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Tyrosine Hydroxylase Phosphorylation In Vivo

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Abbreviations used: AADC, aromatic amino acid decarboxylase; BH_4, tetrahydrobiopterin; BACCs, bovine adrenal chromaffin cells; CaMPKII, calcium and calmodulin stimulated protein kinase II; CDK, cyclin dependent kinase; CPU, caudate putamen; DA, dopamine; DAT, dopamine transporter; 2DG, 2 deoxyglucose; ERK, extracellular signal regulated protein kinase; GDNF, glial cell derived neurotrophic factor; hTH, human tyrosine hydroxylase; KO, knockout; LC, locus coeruleus; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MPFC, medial prefrontal cortex; Mn,

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manganese; MSK, mitogen stimulated protein kinase; NA, noradrenaline; NAC, nucleus acumbens; NSD 1015, *m*-hydroxybenzylhydrazine; NTS, nucleus tractus solitarius; PAGE, polyacrylamide gel electrophoresis; PACAP, pituitary adenylate cyclase activating polypeptide; PK, protein kinase; PP, protein phosphatase; PRAK, p38 regulated and activated protein kinase; PVN, paraventricular nucleus; SDS, sodium dodecyl sulphate; Ser, serine; SN, substantia nigra; TH, tyrosine hydroxylase; VMAT, vesicle monoamine transporter; VTA, ventral tegmental area; WT, wild type.

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Abstract

Tyrosine Hydroxylase (TH) is the rate limiting enzyme in the synthesis of the catecholamines dopamine, noradrenaline and adrenaline. One of the major mechanisms for controlling the activity of TH is protein phosphorylation. TH is phosphorylated at serine (Ser) residues Ser8, Ser19, Ser31 and Ser40. There have been a number of previous reviews focused on TH phosphorylation *in vitro* and *in situ*. This review on TH phosphorylation *in vivo* has three main sections focusing on: (1) the methods used to investigate TH phosphorylation *in vivo*; including the animals used, the sacrifice procedures, the tissue preparation, the measurement of TH protein levels and TH phosphorylation and the measurement of TH activation. (2) the regulation and consequences of TH phosphorylation *in vivo*; including the kinases and phosphatases acting on TH, the stoichiometry of TH phosphorylation, the proteins that bind TH and TH subcellular location. (3) the acute and prolonged changes in TH phosphorylation in specific catecholaminergic tissues; including the adrenal medulla, the nigrostriatal pathway and the mesolimbic pathway.

Introduction

Tyrosine Hydroxylase (TH) is the rate limiting enzyme in the synthesis of the catecholamines dopamine (DA), noradrenaline (NA) and adrenaline. TH activity is controlled by multiple mechanisms, is critical for normal physiology and is implicated in certain neuropathological conditions. A number of reviews on TH have been published (Kumer & Vrana 1996; Fitzpatrick 1999; Flatmark 2000; Pardridge 2005; Benavides-Piccione & DeFelipe 2007; Nakashima *et al.* 2009; Sumi-Ichinose *et al.* 2010; Lenartowski & Goc 2011; Bademci *et al.* 2012; Di Giovanni *et al.* 2012; Khan *et al.* 2012; Tabrez *et al.* 2012; White & Thomas 2012; Nagatsu *et al.* 2018).

One of the major mechanisms for controlling the activity of TH is protein phosphorylation. TH is phosphorylated at serine (Ser) residues Ser8, Ser19, Ser31 and Ser40 and at threonine8 in human TH (Grima *et al.* 1987). Ser/Thr8 will not be discussed in this review, as it is not known to be involved in modulating TH activity and it has not been investigated *in vivo*. The effect of TH phosphorylation at Ser19, Ser31 and Ser40 has been investigated using purified TH *in vitro*, using cell cultures and tissue slices *in situ*, and after obtaining tissue from animals treated *in vivo* with a range of physiological and pathological stressors. An extensive review of the regulation of TH phosphorylation was undertaken well over a decade ago (Dunkley *et al.* 2004). This review focused mainly on *in vitro* and *in situ* studies. Since then some excellent reviews have been published that also focus largely on *in vitro* studies of TH phosphorylation and its consequences (Fujisawa & Okuno 2005; Daubner *et al.* 2011). A comprehensive review on TH phosphorylation *in vivo* in animal models of Parkinson's has recently been published (Johnson *et al.* 2018).

The first purpose of this review is to overview the methods used to investigate TH phosphorylation *in vivo*. The second purpose is to address a series of specific questions related to the regulation and consequences of TH phosphorylation *in vivo*. In order to address these questions it is necessary to selectively update relevant *in vitro* and *in situ* findings. It is assumed that readers have read our earlier review (Dunkley *et al.* 2004) and so the main focus will be on publications since then. The third purpose of the review is to overview the TH phosphorylation responses in specific peripheral and central catecholaminergic tissues. The focus of this section will be on the timing of the TH phosphorylation responses. Acute responses are primarily physiological. Prolonged responses are related to development, responses to drugs or pathology, and ageing. A summary of the major issues will be provided at the end of each section of the review.

Methods used to Investigate TH Phosphorylation and Activation *In Vivo*

A large number of methods are used to investigate TH *in vivo*. The critical variables are the animals used, the procedures used to sacrifice the animals and prepare the tissues, and the procedures used to measure TH protein levels, TH phosphorylation and TH activation. Differences in these methods makes direct comparisons between many *in vivo* studies difficult.

Animals used

Almost all of the studies reported in this review used either male rats or mice. The basal stoichiometry of TH phosphorylation in the brain is very comparable for both species (Salvatore *et al.* 2000), suggesting their overall similarity. However, the genetic background of the animal influences TH. TH protein and the responses to drugs differed between Lewis and Fischer rats in the mesolimbic system (Beitner-Johnson *et al.* 1991; Ortiz *et al.* 1995). There are also differences between Sprague Dawley and Wistar rats in the locus coeruleus (LC) and ventral tegmental area (VTA) (Rosin *et al.* 1995). The age of the animal influences TH. Aged rats show decreased TH protein levels and reduced Ser31 stoichiometry (Salvatore *et al.* 2009b). There is also decreased TH activation and TH phosphorylation responses to 6-hydroxydopamine treatment in aged rats when compared to younger rats (Unnerstall & Ladner 1994).

Sacrifice procedures

When an animal is sacrificed, cells are immediately deprived of oxygen, ATP becomes limited and protein kinases no longer function. In contrast, protein phosphatases and proteolytic enzymes remain active. It is therefore essential to undertake the procedures that cool the tissues as quickly as possible to minimize dephosphorylation and proteolysis. The most rapid methods for sacrificing an animal use focussed microwave irradiation, direct immersion in liquid nitrogen, or cervical dislocation. These procedures are currently unacceptable to many animal ethics committees, but microwave irradiation is clearly superior for preserving the *in vivo* protein phosphorylation status (O'Callaghan & Sriram 2004). Decapitation (Lindgren *et al.* 2001; Sominsky *et al.* 2013) following mild anaesthesia to render the animal unresponsive (Ong *et al.* 2014; Salvatore 2014), or decapitation in conjunction with the use of a Decapicone (Dunkley and Robinson, 2018), are currently used procedures. Anaesthetic overdose is also used, but this takes many minutes to cause death. This can have major effects on TH phosphorylation (Springell *et al.* 2005; Snyder *et al.* 2007), especially when compared to decapitation (Dunkley, unpublished data). However, anaesthetics are required for many *in vivo* studies, where manipulations such as surgical procedures or perfusion are required.

Tissue preparation

The method used to collect the tissue can contribute to the post-mortem changes in TH phosphorylation. For studies on brain tissue, the brain is first removed by dissection of the skull. This takes a variable amount of time depending on exactly how carefully this is undertaken and on the brain region being collected. Brains are always cooled immediately after collection by washing in ice cold isotonic buffer. Specific brain nuclei are collected by one of two main techniques. The first uses non frozen tissue sliced coronally in a brain matrix and dissection of specific nuclei is then undertaken freehand, or with a punch (Salvatore 2014; Salvatore *et al.* 2012b; Ong *et al.* 2014). This method is relatively simple and provides entire brain nuclei in a single punch, but may include more contaminating nuclei. The second uses frozen brains sliced with a freezing microtome and specific nuclei are collected with a punch (Ong *et al.* 2011a). This method requires more than one slice to collect a complete brain nucleus, but has less contaminating nuclei. Collection of the whole adrenal is relatively quick and easy, but dissection of the medulla without cortical tissue takes significantly more time (Ong *et al.* 2014). At this point tissue is generally frozen at -80^{o}C until it can be processed.

Homogenisation of frozen tissue in ice cold sodium dodecyl sulphate (SDS) and boiling prior to polyacrylamide gel electrophoresis (PAGE), ensures that minimum further disruption of TH phosphorylation or proteolysis occurs and it allows analysis of all of the TH in the tissue (Ong *et al.* 2014). However, TH activity is destroyed and catecholamine metabolite levels are not able to be determined (Ong *et al.* 2014). Homogenisation in acid solubilises catecholamines and their metabolites without oxidation. All of the TH protein can be recovered from the pellet using SDS (Salvatore *et al.* 2012b). In this case, the acid destroys TH activity. Tissues can be lysed using a hypotonic solution, but addition of phosphatase and protease inhibitors is essential to preserve TH integrity (Ong *et al.* 2014). TH is a largely soluble protein and generally a supernatant fraction is used (Ong *et al.* 2014). However, there is always TH retained in the pellet leading to a loss of some protein. This is generally less than 10% of total but it may be a functionally unique fraction of TH (see What Proteins Bind to TH and Where is TH Localised?).

TH protein and TH phosphorylation by western blotting

TH protein levels and TH phosphorylation are generally assessed by western blotting. We (Cammarota *et al.* 2003; Gordon *et al.* 2009a) and others (Salvatore *et al.* 2012b) produce in-house phospho-specific antibodies against individual phosphorylation sites on TH, using peptide sequences obtained from earlier studies (Xu *et al.* 1998). Many antibodies are also available from commercial sources, but these vary widely in their sensitivity and specificity (Dunkley and Dickson, unpublished data). Antibodies directed at the N-terminal region of TH should not be used to measure TH protein

levels. This is where phosphorylation occurs and the phosphorylation interfering with antibody binding could give inappropriately low results. We use antibodies directed against the middle and C-terminal portions of TH. TH protein levels must be measured relative to a protein that is unchanged by the stimulus and we generally use beta-actin.

The results for TH phosphorylation at specific sites are typically the optical density values from the scans of the western blots. The basal level of TH phosphorylation is calculated in arbitrary units, with the mean set at 100%. The mean level of TH phosphorylation in the manipulated samples is expressed as a percentage of the mean basal level. Other studies set the mean basal level at 1 and then express the mean level for the manipulated sample as a fold change. Therefore, a 100% value, or a 1-fold change, for a manipulated sample means no change from the basal; while a 200% value for a manipulated sample is equivalent to a 2-fold change from the basal. Alternatively, the optical density values for TH phosphorylation in an individual sample is divided by the optical density value for TH protein levels for that sample. The mean for the basal samples is then set at either 100%, or 1-fold, before comparison with the mean for the manipulated sample. This accounts for any animal variation in TH protein levels, or changes in TH protein levels that may have occurred in response to the manipulation. In a few studies, the stoichiometry of TH phosphorylation at individual phosphorylation sites is determined (see What is the Stoichiometry of TH Phosphorylation?). TH phosphorylation and TH protein levels are determined for each individual sample, relative to standard curves for TH phosphorylated at Ser19, Ser31, Ser40, or for TH protein. Mean TH phosphorylation under basal conditions is then expressed as the number of moles of TH phosphorylated at a particular site relative to the number of moles of TH protein monomer. A value of 0.05, or 5%, means that 5 mols of TH is phosphorylated at a particular phosphorylation site for every 100 mols of TH protein monomer. The mean for the manipulated samples can then be expressed as a %, or as a fold change, relative to the mean basal value.

