosphorylation of Ser^{44}, we prepared mutants of hTH1 and hTH2 where Ser^{19} was converted to alanine so Ser^{19} could not be phosphorylated. We compared the rates of phosphorylation by CaMKII of Ser^{40} in S19A hTH1 with that of Ser^{44} in S19A hTH2. The results in Fig. 6*B* show that CaMKII could phosphorylate Ser^{44} in hTH2 faster than Ser^{40} in hTH1 in the absence of Ser^{19} phosphorylation, but the effect was small, with there being only a 30% increase. This direct effect (30% increase) can only contribute in a minor way to the effect seen in the presence of Ser^{19} phosphorylation (150% increase). We therefore believe that it is the phosphorylation of Ser^{19} that is primarily responsible for the increased rate of Ser^{44} phosphorylation in hTH2. The rate of phosphorylation of Ser^{40} in hTH1 by PKA (which does not phosphorylate Ser^{19}) was the same as the rate of phosphorylation of Ser^{44} in hTH2 by PKA (Fig. 6*C*). Thus the presence of the 4-amino-acid insert in hTH2 does not affect the rate at which PKA phosphorylates Ser^{44} compared with the rate at which PKA phosphorylates Ser^{40} in hTH1.

DISCUSSION

Previously we have shown that phosphorylation of Ser^{19} in TH can potentiate the phosphorylation of Ser^{40} *in vitro* (24) and potentiate the phosphorylation of Ser^{40} and lead to TH activation *in situ* (25). The data reported here extends this work by showing that the phosphorylation of Ser^{31} can also increase the rate of Ser^{40} phosphorylation and TH activation both *in vitro* and *in situ*. The phosphorylation of Ser^{40} had no effect on the rate of phosphorylation of either Ser^{19} or Ser^{31}. Therefore the effect of phosphorylation of Ser^{19} and Ser^{31} is hierarchical in nature. In light of the very modest effect that phosphorylation of Ser^{19} or Ser^{31} have on TH activity we would suggest that the main role of the phosphorylation of these sites is to potentiate the rate of phosphorylation of Ser^{40}.

Phosphorylation of Ser^{31} increased the rate of phosphorylation of Ser^{40} in dopamine-free TH but did not have any effect on the PKA activation of dopamine-bound TH. This is the same as the situation with Ser^{19} where Ser^{19} phosphorylation increased the rate of Ser^{40} phosphorylation in dopamine-free TH (24) but had no effect on the PKA activation of dopamine-bound TH.[4] The phosphorylation of Ser^{40} in dopamine-free TH has very little effect on TH activity (5) and so the question arises as to how Ser^{31} (or Ser^{19}) phosphorylation produces the potentiation of TH activation that we have found in intact cells. In response to a stimulus Ser^{40} phosphorylation in TH increases in the first 2–3 min. Clearly, phosphorylation of Ser^{19} or Ser^{31} does not effect the initial activation of catecholamine-bound TH. If there is continued stimulation the level of Ser^{40}, phosphorylation remains relatively stable (35). This means that after the initial activation phase there is an equilibrium phase where the rate of phosphorylation of Ser^{40} must equal the rate of dephosphorylation of Ser^{40} in order that the level of phosphorylation of Ser^{40} in the cell remains constant. This equilibrium phase is shown diagrammatically in Fig. 7. In the absence of Ser^{31} phosphorylation, the level of Ser^{40} phosphorylation will reach a stable point when the rate of phosphorylation equals the rate of dephosphorylation (Fig. 7, *dotted line*). Phosphorylation of Ser^{31} will increase the rate of phosphorylation of

[4] J. Daniel, P. Dunkley, and P. Dickson, unpublished observations.

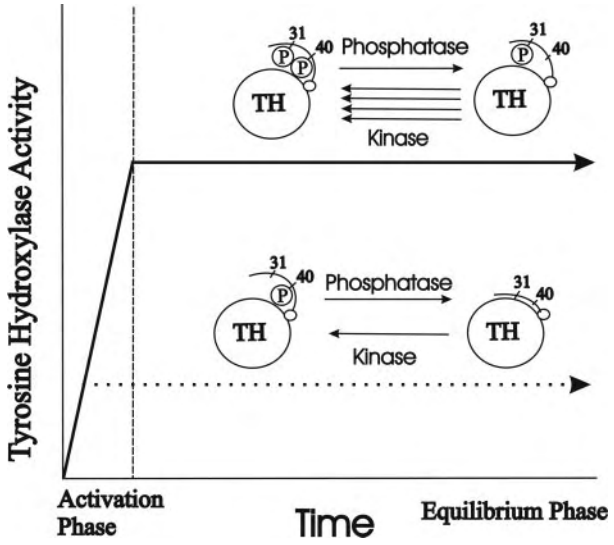

FIGURE 7. **Model for the effect of Ser^{31} phosphorylation in increasing Ser^{40} phosphorylation and TH activity in intact cells.**