TH activation measurements

A number of techniques are used for measuring TH activation *in vivo*. These are TH activity, L-DOPA synthesis, or DA content. TH is the rate-limiting enzyme in the catecholamine synthesis pathway and measurements of L-DOPA and DA synthesis can be used to measure TH activation. The techniques will be specified throughout the review, because it is possible for one measure of TH activation to be altered while another remains unchanged (Kobori *et al.* 2004).

TH activity is measured *in vitro* using the tritiated water release assay (Ong *et al.* 2014; Reinhard *et al.* 1986). This technique has the advantage that it uses optimal conditions for measuring the enzyme activity *in vitro,* including optimal substrate and cofactor concentrations. The results are

not influenced by the *in vivo* concentrations of TH substrate or cofactors, or the activity of other enzymes and catecholamine transport proteins. However, the assay requires that during the preparation of sample from the starting tissue there is no loss of TH protein, inactivation of TH, proteolysis or dephosphorylation. The effects of TH phosphorylation are seen especially for Ser40, as this site directly alters the amount of catecholamine binding and has the largest effect on TH activity (Dunkley *et al.* 2004). There are much smaller effects of Ser31 phosphorylation, and no direct effects of Ser19 phosphorylation.

Measurement of **L-DOPA synthesis** is undertaken *in situ* and *in vivo* in the presence of an inhibitor of aromatic amino acid decarboxylase (AADC), such as *m*-hydroxybenzylhydrazine (NSD-1015). This results in the accumulation of L-DOPA. This technique is used *in situ* with cell cultures, or after isolation of tissue slices. The conditions within the intact cells for TH substrate and cofactor typically reflect the *in vivo* situation (Lindgren *et al.* 2000). The use of the inhibitor is unlikely to be optimal for measurement of TH activity, as build-up of product during the assay decreases TH activity. In spite of that, L-DOPA synthesis is likely to provide a good reflection of relative TH activation. When the measurement of L-DOPA synthesis is undertaken *in vivo* (Salvatore & Pruett 2012) it is necessary for the inhibitor to be completely effective at the concentration used in the tissue region being analysed. The inhibitor should not affect other neuronal systems which themselves have the capacity to modulate TH activity in the catecholaminergic neurons of interest.

TH activation is implied by measuring **DA content**. An excellent account of the methods involved in measuring DA content is available (Salvatore *et al.* 2012b). The use of DA content as a measure of TH activation requires that there is adequate TH substrate and tetrahydrobiopterin cofactor (BH_4) available. It is also required that the experimental manipulations do not alter the activities of aromatic amino acid decarboxylase, DA beta hydroxylase, catecholamine-O-methyl transferase and monoamine oxidase, and that there are no changes to the DA transporter (DAT) and the vesicle monoamine transporter (VMAT). When calculating DA content it is important to determine the recovery of DA accurately and details of how this is done are provided (Salvatore *et al.* 2012b). NA and adrenaline are the major products of TH activity in other brain nuclei and in the adrenal medulla and these catecholamines can also be measured instead of DA. In these cases, the activity of DA beta hydroxylase and phenylethanolamine N-methyltransferase can influence the levels of NA and adrenaline respectively.

One further procedure for measuring TH activation is mentioned here as it is used in a number of *in situ* studies included in the review, but it cannot be adapted for *in vivo* studies. **CO_2 release** requires that there be adequate TH substrate and BH_4 available, that AADC activity be unaltered by the condition(s) of the experiment and that TH and AADC activity be retained during tissue preparation (Meligeni *et al.* 1982; Cheah *et al.* 1999; Salvatore *et al.* 2001).

Whatever the method used to measure TH activation there are two ways of expressing the results. The first is to measure total TH activation; where TH activity, L-DOPA synthesis, or DA content, is expressed relative to total tissue protein. The second is to measure relative TH activation by accounting for changes in the level of TH protein in the tissue sample. It is important to delineate how TH activition is expressed, as TH protein levels varies between animals and can be altered independent of TH phosphorylation by many *in vivo* treatments. We therefore recommend expressing TH activation relative to TH protein content.

Summary of Methods

Two protocols are recommended for analysis of TH phosphorylation and TH activation (Ong et al. 2014; Salvatore 2014), as both use very similar procedures except for their measure of TH activation.

1. *Animals: Adult (3-6 month old) male Sprague Dawley rats that have been habituated to their cages and handling.*
2. *Sacrifice: Mild anaesthesia to render the animals unresponsive followed by decapitation.*
3. *Tissue Preparation: Rapid tissue cooling, isolation of specific catecholaminergic cells from fresh tissue using a brain matrix and punch, followed by freezing the tissue for storage. Tissue homogenisation in hypotonic buffer, or acid, followed by SDS solubilisation of proteins.*
4. *TH protein levels and TH phosphorylation: PAGE for protein fractionation followed by TH protein and TH phosphorylation analysis using western blotting with highly specific antibodies.*
5. *TH activation: Ong et al. uses hypotonic homogenisation of tissue and the supernatant collected is used immediately to measure TH activity under optimal conditions for the enzyme. This procedure favours observing changes in TH activation as a result of TH phosphorylation at Ser40, as this produces much larger changes than equimolar changes in TH phosphorylation at Ser31, while changes at TH phosphorylation at Ser19 do not lead to increased TH activity. Salvatore et al. uses acidic homogenisation of tissue and the supernatant is used for measurement of DA content to reflect TH activation. However, many other mechanisms also contribute to DA content, such as DA–beta-hydroxylase activity. If activation of a particular protein kinase induces one or more of these other mechanisms then the phosphorylation of the TH site altered by this kinase may correlate with DA content, even though TH itself may not have been responsible for all of the change.*
6. *Results: TH phosphorylation and TH activition are best expressed relative to TH protein*

levels to minimize animal variation. The effect of the specific animal manipulation on TH protein levels should also be determined.

Regulation of TH Phosphorylation and its Consequences In Vivo

Most of the studies undertaken on TH phosphorylation *in vivo* are not aimed at investigating the regulation of TH per se. Rather, they focused on the physiological responses to manipulations of catecholaminergic cell groups. In these studies, TH phosphorylation is primarily used as an index of cell activation. However, some studies included information on either the regulation of TH phosphorylation, or on the consequences of TH phosphorylation; such as TH activation, TH binding to specific proteins, or changes in TH localisation. This section of the review will focus on a series of specific questions about TH phosphorylation and its consequences *in vivo*. The data used was gathered from a number of studies using a range of different tissues obtained from both rats and mice. Relevant *in vitro* and *in situ* studies will be included in order to assist with interpretation of the *in vivo* data.

Two modes of TH regulation that have been extensively studied *in vitro* and *in situ* since 2004 are not discussed, as there are few relevant *in vivo* studies. The first is hierarchical phosphorylation of TH (Bevilaqua *et al.* 2001; Bobrovskaya *et al.* 2004; Lehmann *et al.* 2006; Gordon *et al.* 2009a; Toska *et al.* 2002; Royo & Colette Daubner 2006; Kansy *et al.* 2004; Salvatore & Pruett 2012). The second is TH stability and turnover (Toska *et al.* 2002; Royo *et al.* 2005; Moy & Tsai 2004; Obsilova *et al.* 2008; Doskeland & Flatmark 2002; Nakashima *et al.* 2011; Nakashima *et al.* 2016; Nakashima *et al.* 2018; Posser *et al.* 2009; Franco *et al.* 2010; Congo Carbajosa *et al.* 2015; Kawahata *et al.* 2015).

Which Protein Kinases Act on TH?

When investigating the adrenal gland it is possible to isolate only the medulla and therefore ensure that the primary source of protein kinases are those cells that also contain TH. However, this is not the case for any sympathetic or brain tissues. This is because TH containing cells are always surrounded by many non catecholaminergic neurons and glial cells that do not contain TH, but do contain the same protein kinases.

Ser40

In vitro and in situ

TH is phosphorylated at Ser40 by eight different protein kinases *in vitro,* but only three of these, protein kinase (PK) A, PKC and PKG, are confirmed *in situ* (Dunkley *et al.* 2004). Subsequent studies used a range of stimuli to increase TH phosphorylation at Ser40. The Ser40 kinase(s) activated *in situ* included PKA and calcium/calmodulin stimulated protein kinase II (CaMPKII) (Kumar *et al.* 2003), PKA (Fukuda *et al.* 2007; Bobrovskaya *et al.* 2007a), or PKC (Bobrovskaya *et al.* 2007b; Gelain *et al.* 2007). In each study TH activity is increased and this correlated with increased TH phosphorylation at Ser40, but not with TH protein levels, or TH phosphorylation at Ser19 or Ser31.

In some studies there was cross-talk between the Ser40-specific kinase PKA and extracellular signal regulated protein kinase (ERK), one of the protein kinases responsible for Ser31 phosphorylation (see Ser31 below). In PC12 cells (Salvatore *et al.* 2001) and striatal slices (Lindgren *et al.* 2002) PD98059, an inhibitor of ERK phosphorylation, altered TH phosphorylation at Ser40. These effects are not due to a direct effect of PD 98059 on PKA, as this inhibitor did not block the forskolin induced increases in Ser40 that are dependent on PKA. Urocortin2 increased TH phosphorylation at Ser40 in PC12 cells, directly via a PKA pathway and indirectly via a PKA-stimulated ERK pathway. The later pathway is blocked by UO126, another inhibitor of ERK phosphorylation (Nemoto *et al.* 2005). It is likely that the increase in Ser40 phosphorylation is due to ERK-stimulated activation of mitogen activated protein kinase activated protein kinase I (MAPKAPK1) and/or mitogen stimulated protein kinase (MSK1). Both of these kinases can phosphorylate TH at Ser40 *in vitro* (Toska *et al.* 2002; Dunkley *et al.* 2004). These results suggest that ERK stimulated kinases can indirectly lead to phosphorylation of TH at Ser40 *in situ*.

Most studies on TH phosphorylation at Ser40 *in situ* investigated acute changes over the first few minutes to an hour, but some studies were extended to a sustained phase at 24 hours (Bobrovskaya *et al.* 2007b; Bobrovskaya *et al.* 2007a; Gelain *et al.* 2007). In chromaffin cells the increases in Ser40 phosphorylation and TH activity due to acute nicotine stimulation are not blocked by the PKC inhibitor GO6983, while the sustained increases in Ser40 phosphorylation and TH activity are (Bobrovskaya *et al.* 2007b). Sustained phosphorylation of TH at Ser40 and sustained TH activation also occurred in PC12 cells treated with pituitary adenylate cyclase activating polypeptide (PACAP) (Bobrovskaya *et al.* 2007a), or manganese (Mn) (Posser *et al.* 2009). Again, the mechanisms leading to sustained Ser40 phosphorylation and TH activation differed from those generated during acute stimulation. With PACAP stimulation, increased PKA activity is sustained and Ser40 phosphatases are inhibited, while with Mn the protein kinase responsible for the sustained phosphorylation was not identified, but is not PKA or PKC. It is therefore clear that the kinases phosphorylating TH at Ser40 can change over time even with a single stimulus.

In vivo

There is evidence in adrenal medulla that PKA phosphorylates TH at Ser40 *in vivo*. In the adrenal medulla, PKA but not PKC is activated 20 and 60 min after treatment of rats with 2 deoxyglucose (2DG). This correlated with an increase in TH phosphorylation at Ser40 (Bobrovskaya *et al.* 2010). Adrenal medulla PKA is activated 40 min after footshock stress and this correlated with an increase in TH phosphorylation at Ser40 (Ong *et al.* 2014).

A number of stressors were investigated to determine whether sustained phosphorylation of TH at Ser40 also occurred in the adrenal medulla *in vivo.* No evidence is found after 2DG treatment (Bobrovskaya *et al.* 2010), social defeat (Ong *et al.* 2011b), or insulin induced hypoglycaemia (Herlein *et al.* 2006; Senthilkumaran *et al.* 2016). However, when a low dose of lipopolysaccharide (LPS) is administered to neonatal rats on days 3 and 5 postpartum, sustained phosphorylation of adrenal TH occurs at Ser40 24 h after the second dose, without any change in TH protein levels (Ong *et al.* 2012). The sustained increase in TH activity at 24 h could not be assigned to only Ser40 phosphorylation, as Ser31 phosphorylation was also increased at this time.

There is evidence in neurons that PKA, and possibly MAPKAPK1 and/or MSK1, phosphorylates TH at Ser40 *in vivo*. Treatment of morphine dependent rats with naloxone leads to withdrawal induced hyperactivity. In the heart after 90 min, there is an increase in TH protein levels, TH phosphorylation at Ser40, as well as the PKA catalytic subunit (Almela *et al.* 2008). The increases in TH protein levels and TH phosphorylation at Ser40 are blocked by infusion of a PKA inhibitor HA-1004 (Almela *et al.* 2008; Almela *et al.* 2009a; Almela *et al.* 2009b). Acute treatment of mice with haloperidol increases the phosphorylation of TH at both Ser31 and Ser40 in the striatum (Hakansson *et al.* 2004). The increase in Ser31 phosphorylation is completely blocked by IP administration of the ERK phosphorylation inhibitor SL327. There is also a 50% reduction in Ser40 phosphorylation, suggesting a role for MAPKAPK1 and/or MSK1. Whatever the kinase(s) responsible for Ser40 phosphorylation in these studies, the data indicate that the cross-talk between PKA and ERK-stimulated kinases seen *in situ* also occur *in vivo*.

Ser31

In vitro and in situ

TH is phosphorylated at Ser31 by both ERK and cyclin dependent kinase 5 (CDK5) *in vitro* (Dunkley *et al.* 2004). Casein kinase 2 and CDK11 are also able to phosphorylate TH, but the site has not been confirmed as Ser31 (Sachs & Vaillancourt 2004).

A number of studies used UO126 and/or PD098059 to confirm that ERK is the major protein kinase phosphorylating TH at Ser31 *in situ.* In each study blocking Ser31 phosphorylation dcreased either TH activity (Kobori *et al.* 2004; Gelain *et al.* 2007) or CO_2 release (Salvatore *et al.* 2001; Knowles *et al.* 2011). The ERK phosphorylation inhibitors had no effect on TH phosphorylation at Ser40 or Ser19. Interleukin 6 increased ERK activation, Ser31 phosphorylation and TH activity without changes at Ser40 or Ser19 (Jenkins *et al.* 2016). This shows that activation of ERK increases TH phosphorylation at Ser31 and TH activation *in situ*.

Other studies showed that ERK activation correlates with increases in TH phosphorylation at Ser31, but does not lead to increased TH activation (Luke & Hexum 2008) (Aita *et al.* 2010). Knowles also found that the PKC activator PMA increased Ser31 phosphorylation, but did not increase CO_2 release. They concluded that ERK activation is necessary, but not sufficient, for CO_2 release. They suggested that the ERK phosphorylation inhibitors might also be inhibiting other ERK-dependent events that effect CO_2 release, perhaps including BH_4 synthesis (Dal Pra *et al.* 2005). This argument could be applied to those ERK phosphorylation inhibitor studies where CO_2 release is measured, but not when TH activity is measured. Another possible reason for the dissociation between TH phosphorylation at Ser31 and TH activation may be the timing of the two measurements. It is generally found that Ser31 phosphorylation is slow and takes at least 10-30 min to reach maximum levels (Haycock 1990; Bobrovskaya *et al.* 2007b; Bobrovskaya *et al.* 2007a). If CO_2 release or L-DOPA synthesis is measured for too short a period then effects of TH phosphorylation at Ser31 on TH activation could be missed.

CDK5 increases the phosphorylation of TH at Ser31 *in vitro* and *in situ* using PC12 cells and striatal tissues from mice (Kansy *et al.* 2004; Moy & Tsai 2004). CDK5 also increases total TH activity via TH phosphorylation at Ser31. The increase in TH activity was attributed to an increase in TH protein levels due to stabilization of TH turnover and not to activation of the enzyme. The situation with CDK5 is complicated by the fact that this kinase reduced ERK phosphorylation and decreased phosphorylation of TH at Ser31. CDK5 can therefore increase or decrease TH phosphorylation at Ser31 depending on the stimulus and the specific signal transduction pathways operating in the cell.

In vivo

There is evidence in adrenal medulla that ERK and possibly CDK5 phosphorylates TH at Ser31 *in vivo*. In adrenal medulla CDKs are activated 20 min after 2DG treatment of rats, while ERK is activated after 60 min, and these changes correlate with an increase in TH phosphorylation at Ser31 (Bobrovskaya *et al.* 2010). ERK is activated from 10-40 min after footshock and Ser31

phosphorylation is increased significantly at 20-40 min (Ong *et al.* 2014). In neonatal adrenal medulla, TH phosphorylation at Ser31 is increased 24 h after a second low dose of LPS, indicating that the activity of Ser31 kinase(s) can be sustained (Ong *et al.* 2012).

There is evidence in neurons that ERK phosphorylates TH at Ser31 *in vivo*. Acute treatment of mice with haloperidol increases TH phosphorylation at Ser31 in the striatum. This is completely blocked by IP administration of the ERK phosphorylation inhibitor SL327 (Hakansson *et al.* 2004). Injection of glial cell derived neurotrophic factor (GDNF) into the striatum of 2 year old-rats increases TH phosphorylation at Ser31 in the striatum and substantia nigra (SN) 30 days later; this correlates with increased ERK phosphorylation (Salvatore *et al.* 2004). Nucleus tractus solitarius (NTS) neurons become hyperactive during morphine withdrawal and TH phosphorylation at Ser31 increases in both the NTS and paraventricular nucleus (PVN); these increases are reduced in the presence of the ERK phosphorylation inhibitor SL327 (Nunez *et al.* 2007).

There is evidence in neurons that CDK5 also phosphorylates TH at Ser31 *in vivo*. Cocaine self-administration causes an increase in TH phosphorylation at Ser31 in striatal neurons and this correlates with an increase in CDK5 activity, with no increase in ERK activity (Kansy *et al.* 2004). There is an increase in TH phosphorylation at Ser31 in the SN of transgenic mice with increased CDK5 activity (Moy & Tsai 2004). However, CDK5 and ERK can each increase the expression of the other. This suggests that it it will not always be possible to determine exactly which kinase is responsible for Ser31 phosphorylation *in vivo*.

Ser19

In vitro and in situ

TH is phosphorylated at Ser19 by CaMPKII, p38 regulated and activated protein kinase (PRAK) and MAPKAPK2 *in vitro* (Dunkley *et al.* 2004). Evidence for these kinases altering TH phosphorylation *in situ* is limited (Dunkley *et al.* 2004). Kobori et al suggested that Ser19 could be phosphorylated by CaMPKII *in situ.* Serum deprivation of a human neuroblastoma cell line increases TH phosphorylation at Ser19, but not at Ser31 or Ser40 (Kobori *et al.* 2004; Kobori *et al.* 2006). CaMPKII is activated over the same period, as defined by threonine 286 autophosphorylation (Kobori *et al.* 2004; Kobori *et al.* 2006). Ser19 is phosphorylated *in situ* by a p38-stimulated protein kinase, most likely MAPKAPK2 (Bobrovskaya *et al.* 2004). There is a concomitant increase in TH activity, but this was attributed to an indirect action of Ser19 phosphorylation leading to an increase in TH phosphorylation at Ser40.

In vivo

Ser19 phosphorylation is correlated with calcium uptake into nerve terminals *in vivo* suggesting a role for CaMPKII (Salvatore *et al.* 2012a). There is indirect evidence for CaMPKII acting on Ser19, as knockout (KO) of the CaMPKII inhibiting protein IRBIT leads to increased TH phosphorylation at Ser19 in the VTA (Kawaai *et al.* 2015). Social defeat causes an increase in TH phosphorylation at Ser19 in the VTA 24h later, indicating that the activity of Ser19 kinase(s) can be sustained (Ong *et al.* 2011a).

Which Protein Phosphatases Act on TH?

PP2A and PP2C are the two protein phosphatases that dephosphorylate TH *in vitro.* PP2A is the predominant phosphatase *in situ* and it dephosphorylates Ser40, Ser31 and Ser19 in TH (Dunkley *et al.* 2004). All three TH phosphorylation sites will be discussed together. Other protein phosphatases have similar inhibition profiles to PP2A and these phosphatases have not been adequately investigated in respect to TH phosphorylation (Cohen 1997; Honkanen & Golden 2002).

In vitro and in situ

PP2A with the B*'B* regulatory subunit dephosphorylates TH at Ser19, Ser31 and Ser40 *in vitro* (Saraf *et al.* 2007). This is due to Glu153 in the B*'B* regulatory subunit of PP2A interacting with Arg37 and Arg38 in TH (Saraf *et al.* 2010). PP2A also dephosphorylates TH *in situ* in PC12 cells (Saraf *et al.* 2007; Saraf *et al.* 2010). Transient or stable transfection of the PP2A B*'B* regulatory subunit leads to dephosphorylation of TH at Ser40 and decreased CO_2 release. Other PP2A subunits are less effective, or have no effect. Silencing the endogenous expression of the B*'B* regulatory subunit increases TH phosphorylation at Ser40 and CO_2 release (Saraf *et al.* 2007; Saraf *et al.* 2010).

PP2A is physically associated with PKCdelta *in vitro* and the activity of PP2A is decreased by PKCdelta phosphorylation (Zhang *et al.* 2007a). Inhibition of PKCdelta in a neuronal cell cultures increases the phosphorylation of TH at Ser40, L-DOPA synthesis and DA content and this correlates with decreased PP2A activity (Zhang *et al.* 2007a). Treatment of N27 dopaminergic cells with Mn causes a decrease in TH activity and this correlates with PKCdelta activation and increased PP2A activity (Zhang *et al.* 2011).