Ser^{40} in catecholamine-free TH. This will have the effect of altering the equilibrium to favor the phosphorylated form and so the level of Ser^{40} phosphorylation will increase. Ultimately a new equilibrium will be established but it will be at higher level of Ser^{40} phosphorylation than in the absence of Ser^{31} phosphorylation (Fig. 7, *solid line*). Therefore we believe that the role of Ser^{31} (and Ser^{19}) phosphorylation in catecholamine-free TH is to increase the rate of rephosphorylation of Ser^{40} in TH after it has been dephosphorylated (but before dopamine is rebound). This will have the effect of increasing Ser^{40} phosphorylation in intact cells and, in particular, reducing the possibility of the catecholamine binding to the dephosphorylated TH and reinhibiting TH.

This work also provides a major insight into the way the human TH isoforms are regulated. Previous work has failed to identify any significant differences in the activity or regulation of the four human TH isoforms (12, 21–23). The fact that the only sequence difference among the four isoforms is around Ser^{31} in hTH1 suggests that the effect of Ser^{31} (or its equivalent) phosphorylation in increasing Ser^{40} (or its equivalent) phosphorylation may be different in different isoforms. This was in fact the case with the strong potentiation via Ser^{31} phosphorylation being only found in the hTH1 isoform. The other major human TH isoform hTH2 showed stronger potentiation via Ser^{19} phosphorylation than hTH1. How would these differences between the two major TH isoforms impact on the regulation of TH in cells? When cells are stimulated either via depolarization or receptor-mediated mechanisms the level of phosphorylation of Ser^{19} increases rapidly and thereafter starts to decrease (3). In contrast, the level of Ser^{31} phosphorylation only increases after extended stimulation of the cell (3). This means that in response to short term stimuli, hTH2 will be activated to a greater extent than hTH1 because of the stronger potentiation via Ser^{19} phosphorylation. When cells are stimulated for a longer period of time, the ERK pathway is activated. Activation of the ERK pathway will strongly potentiate the activation of hTH1 but will have no effect on hTH2. Therefore hTH1 and hTH2 can be differentially activated depending on the length of the stimulus involved.

Hierarchical phosphorylation via Ser^{31} may also provide a mechanism by which hTH1, or the TH in other mammalian species that are homologous to hTH1, may be differentially activated in different tissues. We have shown here that in unstimulated adrenal chromaffin cells the stoichiometry of Ser^{31} phosphorylation is very low with only 4% of TH phosphorylated at Ser^{31}. This is similar to the results from unstimulated adrenal-derived PC12 cells where only 7% of TH molecules are phosphorylated at Ser^{31} (36). In contrast, the basal level of Ser^{31} phosphorylation was much higher in the brain with as much as 32% of TH phosphorylated at Ser^{31} in the striatum (36). This would mean that in response to short term stimuli the high stoichiometry of Ser^{31} phosphorylation the striatum would lead to strong potentiation of hTH1 activation, whereas this would not occur in adrenal cells where the stoichiometry of Ser^{31} phosphorylation is very low. For many years it was thought that ERK was the only kinase that could phosphorylate Ser^{31} in TH. The recent discovery that Ser^{31} can also be phosphorylated by CDK5 (14, 37) means that this potentiation of activation of hTH1 can be modulated by at least two different pathways and so could provide the basis of quite complex regulation of hTH1.

In summary we have shown the Ser^{31} phosphorylation can strongly potentiate Ser^{40} phosphorylation and TH activation. This has also provided for the first time a mechanism by which the different human TH isoforms are differentially regulated.

Acknowledgments—We thank Melissa Musicka for technical assistance. We thank Melanie Cobb for the kind gift of the ERK2 expression plasmid NPT7-5 His-ERK2-R4F and Jaques Mallet for the kind gift of the hTH1 and hTH3 expression plasmids. We also thank Phil Marley and Steve Bunn for reading the manuscript.

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The Low Affinity Dopamine Binding Site on Tyrosine Hydroxylase: The Role of the N-Terminus and In Situ Regulation of Enzyme Activity

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Neurochemical Research, Vol. 34, Number 10 / October, 2009, p. 1830-1837.

Abstract Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis, is inhibited in vitro by catecholamines binding to two distinct sites on the enzyme. The N-terminal regulatory domain of TH contributes to dopamine binding to the high affinity site of the enzyme. We prepared an N-terminal deletion mutant of TH to examine the role of the N-terminal domain in dopamine binding to the low affinity site. Deletion of the N-terminus of TH removes the high affinity dopamine binding site, but does not affect dopamine binding to the low affinity site. The role of the low affinity site in situ was examined by incubating PC12 cells with L-DOPA to increase the cytosolic catecholamine concentration. This resulted in an inhibition of TH activity in situ under both basal conditions and conditions that promoted the phosphorylation of Ser40. Therefore the low affinity site is active in situ regardless of the phosphorylation status of Ser40.

doi: 10.1007/s11064-009-9989-5