PP2A activity is increased by alpha-synuclein. Overexpression of alpha-synuclein in MN9D cells decreases TH phosphorylation at Ser40 and DA content (Perez *et al.* 2002; Peng *et al.* 2005; Hua *et al.* 2015; Liu *et al.* 2008a). Silencing alpha-synuclein expression increases TH phosphorylation at

Ser40 and DA content (Liu *et al.* 2008a). The decreases in TH phosphorylation at Ser40 are not due to decreases in PKA activity, but rather to increases in PP2A activity (Peng *et al.* 2005). This increase correlates with increases in PP2A methylation (Hua *et al.* 2015). Amphetamine stimulation of SK–N–SH cells up regulates alpha-synuclein levels and decreases TH phosphorylation at Ser40 (Klongpanichapak *et al.* 2008). None of these studies investigated the phosphorylation of TH at Ser31 or Ser19. However, overexpression of WT alpha-synuclein in MN9D cells decreases TH phosphorylation at Ser19, via PP2A activation (Lou *et al.* 2010).

A number of different mechanisms might contribute to the effects of alpha-synuclein overexpression on decreasing TH phosphorylation and decreasing DA content (Daubner *et al.* 2011). There are are only minor or no effects of alpha-synuclein on TH protein levels in MN9D cells (Peng *et al.* 2005; Lou *et al.* 2010). Others found decreases in TH protein levels as a result of cell death, after overexpression of alpha-synuclein, but this is not the case in rat primary mesencephalic cultures, or in a rat dopaminergic cell line (Zhou *et al.* 2000). There can be a direct effect of alpha-synuclein on TH. Alpha-synuclein interacts with TH in brain striatal and MN9D cell supernatants, as judged by co-immunoprecipitation, and alpha-synuclein is able to inhibit the *in vitro* activity of TH (Perez *et al.* 2002). Alpha-synuclein also binds to 14-3-3 proteins and overexpression of alpha-synuclein can lead to decreases in 14-3-3 protein available to bind to TH, increasing the chances of TH dephosphorylation (Kleppe *et al.* 2001; Peng *et al.* 2005). Peng et al. attributed the increase in PP2A activity to a possible interaction between alpha-synuclein and PP2A, as each protein can be coimmunoprecipitated with specific antibodies to the other protein (Peng *et al.* 2005). Alpha-synuclein could also interact with the PP2A methylases or demethylases (Hua *et al.* 2015). Overexpression of S129D alpha-synuclein phosphomimic increases TH phosphorylation, indicating that phosphorylation of alpha-synuclein is able to reverse the effects of overexpression of alpha-synuclein on TH (Lou *et al.* 2010; Wu *et al.* 2011).

In vivo

In the SN of PKCdelta KO mice PP2A activity is decreased. This correlated with an increase in Ser40 phosphorylation and increased DA levels in the striatum (Zhang *et al.* 2007a). Treatment of mice with methamphetamine resulted in a decrease in TH phosphorylation at Ser40 relative to TH protein levels in the striatum (Shin *et al.* 2011). This correlated with an increase in PP2A activity most likely due to increased PKCdelta activity (Dang *et al.* 2015). When a single high dose of LPS is administered to adult rats, Ser40 is dephosphorylated 24 h later and although PKA is still activated, this is overridden by a sustained increase in PP2A activity (Ong et al. 2016).

A number of studies support a role for alpha-synuclein activation of PP2A *in vivo*. There is a decreased TH phosphorylation at Ser19 and TH activity and an increase in PP2A activity in the WT mice compared to alpha-synuclein KO mice (Lou *et al.* 2010). There are no changes in either TH or PP2A protein (Lou *et al.* 2010). Alpha-synuclein overexpressing mice showed decreased levels of TH phosphorylated at Ser19 and Ser40, decreased TH activity, but no changes in TH protein levels in the striatum. There is also decreased TH phosphorylation at Ser19 in the SN. In the olfactory bulb in alpha-synuclein overexpressing mice there is decreased TH phosphorylation at Ser19, decreased TH activity and increased PP2A activity. The decrease in TH activity is presumably due to decreases in Ser31 and/or Ser 40 phosphorylation.

In contrast, other studies do not support a role for alpha-synuclein activation of PP2A *in vivo*. Alpha-synuclein KO mice show no differences from wild type (WT) mice in TH protein levels, TH phosphorylation at Ser40, Ser31, or Ser19, or TH activity. This occurs under basal conditions and in response to nigrostriatal activation of DA neurons with raclopride (Drolet *et al.* 2006). Overexpression of alpha-synuclein increased both TH protein and TH phosphorylation at Ser40 in the striatum, when compared to WT controls (Hua *et al.* 2015). Transducing alpha-synuclein into the olfactory bulbs of alpha-synuclein KO mice with human lentivirus increased TH phosphorylation at Ser19 (with "nearly identical" results for Ser31 and Ser40) without decreasing TH protein levels (Alerte *et al.* 2008).

What is the Stoichiometry of TH Phosphorylation?

In this section of the review the mean basal stoichiometry for TH phosphorylated at a particular site is expressed as a percentage. A value of 5% means that 5 moles of phosphate is present at a particular site for every 100 moles of TH protein monomer. Theoretical fold increases in TH phosphorylation above basal are rounded up to the nearest whole number. Ser40

In vitro and in situ

In vitro studies on TH phosphorylation and TH activation were reviewed in detail (Dunkley *et al.* 2004). In summary recombinant TH is active even though it is not phosphorylated and this is because it contains no bound catecholamines. Phosphorylation of recombinant TH does not lead to increases in TH activity, unless the concentration of BH_4 is limiting and well below that routinely used in the TH activity assay. Addition of catecholamines to recombinant TH leads to TH inhibition. Phosphorylation of TH at Ser40 using PKA displaces the bound catecholamines and activates TH. Since 2004 a number of *in vitro* studies with recombinant TH have been undertaken that provide further insight into TH phosphorylation at Ser40 and catecholamine binding. It is confirmed that Ser8, Ser19, or Ser31

phosphorylation has no effect on catecholamine binding (Royo *et al.* 2005). There are four human isoforms of TH and these differ in the number of amino acids inserted to the N-terminal side of Ser31 when compared to the rat enzyme. hTH1 is the same as the rat in this region, hTH2 has an insertion of 4 amino acids, hTH3 has an insertion of 27 amino acids and hTH4 has an insertion of 31 amino acids; including both the 4 amino acids in hTH2 and the 27 amino acids in hTH3 (Le Bourdelles *et al.* 1988). Phosphorylation of the Ser40 equivalent site in each of the human isoforms decreases dopamine binding by two orders of magnitude. This indicates that the inserts do not alter the relationship between the Ser40 phosphorylation site and the high affinity catecholamine binding site (Sura *et al.* 2004). A second low affinity catecholamine binding site is found on TH and binding of catecholamines at this second site decreases TH activity (Gordon *et al.* 2008; Gordon *et al.* 2009b). This is likely to be physiologically important at high catecholamine concentrations (Briggs *et al.* 2011), but as TH phosphorylation at Ser40 did not alter catecholamine binding at the low affinity site it will not be further discussed.

X-ray crystallography indicated that TH is a tetramer made up of a dimer of dimers (Goodwill *et al.* 1997) and this structure also exists in solution (Kumer & Vrana 1996). It was suggested that only one catecholamine bound to each dimer is sufficient to inhibit TH through the high affinity catecholamine binding site (Fujisawa & Okuno 2005). This is consistent with a stoichiometry of catecholamine binding to TH of approximately 50% seen in other studies (Daubner *et al.* 1992; Gordon *et al.* 2008). It was further suggested that both subunits on each dimer needed to be phosphorylated at Ser40 to release bound catecholamine and fully activate TH (Fujisawa & Okuno 2005). This is consistent with approximately 30% of TH being readily phosphorylated without increasing TH activity (P Dickson, Unpublished data) and the very high levels of PKA being required to phosphorylate cateholamine-bound TH to 100% (Lehmann *et al.* 2006).

What proportion of TH is phosphorylated and how many subunits in TH need to be phosphorylated to activate TH *in situ*? Basal TH phosphorylation stoichiometry at Ser40 is approximately 3% in both PC12 cells (Salvatore *et al.* 2001) and BACCs (Bobrovskaya *et al.* 2004). TH is not phosphorylated at more than one site per subunit under basal conditions in PC12D cells, indicating that when Ser40 is phosphorylated neither Ser31 nor Ser19 are also phosphorylated on the same subunit (Nakashima *et al.* 2016). Forskolin only increases TH phosphorylation at Ser40 in PC12 cells. Low concentrations of Forskolin (0.03 uM) increases Ser40 phosphorylation to approximately 6%, but does not increase CO_2 release. Medium concentrations (0.3 uM) increases Ser40 phosphorylation to approximately 9% and increases CO_2 release approximately1.7-fold (Salvatore *et al.* 2001). The increase in Ser40 phosphorylation at low concentrations of Forskolin is most likely to have been on enzyme that was catecholamine free, which would not increase TH activity. Alternatively, it could have been on catecholamine containing TH, but if there is a threshold of phosphorylation required to release the catecholamine then that was not reached. It is also possible

that there is an increase in TH activity, but that is insufficient to lead to measurable CO_2 release. In BACCs high concentrations of Forskolin (1 uM) increases TH phosphorylation stoichiometry at only Ser40 to approximately 13% and increases TH activity approximately 3-fold (Bobrovskaya *et al.* 2004). This increase in TH activity is greater than the fold increase calculated, if a Vmax value of 53 is used for DA-bound TH phosphorylated at Ser40 and a Vmax value of 3 is used for DA-bound TH (Daubner *et al.* 1992).

TH is therefore activated *in situ* to a smaller or a larger extent than anticipated from *in vitro* studies depending on the cell type, the concentration of Forskolin used and the procedure used to measure TH activation. Further work is required to clarify exactly what is happening *in situ*.

In vivo

The adrenal medulla needs large stores of catecholamines to release into the blood during stressful events and it does not have a catecholamine reuptake system. It therefore maintains high levels of TH phosphorylation (Saraf *et al.* 2007), TH activity (Okuno & Fujisawa 1985) and cytosolic catecholamines (Mosharov *et al.* 2006) to maintain catecholamine stores. The basal stoichiometry of TH phosphorylation at Ser40 *in vivo* is very high at 18% when compared to 3% for primary chromaffin cells in culture (Table 1). The adrenal medulla cells *in vivo* are presumably activated by the splanchnic nerve and/or hormones leading to increases in basal TH phosphorylation at Ser40. The B′*B* regulatory subunit of PP2A is not found in the adrenal medulla and there is much less Ser40 phosphatase activity than in the brain (Saraf *et al.* 2007), suggesting that Ser40 kinase(s) drive this increased basal TH phosphorylation *in vivo*. For the adrenal medulla it is theoretically possible to increase TH phosphorylation at Ser40 by 6-fold. In our laboratory we saw a maximum increase of 2.5-fold in the adrenal medulla in response to LPS. This shows that the maximum stoichiometry achieved for Ser40 *in vivo* is approximately 45% (Table 2). These calculations make the assumption that there is no change in the Ser40 phosphorylation stoichiometry between the home cage controls used to measure basal stoichiometry and the saline injected or sham treatment controls used for the various stimuli. This is generally the case in the only study from our laboratory where both a home cage basal control and a stimulation control are included (Ong *et al.* 2011a). An increase in TH phosphorylation in the adrenal medulla *in vivo* at only Ser40 by 1.5-fold increases TH activity by 2-fold (Ong *et al.* 2012).

Brain neurons have smaller stores of catecholamines, when compared to the adrenal medulla. Brain neurons have transport proteins that can reuptake released catecholamines. They also have lower levels of TH activity and cytosolic catecholamines (Mosharov *et al.* 2006). The basal stoichiometry of TH phosphorylation at Ser40 in neuron cell bodies from the SN, VTA, hypothalamus

and LC is always very low, with only 1.6-6% of the total TH protein being phosphorylated (Salvatore *et al.* 2000; Salvatore *et al.* 2009b; Keller *et al.* 2011; Salvatore *et al.* 2012b; Salvatore & Pruett 2012; Ong *et al.* 2014)(Table 1). For neuron cell bodies it is theoretically possible to increase TH phosphorylation at Ser40 by up to 24-fold. In our laboratory we saw a maximum increase of 1.6-fold in response to hydralazine in the VTA and Salvatore saw a maximum increase of 2.4-fold in response to haloperidol in the SN. This shows that the maximum stoichiometry achieved for Ser40 *in vivo* is approximately 8% (Table 2). A larger increase of 7.4-fold is seen for Ser40 in response to NSD1015, but this is not a physiological situation (Salvatore & Pruett 2012). In neuron cell bodies *in vivo*, limitations in the activity of Ser40 kinases relative to Ser40 phosphatases and the presence of bound catecholamines on TH substantially limit the capacity to phosphorylate TH at Ser40. This data is consistent with the early immunohistochemical studies where Ser40 phosphorylation is difficult to observe in the absence of PKA activators (Xu *et al.* 1998). An increase in TH phosphorylation in the VTA at only Ser40 by 1.4-fold increases TH activity by 2-fold (Ong *et al.* 2017a).

The basal stoichiometry of TH phosphorylation at Ser40 in neuron terminals of the striatum, nucleus acumbens (NAC) and medial prefrontal cortex (MPFC) is also low and ranged from 2.6- 5% (Salvatore *et al.* 2000; Ong *et al.* 2014)(Table 1). For neuron terminals it is theoretically possible to increase TH phosphorylation at Ser40 by up to 31-fold. Salvatore saw a maximum increase of 2.5-fold in response to clozapine in the NAC. This shows that the maximum stoichiometry achieved for Ser40 *in vivo* is approximately 13% (Table 2). Immunohistochemistry studies found that the B'*B* regulatory subunit of PP2A is present in almost all TH positive neurons and is highly expressed in cell bodies and dendrites, but is virtually absent from axons and presynaptic terminals (Saraf *et al.* 2007). As the basal stoichiometry of TH phosphorylation at Ser40 in cell bodies and terminals is similar it means that the basal phosphorylation cannot therefore be primarily controlled by PP2A. This implies that either the Ser40 kinases are more active in the cell body to balance the higher levels of PP2A, or are less active in the nerve terminals.

Ser31

In vitro and in situ

When TH is phosphorylated by ERK *in vitro* the stoichiometry is approximately 50% (Sutherland *et al.* 1993; Halloran & Vulliet 1994; Kansy *et al.* 2004; Lehmann *et al.* 2006). When CDK5 is used the stoichiometry is also approximately 50% (Kansy *et al.* 2004). The reason(s) for the low stoichiometry of Ser31 phosphorylation *in vitro* in most studies is not clear, but it may indicate that the first subunit in each TH dimer is easier to phosphorylate at Ser31 than the second subunit. Using PC12 cells the basal level of TH phosphorylation *in situ* is 9% for Ser31 and treatment with high potassium increased

TH phosphorylation to 27% (Salvatore *et al.* 2001). This stoichiometry is substantially higher than for Ser40 and reflects the fact that catecholamine binding to TH does not alter the ability of ERK to phosphorylate Ser31 (Royo *et al.* 2005; Lehmann *et al.* 2006).

ERK phosphorylates hTH3 and hTH4 at the sites equivalent to Ser31 to a stoichiometry of approximately 100%, while the hTH2 isoform is not able to be phosphorylated at the Ser31-equivalent site (Ser35) (Sutherland *et al.* 1993; Lehmann *et al.* 2006). Substitution of amino acids immediately adjacent to Ser31 clearly changes the amino acids required for ERK substrate specificity and either increases or decreases the extent of TH phosphorylation relative to hTH1. hTH1 and hTH2 account for over 90% of the TH protein, and they are present in approximately equal protein levels (Haycock 2002). This means that almost half of the TH in humans cannot be activated by ERK-stimulated Ser31 phosphorylation. It would be of interest to know whether CDK5 could phosphorylate hTH2 at Ser35 either *in vitro* or *in situ*.

In vivo

For the adrenal medulla the basal TH phosphorylation stoichiometry at Ser31 is 6% (Table 1), which was lower than that observed in chromaffin cells in culture. For the adrenal medulla it is theoretically possible to increase TH phosphorylation at Ser31 by 17-fold. In our laboratory we saw a maximum increase of 9-fold in response to 2DG in the adrenal medulla. This shows that the maximum stoichiometry achieved for Ser31 *in vivo* is approximately 54% (Table 2). This is similar to the maximal stoichiometry achieved *in vitro* with ERK and CDK5. In the adrenal medulla an increase in TH phosphorylation at only Ser31 by 1.6-fold increases TH activity by 2.2-fold (Ong *et al.* 2014).

The basal TH phosphorylation stoichiometry at Ser31for neuron cell body regions of the brain, including the SN, VTA, hypothalamus and LC, is between 6-13% (Table 1). This is higher than for Ser40. For neuron cell bodies it is theoretically possible to increase TH phosphorylation at Ser31 by 10-fold. In our laboratory we saw a maximum increase of 2.8-fold in response to social defeat in the LC and Salvatore saw a maximum increase of 3.2-fold in response to GDNF in the SN. This shows that maximum stoichiometry achieved for Ser31 *in vivo* is approximately 28% (Table 2).

For the nerve terminal regions of the brain, including the striatum, MPFC and the NAC the basal phosphorylation stoichiometry at Ser31 is between 21-36% (Table 1), indicating that Ser31 kinases are more active relative to Ser31 phosphatases at nerve terminals *in vivo* when compared to their corresponding cell bodies. For neuron terminals it is theoretically possible to increase TH phosphorylation at Ser31 by 3-fold. In our laboratory we saw a maximum increase of 2.1-fold in response to footshock in the MPFC and Salvatore saw a maximum increase of 2.8-fold in response to raclopride in the NAC. This shows that the maximum stoichiometry achieved for Ser31 *in vivo* is

approximately 71% (Table 2). This is greater than the stoichiometry achieved *in vitro* with ERK and CDK5. In the MPFC an increase in TH phosphorylation at only Ser31 by 2.2-fold increases TH activity by 2.0-fold (Ong *et al.* 2014).

Ser31 phosphorylation correlated with basal DA content in the SN, VTA, striatum and NAC, even though TH phosphorylation stoichiometry at Ser31 is much lower in the cell bodies when compared to the nerve terminals (Salvatore & Pruett 2012). It was argued that this is because Ser31 phosphorylation is likely to be important in the cell body regions such as the SN and VTA that are more reliant on de novo synthesis due to reduced DA reuptake capacity.

Ser19

In vitro and in situ

TH is phosphorylated *in vitro* at Ser19 by CaMPKII to 100% (Fujisawa & Okuno 2005).

Basal TH phosphorylation stoichiometry at Ser19 in PC12 cells is 5% (Salvatore *et al.* 2001) and in BACCs is 12% (Bobrovskaya *et al.* 2004). High potassium increases Ser19 phosphorylation to 20% in PC12 cells and anisomycin increases Ser19 phosphorylation to 24% in BACCs. Therefore stimulated Ser19 phosphorylation *in situ* did not approach the stoichiometric levels achievable *in vitro*.

In vivo

For the adrenal medulla basal TH phosphorylation at Ser19 is 33% (Table 1). This is much higher than for Ser19 in chromaffin cells in culture and for Ser40, or Ser31, *in vivo*. For the adrenal medulla it is theoretically possible to increase TH phosphorylation at Ser19 by 3-fold. In our laboratory we saw a maximum increase of 1.5-fold in response to LPS. This shows that maximum stoichiometry achieved for Ser19 *in vivo* is approximately 50% (Table 2). This is less than the 100% achieved *in vitro* with CaMPKII, but is very much higher than the stoichiometry observed *in situ*.

For the cell body regions of the brain, including the SN, VTA, hypothalamus and LC, the basal TH phosphorylation stoichiometry at Ser19 varies between 13-36% (Table 1). This data is consistent with the early immunohistochemical studies in the brain where TH phosphorylation at Ser19 is always detectable along with TH protein (Xu *et al.* 1998). For the neuronal cell body regions it is theoretically possible to increase TH phosphorylation at Ser19 by 4-fold. In our laboratory we saw a maximum increase of 2.5-fold with hydralazine in the VTA and Salvatore saw a maximum increase of 2.5 in response to haloperidol in the SN. This shows that maximum stoichiometry achieved for Ser19 *in vivo* is approximately 65% (Table 2).

For the nerve terminal regions of the brain, including the striatum and the NAC, the basal TH phosphorylation stoichiometry at Ser19 is between 10-16% (Table 1). This suggests that Ser19 kinases are less active relative to Ser19 phosphatases at nerve terminals *in vivo* when compared to their corresponding cell bodies. For the neuronal cell body regions it is theoretically possible to increase TH phosphorylation at Ser19 by 9-fold. In our laboratory we saw a maximum increase of 1.8-fold with hydralazine in the MPFC and Salvatore saw a maximum increase of 2.5 in response to haloperidol in the striatum. This shows that maximum stoichiometry achieved for Ser19 *in vivo* is approximately 20% (Table 2).

None of the *in vivo* studies showed an increase in TH phosphorylation at only Ser19 leading to TH activation.

Ser19, Ser31 and Ser40

In situ

The basal phosphorylation of TH in PC12 cells is Ser40 3%, Ser31 9% and Ser19 5% (Salvatore *et al.* 2001). This means that if each site is phosphorylated on a different TH subunit then 17% of TH is phosphorylated. In PC12D cells under basal conditions the proportion of TH phosphorylated at a single site (either Ser19, Ser31 or Ser40) is approximately 20 % and there is no phosphorylation at two or three sites, as determined by phosphate affinity SDS-PAGE using Phos-Tag^{TM} acrylamide (Nakashima *et al.* 2016). These results suggest that under basal conditions *in situ* individual TH subunits are not phosphorylated at more than one site.

In vivo

The basal phosphorylation of TH in adrenal chromaffin cells is Ser40 18%, Ser31 6% and Ser19 33% (Ong *et al.* 2014). This means that if each site is phosphorylated on a different TH subunit then 57% of TH is phosphorylated. The maximum phosphorylation of TH in adrenal gland is Ser40 45%, Ser31 54% and Ser19 50% (Table 2). If all three sites are maximally phosphorylated then more than one phosphorylation site must exist on some TH subunits. However, there is no evidence to date as to how many sites are phosphorylated on individual TH subunits *in vivo*. The Phos-Tag^{TM} acrylamide procedure would be valuable for answering this question.

What Proteins Bind to TH? Where is TH Localised?

A number of proteins, including 14-3-3, alpha-synuclein, PP2A, AADC, GTP cyclohydrolase, VMAT and DJ-1, bind to TH, *in vitro* or *in situ*, and modulate TH activity and/or TH subcellular location (Fujisawa & Okuno 2005; Daubner *et al.* 2011). In this review the section on "Which Protein Phosphatases Act on TH?" covered both PP2A and alpha synuclein interactions with TH. There is clear evidence that PP2A acted on TH and therefore PP2A must bind to phosphorylated TH *in vivo*, but there is no clear evidence that the actions of alpha synuclein on TH *in vivo* are due to alpha-synuclein binding directly to TH. TH is also bound to CaMPKII *in vitro* in a phosphorylation dependent manner (Skelding *et al.* 2010).

14-3-3

The first protein found to bind to TH directly and increase its activity is the 14-3-3 protein (Yamauchi *et al.* 1981; Kleppe *et al.* 2001; Fujisawa & Okuno 2005; Daubner *et al.* 2011; Ichimura *et al.* 1987). *In vitro* studies using cloned TH found that the 14-3-3 protein binds to TH only if TH is phosphorylated at Ser19, or Ser40. Phosphorylation of both Ser19 and Ser40 further increases the 14-3-3 proteins affinity for TH (Kleppe *et al.* 2001; Toska *et al.* 2002). Cross immunoprecipitation experiments suggest that 14-3-3 epsilon protein interacts with TH phosphorylated at Ser19 *in situ* (Wang *et al.* 2009). SiRNA knockdown of 14-3-3epsilon *in situ* decreases TH activity and DA content (Wang *et al.* 2009). Different 14-3-3 isoforms activated phosphorylated TH to different extents, with some isoforms leading to no activation (Kleppe *et al.* 2001; Wang *et al.* 2009; Toska *et al.* 2002). Transient overexpression of CDK11p110 decreased the interaction between14-3-3 protein and TH, possibly due to phosphorylation of the 14-3-3 protein (Sachs & Vaillancourt 2004). The mechanism for TH activation by the 14-3-3 proteins was speculated to include TH stabilization against proteolysis, conformational distortion of TH, hierarchical phosphorylation of Ser40 due to prior Ser19 phosphorylation, or inhibition of PP2A dephosphorylation of TH (Kleppe *et al.* 2001; Toska *et al.* 2002; Obsilova *et al.* 2008; Halskau *et al.* 2009; Daubner *et al.* 2011). The N-terminal domain of Ser19 phosphorylated TH binds to membranes with enhanced affinity in the presence of 14-3-3 proteins (Halskau *et al.* 2009) and this could modulate its localisation and/or its activity.

We know of no studies confirming a role for a direct binding of 14-3-3 protein to phosphorylated Ser19, or Ser40, *in vivo* leading to TH activation.

GTPCH, VMAT, AADC, DJ-1 and Hsc70

In Situ

TH is primarily (>90%) a soluble protein in the adrenal chromaffin cells, based on subcellular fractionation studies, but some TH is always associated with membranes (Haycock *et al.* 1985; Kuhn *et al.* 1990). Daubner suggested that the differences in sequence of the four human TH isoforms might lead to differential binding to selected proteins (Daubner *et al.* 2011). TH is found associated with large dense core vesicles in adrenal chromaffin cells (Kuhn *et al.* 1990) and directly bound to VMAT1 in PC12 cells (Cartier *et al.* 2010). TH also binds directly to VMAT2 at multiple domains in rat striatal lysates. TH, VMAT2 and AADC are functionally associated with synaptic vesicles isolated from striatum; the vesicle-bound TH is active and together with AADC produces DA that is taken into the vesicles by VMAT2 (Cartier *et al.* 2010). TH binds directly to the molecular chaperone Hsc70, which targetes TH to form a complex with VMAT2, AADC and synaptic vesicles (Parra *et al.* 2016). Hsc70 also activates TH. TH and AADC bind to DJ-1 oxidised at cysteine 106 leading to increases in TH activity (Ishikawa *et al.* 2009).

TH phosphorylation alters its binding to a range of proteins. TH binds GTP Cyclohydrolase 1, an enzyme responsible for production of BH_4, and this interaction is dependent on TH phosphorylation by PKA (Bowling *et al.* 2008). Nakashima found using immunohistochemistry that in PC12D cells TH phosphorylated at Ser31 and Ser40 is mainly cytoplasmic, while TH phosphorylated at Ser19 is mainly nuclear (Nakashima *et al.* 2011). Similar results are also found with undifferentiated PC12Adh cells treated with Triton X100 (Jorge-Finnigan *et al.* 2017). The TH N-terminal region contains two nuclear localisation sequences and inhibition of nuclear import increases Ser19 phosphorylation (Nakashima *et al.* 2018). When PC12* cells were incubated with saponin to preserve protein-protein interactions TH phosphorylated at Ser31 is found in a perinuclear location associated with synaptic vesicle markers and this association is blocked by inhibitors of Ser31 phosphorylation (Jorge-Finnigan *et al.* 2017). Ser31 phosphorylated TH is likely to be binding to VMAT2 and alpha synuclein and not to the lipid membranes of vesicles. TH phosphorylation at Ser31 facilitates transport of TH from the soma to the nerve terminals via the microtubule network (Jorge-Finnigan *et al.* 2017).

In vivo

Early studies on TH distribution suggest that the cytosol was the prominent location of the enzyme, especially in the SN and VTA, while membrane associated TH is also found in the terminal regions of the striatum and NAC (Cartier *et al.* 2010). Immunocytochemical studies show that TH and Hsc70 colocalize in SN and VTA neurons (Parra *et al.* 2016). TH phosphorylation at Ser31 is higher in nerve

terminals than in cell bodies. This is consistent with TH phosphorylated at Ser31 being involved in binding to SVs in protein complexes and also with this phosphorylated form of TH being involved in vesicle trafficking (Jorge-Finnigan *et al.* 2017).

Summary of TH Regulation

1. *In vivo protein kinases: There is evidence that: PKA phosphorylates TH at Ser40 in the adrenal medulla, heart sympathetic neurons and the striatum: MAPKAPK1 and/or MSK1 phosphorylates TH at Ser40 in the striatum: ERK and possibly CDK5 phosphorylate TH at Ser31 in the adrenal medulla and in striatal neurons: CaMPKII phosphorylates TH at Ser19 in the VTA. Sustained phosphorylation of TH at Ser40, Ser31 and Ser19 only occurs in the adrenal medulla in response to specific stimuli.*

2. *In vivo protein phosphatases: There is evidence in neurons that PP2A acts on at least Ser40 and Ser19 in vivo. PP2A activity is increased by alpha-synuclein in situ, but there is no consistency in the data in relation to the effect of alpha-synuclein on TH phosphorylation in vivo.*

3. *In vivo phosphorylation stoichiometry: Basal Ser40 phosphorylation stoichiometry is very high in the adrenal medulla relative to cultured adrenal chromaffin cells. Basal Ser40 phosphorylation stoichiometry is very low in neurons, where there was little difference between cell bodies and their terminals. A range of stimuli increase TH phosphorylation at Ser40 in both adrenal medulla and neurons, but the maximum stoichiometry achieved is still very low relative to that observed in vitro. An increase in only Ser40 phosphorylation stoichiometry in vivo is sufficient to activate TH.*

   Basal Ser31 phosphorylation stoichiometry is low in the adrenal medulla and in nerve cell bodies, but is much higher in nerve terminals. A range of stimuli increase TH phosphorylation at Ser31 in both adrenal medulla and neurons. The maximum stoichiometry in adrenals is similar to that achieved in vitro, but is less for neuronal cell bodies and greater for nerve terminals. An increase in only Ser31 phosphorylation stoichiometry in vivo is sufficient to activate TH. Human isoform hTH2 cannot be phosphorylated at the Ser31 equivalent site by ERK and therefore almost half of the TH protein in humans cannot be activated by this kinase.

   Basal Ser19 phosphorylation stoichiometry is very high in the adrenal medulla and in nerve cell bodies, but is low in nerve terminals. A range of stimuli increased TH phosphorylation at Ser19 in adrenals and neurons and the maximum stoichiometry achieved is much higher than

in situ, but lower than that in vitro. There is no evidence that only Ser19 phosphorylation can activate TH in vivo.

4. *In vivo protein binding and location: There is substantial in situ evidence that TH binds to a number of proteins, other than protein kinases and phosphatases, and that this binding can alter TH localisation within cells, or TH activity. However, there is little evidence that these protein associations occur in vivo and are physiologically relevant, even for the 14-3-3 protein.*
5.

Acute and Prolonged TH Phosphorylation Changes in Specific Catecholaminergic Cells In Vivo

A major focus of this section will be on the timing and duration of TH phosphorylation changes observed in specific sympathetic and brain regions in response to a range of stimuli, or ageing. Some studies investigated Ser40, Ser31 and Ser19, but many only investigated one site. When there are only a few references for a particular sympathetic (heart, carotid bodies, retinal amacrine cells) or brain region (cortex, hypothalamus, brain stem, LC, NTS, PVN) the papers will not be discussed in the text, but the basic findings are listed in Table 3. Human studies where post-mortem tissue has been used and studies that have relied primarily on immunohistochemistry will not be included. Readers are also referred to an excellent review on TH responses in animal models of Parkinsons's disease which includes both sympathetic (heart, GIT and adrenal) and brain (nigrostriatal pathway, LC, medulla oblongata, olfactory bulb), the details of which will not be repeated here (Johnson *et al.* 2018).

Acute TH Phosphorylation

Acute TH phosphorylation changes generally occur while the stimulus is still present. They are likely to be due to maintenance of protein kinase and/or phosphatase activity changes, induced by physiological responses to the stimulus that begin within minutes and only last from minutes to hours.

Adrenal Medulla

When sympathetic systems are activated and catecholamines secreted, TH phosphorylation and TH activity are increased to maintain a constant level of catecholamines in the tissues (Zigmond et al., 1989).

In situ studies with cultured PC12 cells and primary BACCs stimulated with nicotine, angiotensin II, or histamine, found that Ser19 phosphorylation occurs within minutes (Haycock 1990; Bobrovskaya *et al.* 2001; Cammarota *et al.* 2003; Dunkley *et al.* 2004). Ser40 phosphorylation followed and is maximal within 5 min. Ser31 phosphorylation is delayed and is maximal by about 10 min (Haycock 1990; Bobrovskaya *et al.* 2001; Cammarota *et al.* 2003; Dunkley *et al.* 2004). TH activity correlates with Ser40 phosphorylation in response to nicotine (Dunkley *et al.* 2004) and with Ser31 phosphorylation in response to histamine (Cammarota *et al.* 2003). Studies with perfused rat adrenal glands found that TH phosphorylation and activation depends on the stimulus (Haycock & Wakade 1992); electrical stimulation at 10 Hz for 30 s leads to Ser19 and Ser40 phosphorylation, while 1 Hz for 5 min is required to increase Ser31 phosphorylation. Vasoactive intestinal polypeptide stimulation leads to increased Ser40 phosphorylation at 3 min, while nicotine and muscarine leads to Ser19, Ser31 and Ser40 being phosphorylated at 3 min. In these experiments, TH activity correlates with Ser40 phosphorylation and not with Ser31 or Ser19 phosphorylation.

In vivo studies on rat adrenals included the use of the psychological stressors social defeat (Ong *et al.* 2011a) and immobilisation (Ong *et al.* 2011b), the physical stressor electrical footshock (Ong *et al.* 2014), or the metabolic stressors 2DG (Bobrovskaya *et al.* 2010) and insulin induced hypoglycaemia (Senthilkumaran *et al.* 2016; Senthilkumaran & Bobrovskaya 2017). Overall the results from these studies suggest that physiological changes in TH phosphorylation *in vivo* are substantially delayed when compared to the *in situ* studies. The maximum phosphorylation levels for Ser40 occur between 20-40 min after the stimulus and for Ser31 occur between 10-90 min. In some studies Ser31 phosphorylation preceded Ser40 phosphorylation. This did not occur *in situ*. The largest fold increases observed were for Ser31 phosphorylation, with modest increases for Ser40 phosphorylation and no increases for Ser19 phosphorylation. It may be that changes in Ser19 phosphorylation always occur at earlier or later times. Psychological stimuli produce very modest changes in TH phosphorylation, while metabolic stressors produce much larger responses, especially for Ser31. TH activity was not determined in most of these studies. However, with footshock stress TH phosphorylation at Ser31 correlates with TH activity after 20-30 min in the adrenal medulla. TH phosphorylation at Ser40 is delayed until 40 min and this led to a further increase in TH activity (Ong *et al.* 2014).

Nigrostriatal pathways

SN:

In vivo studies with haloperidol treatment for 30-40 min increases phosphorylation of TH at Ser19, Ser31 and Ser40 (Salvatore *et al.* 2000). Insulin induced hypoglycaemia increases phosphorylation of TH at Ser19 and Ser31 after 60 and 90 min (Senthilkumaran *et al.* 2016). Social defeat for 60 min increases TH phosphorylation at Ser40, 10 min after treatment (Ong *et al.* 2011a). TH activation was not determined in these studies.

Striatum:

In situ studies using striatal slices exposed to NMDA, D2 receptor activators, or depolarising agents found increases in TH phosphorylation at Ser40 that correlated with changes in TH activity and L-DOPA synthesis (Lindgren *et al.* 2000; Lindgren *et al.* 2001; Lindgren *et al.* 2002; Lindgren *et al.* 2003).

In vivo studies using electrical stimulation of the medial forebrain bundle for 20 min (Haycock & Haycock 1991), or acute haloperidol treatment for 30-40 min (Salvatore *et al.* 2000), increases striatal TH phosphorylation at Ser19, Ser31 and Ser40. Gamma-butrylactone treatment for 35 min increases striatal TH phosphorylation at only Ser19 and Ser40 (Lew *et al.* 1999). High frequency subthalamic nucleus stimulation for 2 h increases striatal TH phosphorylation at only Ser19 (Reese *et al.* 2008). In contrast, cocaine treatment decreases TH phosphorylation at Ser19, Ser31 and Ser40 in the caudate (Jedynak *et al.* 2002). The decreases at each site occur over different time courses up to 120 min; with Ser40 decreasing most rapidly and Ser31 decreasing at a much slower rate. Haloperidol or gamma-butyrolactone treatment both increase TH activity and DA content in the striatum and the increases in Ser40 phosphorylation correlate with these changes (Salvatore *et al.* 2000; Lew *et al.* 1999). High doses of cocaine decreases L-DOPA synthesis in the caudate after 40 min. However, both low and high doses of cocaine equally decrease TH phosphorylation suggesting that the changes in TH activity are not entirely due to the changes in TH phosphorylation (Jedynak *et al.* 2002).

Mesolimbic and Mesocortical pathways

VTA:

There is no effect of footshock, or time (10, 20, 40 min), on TH phosphorylation at Ser19, Ser31 or Ser40, or on TH activity (Ong *et al.* 2014). There is no effect of insulin induced hypoglycaemia on TH phosphorylation after 30, 60 or 90 min at any site, except for a small increase in TH

phosphorylation at Ser31 after 90 min (Senthilkumaran *et al.* 2016). Cocaine addicted rats show an increase in TH phosphorylation at Ser40 when cocaine is reintroduced (Yao *et al.* 2010). However, acute administration of cocaine leads to decreases in TH phosphorylation at Ser19 after 40 min (Jedynak *et al.* 2002). Earlier studies had shown that acute cocaine has no effect on TH activity (Baumann *et al.* 1993).

NAC:

Acute administration of cocaine leads to decreases in TH phosphorylation at Ser19, Se31 and Ser40 after 40 min (Jedynak *et al.* 2002). L-DOPA synthesis is decreased at the same time. The TH phosphorylation site responsible could not be identified as all three sites were affected.

MPFC:

Footshock causes a significant increase in the Ser31 phosphorylation after 40 mins and this correlated with an increase in TH activity (Ong *et al.* 2014). There were no other effects on TH protein levels or TH phosphorylation at Ser19 or Ser40.

Prolonged TH Phosphorylation

Prolonged TH phosphorylation changes fall into four categories depending on the the age at which the stimulus is presented and the period of exposure to the stimulus.

1. Studies where the change is always there throughout development and in later life, including KOs, transgenics, hybrid strains and specifically bred animals.
2. Studies where the stimulus is present for a prolonged period.
3. Studies where exposure to the stimulus is relatively brief, but tissues are collected after a prolonged period.
4. Studies on ageing where no specific stimulus has been applied.

The following sections are organized with a paragraph for each of these categories within the specific tissue being discussed. In all four categories prolonged TH phosphorylation changes are likely to be due to gene expression changes that begin within days and last from weeks to years.

Adrenal Medulla

1: N-type Ca^{2+} channel alpha1B-deficient mice showed increases in TH protein and TH phosphorylation at Ser40 (Takahashi & Nagasu 2006).

2: Exposure of male rats to a high fat diet from PND 21 increases TH phosphorylation at Ser40 after 19 weeks (Bobrovskaya *et al.* 2013). This diet also reverses the effects of maternal separation (Bobrovskaya *et al.* 2013).

3: Immune challenge with low doses of LPS in 5 day postnatal ratsmarkedly increases TH phosphorylation at Ser19, Ser31 and Ser40 at day 50 (adolescent) and at day 85 (adult) (Sominsky *et al.* 2013). It also increases TH activity at both times (Sominsky *et al.* 2013). This is the first stimulus to clearly show an increase in TH phosphorylation at Ser19 in the adrenal medulla *in vivo*. In contrast immune challenge of adult rats with a single higher dose of LPS decreases TH phosphorylation at Ser40 and Ser31 and TH activity after 1 day (Ong *et al.* 2017b). This is due to PP2A activation, despite PKA and PKC being activated at that time. None of the phosphorylation changes last beyond a week (Ong *et al.* 2017b). Maternal separation of female rats for three hours a day from PND 2-14 leads to decreases in TH phosphorylation at Ser40 19 weeks later (Bobrovskaya *et al.* 2013).

Nigrostriatal Pathways

SN:

1: A number of changes in TH phosphorylation are observed in transgenic and KO mice, compared with wild type mice, as a result of changes that occur throughout development. Increases in TH phosphorylation at Ser40 are found in PKC delta KO mice (Zhang *et al.* 2007b). DA and DOPAC levels are also both increased (Zhang *et al.* 2007b). TH phosphorylation at Ser31 is significantly increased in microsomal epoxide hydrolase KO mice (Liu *et al.* 2008b). Alpha-synuclein KO mice show elevated TH phosphorylation at Ser19 and TH activity, while overexpression of alpha-synuclein show decreases in TH phosphorylation at Ser19 and Ser40 and TH activity (Lou *et al.* 2010).

2: Calorie restriction for 6 months in aged rats had no effect on TH phosphorylation, despite an increase in TH protein levels (Salvatore *et al.* 2017).

3: When aged rats are treated with a single injection of GDNF into the striatum and then sacrificed 30 days later there is an increase in TH phosphorylation at Ser31 in the ipsilateral SN. There are no changes in TH protein levels, or TH phosphorylation at Ser19 or Ser40 at this time (Salvatore *et al.* 2004). In a subsequent study TH phosphorylation at Ser31 in the ipsilateral SN was again increased by GDNF, despite a decrease in TH protein levels, and the contralateral SN showing an increase in TH phosphorylation at Ser31 (Salvatore *et al.* 2009a).

4: Ageing causes loss in locomotor activity as measured by a number of parameters in Brown-Norway Fisher 344 F1 hybrid rats and this correlates with DA content (Salvatore *et al.* 2009b). In a subsequent study TH phosphorylation at Ser19, Ser31 and Ser40 decreases during ageing from 6 months to 18-24 months (Salvatore & Pruett 2012). The decreases must have occurred mostly between 6 and 12 months, as there was no TH phosphorylation changes for any site between 12 and 18 months (Salvatore *et al.* 2017).

Striatum and CPU:

1: KO mice lacking the iron regulatory protein-2 throughout development show evidence of DA neuronal loss, including a decrease in TH protein levels after 16-19 months (Salvatore *et al.* 2005). There is a concomitant increase in TH phosphorylation at Ser40, but not at Ser31 or 19, and an increase in DA content relative to TH protein levels. It was concluded that iron misregulation led to an increase in the loss of some striatal DA neurons and that an increase in TH phosphorylation at Ser40 led to an increases in DA content in the remaining neurons.

2: Chronic restriction in adult male rats for 15 days had substantial and complex effects on the DA neurons when compared to *ad libitum* feeding (Pan *et al.* 2006). There is a decrease in L-DOPA synthesis, which correlated with a decrease in TH phosphorylation at Ser40 phosphorylation (Pan *et al.* 2006). Calorie restriction for 6 months in aged rats increases TH phosphorylation at Ser31, despite a decrease in TH protein levels (Salvatore *et al.* 2017).

3: Mn treatment of neonatal rats between postnatal days 8-12 showed a dose dependent increase 48 h after the last treatment in the TH protein levels and TH phosphorylation at Ser19, Ser31 and Ser40 (Peres *et al.* 2016). 70 days after the last treatment high doses of Mn causes a decrease in TH protein levels and TH phosphorylation at Ser19 and Ser40, while TH phosphorylation at Ser31 increases. When aged rats are treated with a single injection of GDNF into the striatum and then sacrificed 30 days later there there is an increase in TH phosphorylation at Ser19, Ser31 and Ser40, despite a decrease in TH protein levels (Salvatore *et al.* 2004). In a subsequent study there is an increase in TH phosphorylation at Ser19 and Ser31, but in this instance there was no increase in Ser40 (Salvatore *et al.* 2009a).

4: Normal ageing for 6, 18 or 24 months (Cruz-Muros *et al.* 2007) induces an age dependent loss in DA content in the ventral and the dorsal striatum (Cruz-Muros *et al.* 2007). The ventral striatum shows a loss of TH activity and L-DOPA at both 18 and 24 months that is not seen in the dorsal striatum. After both 18 and 24 months TH phosphorylation at Ser31 is decreased in both the ventral and dorsal striatum. This suggests that TH phosphorylation at Ser31 is unlikely to be involved

in the decrease in TH activity. TH phosphorylation at Ser19 and Ser40 are both increased at 18 and 24 months in the ventral striatum and not in the dorsal striatum, also suggesting that these sites are also not contributing TH activity. In contrast TH phosphorylation at Ser19, Ser31 and Ser40 is unchanged during ageing in BNF1 rats from 6 months to 18-24 months (Salvatore & Pruett 2012) and from 12-18 months (Salvatore *et al.* 2017).

Mesolimbic pathways

VTA and NAC:

1: Maternal neglect was exhibited in a population of female mice that had been bred for this purpose (Gammie *et al.* 2008). The mothers DA neurons are activated in the zona incerta, VTA and NAC, as judged by cFos expression, but TH phosphorylation at Ser40 was only increased in the zona incerta.

2: There was a significant increase in the TH phosphorylation at Ser40 in the VTA of rats exposed to an iron deficient diet from PND 5 to day 65 (Connor *et al.* 2009).

4: Ageing decreases TH phosphorylation at Ser19, Ser31 and Ser40 in the VTA, but only decreased Ser19 phosphorylation in the NAC (Salvatore & Pruett 2012).

Cell Bodies versus Nerve terminals

The control of TH phosphorylation and DA synthesis differed between the SN and VTA cell bodies and their respective terminals in the striatum and the NAC after acute exposure to a TH inhibitor for 90 min (Salvatore & Pruett 2012), prolonged exposure to a DA transport KO (Salvatore *et al.* 2016), or prolonged calorie restriction (Salvatore *et al.* 2017). Differences also occurred between the SN and the striatum in response to ageing (Salvatore & Pruett 2012).

Summary of Acute and Prolonged TH Phosphorylation Changes

1. *Acute changes in TH phosphorylation occur in vivo in response to a number of stimuli in the adrenal medulla, nigrostriatal pathway and mesolimbic pathways. When compared to in situ studies the in vivo changes are generally delayed and modest. They did not always follow the in situ pattern of TH phosphorylation at Ser19 being followed by Ser40 then Ser31. In the adrenal medulla Ser19 phosphorylation is not altered at the times investigated. In the nigrostriatal pathway, the mesolimbic pathway and the mesocortical pathway there is a range of responses for all three TH phosphorylation sites, including increases and decreases depending on the stimulus. As with striatal slices in situ TH phosphorylation at Ser40 in the*

striatum generally correlated with TH activation. TH phosphorylation changes at Ser31 correlated with TH activation in the prefrontal cortex.

2. *Prolonged changes in TH phosphorylation occur in response to a number of stimuli in the adrenal medulla, nigrostriatal pathway and mesolimbic pathways in vivo. However, in many studies not all sites were investigated. When compared to acute responses, the changes can last for months, are often substantial and often led to changes in TH activation. Two classes of prolonged changes in TH phosphorylation occur in both the adrenal medulla and in the brain. Firstly, there is continuous activation, such as in KO, transgenic or specifically bred animals, or where there is a continuous change in dietary fat, calories or Fe. Secondly, there is a brief exposure during a susceptible period, such as an early post natal immune challenge, maternal separation, or Mn treatment; or a GDNF challenge in aged animals. Ageing leads to decreases in DA metabolism in the SN and striatum. Ageing generates prolonged changes in TH phosphorylation that includes increases, decreases and no change being observed. The specific change depends on the rat species, the exact age of the animals and the specific tissue regions examined.*

3. *In response to a range of stimuli the cell bodies of the nigrostriatal and mesolimbic pathways show acute and prolonged TH phosphorylation responses that are independent of their terminal fields.*

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Table 1 Basal TH Phosphorylation Stoichiometry

Region	Ser19 %	Ser31 %	Ser40 %	TH Protein+	Reference
SN	~26	~8	~3	0.27	(Salvatore *et al.* 2000)*
SN	13	9	4.5		(Salvatore *et al.* 2009b)
SN	13	11	5		(Keller *et al.* 2011)
SN	ND	6.9	1.6		(Salvatore & Pruett 2012)
SN	**17.3**	**8.7**	**3.5**		**Mean**
VTA	~25	~12	~3	1.00	(Salvatore *et al.* 2000)*
VTA	25	10	3.5		(Salvatore *et al.* 2009b)
VTA	ND	10.4	5.1		(Salvatore & Pruett 2012)
VTA	28	13	6	0.68	(Ong *et al.* 2014)
VTA	**26**	**11.4**	**4.4**		**Mean**
Hypothalamus	**26**	**13**	**5**	**0.07**	**(Salvatore *et al.* 2000)***
LC	**36**	**6**	**3**	**0.27**	**(Ong *et al.* 2014)**
Striatum	~10	~31	~3	0.42	(Salvatore *et al.* 2000)*
Striatum	6	33	2.2		(Salvatore *et al.* 2009b)
Striatum	ND	29	2.5		(Salvatore & Pruett 2012)
Striatum	**8**	**31**	**2.6**		**Mean**
NAC	~16	~21	~3	0.28	(Salvatore *et al.* 2000)*
NAC	12	36	4.9		(Salvatore *et al.* 2009b)
NAC	ND	18.3	7.0		(Salvatore & Pruett 2012)
NAC	**14**	**25.1**	**5**		**Mean**
MPFC	**11**	**34**	**3**	**0.17**	**(Ong *et al.* 2014)**
Adrenal Medulla	**33**	**6**	**18**	**2.26**	**(Ong *et al.* 2014)**

Values in the Table for Ser19, Ser31 and Ser40 are the stoichiometry of phosphorylation at each individual site expressed as %, where a value of 5 means that 5 moles of Ser phosphorylation occurs for every 100 moles of TH protein monomer.

+ TH protein levels, in ng, relative to µg total protein

*~ values shown for stoichiometry were estimated from a graph in the publication where no Table of

data was available.

ND, not done

Table 2 Maximum TH Phosphorylation Stoichiometry

Region	pSer19	pSer31	pSer40	Reference
Adrenal Medulla	1.5[1]	9[2]	2.5[1]	Maximum Fold increase
Adrenal Medulla	50%	54%	45%	Maximum Stoichiometry
Neuron Cell Bodies	2.5[3]	~3.2[4,*]	2.4[5]	Maximum Fold increase
Neuron Cell Bodies	65%	28%	8%	Maximum Stoichiometry
Neuron Terminals	1.8[3]	2.1[6]	2.5[5]	Maximum Fold increase
Neuron Terminals	20%	71%	13%	Maximum Stoichiometry

Maximum Fold increases were reported in the following studies for tissues where a basal stoichiometry was available (see Table 1). No larger fold increase was seen for the adrenal, the neuron cell bodies, or neuron terminals when scanning other papers from the laboratories where basal stoichiometry was determined.

1 (Sominsky *et al.* 2013) LPS stimulation at 85 days in the adrenal (Ser19 and Ser40)

2 (Bobrovskaya *et al.* 2010) 2DG stimulation at 60 min in the adrenal (Ser31)

3 (Damanhuri *et al.* 2012) Hydralazine stimulation at 30 min in the MPFC (Ser19) or VTA (Ser19 and Ser40)

4 (Salvatore *et al.* 2009a) GDNF stimulation at 30 days in the SN (Ser31)

5 (Salvatore *et al.* 2000) Haloperidol stimulation at 30 min in the SN, or clozapine stimulation in the NAC (Ser40)

6 (Ong *et al.* 2014) Footshock at 40 min in the MPFC (Ser31 and Ser40)

*~ values shown for stoichiometry were estimated from a graph in the publication where no Table of data was available.

Maximum Stoichiometry was determined by multiplying the average basal stoichiometry in Table 1 (Bold) for the appropriate tissue by the Maximal Fold increase observed in the cited reference. Values were rounded up to the nearest whole number.

Table 3 Specific Catecholaminergic Cell Types *In Vivo*

Region	**Treatment**	**TH Protein**	**TH phosphorylation**	**TH activation**	
Acute					
Heart	Naloxone treatment of morphine dependent rats	Increased at 60 and 90 mins	Ser40 increased at 60 and 90 mins Ser31 increased at 90 mins	Increased TH activity at 60 mins correlates to Ser40	(Gonzalez-Cuello *et al.* 2004 ; Almela *et al.* 2007; Almela *et al.* 2008)
Retinal Amacrine cells	Dark and Light for 20-30 min; +/- GABA and Glycine antagonists	ND	Ser19, Ser31 and Ser40 all increased in Light and in response to drugs	Increased L-Dopa synthesis in Light	(Witkovsky *et al.* 2000; Witkovsky *et al.* 2004)
Hypothalamic TIDA neurons	Progesterone for 5 h period	Unchanged	Ser19, Ser31 and Ser40 all decreased	Decreased DA synthesis	(Arbogast & Voogt 2002; Liu & Arbogast 2008; Liu & Arbogast 2010)
Locus Coerulus	Social Defeat for 3 consec utive 20 min periods	ND	Increased Ser31 and Ser40 at 10 min after final defeat	ND	(Ong *et al.* 2011a)
	Immobilisation for 10, 20 or 40 min	Unchanged	Increased Ser31 at 10 min	ND	(Ong *et al.* 2011b)
	Footshock for 10 , 20 or 40 min	Unchanged	Increased Ser31 at 20 and 40 min; Decreased Ser19 at 40 min	Increased TH activity at 40 min but not 20 min	(Ong *et al.* 2011b; Ong *et al.* 2014)
	Insulin induced hypoglycaemia for 10-120 min	Unchanged	Unchanged between 30-90 min	ND	(Senthilkumaran *et al.* 2016)
NTS	Naloxone treatement of Morphine	Increased at 90 min after naloxone	Ser31 increased at 90 min after naloxone	ND	(Nunez *et al.* 2007)

	dependent rats				
PVN	Naloxone treatement of Morphine dependent rats	Unchanged	Ser31 increased at 90 min after naloxone	TH activity increased at 90 min	(Nunez *et al.* 2007)
NTS and PVN	Naloxone after adrenalectomy and corticosterone	Unchanged	Unchanged	Unchanged	(Nunez *et al.* 2009)
NTS	Naloxone treatement of Morphine dependent rats	ND	Increased Ser31 and 40 at 30 min	ND	(Navarro-Zaragoza *et al.* 2011)
Prolonged					
Carotid Bodies	Hypoxia-Chronic vs Intermittent from 1-30 days	Increased at 1-30 days	Ser19, Ser31 and Ser40 increased at 1-30 days	ND	(Hui *et al.* 2003)
Brain Stem Medulla and Pons	Intermittent hypoxia for 10 days		Increased Ser31 and Ser40 in medulla not pons	Increased TH activity and DA synthesis	(Raghuraman *et al.* 2009; Raghuraman *et al.* 2012)
Cortex	Prolonged hypoxia from 6 h to 7 day		Ser40 increased only at 7 days	TH activity increased at 1-3 days	(Gozal *et al.* 2005)